Antimicrobial Properties of Traditional Medicine Used for Treatment of HIV/AIDS and its Opportunistic Infections

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Antimicrobial Properties of a Traditional Medicine Used for the Treatment of HIV/AIDS and its Opportunistic Infections

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ABSTRACT

This study was conducted to establish the scientific basis of the reported ethnomedicinal use of *Ihlamvu laseAfrika* (*IHL*) against Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Virus (AIDS) related infections. *IHL* is believed to have a positive effect on AIDS however this has neither been clinically nor laboratory proven. Such effect can either be directly due to *IHL*'s inhibition of the virus causing AIDS or indirectly by the inhibition of organisms causing opportunistic infections. Experiments were carried out to test for the effect of *IHL* against *Cryptococcus neoformans*, *Candida albicans*, Herpes Simplex Virus (HSV), *Mycobacterium tuberculosis* (*MTB*) and HIV.

The toxicity of *IHL* was determined by means of three assays. Using the Trypan Blue Dye exclusion test, an aqueous mixture of *IHL* was tested on Vero cells (African Green Monkey) for acute toxicity at two concentrations. Cell membranes compromised by *IHL* would take up dye and eventually spill their contents. Vero cells that were exposed to 1µg/mL and 100µg/mL concentrations of *IHL* for 7 hours resulted in (8.9±0.15) % and (98.7±0.84) % cell viability (n=3), respectively. When the duration of incubation increased to 48 hours, percentage cell viability of 1µg/mL and 100µg/mL concentrations were (98.3±0.50) and (98.2±0.50) respectively.

The second cytotoxicity test involved incorporation an aqueous mixture of *IHL* onto 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT). Cells were incubated in *IHL* for 24 and 48 hours resulting in a decrease in cell viability in a dose-dependent manner. At the lowest *IHL* concentration (0.1µg/mL) the cell viability was 80% and 78.5% after 24 and 48 hours incubations, respectively whereas at the highest concentration (1000µg/mL) was used in 24 and 48 hours incubation, cell viability was 50% and 80% respectively.

The third cytotoxicity test called glutathione (GSH) focused on antioxidant level. The aim was to determine the highest concentration at which cells starts dying, concentrations used

were 0.23; 0.46; 0.94; 1.88; 3.75; 7.50; 15.0 and 30.0 mg/mL. The results showed that the antioxidants levels were reduced in proportions relative to *IHL* concentration levels. The safe and effective dose of *IHL* obtained was 1.88mg/mL.

The second objective of the study was to determine *IHL*'s active principle that is capable of inhibiting growth of *C. albicans* and *C. neoformans,* HSV, *MTB* and HIV. Solvents such as methanol, ethanol and acetone were utilized including an aqueous extract to extract it. The most suitable extract to inhibit the proliferation of the aforementioned organisms needed to be established. Upon its establishment, it was then used to determine the minimum inhibitory concentration (MIC). This was done in all susceptibility tests except for HIV whereby a 'neat substance' was used. In the case of HSV a causative agent for herpes, its susceptibility towards several *IHL* extracts was assessed with real-time polymerase chain reaction (RT-PCR). PCR attenuates specific site of DNA and quantifies viral load and the focus was the UL30 position which is targeted by most drugs. When comparing all solvent extracts as well as an aqueous extract of similar concentration, it was found that the methanol extract emerged as the strongest viral inhibitor with the lowest viral yield, and its threshold value, *Ct* = 18.4± 0.86 while the *IHL* concentration was 1.88mg/mL. The MIC of the methanol extract was 1.25mg/mL and *Ct*=18.9±1.14. An acetone extract proved to be the weakest thus its viral load was the highest, its *Ct*= (8.50±1.33) whilst *IHL* concentration was 1.88mg/mL.

Cryptococcus neoformans known for causing meningitis and encephalitis in AIDS patients and *C. albicans* a causative agent for vaginal and oral thrush were two opportunistic infections tested for susceptibility towards *IHL*. The disk diffusion method was used for both fungal organisms. The best suited solvent extract was established and then used to determine the MIC. An aqueous extract showed the best activity with the inhibition zones of (10.5±1.642) mm when tested against *C. albicans* followed by ethanol extract (9.2±0.676) mm while acetone

extract (8.80 ±1.21) mm had the lowest activity. The MIC of *IHL*'s aqueous extract was 1.0mg/mL and the corresponding zone of inhibition was (10.6±1.34) mm.

When *C. neoformans* was tested for susceptibility against various *IHL* solvent extracts, the *IHL*'s aqueous extract had inhibition zones of (21.1±2.40) mm thus emerged as the strongest followed by methanol extract (10.3±0.43) mm while ethyl acetate extract was least active (7.13±0.33) mm. The MIC of the aqueous extract was 1.0mg/mL and its corresponding zone of inhibition was (11.4±0.55) mm. Furthermore, the growth inhibition of both *C. neoformans* and *C. albicans* by *IHL*'s aqueous extract were confirmed in liquid media with broth microdilution method. This technique tends to mimic what is likely to happen in a biological fluid. The results obtained depicted a dose-dependent response and both organisms shared a common MIC of 2.0mg/mL. From the broth microtitre plate aliquots samples were plated onto agar and used to further determine the minimum lethal concentration (MLC). The MLC essentially determines the antifungal concentration of an agent at which no colonies displayed visible growth. The MLC's of *IHL* towards *C. albicans* and *C. neoformans* were 32 and 8 mg/mL respectively. *IHL* proved fungicidal at higher concentrations and fungistatic at low concentrations.

Further susceptibility tests of *IHL* extracts were carried out on bacterial pathogens such as the *MTB*, a causative agent for Tuberculosis with 1% proportion method. This method seeks to determine if isolates are resistant if colonies grown in the presence of drugs are greater or equal to 1% of colonies grown in drug-free control quadrant. The best solvent extract was determined and then used to determine the MIC. Acetone extract results were 0.2% meaning that it strongly inhibited growth of *MTB* better than ethyl acetate (5%) and others the worst results were that of an aqueous extract (113%). A confirmation exercise was done with an agar dilution method. All extracts were incorporated onto agar and *MTB* colonies growing relative to negative controls after 21 days of incubation meant resistance while no growth meant susceptible. The *MTB* strain again proved susceptible towards the acetone extract but resistant towards methanol, ethanol, and

aqueous extracts. The dichloromethane and ethyl acetate extracts seemed to have damaged the polypropylene plates rendering results null and void. Using agar dilution method, an MIC of an acetone extract was 16mg/mL.

An aqueous extract was used for assessing HIV for susceptibility towards *IHL*. The quantitation of viral results were carried out on a spectrophotometer and a second generation tetrazolium dye (XTT) was used. The results showed that approximately -3.29 dilution of the aqueous extract did not protect cells. On the contrary, it proved to be toxic to both uninfected and infected cells. Moreover at low doses the extract demonstrated 50% protection towards uninfected cells.

The third objective entailed the assessment of reproducibility of *IHL* that is routinely prepared by the Traditional Health Practitioner (THP). Batch to batch reproducibility is always a concern especially since traditional medicine is manufactured without any traceable set of standards. Two *IHL* samples that were prepared on different dates were assessed. Using a thin layer chromatography (TLC) a striking resemblance in the two samples was established visually by way of fractions produced. However, since TLC is a qualitative tool, it was incumbent that an instrument that doesn't separate sample's chemical constituents was used. The results produced by nuclear magnetic resonance (NMR) confirmed similarities in the two batch of *IHL* samples produced on different dates as it was the case with TLC. Peak intensity and the number of peaks in the chromatogram was a mirror image of the other thus confirming consistency in *IHL* preparation.

The susceptibility tests of *IHL* towards viruses, bacteria and fungal pathogens present reasons why *IHL* is regarded as a non-specific repressor of pathogens people living with AIDS (PLWA) present with. The fourth objective of the study entailed the establishment of active principles responsible for the aforementioned activities. The acquisition of chemical fingerprints

and their analysis was carried out on an Ultra Performance Liquid Chromatography Mass Spectrometer (UPLC-MS). The substances thought to be responsible for antimicrobial activities included:- thalebanin B, methyillukumbin A, kuguacin J, mauritine H, 2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione, isoferuloyllpeol, diosindigo A, kuguacin R, verbascoside, kuguacin B and nuciferin. Further confirmation studies are needed on fractions to identify their chemical makeup as well as their activities on all of the aforementioned microorganisms.

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List of Abbreviations

+ve..... positive

°C..... Degrees Celsius

µL..... microliter

^{13}C..... Carbon isotope

^{1}H..... Hydrogen

ABC..... ATP-binding cassette

Acet..... acetone

ACV..... Acyclovir

AIDS..... Acquired Immunodeficiency Syndrome

Alu Si..... Aliminium silicone

AMB..... Amphotericin B

AR..... Analytical Reagent

ARV..... antiretrovirals

ATM..... African Traditional Medicine

AZT..... Azidothymidine/Zidovudine

BMLE..... ..... bitter melon leaf extract

CA..... Capsid

CBD..... Conventional of Biological Diversity

cDNA ..... complementary deoxyribonucleic acid

CFU..... Colony forming units

CO_2..... Carbon Dioxide

COSY..... Correlation spectroscopy

CPE..... cytopathic effect

CsA..... Cyclosporine A

Ct..... cycle threshold

CYP_{450}..... Cytochrome P450

DAD..... Diode Array Detector

DCM..... Dichloromethane

DNA..... Deoxyribonucleic acid

DNAse..... deoxyribonuclease

EA..... Ethyl acetate

EC_{50}..... Median Effective Concentration

EDTA..... Ethylenediaminetetraacetic acid

ELISA..... Enzyme-Linked Immuno Assay

EMEM..... Minimum essential medium eagle

Et_2O..... Diethyl ether

EtOAc..... Ethyl ethanoate

EtOH..... Ethanol

FCS..... Fetal Calf Serum

GC..... Gas Chromatography

GSH..... Reduced glutathione

H_2O..... Water

HAART..... Highly Active Antiretroviral Therapy

HDMS..... High Definition Mass Spectrometer

HIV..... Human Immunodeficiency Virus

HMBC..... Heteronuclear Multiple Bond Correlation

HMQC..... Heteronuclear Multiple Quantum Coherence

HRESIMS..... High Resolution Electronspray Interface Mass Spectrometry

HSV..... Herpes Simplex Virus

IALCH..... Inkosi Albert Luthuli Central Hospital

IC_{50}..... Median Inhibitory Concentration

IC_{50}/EC_{50}..... Selectivity index

IKS..... Indigenous Knowledge Systems

IN..... Integrase

INH..... Isoniazid

IUATLD..... International Union against Tuberculosis and Lung Disease

kV..... kilovoltage

L/h..... liters per hour

m/z..... mass to charge ratio

MA..... Matrix

MDR..... Multidrug resistant

MeOH..... Methanol

mg/mL..... milligrams per millimeters

$MgCl_2$..... magnesium chloride

MIC..... Minimum Inhibitory Concentrations

mL..... millimeters

MLC..... Minimum Lethal Concentration

mM..... millimolar

MOPS..... Morpholinepropanesulfonic acid

MOU..... Memorandum of Understanding

MTB..... *Mycobacterium tuberculosis*

MTT.....Methylthiazol tetrazolium

N..... No

N..... Nitrogen

Na..... Sodium

NC..... Nucleocapsid

NEMA..... National Environment Management Act

NIAID..... National Institute of Allergy and Infectious Diseases

NIH.....National Institute of Health

NMR.....Nuclear Magnetic Resonance

NRF..... National Research Foundation

O..... Oxygen

OADC..... Oleic acid albumen dextrose catalase

OD..... Optical density

PBS..... Phosphate buffer saline

PCR..... Polymer Chain Reaction

PEPFAR..... President's Emergency Plan for AIDS Relief

P-gp..... P-glycoprotein

PLWA..... People Living With AIDS

PR..... Protease

PRA..... plaque reduction assay

PTFE..... Polytetrafluoroethylene

R..... resistant

RIF..... rifampicin

RIF/Solv..... Rifampicin in solvent

RNA..... Ribonucleic acid

RPMI..... Roswell Park Memorial Institute

RT..... Reverse Transcriptase

RT..... room temperature

RT-PCR..... Real Time Polymer Chain Reaction

S..... susceptible

S..... Sulphur

SADC ..... Southern African Development Countries

SDA..... Sabourad dextrose agar

Spp..... species

STD..... Sexually transmitted diseases

STI..... Sexually Transmitted Infections

SU ..... Surface unit

TAACF..... Tuberculosis Antimicrobial Acquisition and Coordination Facility

$TCID_{50}$..... Median Tissue Culture Infective Dose

TCM.....Traditional Chinese medicine

THP.....Traditional Health Practitioner

TLC.....Thin Layer Chromatography

TM..... Transmembrane unit

TM/Solv.....Traditional medicine in solvent

TMs/CAMs..... Traditional Medicine/Complementary Alternative Medicine

TOCSY..... Total Correlation Spectroscopy

TOF.....Time of flight

TRIP..... Trade-Related Aspects of Intellectual Property

UK..... United Kingdom

UKZN..... University of KwaZulu-Natal

UPLC-MS..... Ultra Perfomance Liquid Chromatography Mass Spectrometer

USA..... United States of America

UV..... Ultra Violet

-ve..... negative

WHO..... World Health Organization

XDR-TB..... Extreme drug resistant tuberculosis

XTT..... Methylthiazol tetrazolium (water soluble)

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Appendix 1

Chapter 1

INTRODUCTION

Chapter 1

Introduction

Infection with Human Immunodeficiency Virus (HIV) an etiologic agent of the Acquired Immune Deficiency Syndrome (AIDS) is a global phenomenon (Gurib-Fakim, 2006). AIDS can be described as a complex array of disorders due to deterioration of the immune system caused by the HIV leading the individual being susceptible to opportunistic infections and tumours (Marx, 1982). HIV targets the immune system especially macrophages and helper T cells wherein proliferation takes place before dispersal into new viral host. During viral replication the immune system is severely damaged by production of new virions and more so with every infection hence millions of new virions are produced each day. The production of antibodies and T cells to fight infections remains ineffective against the rate of viral production with subsequent appearance of cancer associated with AIDS and secondary opportunistic infections (Gurib-Fakim, 2006).

Current treatment options be it orthodox drugs or even vaccines do not cure AIDS, however, retards its progression. Previous studies attribute antimicrobial activities to secondary metabolites. At present from 36 000 extracts that have been screened by the National Cancer Institute of USA, approximately 3600 displayed anti-HIV activity (Gurib-Fakim, 2006).

Traditional medicine practice is most prevalent amongst South African population for managing the physical and psychological and social health needs (Rabe and van Staden,

1997). Natural products have become the subject of intense investigation recently in relation to conservation and as to whether their application are supported by their intended pharmacological effects or merely based on tales of the ancient (Cunningham, 1988; Locher *et al*, 1995; Williams, 1996). Undoubtedly traditional medicine's application is quickly gaining momentum as an alternative means of health care. Hence there is a need to screen medicinal plants for activity. The purpose of this study was to investigate South African medicinal plant product known as *Ihlamvu laseAfrika* (*IHL*) for potential antimicrobial activity by preliminary bioassay screening and metabolites profiling.

The dissertation is written such that each chapter explores antimicrobial properties of *IHL* on a specific organism/s. There are three sections/chapters such as literature review (Chapter 2), Chapter 3 (investigated secondary metabolites possibly responsible for the aforementioned antimicrobial properties) and Cytotoxicity assay on Chapter 4 which are the only exceptions. However, Chapter 5 investigated antiherpes properties of our test substance. In chapter 6 the antifungal properties of the test substance were also investigated. *Cryptococcus neoformans* and *Candida albicans* were two microorganisms tested for *IHL* susceptibility. Antimycobacterium properties tested on *MTB* strain with two techniques such as 1% proportion method and agar dilution was covered on chapter 7. In chapter 8 the anti-HIV properties were the last susceptibility assay to be explored. The last chapter gave an overall discussion and conclusion.

Briefly the study aimed at investigating the following *IHL* properties:-

(a) anti-HSV

(b) antifungal (*C. albicans* and *C. neoformans*)

(c) antimycobacterial tuberculosis (*MTB*)

(d) anti-HIV and

(e) chemical profile of essential compounds possibly responsible for antimicrobial activities of *IHL*.

Chapter 2

Literature Review

Literature Review

2.1 Background

2.1.1 What is traditional medicine?

The World Health Organization (WHO) gave the following definition to African traditional medicine:

"The sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" (WHO, 2008).

This definition sets an immediate distinction between science-based approaches to treatment to the traditional way. According to Steinglass (2002), an African approach to healing is holistic such that an ill person's spiritual and physical well-being are simultaneously treated. According to Ayurveda and Traditional Chinese medicine (TCM) systems, the focus is also on the affected individual rather than on the disease (Patwardhan *et al*, 2005). Alternately, the science-based approach or biomedicine would label traditional medicine as phytotherapy. Hence, the medical anthropologists would associate traditional medicine and its practitioners with ethnomedicine (Nitchter, 1992). Nevertheless, practitioners of medicine are generally subdivided into diviners (mediums) and healers (Joles *et al*, 2000; Steinglass, 2002). Diviners diagnose the sickness through spiritual interventions (ancestral) whereas healers

provide medicines (Jolles *et al*, 2000). Healing is approached as a calling that is passed down from generation to generation and it would turn into a family business (Pujol, 1990; Liu, 2005). However, only one individual within the family would receive the gift also referred to as "*isikhwama*" – the bag. Direct translation takes away the essence to description since the bag is rather a trust that becomes a closely guarded secret. Scientific studies simply tap into isolating or perhaps modify the plant's active compounds (Gilani *et al*, 2005; Patwardhan, 2005; van Vuuren, 2008). Research into traditional medicine is done in a bid to further add into the information pool hence setting a stage to a more novel entity discovery. More therapeutics are necessary in the midst of drugs shortage inadvertently attributed to drug-resistance especially seen with Acquired Immunodeficiency Syndrome (AIDS) and treatment defaults patients.

The abolishment of the traditional medicine practice by the colonial and apartheid regimes in South Africa altered a cultural landscape setting up a platform through which sadistic practices by some charlatans under the banner of traditional medicine also flourished. The two legislations, the Witchcraft Suppression Act of 1957 and Witchcraft Suppression Amendment of 1970 vehemently prohibited traditional health practices, however its abolishment could be backtracked to 1891 (Jolles *et al*, 2000). Meanwhile the encroachment of western healthcare system nullified its competition which saw traditional health practices slowly becoming extinct (Jolles *et al*, 2000).

2.2 Benefits of Traditional Medicines

From prerecorded history until recently, southern African people especially the rural and peri-urban dwellers would mostly derive health benefits, nutrition and material resources from indigenous plants (Pujol, 1990). Health challenges which include coughs, wounds, cuts and abrasions, intestinal and gut disorders, migraine, skin infections, snake and insects bites etc have been managed with natural product be it fauna or flora based products. (Pujol, 1990; Liu, 2005).

2.3 Plant-derived drugs

Medicinal plant products including their derivatives and analogs contributed over 50% of clinically used drugs in which 25% of that total is a contribution from higher-plants (Balandrin *et al*, 1993). According to folklore, records sampled from several cultures around the world, give crucial leads to active therapeutic properties (Bohonos *et al*, 1966; Swain, 1972; Goldstein *et al*, 1974; Lewis *et al*, 1977; Balandrin *et al*, 1985; Duke *et al*, 1985; Balandrin *et al*, 1988; Tyler *et al*, 1988; Kinghorn, 1992; Kinghorn, 1993).

Paclitaxel (also called Taxol®) is a classic example of a plant derived drug that is commercially available. It was discovered in 1982 from Pacific yew and is presently used as a refractory ovarian cancer drug (Cragg *et al*, 1993). Paclitaxel is a taxane diterpenoid. Etoposide is another example of a semisynthetic antineoplastic agent that is also commercially available and it is derived from podophyllotoxin, a mayapple

constituent (Balandrin *et al*, 1993). The mayapple plant (*Podophyllum peltatum* L.) species is applied in chemotherapy for refractory testicular cancer treatment, small cell lung cancer, nonlymphocytic leukemias and non-Hodgkin's lymphoma (Horwitz *et al*, 1977; Cabanillas, 1979; Issell *et al*, 1979; Radice *et al*, 1979; Budavari *et al*,, 1989; Hussar, 1984). Atracurium besylate adds to the list of new plant derived drugs. It soothes skeletal muscle and it is both structurally and functionally closely linked to curare alkaloids (Budavari *et al*, 1989; Hussar, 1984). The Δ^9-tetrahydrocannabinol is a drug that is derived from *Cannabis sativa* L. commonly known as marijuana plant or dagga and has found useful application in cancer chemotherapy (Duke *et al*, 1985; Budavari *et al*, 1989; Anon, 1985). Furthermore, a steroid called digitoxin that is derived from *Digitalis purpure* L. plant and *Digitalis lanata Ehrhart* (foxgloves) is used clinically as cardiotonic glycoside. Opium which is an alkaloid also known clinically as codeine or morphine, is derived from *Papaver somniferum* L. and is used for pain management. The second commercially available alkaloid drug is reserpine which is extracted from *Rauvolfia serpentia* L. (East India snakeroot) is used as an antihypertensive psychotropic drug. The third alkaloid drug is vinblastine or vincristine which is derived from *Caranthus roseus* L. (Madagascar rosy periwinkle) plant and is used as an anticancer drug. Physostimine is a forth alkaloid drug taken from *Physostigma venenosum* Balfour (Calabar bean) and it functions as a cholinergic or a parasympathomimetic drug. Another plant-derived drug that acts as a parasympathomimetic is pilocarpine which is derived from *Pilocarpus jaborandi* Holmes and other related species.

Furthermore there is an antimalarial and cardiac antiarhythmic drug known as quinine or quinidine that is manufactured from Cinchona species whose bark is richly filled with alkaloids. Other than synthetic drugs, gout has been treated with a plant-derived drug called colchicine. Colchicine comes from the *Colchicine autumnale* L species. Another drug which is used as a recreational drug besides marijuana and morphine is cocaine. Cocaine is derived from the leaves of *Erythroxylum coca* Lamarck and its intended function was to find application as a local anesthetic. (Lewis *et al*, 1977; Tyler *et al*, 1988; Farnsworth, 1973; Farnsworth *et al*, 1976; Farnsworth, 1977; Farnsworth *et al*, 1977; Farnsworth, 1966). The last but not least alkaloid drug is d-Tubocurarine, another drug for soothing muscles that is derived from plant such as *Strychnos toxifera* Bentham and *Chondodendron tomentosum* Ruizet Pavon (curare) (Budavari *et al*, 1989; Hussar, 1984). The Chinese developed *Qinghaosu* also known as artemisinin drug which acts as an antimalaria agent and it is taken from *Atermisia annua* L. plant (Klayman *et al*, 1984; Klayman, 1985; Nair *et al*, 1986). There are also compounds such as organosulphur that were investigated from plants such as garlic and onions for their potential utilization as cardiovascular agents (Block *et al*, 1984; Block , 1985) as well as ellagic acid, *p-carotene* and vitamin E (tocopherol) that were investigated for further usage as prototype antimutagenic and anticancer properties (Budavari *et al*, 1989).

Secondary metabolites have in some cases been used as models or templates through which absolute synthesis of new drug entities were based. These entities include belladonna alkaloids such as atropine, physostigmine, quinine, cocaine, gramine,

opiates (examples are codeine and morphine), papaverine, salicyclic acid were all derived from these models. Another class feature includes anticholinegernics, anticholinesterases, antimalaria agents, benzoicaine, procaine, lidocaine (xylocaine), and local anesthetics, analgesics (Talwin), propoxyphene (Darvon) methadone and mereperidine, verapamil and aspirin (acetylsalicyclic acid) (Lewis *et al*, 1977; Roche, 1977; Cassady *et al*, 1980; Gund *et al*, 1980). Similarly in the Unites States (US) the furanochromone analogue that is derived from *Ammi visnaga* (L.) Lam fruit which was commercialized as bronchodilator and coronary medicine led to sodium chromoglycate synthesis also referred to as chromolyn sodium. Chromolyn is currently marketed as a bronchodilator however the main advantage with chromolyn is its antiallergenic properties (Sneader, 1985). In a similar example, the galegine alkaloid also an active ingredient of *Galega officinalis* L. was clinically prescribed for diabetic patients. However, due to its toxicity to humans, several compounds synthesized led to metformin which is structurally and pharmacologically similar to galegine and thus is used as an antidiabetic drug (Sneader, 1985). Hence the abovementioned secondary metabolites illustrate the importance of traditional medicine, their secondary metabolites, especially the active principles, and the role they play in modern drug synthesis.

2.4 Treatment with traditional medicine

According to African tradition, diseases are believed to be inflictions from supernatural beings, through spiritual, entities or ancestral spirit, living people, animals, plants and "pollutants". Disobedience manifests into disorder or entropy in

one's life coupled to a host of misfortunes. Sacrificing of animals, and administration of some medicinal plants that are specially formulated by well renowned healers, help restore normality (Kale, 1995). It is important to note that THPs treat symptoms as opposed to disease entity. Healers lack pathology and patterns of diseases progression hence they control symptoms (Leung, 2004). Alternately, diseases can also be naturally occurring such as sexually transmitted but to manage symptoms even for minor breakout can be a daunting task. Failure to do so can lead one to sort counsel from the elders however, one has to contend with discipline. Inability to sort out the problem, the elders would make reference to a THP who is endowed with signs and symptoms who would then apply the appropriate intervention.

Euclea natalensis is shrub that is mostly found inland and the coastal regions or generally in southern Africa (van Wyk *et al*, 1997). Medicines derived from root infusions were taken for bacterial infections by indigenous people of southern Africa (Watt *et al*, 1962). However, the plant's medicinal properties includes treatment of urinary tracts infections, sexually transmitted diseases and dysmenorrhoea. In some parts of the country it is used as a "*miswak*" or toothbrush (Stander *et al*, 1991). The indigenous people of KwaZulu-Natal, use the root portion for Tuberculosis (TB)-related symptoms which include, bronchitis, pleurisy and asthma (Watt *et al*, 1962). Activity of this plant is attributed to secondary metabolites for example, the naphthoquinones, dihydroxyursanoic acids (lactones derivatives), triterpenoids and tetrahydroxyflavanone arabinopyranoside (van der Vijver *et al*, 1974, Ferreira *et al*, 1977, Lall *et al*, 2001).

2.4.1 Use of traditional medicine (TM) to treat Herpes Simplex Virus

According to World Health Organization (WHO) traditional medicine can either be composed of a single plant or a combination of several plants, animals and minerals (WHO, 2008). In order to manage Herpes Simplex Virus (HSV) breakouts, local THPs have a wide variety of medicinal plants to choose from, depending on their geographic location. *Helichrysum aureonitens* Sch Bip. (*Asteraceae*) is one medicinal plant that is predominantly found in southern Africa, particularly in KwaZulu-Natal region (Hilliard, 1983). According to Meyer *et al* (1996), an extract of this plant would be topically applied to affected areas. Herpes zoster has been treated with exudates of *H. aureonitens* which treated skin infections by topically applying it to affected areas.

2.4.2 Use of TM to treat *Mycobacterium tuberculosis* (*MTB*)

To offer relief from symptoms due to *MTB* which may include incessant coughing, weight loss, loss of appetite and night sweats, the traditional healer may administer extracts made from leaf, bark and root of *Acacia nilota* or *Combretum kraussii* and *Euclea Natalensis* species to patients (Lall *et al*, 1999).

2.4.3 Use of TM to treat Fungal Infections (*Candida albicans*)

Common infections that are closely linked to *C. albicans* clinically manifest as pseudomembraneous (thrush), erythematous and hyperplastic variants to linear gingival erythema that often occur to immunocompromized individuals (Thamburan

et al, 2006). *C. albicans* is a commensal organism that is not particularly associated with morbidity however, upon exceeding one third of mouth and gut flora which is an acceptable level to normal hosts, it can intervene with nutritional intake (Fichtenbaum *et al*, 2000). At this point a THP may opt for extracts taken from *Tubalghia alliacea* (Thamburan *et al*, 2006) of South African origin.

2.4.4 Use of TM to treat Acquired Immunodeficiency Syndrome (AIDS)

When immunocompromized patients present with clinical symptoms befitting AIDS, the most trusted phytotherapeutic agent that is highly recommended would most probably be *Hypoxis hemerocallidea* and *Sutherlandia frutescens* extracts. The two plants extracts were endorsed by the South African Health Ministries in a fight against HIV since it displayed extensive immunostimulation properties (SADC, 2002).

Treating of symptoms constitute one branch of traditional medicine involving a healer (*inyanga*). The second branch entails seeking the main cause of illness and dispensing treatment and it involves a consultation with a diviner (*Isangoma*). *Sangomas* would diagnose by "throwing bones" which would tell of the past and present and foretell the future events (Pujol, 1990). Hence traditional medicine substantially introduces intricate relations that unite the living with the unseen and ultimately gives guidance that would ensure safe passage in life.

2.5 HIV/AIDS treatment options

As it is observed in treatment of several chronic disorders with traditional interventions, a trend is also noticeable in traditional medicine usage against Human Immunodefiency Virus (HIV)/AIDS (Sukati *et al*, 2005; Bodeker *et al*, 2000; Josephs *et al*, 2007; Manfredi, *et al*, 1999). The reasons vary from developed to developing countries.

Lack of access to antiretrovirals (ARV)'s created much dependency towards natural products amongst people living with AIDS (PLWA) in order to manage symptoms. High cost of drugs also became a contentious issue however as soon as they became available, high cost price made them inaccessible due to high levels of unemployment. This therefore meant high viral load was to go rampant on the resource poor communities (Ivers *et al*, 2005). This prompted WHO to introduce a 3 by 5 initiative which essentially meant to ensure that 3 million PLWA would have access to ARV's by 2005. The initiative was coupled to ARV's price reduction (Peres-Casas *et al*, 2001) as a result of the World Trade Organization signing an agreement on Trade-Related Aspects of Intellectual Property rights (TRIPs) that would ensure increased access to ARV's an aid geared for Southern African Development Community (SADC) region.

Notwithstanding the aforementioned initiative by the WHO, reliance on natural products remained a formidable challenge to the Highly Active Antiretrovirals Therapy (HAART) program (Langewitz *et al*, 1994; Dahab *et al*, 2008). Media would report on potential immunostimulants with absolutely no side effects. Subsequently

patients on ARVs would terminate HAART regiment only to ensue with traditional medicine/complementary and alternative medicine (TMs/CAMs) interventions due to perceived lack of side effects. Such statements remain invalid with most TM/CAM that have not been clinically tested hence it only remains a marketing gimmick on the part of some THPs. When patients suddenly stop taking ARV's and switch to TMs it becomes a serious life threatening undertaking. The presence of regulatory bodies would therefore serve as a deterrent to such a robust and irresponsible marketing tactic (WHO, 2005). A combination of both therapeutic agents, TM/CAM and HAART has been taken up by another group living with HI virus for viral suppression disregarding negative impact this might have on their livelihood and as well as the impact of dual therapeutics effect on drug metabolism (Josephs *et al*, 2007; Babb *et al*, 2007; Mill *et al*, 2005). General studies revealed overwhelming evidence that proves drug interaction between two therapeutic agents (Mills *et al*, 2004) which therefore prompt for TM-HAART interaction studies to be conducted immediately.

2.6 Globalization and Traditional medicine

With globalization of all human aspects saw floodgates of trade being widely opened and information exchange on an increase, traditional medicine has also undergone total revolution. The suppressive laws previously used to deny custom practices were abolished and governments embraced change and reinstated traditional health practices as a right enshrined in the constitution of the land, as the "right to health". As a result a number of organizations such as KwaZulu-Natal Traditional Healers

Council (eThekwini Local Branch), *Mwelela Kweliphesheya* & *Umgogodla Wesizwe*, and African Dingaka Association etc freely practiced the trade. Instead of seeing THP totally collapsing, traditional medicine made a robust return. It gained momentum rather and acquired more attention than previous practices, HIV/AIDS is held accountable. Furthermore traditional medicine is a system that is deeply entrenched within African culture despite westernization even amongst the youth (Sherrif, 1996). Households continue to utilize traditional medicine as a primary health care (Hardon *et al*, 2008). The youth especially those in trouble with the law would seek counsel from THP's to win major cases. Teenage pregnancy which is most prevalent in townships strongly relied on local THPs which prompted government to legalize abortion otherwise illegal abortion became another "assisted suicide" in African townships. Almost 60% of babies were delivered with the help of local midwives (Karim *et al*, 1994). I would argue however, that traditional medicine utilization is presently most prevalent in urban areas as opposed to rural areas. It is cultivated and prepared in rural areas but most utilization is found in urban areas. With urbanization and globalization of the entire country, rural areas are becoming obsolete, hence the mushrooming of informal settlement in cities which subsequently introduces rural practices to major cities including informal trade (Dauskart, 1990).

Job seekers migrate to urban areas for career opportunities whereby THPs would dispense lucky charms for career opportunities and recreational medicine to help maintain family life (aphrodisiacs). Zimbabweans have coined a term, "Central Lock System". It is a spell that is cast to unsuspecting party thus discourages extramarital

affairs (Mail and Guardian, 2001). It is not unusual on every street corner to observe flyers dispensed or pasted on robots advertising services of a powerful healer from distant shores. Scales have drastically tilted in favour of traditional practices which saw its support rising in scales of 200 000: 25 000, THP's vs general practitioners (GP's) respectively (Karim *et al*, 1994). Monies injected to boost country's economy and stimulate job creation through the trade were estimated to 3.4 USD through sales of 525 tonnes of plant materials (Mander, 1998; Dold *et al*, 2002).

Further development which appeals to the trade support includes digital migration from word of mouth to most robust marketing tool involving popular gadgets i.e. cellphones. Other means THP's have employed to reach the masses include the internet, newspaper and radio (Bonora, 2001). Local newspapers are richly filled with adverts and often disturbing news involving slaughtering for human body part constituting "*muthi* killings", a practice that is spiraling out of control. "*Muthi"* killings are motivated by numerous factors practiced by dubious characters that seek to undermine the trade. Such atrocities are countrywide however they are most prevalent in Limpopo, southern KwaZulu-Natal and the Eastern Cape and until recently in the Midlands (Pietermaritzburg) judging from publicity in the news. "*Muthi*" simply means a substance that could be of either fauna or flora origins which is administered to patients in distress by an experienced THP (Ashforth, 2005). The infiltration of traditional health practices with witchcraft (*abathakathi*) who openly advertise such services to public is a disservice not only the trade but to the spirit of "*ubuntu"* which is inherent in African traditional medicine which mocks the

country's constitution. The use "*tokoloshes"* to fetch money is tantamount to theft and robbery. "*Tokoloshes*" are "small boys like creatures" that are only visible to children of tender age which witches use to execute a special task which is usually rewarded by spilling of blood (Ndhlala, 2009).

2.7 Research into Traditional Medicine in South Africa

Southern Africa is home to 30 000 plant species of which 3 000 species have medicinal properties (van Wyk *et al*, 1997). Furthermore, South Africa has culturally diverse societies each having unique customs however, despite cultural diversity, traditional medicine plays a pivotal role nonetheless. In addition to this, the preservation of indigenous knowledge systems that is prevalent through all the cultures is oral and it has been incessantly practiced without fail from generation to generation (Hutchings *et al*, 1994; van Wyk *et al*, 1997). Hence there are currently 200 000 THP who render services to their respective communities (van Wyk *et al*, 1997). Traditional medicine traded annually can be estimated to 20 000 tons. Furthermore, the informal trading with traditional medicine at low cost gives the practice an added advantage as opposed to the western system (Taylor *et al*, 2001). As a result there are 80% South Africans who derive benefits from use of traditional medicines despite the improved availability of orthodox drugs (Shale *et al*, 1999).

South African botanists have compiled a list of indigenous plant species, however critical information related to efficacy and medicinal properties has still been minimal. Countries such as Germany and China developed ways of classifying

traditional medicines that have proved useful over time. China developed a pharmacopeia on traditional medicines whereas Germany established monographs (Keller, 1991). South Africa compiled a list of medicinal products and their constituents, a work done by Nair *et al* (Nair *et al*, 2006). Data compiled from product is crucial for their commercialization.

Research done on natural products in South Africa focuses both on *in vitro*, *in vivo* studies as well as on safety and efficacy (Cocks *et al*, 2002). A new trend is developing worldwide that seeks to integrate traditional medicine with primary health care (Fennell *et al*, 2004).

The streamlining of traditional health practices into mainstream healthcare system in South Africa started in the 90's post apartheid era. A group of scientists were allotted a task of evaluating the health system and the proposed a model (Freeman *et al*, 1992).

The period between 1999 and 2006 had had a tremendous research output that provided clinical evidence of phytotherapeutic nature. Contribution towards knowledge production by South African scientists as witnessed in Journal of Ethnopharmacology publications from 1980 to 1994 was c.a 10-20%. However, there has been dramatic change in contributions recently; the percentage contribution has reached an alarming 55% (Light *et al*, 2005). Claims made by some THPs that they treat HIV/AIDS (City Press, 2001; Mercury, 2000) and their preparedness into submitting their medicines to be scientifically validated for antimicrobial and

properties raised a serious curiosity to science communities. The subsequent research validated some claims and it was observed that traditional medicines have antimicrobial properties. In case of AIDS, it remained to be proven whether TMs exerts their activities by inhibiting growth of HIV causing AIDS or indirectly through inhibition of microbes causing secondary opportunistic diseases. Some publications made by a number of research institutes and laboratories garnered interest into the subject, marking growing interest into research of TMs (Hutchings *et al*, 1994; van Wyk *et al*, 1997). South African government through National Research Foundation (NRF) answered a call and made a generous contribution (R15 million/pa) towards research into Indigenous Knowledge Systems (IKS) (Mulholland, 2005). More contributions have been received from the international communities such as President's Emergency Plan for AIDS Relief (PEPFAR) based in the US. PEPFAR's mandate is to help in a fight against AIDS, malaria and TB to impoverished nations of the world.

According to statistics less than 5% investigations have been conducted regarding "plants and antimicrobial" of South African origins (table 2.0), according to Scopus and Science Direct. Hence more research is required for knowledge production especially in the field of antimicrobial properties, otherwise South Africa research input on a global scale is minimal (Light *et al*, 2005).

Table 2.0: A display of some prolific authors whose research have been severally cited.

Publications	Authors
Antimicrobials research	van Wyk (2002)
Antimicrobial Screening	Rabe and van Staden (1997); Lin *et al*, 1999; McGaw *et al*, 2000; Kelmanson *et al*, 2000; Motsei *et al*, 2003; Eldeen *et al*, 2005; Buwa and van Staden, 2006; McGaw and Eloff, 2005
Antimicrobial Reports	Meyer and Afolayan, 1995; Meyer and Dilika, 1996; Meyer *et al*, 1997; Dilika *et al*, 1997; Afolayan and Meyer, 1995; Mathekga and Meyer, 1998; Lourens *et al*, 2004; van Vuuren *et al*, 2006
Antimicrobial (STD)	Tshikalange *et al*, 2005; Lall and Meyer 1999
Antimicrobial Activities	Eloff, 1999; Martini *et al*, 2004; Eloff *et al*, 2005
MIC	Eloff 1998a
Chemical Profiling	Rabe and van Staden 2000; Drewes *et al*, 2005; Drewes *et al*, 2006
Antimicrobial	Louw *et al*, 2002; Lewu *et al*, 2006

The amount of research done on South African plant species still remains insufficient for the World Health Organization to establish monographs upon which guidelines for phytotherapeutics could be based upon.

Studies revealed a dramatic increase in traditional medicine usage reaching an alarming $60 billion figure the world over (WHO Fact Sheet *No. 134*, 2008). Further consumption per country is also outlined in the table 2.1 below:

Table 2.1: Consumption of traditional medicine per country

Country	TM % Use	Annual Spending	Frequency of Use
China	30-50	N/A	N/A
Europe	>50	N/A	>1
North America	>50	N/A	>1
Dev Countries	>50	N/A	>1
Germany	90	N/A	>1
UK	N/A	$320m	>1
Ghana	60	N/A	>1
Mali	60	N/A	>1
Nigeria	60	N/A	>1
Zambia	60	N/A	>1
USA	N/A	$150m	>1
RSA*	75	$76.9m	>1

(* Food Agricultural Organization, 1998; WHO fact sheet No. 134, 2008, more than once is abbreviated: >1).

2.8 Biodiversity and legislation

Ethno-botanical knowledge has been the source to sizeable drug discoveries (Kartal, 2007). Over the past plenty of national corporations have exploited indigenous resources through which drugs of commercial value have been produced without compensating IKS holders for knowledge transferred. Statutory bodies such as Conventional of Biological Diversity (CBD) have thus been mandated to assign ownership of biodiversity to individuals or community and to accord rights to protect this knowledge (Ng'etich, 2005).

In South Africa an act also referred to as the National Environment Management Act (NEMA), the Biodiversity Act of 2004 as set out in the Government Gazette of 2008 No: 30739 seeks to regulate the following:-

i) To further regulate the permit system since the system applies to bio-prospecting that involves any indigenous biological resources or export from the Republic of any indigenous biological resources for the purpose of bio-prospecting or any other kind of research;

ii) To set out the content of the requirements and the criteria for benefit-sharing and material transfer agreement.

2.9 Intellectual Property Rights

Whether new products, new technologies or even active compounds are derived from use of indigenous knowledge, legislation needs to ensure that those communities who have historical guardianship will reap benefits derived from their products or new discoveries. The indigenous knowledge holders may have been previously excluded on opportunities that realize full value of their indigenous resources but current legislation (NEMA) protects and also unlock future benefits in a way that ensures sustainable development of the respective communities. Otherwise bio-piracy would run unabated. Bio-piracy applies to commercially viable products that are derived from traditional knowledge without returns accorded to indigenous knowledge holders. This act is rife in developing countries (Udgaonkar, 2004).

The South African government issued explicit definition to IK as set out in the Government Gazette No: 30739 issue of 2008 where it is defined as, “indigenous use or knowledge” include knowledge of, discoveries about or the traditional use of indigenous biological resources, if that knowledge, discovery or use has initiated or

will contribute to or form part of a proposed bio-prospecting or research project to which an application for a permit relates.

2.10 Bio-prospecting and legislation

Bio-prospecting is interpreted as a process whereby local or foreign researchers, intentionally seek amongst the wealth of a country's indigenous resource, those opportunities represented by species' and active compounds contained in a species which will result in successful commercial exploitation, most often and tragically so, outside the country of its origin, an exclusive benefit of a few people unrelated to that country (MBB Consulting *et al*, 2006).

The NEMA act seeks to act against overexploitation of the countries' natural resources also whilst ensuring that indigenous knowledge holders reap benefits in cases where commercial benefits arise from the utilization of indigenous resources.

2.11 Trends in traditional medicine

South Africa is one of the countries in the world experiencing an increased usage of traditional medicine in recent years as it can be observed on informal street vendors and some pharmacies marketing these products. Traditional medicines application is widespread (Thring *et al*, 2006). It is probably because it is firstly a cultural practice dating from time immemorial before the encroachment of western system of healing and practice. Secondly, the shortage of medical personnel attributed to its widespread use. At present there is 1:40000, doctor to patient ratio whereas regarding THPs it is estimated at 50:400 which means easy access to traditional healers (Karim *et al*, 1994).

Contrary to popular belief, the market for these products is not poverty driven as it has been reported elsewhere but accessibility and affordability dictate trends instead (Mander, 1998). Sales volume of these products proves it to be a very lucrative although informal business. The Eastern Cape is one of low socio developed region in South Africa. People in this region rely on traditional medicine mostly for diseases treatment as well as personal well-being hence traditional medicine finds most uses in this region (Cocks *et al*, 2002). The annual revenue generated by this sector is estimated at R500million. Recent statistical findings have estimated a 54% increase in the trade of medicines in this region leading on sales is *Hypoxis hemerocallidea* (African potato) used by people living with AIDS (Dold *et al*, 2002). Not only has this generated much research on African potato but to traditional medicine at large and South Africa is also one of the leading researchers (Light *et al*, 2005).

The THP Bill remains South Africa's health initiative to curb the scourge of AIDS. The SADC leaders also adopted and ratified a health strategy in a fight against AIDS (Baleta, 1998). The collaborative work began to see THPs acquiring skills such as educators, counselors and disseminate information on HIV and STI's to their respective communities and amongst their peers (King, 2000; McMillen *et al*, 1999; Nakyanzi, 1999).

2.12 Safety assessment of Traditional Medicines

General safety evaluation in traditional medicine is purely based on the long historical period of use. Traditional medicine in South Africa was banned by previous apartheid

regime. As a matter of fact this is a turning point in traditional medicine history because the two acts enacted, Witchcraft Suppression Act of 1957 and Witchcraft Suppression Amendment Act of 1970 explicitly prohibited traditional healing practices (Jolles *et al*, 2000). Ironically the trade now suffers hangover from past degradation seeing that it now often carries horror stories associated to witchcraft that further tarnish the trade by the very people who are supposed to benefit from it. This explains the secrecy even unto the level of consumers benefiting from these aids (Martin-Facklam *et al*, 2004). This makes data collection impossible especially for treatment efficacy studies.

The increased research on TMs/CAMs saw WHO Western Pacific region stipulating guidelines to follow for safety and efficacy evaluation (WHO Western Pacific region, 2008). Many countries seem to display a serious lack of vigilance with regards to traditional healthcare practices due to unstructured regulatory bodies (Schilter *et al*, 2003; Bast *et al*, 2002). A comprehensive approach towards the subject should cover the following viz:- medicinal usage, chemical data, toxicological studies, pharmacological studies, intervention trials, epidemiological studies, patients profiles/case records, and post- marketing surveillance (Schilter *et al*, 2003). Had there been sufficient regulation of TMs/CAMs, post-marketing surveillance could be readily available had products been registered. Safety and efficacy on commonly used natural products, especially in developed countries, is accessible via literature (refer table 2.0). Hence some aspects such as adverse health risks, side effects and treatment interaction are sufficiently covered (Magee, 2005). Studies on side effects and

adverse reactions on TMs/CAMs were compiled based on fatal cases previously reported on some plant products (Steward *et al*, 1999).

Ihlamvu laseAfrika (IHL) is a polyherbal mixture (courtesy of Baba Thabethe) that is regarded as a non-specific growth repressor of pathogens a person infected with HIV/AIDS presents with. However, certain factors need careful consideration regarding TM intended benefits. Seasonal changes, for example may affect pharmacological profile of medicinal plants. These changes can be exhibited within changes in the genome, the transcription, expression and post-translation modifications of proteins as well as secondary metabolites (Moco *et al*, 2007). Chemical consistency is important in ensuring efficacy and consumer safety (Sahoo *et al*, 2010). In the next chapter, cytotoxicity studies regarding use of *IHL* would be dealt with. The chapter aims at establishing cytotoxicity of *IHL* on the basic functions of the cell that can be quantified relative to levels of cellular damage. The second aim serves to determine the safe and effective dose to be used throughout the Glutathione (GSH) assay.

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Chapter 3

Chemical Profile analysis of *IHL*

3.0 Abstract

Introduction: New chemical entities with novel mechanism of action that prove useful against multi-drug resistant organisms are mostly plant based and they present as secondary metabolites (Ballel *et al*, 2005; Pereira *et al*, 2005). Plants utilize secondary metabolites for protection however, these chemical entities have also been utilized by man from time immemorial. Lack of standardized TM preparation, inconsistencies may set in and also material used may become extinct. Hence to sustain life certain measures need to be taken to preserve and sustain them.

Objective: To verify batch to batch reproducibility using thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) and to profile secondary metabolites using UPLC-MS.

Materials and Methods: Samples E and F would be analyzed with TLC and visualized under UV at 254 and 366nm and furthermore confirm with NMR. After establishing similarities, one sample would undertake chemical profiling with UPLC-MS.

Results: TLC established striking similarities with samples E and F. Further resemblance was confirmed with NMR. Chemical profiling of *IHL* enabled establishment of the following chemical entities viz:- thalebanin B, methyillukumbin A, Kuguacin J, mauritine H, 2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione, Kuguacin J, Isoferuloyllpeol, Diosindigo A, Kuguacin R, verbascoside, Kuguacin B and nuciferin

Conclusion: It has been proven that *IHL* possesses antimicrobial properties that could possibly be used to manage HIV/AIDS and its secondary opportunistic infections. Furthermore despite serious lack of standardized operational processes and techniques

utilized by THPs during their medicinal preparations, consistency within batches manufactured on different dates was established.

3.1 Introduction

3.1.1 Metabolites Profiling (UPLC-MS) and Quality Control (NMR and TLC)

There is a growing interest and a great potential for drug development from plants (Cox *et al*., 1994; Farnsworth, 1993). This is necessitated by drug resistance experienced with current drug interventions especially witnessed with HIV/AIDS and TB patients. Resistance however inadvertently exhausts current drug regimen such that new options need exploration. New chemical entities with novel mechanism of action that proves useful against multi-drug resistant organisms are mostly plant based (Ballel *et al*, 2005; Pereira *et al*, 2005).

A traditional medicine called *IHL* that is of anecdotal use by HIV/AIDS patients in KwaZulu-Natal province was studied. Plants utilize secondary metabolites for protection, however, the secondary metabolites have also found useful role to man although not all plants have been researched. Successful screening for antimicrobial activity against test plant as well as biosafety assays led to further tests. One of the objectives of this chapter is to identify the bioactive components present in *IHL* that are thought to have antimicrobial activities.

3.1.2 Secondary metabolites

Metabolites can be divided into two, namely primary and secondary metabolites. The plant primary metabolites are responsible for plant growth and development whereas secondary metabolites are species specific such that in tissues, cells they are involved in plant development (Verpoorte *et al*, 2007). To withstand changing seasons, secondary metabolites curtail for such changes. As an adaptation strategy, plants have developed a complex machinery responsible for abiotic (light, UV, water) and biotic (parasites, herbivores and attacks from pathogens) stress factors (Hall, 2006). Furthermore, primary metabolites serve metabolic functions (agricultural yields) whereas secondary metabolites are responsible for colours, flavours and also circumvent stress factors (Verpoorte *et al*, 2007). Moreover, it is secondary metabolites that man derive health-benefits from, presented as diospyrins and others in various plant species. In order to establish the type of metabolites present, several analytical techniques have been employed. Firstly there is "metabolic fingerprinting". This is high throughput qualitative screening for metabolic constituents. Sample preparation and analysis is relatively rapid and it is a simple technique. Alternatively, there is "metabolic profiling" technique involving identification, quantification of secondary metabolites. To be borne to mind that the data presented with this technique represents a "snapshot" of metabolic information confined to time and space. Stress factors contribute largely to different expression of different secondary metabolites. Notwithstanding the fact that samples taken from different sources can still compare however, the conclusion reached should be clearly based on the initial sample differences (Hall, 2006).

3.1.3 Analytical techniques applied in secondary metabolites studies

There are five major techniques that are presently utilized for profiling viz:- H/UPLC-MS, GC-MS, TLC-UV, MS^n and NMR (Verpoorte *et al*, 2007). NMR and mass spectrometers are regarded as the primary tools that can be universally applied. However, the two technologies display some distinctive characteristics as well as disadvantages considering a wide array of metabolites detected, their resolution and sensitivity (Verpoorte *et al*, 2007). As a result, the current techniques fall short in detecting both qualitative and quantitative data presented by all metabolites in tissue extract. Hence, a "profile" of a specific group is subsequently identified and quantified if not, "fingerprint" of a particular metabolite is subjected to pattern analysis (Ratcliffe *et al*, 2005). In this investigation, the chemical profiling would be carried out on UPLC-MS, hence this technique is emphasized.

3.1.4 Quality Control with Thin Layer Chromatography (TLC)

TLC is one of the important technique used in secondary metabolites studies however, its importance is fading with time (Merfort, 2002). This technique however, provides crucial information about the type of metabolites present. In order to visualize TLC fractions, numerous reagents can be utilized such as vanillin/o-phosphoric acid, anisaldehyde or p-dimethylaminobenzaldehyde-sulfuric acid, sulfuric acid, resorcin-sulfuric or phosphoric acid, aluminium chloride or hydroxyl-amine (Kery *et al*, 1987; Kelsey *et al*, 1973; Drozdz *et al*, 1978; Picman *et al*, 1980; Villar *et al*, 1984; Nowak, 1993)

Preliminary TLC screening of two sample batches was done in order to establish reproducibility, an important quality control exercise.

3.2 The study's objective:

1. To verify batch to batch reproducibility with:
    a) Thin Layer Chromatography (TLC)
    b) Nuclear Magnetic Resonance (NMR)
2. To do chemical fingerprinting of *IHL* with UPLC-MS

3.3 Materials and Methods

3.3.1 TLC Chemicals

1. Methanol, gradient grade (Merck).

2. Formic acid, spectroscopic grade (Fluka).

3. Water, triple deionized, from the Milli-Q purification system (Millipore, Bedford, MA), resistivity 18.0 MΩ-cm, filtered through 0.2 μm membrane filter.

4. Acetonitrile, ultra gradient HPLC grade (Merck).

5. Sulphuric acid (Merck)

6. Vanillin Spray (Sigma-Aldrich)

3.3.1.1 Sample Preparation and Plate Development

The two lyophilized products of equal quantities were reconstituted in aqueous medium. The two sample assigned labels UKZN-201-12001E and UKZN-201-12001F were shortened to E and an F respectively. The two samples were subjected to thin layer chromatography (TLC) analysis. TLC is regarded as the simplest and rapid method for detecting plant constituents in metabolomics studies. Approximately 5μL of compound mixtures E and F were neatly applied onto silica gel plate (Alu Si G/UV254).The plate

was hairdryed to eliminate streaking due to aqueous medium. A mobile phase composed of (90: 9.5:0.5) chloroform/methanol/acetic acid was used. The plate was furthermore sprayed with vanillin (1% alcoholic vanillin and 10% sulphuric acid). For improved visualization the plate was heated in the oven for almost 20 minutes at 100°C. The plates were viewed under UV 254 and 366nm.

3.3.2 Quality Control with Nuclear Magnetic Resonance 600 Varian (NMR)

3.3.2.1 Principle

Nuclear magnetic resonance principle is based upon nuclei spin in an external magnetic field. In the absence of the magnetic field, there's a random orientation of nuclei spins. However, in case of a strong magnetic field, the nuclei spin is reoriented such that it is aligned with the field or against it. Orientation that's parallel to alignment of applied forces has lower energy. When nuclei are irradiated with RF radiation, the lower energy nuclei move to high state and it is said to be in resonance, hence the term nuclei magnetic resonance.

3.3.2.2 Methods NMR Sample Preparation

Ten milliliters of methanol-CD3OD (Sigma-Aldrich Grade) were used to rehydrate lyophilized products (E and F). Each of the two products was transferred onto NMR capillary tubes that was then placed into NMR port and analyzed for protons -^{1}H presence.

3.3.2.3 NMR parameters

The sample analysis was done with an Inova spectrometer operating at 8445.0Hz for ^{1}H frequency (Varian). Spectra were collected via solvent suppression pulse sequence based on one dimensional nuclear Overhauser effect done to saturate residual [^{1}H] water proton signal (acquisition time - 1.94 min, tof - 1199.4 and tpwr - 54). Transients were collected over spectral width of 10ppm at 25°C.

3.3.3 Chemical Profiling with Ultra-Performance Liquid Chromatography-Mass Spectrometer (UPLC-MS)

3.2.3.1 Principle:

High performance liquid chromatography mass spectroscopy is basically the separation of a mixture with HPLC system preferably using reverse-phase column. In such an instance, the analyte is ionized with an appropriate ion source by various methods (ESI or electron spray ionization, APCI or chemical ionization under atmospheric pressure or in multi-mode source by various methods) followed by partial fragmentation. Soon after acceleration they are deflected by a magnetic field which resolves them according to their mass.

3.3.3.2 UPLC-MS: Instrumentation and Software

The UPLC-TOF-DAD-MS system: UPLC Waters Acquity instrument was coupled to an Acquity DAD (diode array detector) and a Synapt HDMS detector (tandem

Time-of-flight mass spectrometer). The mass spectrometer detector used an electrospray ion source (ESI). The Synapt HDMS system was operated on positive and negative

ionization modes. An aquity UPLC BEH C_{18} column of 1.7µm particle size and 1.0X50mm column dimensions, with a column pre-filter were used. The system used the MassLynx 4.1 instrument software (Waters).An XCMS program for mass peaks extraction and alignment is an incorporated program used for data analysis.

3.3.3.4 Sample Preparation

Random collections of fresh samples from a traditional healer took place on different dates and these were tested for consistency. From each batch a sample was aliquoted into 50mL centrifuge tube, filtered through a 0.22µm PTFE filters and labeled. Quality checks of the two aqueous samples labeled UKZN-201-12001E and UKZN-201-12001F were done on Ultra Performance Liquid Chromatography- Time of Flight- Diode Array Detector-Mass Spec (UPLC- TOF-DAD-MS).

3.3.3.5 The UPLC parameters

There was a 30 minutes sample runtime. The system mobile phase was set as discussed above. A sample injection volume of 1µL was used. The pump flow rate was 0.3mL/min. The temperature of an autosampler was set to 12°C. The column temperature was set to 35°C. The injection needle washed with 200µL of the strong needle wash solution and 600µL weak solution. The column was equilibrated for at least an hour before use.

3.3.3.6 Diode Array Detector

The diode array (DAD) detector was connected in series with Synapt HDMS that was fitted with an ESI 3000 which operated on a V-mode. UV-Visible absorbance spectra

complemented the MS with regards to compounds identification. An Acquity UPLC DAD detector was operated within 220 to 500 nm range.

3.3.3.7 Time of Flight (TOF) parameters

The TOF was operated in the V-mode at a high mass resolution. Spectral acquisition was set from 100 to 1000 m/z with no preset target with scan duration of 0.4 sec. The MS parameter were set such that the capillary voltage was 3.2kV, cone voltage 35kV, source temperature 120°C, the source temperature was 120°C and the desolvation temperature 300°C. Nitrogen had a flow rate of 25L/h.

3.3.3.8 High Resolution Mass Spectrometry (HRMS)

The high resolution mass spectra could be found on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometry (Waters, MA, USA). It is normally equipped with an electronspray interface hence it also sometimes referred to as High Resolution Electronspray Interface Mass Spectrometry (HRESIMS) which is coupled to an Aquity UPLC-MS (Waters, MA, USA). The electronspray interface conditions are as follows: Voltage: 2800, Cone voltage: 40V, MCP Detector voltage: 2650V, Source temperature 120°C, Desolvation gas flow: 550 l/h, Desolvation temperature 250°C, Cone gas flow: 10 l/h. Both positive and negative modes were used for detection in m/z range of 100 to 1000 and scan time was set to 0.25 s in centroid mode.

3.4 Results and Discussion

3.4.1 Batch-to-batch reproducibility with TLC

In order to ensure sample reproducibility for every batch of *IHL* produced, quality control check was incumbent. Local traditional practitioners often do not adhere to aseptic practices since they have no laboratories. Sample preparations and chemical constituents of traditional medicines remain undisclosed thus making important documents such as standard operation procedures non-existent. Hence some governments took unilateral decision to streamline traditional medicines into the mainstream healthcare system in order to regulate them as public entities. That entailed research initiations that would amicably introduce policy issues and quality control measures regarding product development. A bioassay such as thin layer chromatography (TLC) was amongst the techniques adopted for the establishment of batch-to-batch reproducibility, particularly for this product. The TLC technique displayed optimal sample reproducibility hence it proved to be an effective and reliable qualitative tool (figure 3.1).

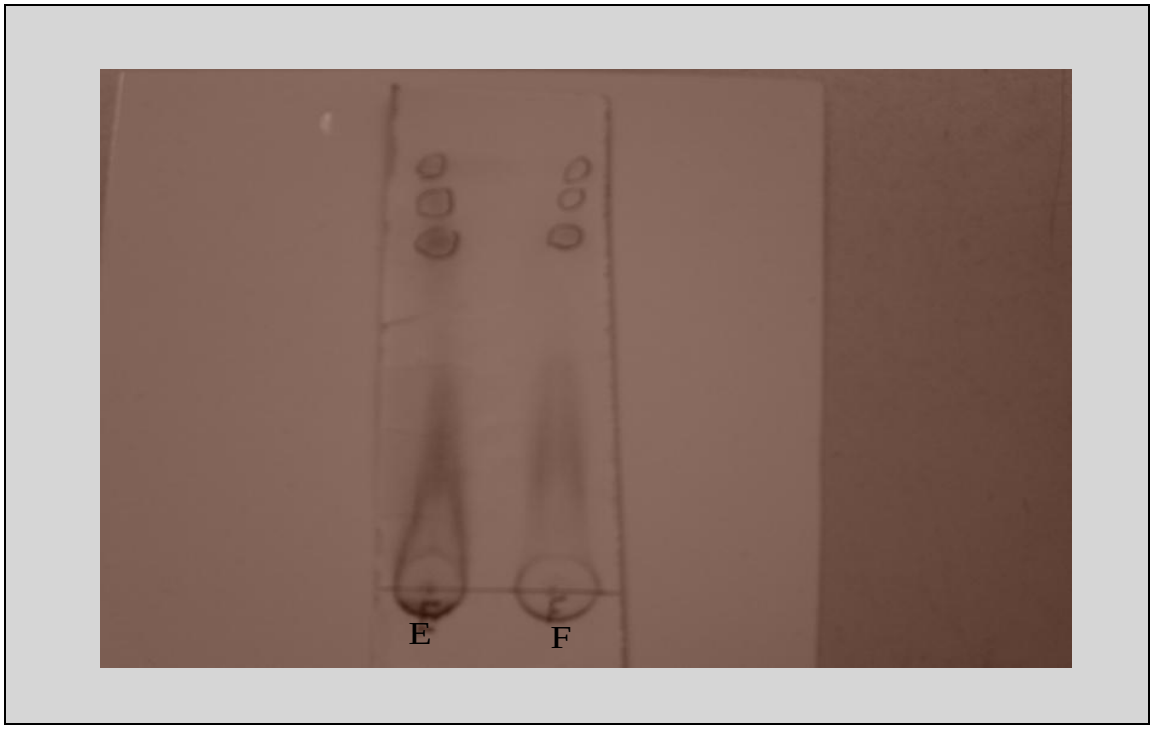

Figure 3.1: Fractions of *IHL* eluted onto TLC plate a test done to compare sample E and F. Batch to batch reproducibility of *IHL* with this technique revealed strong similarities of the two samples denoted by E and F. The two samples were apparently prepared on different dates. The letters E and F were conveniently used otherwise the two samples were identified as UKZN-201-12001(**E**) and UKZN-201-12001 (**F**).

Sample E and F migrated equal distances from the solvent front suggesting that the polarities of the two samples to be identical. Furthermore, when the TLC plates were sprayed with vanillin, the brownish purple fractions became apparent. The darker the sample spot the more concentrated the sample as less UV tends to be more adsorbed (Hess, 2004).The bioautographic technique is capable of producing useful data which helps identify bioactive principle which can be identified by their unique staining

reagents. Major details such as medicinal types used could further be deduced. However, it is important to note that TLC is only a qualitative tool (Hess, 2004) hence the utilization of NMR which does not separate chemical constituents had to suffice (Negussie, 2009). As the samples were loaded onto TLC plate, an identical and clear separation of compounds from the component mixtures suggested definite samples of the same source.

3.4.2 Batch-to-batch reproducibility with NMR

Further confirmation test testing for similarity between sample E and F was also carried out on an NMR and results were deduced chromatographically.

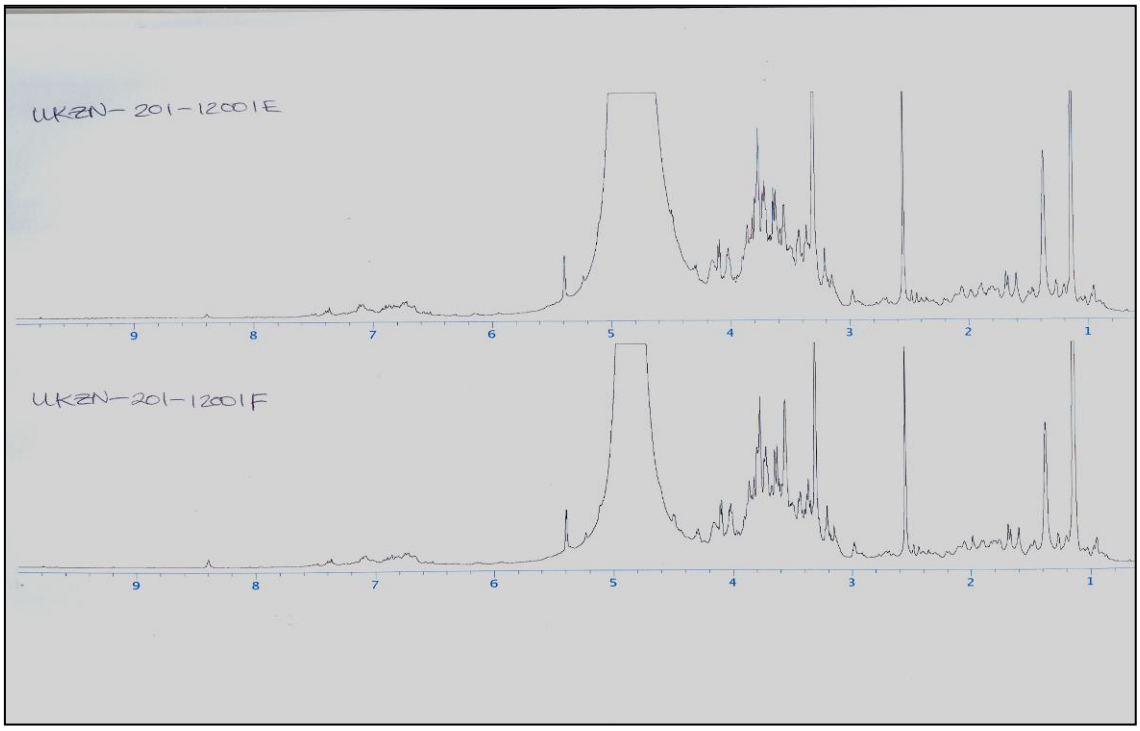

Figure 3.2: The NMR spectrum verifying similarities between sample E and F.

Through visual assessment of the two superimposed sample chromatograms, there appeared similar reproducible peak intensity and peak characters, the retention times and peaks' magnitude also concurred with one another (figure 3.2).

NMR technique is an ideal tool for structural elucidation since it does not separate the analyte hence it remains reusable. As a result NMR is closely identified as a universal detector (Negussie, 2009) and therefore can be ideal for quality control situation.

The process involving structural elucidation entails an output signal identified as a characteristic spectrum which is unique for individual atoms and molecules. NMR is not quite sensitive such that a substantial amount of a sample (> 1mg of 1000Da substance) is needed for structural elucidation (Neuhof *et al*, 2005). Another major setback with NMR is its inability to detect low abundant molecules and subsequent detection of stereochemistry. Hence, for effective utilization of NMR it has to be supported by mass spectrometers and chiral chromatography tools (Neuhof *et al*, 2005). Important data that is incumbent for structural elucidation is based on homonuclear NMR, heteronuclear NMR, HMQC-TOCSY and HMBC variables. The homonuclear NMR would provide details on spin characteristics hence it grouped together parts of molecule. Heteronuclear NMR would furnish with fingerprint details of a molecule thus provide data on multiplicity. A HMQC-TOCSY would put together particularly the spin details with dispersion of the heteronuclear spectra. Eventually ^{1}H, ^{13}C, COSY, HMQC and HMBC would determine the compound which would eventually be conferred to mass spectroscopy output (Neuhof *et al*, 2005).

The reproducibility of the prepared samples was excellent and the NMR spectral analysis of these samples suggested they share the same grouping, spins, bonds and subsequently structures that characterize secondary metabolites to be revealed in UPLC-MS chemical profiling. The ^{1}H-NMR revealed all characteristic resonances for both E and F samples of *IHL*. The high resolution mass provided by homonuclear NMR is ideally suited for molecular weight higher than 800 otherwise results tends to be ambiguous. The molecular weights (221 - 656) for active compounds of *IHL* did not contradict one another.

In conclusion, NMR was shown to represent a rapid and effective technique for the characterization of complex mixtures similar to that of *IHL*. There was an apparent ease of detection of many compounds that were present in different amounts, with relatively less and quick sample preparation.

3.4.3 Chemical fingerprinting with UPLC-TOF-DAD-MS

Having established similarities of the two samples, one of them (samples E) was submitted for chemical profiling and the chromatogram generated is shown. Furthermore snapshots of individual peaks constituting the chromatogram were highlighted. The chemical fingerprints were generated by highlighting the apex of individual peak and these were used to elucidate the possible structure of each peak with the highest precision.

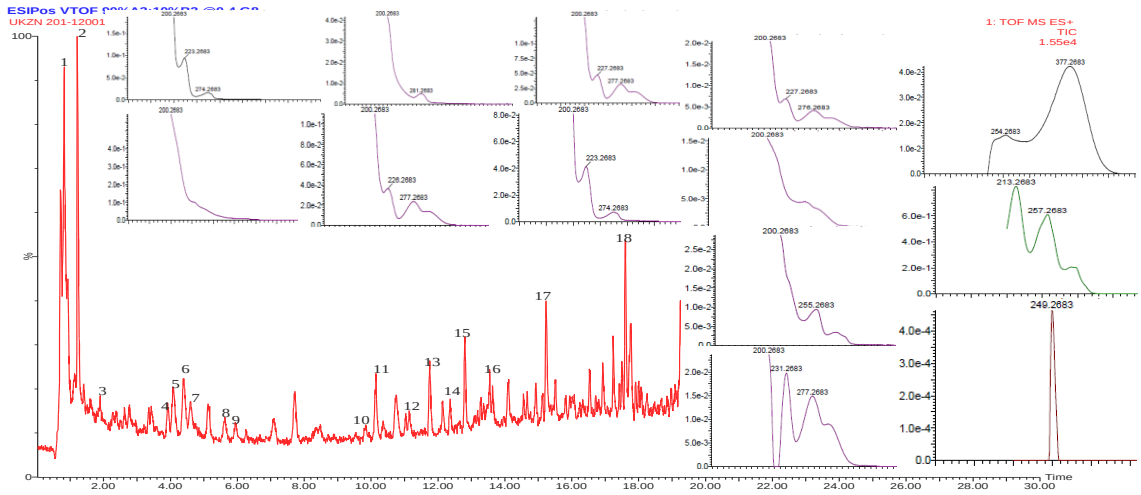

Figure 3.3: Chromatogram representing 12 major peaks of *IHL* as identified on the UPLC-MS chromatogram.

3.4.4 Chemical fingerprints and Proposed Structures of Identified Compounds

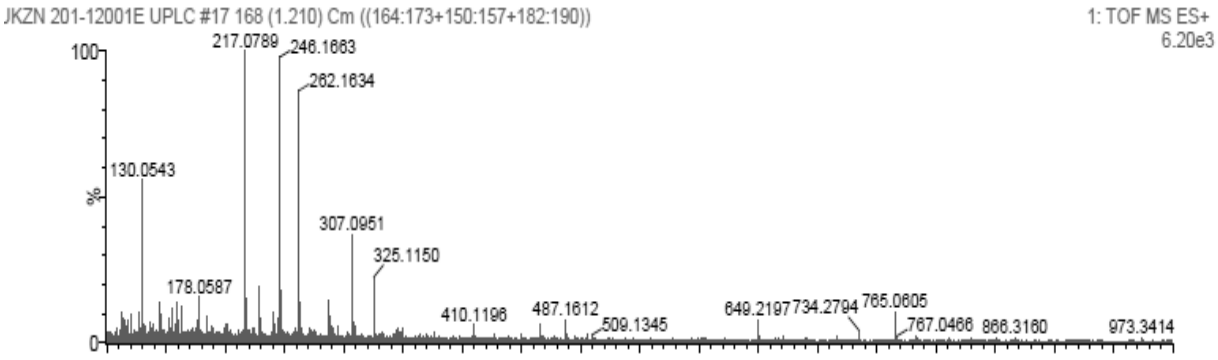

Figure 3.4: High resolution ESI-TOF-MS spectrum in positive mode

Compound 1

Name:	**Thalebanin B**
Synonym(s):	3-Methylbutanoic acid *N*-methyl-*N*-(2-phenylethenyl)amide.
Molecular Formula:	$C_{14}H_{19}NO$
Molecular Weight:	217.310
Accurate Mass:	217.146664
Percentage Composition:	C 77.38%; H 8.81%; N 6.45%; O 7.36%
HRESIMS *m/z*	217.0789, $[M]^+$, -(calculated for $C_{14}H_{19}NO$, 217.1467).

Compound 1: The high resolution mass spectrum HRESIMS in positive mode provided a pseudomolecular ion at m/z 217.0789 $[M]^+$, calculated for $C_{14}H_{19}NO$, 217.1467. The deduced molecular formula of $C_{14}H_{19}NO$ corresponds to the pseudo-molecular ion of compound 1 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Thalebanin B, a glycomis amide that was isolated from *Glycosmis crassifolia* plant (Greger *et al*, 1996)

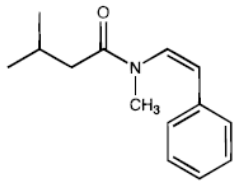

Figure 3.4.1: Proposed structure of Thalebanin

Thalebanin B is a one of the secondary metabolites found present in *IHL*. Thalebanin is a new phenethyl/styrylamine-derived amide isolated from lipophilic leaf extracts of *Glycosmis* cf. *mauritiana*, *Glycosmis* cf. *cyanocarpa*, and *Glycosmis crassifolia.* Thalebanin B extraction from dried leaves was first carried out with EtOAc and later purified with EtOAc in hexane. Thalebanin B displayed significant antifungal as well as insecticidal activity against *Cladosporium herbarum* and *Spodoptera littoralis*, respectively (Greger *et al*, 1996).

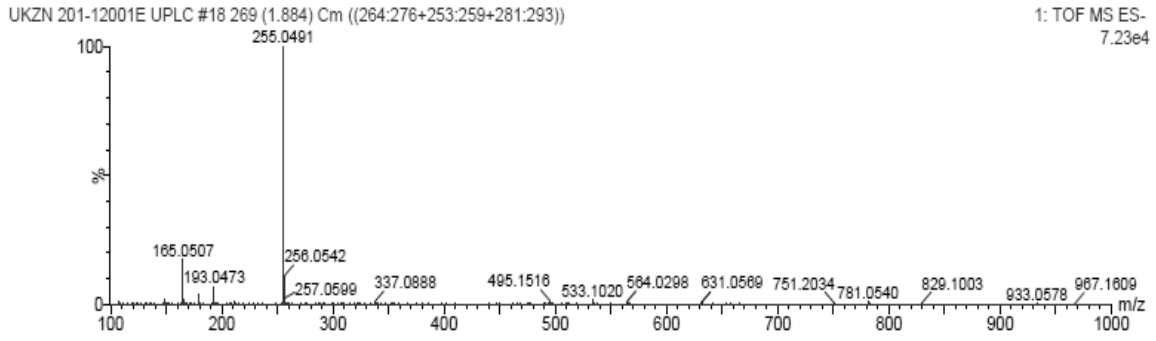

Figure 3.5: High Resolution ESI-TOF-MS spectrum in negative mode

Compound 2

Name:	**2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione**
Molecular formula:	$C_{16}H_{17}NO_2$
Molecular mass:	255.2914
Accurate mass:	255.3142
HRESIMS *m/z*	256.322 $[M+H]^+$, (calculated for $C_{16}H_{18}NO_2$, 256.322)

Compound 2: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 256.322 $[M+H]^+$, calculated for $C_{16}H_{18}NO_2$, 255.322. The deduced molecular formula of $C_{13}H_{17}NOS$ corresponds to the pseudo-molecular ion of compound 2 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several *2-methyl-3-(piperidin-1-*

yl) naphthalene-1,4-dione, a naphthoquinone that was isolated from *E. natalensis* plant (Mital *et al*, 2010).

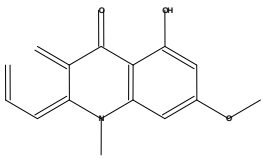

Figure 3.5.1: Proposed structure of 2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione

2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione: A new in vitro technique utilized for antimycobacterial tuberculosis screening for new drug research and development was carried out on Tuberculosis antimicrobial acquisition and coordination facility (TAACF)(Collins *et al*, 1997). When anti-*MTB* agent recorded IC90<10mg/mL, it was considered active. 2-methyl-3-(piperidin-1-yl) naphthalene-1,4-dione displayed antitubercular activity within 2.40 to 10.88mg/mL range (Mital *et al*, 2010) This compound isolated from *E. natalensis* plant (Mital *et al*, 2010), constituted one of active principles found in *IHL*.

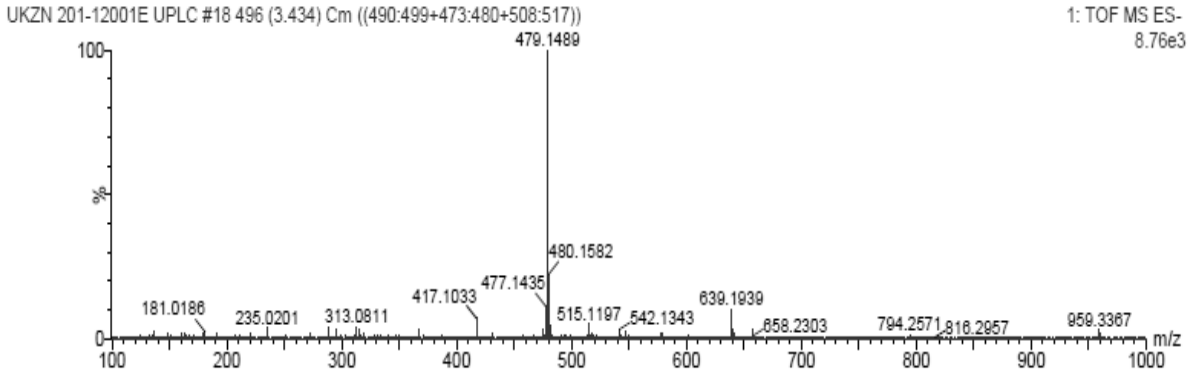

Figure 3.6: High resolution ESI-TOF-MS spectrum in negative mode

Compound 3

Name:	**Kuguacin B**
Synonym(s):	3,25-Dihydroxycucurbita-5,23-dien-7-one.
Molecular Formula:	$C_{30}H_{48}O_3$
Molecular Weight:	456.707
Accurate Mass:	456.360345
Percentage Composition:	C 78.90%; H 10.59%; O 10.51%
Physical Description:	Crystal.
HRESIMS *m/z*	479.3511 $[M+Na]^+$, (calculated for $C_{30}H_{48}O_3Na$, 479.3501).

Compound 3: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 479.3511 $[M+Na]^+$, calculated for $C_{30}H_{48}O_3Na$, 479.3501.

The deduced molecular formula of $C_{30}H_{48}O_3$ corresponds to the pseudo-molecular ion of compound 3 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several **Kuguacin B**, a cucurbitacins that was isolated from *Momordica charantia,* (Chen *et al*, 2008).

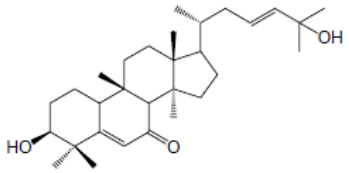

Figure 3.6.1: Proposed structure of Kuguacin B

Compound 3: Kuguacin B is one of the five potentially bioactive cucurbitacins that was extracted with MeOH from the root of *Momordica charantia* L. that proved to possess anti-HIV properties. The study was done in Yunnan, China by Chen and co-workers utilizing indigenous *Momordica charantia* L. species. An *in vitro* investigation into anti-HIV properties of Kuguacin B on C8166 cells however revealed weak anti-HIV activity (EC_{50}= 12.08µg/mL), its selectivity index and cytotoxicity were both 30.9 and 37.35µg/mL respectively (Chen *et al*, 2008).

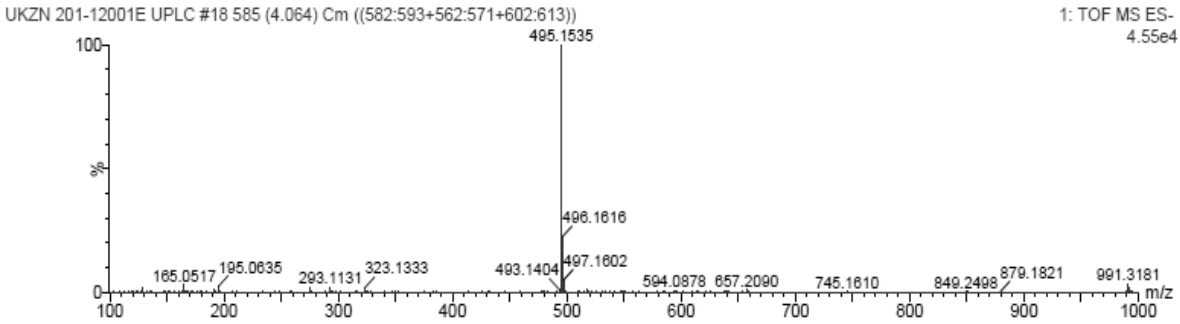

Figure 3.7: High resolution ESI-TOF-MS spectrum in negative mode

Compound 4

Name:	**Kuguacin R**
Synonym:	5β, 19-epoxycucurbita-6,23-diene-3 β, 19,25-triol
Molecular Formula:	$C_{30}H_{48}O_4$
Molecular Weight:	472.707
Accurate Mass:	472.35526
Percentage Composition:	C 76.23%; H 10.23%; O 13.54%
HRESIMS *m/z*	472.35526 $[M+Na]^+$,-(calculated for $C_{30}H_{48}O_4Na$, 495.1535).

Compound 4: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 472.3553 $[M-Na]^-$, calculated for $C_{30}H_{48}O_4Na$, 495.1535. The deduced molecular formula of $C_{30}H_{48}O_4$ corresponds to the pseudo-molecular ion of compound 4 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the

literature, this molecular formula corresponds to that of several **Kuguacin R**, a cucurbitane triterpenoid that was isolated from *Momordica foetida Schum* (Mulholland *et al*, 1997).

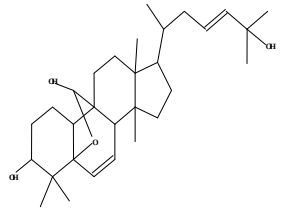

Figure 3.7.1: Proposed structure of Kuguacin R

Kuguacin R is cucurbitane triterpenoid compound that was isolated from *Momordica charantia* (Chen *et al*, 2009). 407mg, 0.000457% fraction obtained over silica gel CC were achived with CHCl3/MeOH from 40:1 to 20:1 and MeOH/H_2O in 60:40 to 75: 25 ratios and then Sephadex LH-20 (MeOH). Kuguacin B together with three other cucurbitans displayed antiviral activity *in vitro* that was measured at 23.7µg/mL. The selectivity index (IC_{50}/EC_{50}) and cytotoxicity were both 8.4 and >200 µg/mL, respectively (Chen *et al*, 2009). It was noticed that some literature cited in this work would elaborate on solvents as well as portions of plants used while some could not.

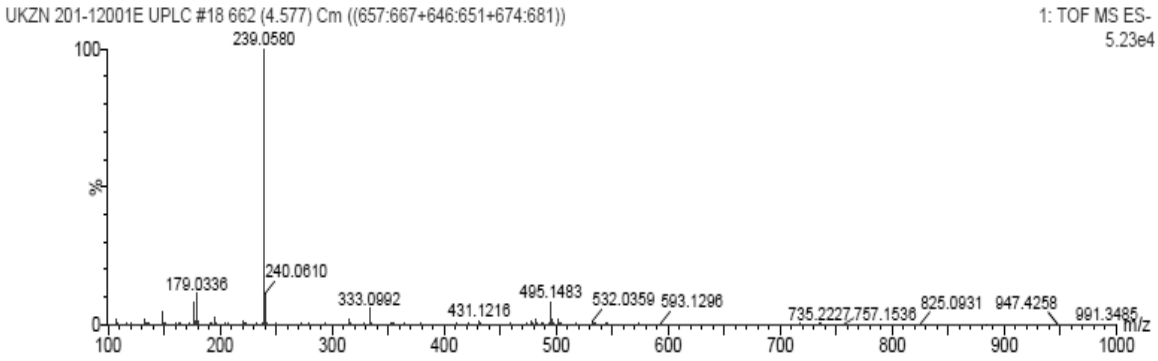

Figure 3.8: High resolution ESI-TOF-MS spectrum in negative mode

Compound 5

Name:	**Methylillukumbin A**.
Molecular Formula:	$C_{13}H_{15}NOS$
Molecular Weight:	233.334
Accurate Mass:	233.087434
Percentage Composition:	C 66.92%; H 6.48%; N 6.00%; O 6.86%; S 13.74%
HRESIMS *m/z*	239.0580 $[M+6H]^+$, (calculated for $C_{13}H_{21}NOS$, 239.134234)

Compound 5: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 239.0580 $[M+6H]^-$, calculated for $C_{13}H_{21}NOS$, 239.134234. The deduced molecular formula of $C_{13}H_{15}NOS$ corresponds to the pseudo-molecular ion of compound 5 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several

Methylillukumbin A, a amide that was isolated from *Glycomis mauritiana* (Greger *et al*, 1996).

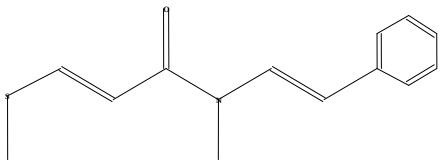

Figure 3.8.1: Proposed structure of Methylillukumbin A

Compound 5: Methylillukumbin A is active principle that was isolated from *Glycomis mauritiana*. The isolated fractions of *G. mauritiana* prepared with Et_2O in hexane, displayed antifungal characteristics according to a study that was done in Sri Lanka (Greger *et al*, 1996). The study further revealed that all amides derived from methylthiopropenoic acid that were joined to styryl were strongly antifungal than phenethyl-amine. The flower of this plant was chewed for toothaches and pyorrhea by Xhosas (Watt and Breyer-Brandwijk, 1962).

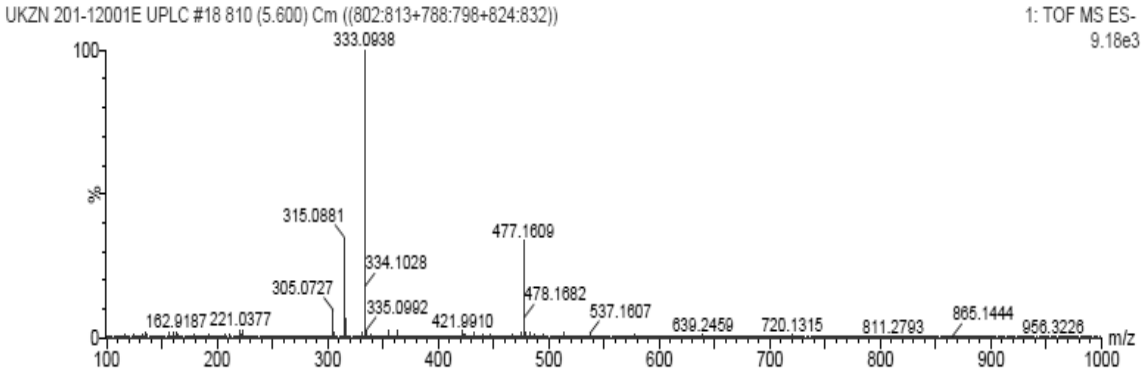

Figure 3.9: High resolution ESI-TOF-MS spectrum in negative mode

Compound 6

Name:	**3,5-Dihydroxy-4′,7-dimethoxyhomoisoflavanone.**
Molecular Formula:	$C_{18}H_{18}O_6$
Molecular Weight:	330.337
Accurate Mass:	330.11034
Percentage Composition:	C 65.45%; H 5.49%; O 29.06%
Physical Description:	Amorphous.
HRESIMS *m/z*	333.0938 $[M+3H]^+$, calculated for $C_{18}H_{21}O_6$, 333.13374

Compound 6: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 333.0938 $[M+3H]^-$, calculated for $C_{18}H_{21}O_6$, 333.13374. The deduced molecular formula of $C_{18}H_{18}O_6$ corresponds to the pseudo-molecular ion of

compound 6 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several **3,5-Dihydroxy-4′,7-dimethoxyhomoisoflavanone**, a homoisoflavonoid that was isolated from *Eucomis bicolor* (Jiang *et al*, 2007, Bangani *et al*, 1999, Crouch *et al*, 1999).

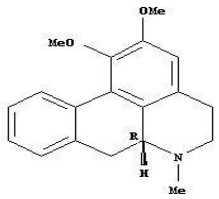

Figure 3.9.1: Proposed structure of 3,5-dihydroxy-4,7-dimethoxyhomoisoflavonone

Compound 6: Homoisoflavonoids such as 3,5-dihydroxy-4,7-dimethoxyhomoisoflavonone forms a special type of flavonoids that are commonly found in Liliaceae family with several biological functions. Homoisoflavonoids have been identified amongst family of Liliaceae such as Ophiopogon, Polygonatum, Scilla, Eucomis (*E. bicolor*) and Muscari (Jiang *et al*, 2007, Bangani *et al*, 1999, Crouch *et al*, 1999). 3,5-dihydroxy-4,7-dimethoxyhomoisoflavonone is amongst several homoisoflavonoids that have been extracted with pure hot ethanol by refluxing and it was proven to possess several biological function that may include anti-inflammatory, antibacterial, antihistaminic, antimutagenic and angioprotective activities (Jiang *et al*, 2007). However, homoisoflavonoids have also displayed potential phophodiesterase inhibition (Jiang *et al*, 2007). In Chinese medicine such as *Mai-Dong*, homoisoflavonoids identified from *Ophiopogon japonicus* are specially featured as clinical tonic drug because of their therapeutic effect (Zhou *et al*, 2008, Kou *et al*, 2005, Kou *et al*, 2006).

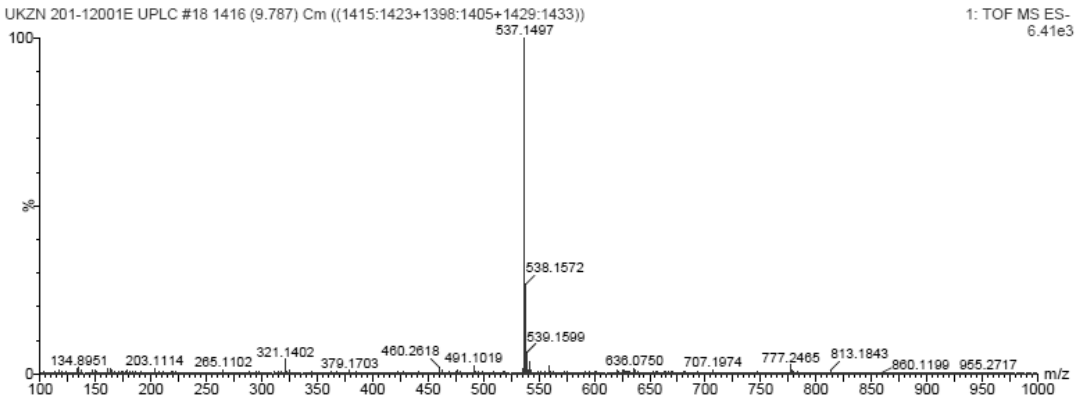

Figure 3.10: High resolution ESI-TOF-MS spectrum in negative mode

Compound 7

Name:	**Anhydrocochlioquinone A**
Molecular Formula:	$C_{30}H_{42}O_7$
Molecular Weight:	514.658
Accurate Mass:	514.293055
Percentage Composition:	C 70.01%; H 8.23%; O 21.76%
Physical Description:	Amorphous red solid
HRESISM *m/z*	537.2826 $[M+Na]^+$, calculated for $C_{30}H_{42}O_7Na$, 537.2826

Compound 7: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 537.1497 $[M+Na]^+$, calculated for $C_{33}H_{54}O_4$ Na, 537.4016). The deduced molecular formula of $C_{30}H_{42}O_7$ corresponds to the pseudo-molecular ion of compound 7 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the

literature, this molecular formula corresponds to that of several Anhydrocochlioquinone A , a quinone that was isolated from Bipolaris oryzae (Phuwapraisirisan *et al*, 2007).

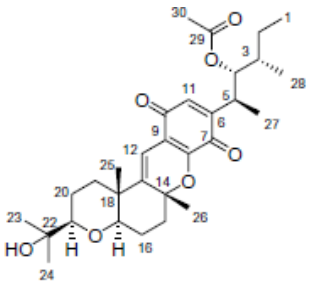

Figure 3.10.1: Proposed structure of Anhydrocochlioquinone A

Anhydrocochlioquinone A: A study conducted on Bipolaris oryzae led to the discovery of new antitumour properties for the first time (Phuwapraisirisan *et al*, 2007). Amongst compounds isolated through bioassay-guided fractionation of the EtOAc, anhydrocochlioquinone A was one of the antitumour compounds. Previous studies have reported on cochlioquinones as anti-angiogenic agents (Jung *et al*, 2003) as well as antagonists for human chemokine receptor CCR5, the important drug targets of most anti-HIV agents (Yoganathan *et al*, 2004). Cochlioquinones however displayed moderate to weak cytotoxicity hence displaying anti-cancer activity which could be achieved either via anti-angiogenesis or apoptosis. Nevertheless, cochlioquinone A inhibited diacylglycerol kinase enzyme that is crucial for the induction of apoptosis in melanoma cells (Machida *et al*, 1995, Yanagisawa *et al*, 2007).

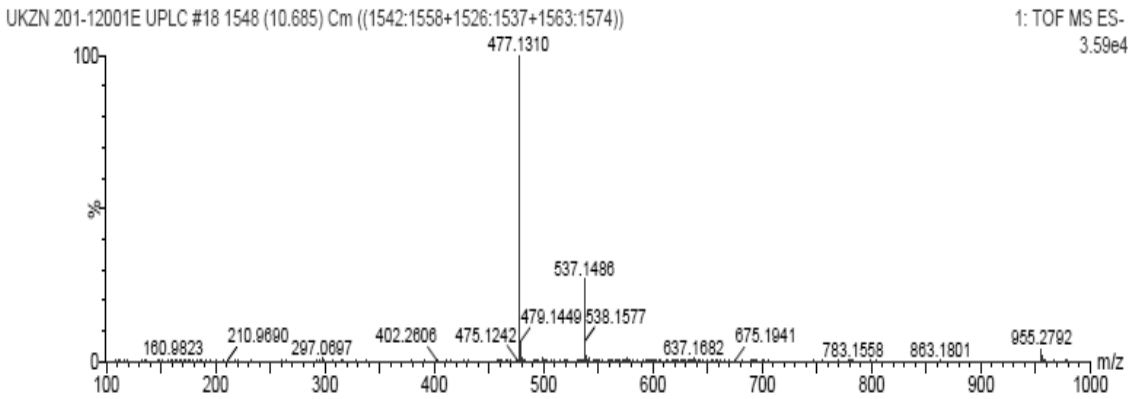

Figure 3.11: High resolution ESI-TOF-MS spectrum in negative mode

Compound 8

Name:	**Kuguacin J**
Synonym	(23E)-3b-hydroxy-7b-methoxycucurbita-5,23,25-trien-19-al
Molecular Formula:	$C_{30}H_{46}O_3$
Molecular Weight:	456.707
Accurate Mass:	456.360345
Percentage Composition:	C 78.90%; H 10.59%; O 10.51%
Physical Description:	Crystal (MeOH)
HRESIMS *m/z*	477.3603 $[M+Na]^+$, (calculated for $C_{30}H_{45}O_3Na$, 477.3603)

Compound 8: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 477.1310 $[M+Na]^-$, calculated for $C_{30}H_{45}O_3Na$, 477.3603. The deduced molecular formula of $C_{30}H_{46}O_3$ corresponds to the pseudo-molecular ion of compound 8 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Kuguacin J, a sterol that was isolated from *Momordica charantia* (Kimura *et al*, 2005).

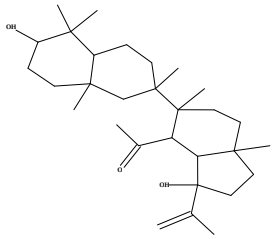

Figure 3.11.1: Proposed structure of Kuguacin J

Kuguacin J: The current drug regiment seems to loose potency when particularly managing multidrug resistance especially witnessed in cancer patients who undergo chemotherapy (Pitchakarn *et al*, 2011). Resistance displayed by patients undergoing chemotherapy is mainly associated with the overexpression of ATP-binding cassette (ABC) drug transporters such as P-glycoprotein (P-gp) that is responsible for effluxing drugs from cancerous cells (Anuchapreeda *et al*, 2002, Larsen *et al*, 2000, Lehnert, 1998, Schoenlein *et al*, 1992). Pitchakarn and co-workers have previously established that *Momordica charantia,* bitter melon leaf extract (BMLE) could reverse MDR phenotype through onsite upregulation of intracellular accumulation of chemotherapeutic drugs. Kuguacin J has proved to be the main principle found in BMLE also established that enhanced sensitivity to vinblastine and paclitaxel in KB-V1 cells (Pitchakarn *et al*, 2011) through inhibition of drug transport activity of P-gp thereby upregulated onsite accumulation of rhodamine and calcein Am in cells. The study further revealed that

kuguacin J which was subfractioned with 95% EtOH also inhibited [125I]-iodoarylazidoprazosin into P-gp in a dose-dependant manner which signifies that kuguacin J directly interact with the agent's drug binding site.

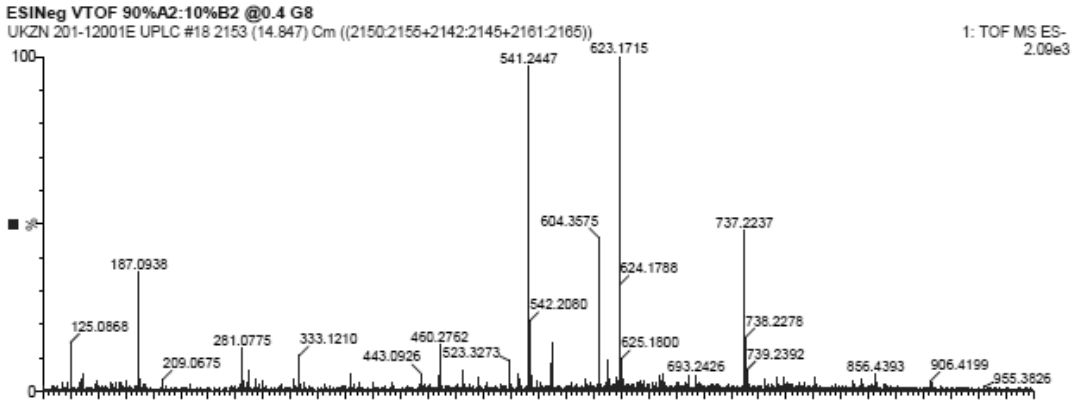

Figure 3.12: High resolution ESI-TOF-MS spectrum in positive mode

Compound 9

Name:	Verbascoside
Synonym(s):	Acteoside/Kusaginin.
Molecular Formula:	C29H36O15
Molecular Weight:	624.594
Accurate Mass:	624.205425
Percentage Composition:	C 55.77%; H 5.81%; O 38.42%
Physical Description:	Yellow powder
HRESIMS m/z	623.1489 [M-H]-, calculated for C29H35O15, 623.1976

Compound 9: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 623.1489, $[M-H]^-$, calculated for $C_{29}H_{35}O_{15,}$ 623.1976. The deduced molecular formula of $C_{29}H_{35}O_{15}$ corresponds to the pseudo-molecular ion of compound 9 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Verbascoside, a phenolic antioxidant that was isolated *Clerodendrum hirsutum* (Cooper *et al*, 1980, Pardo *et al*, 1993).

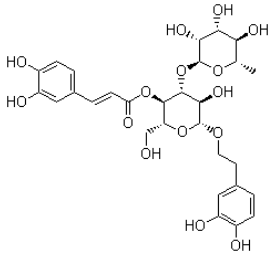

Figure 3.12.1: Proposed structure of Verbascoside

Verbascoside is an active principle that was isolated from *Clerodendrum hirsutum* species exhibiting antimicrobial properties (Cooper *et al*, 1980, Pardo *et al*, 1993). Dirdry *et al*. (1999) discovered antibacterial properties of the same species whereby verbascoside proved active against both Gram positive and Gram negative bacteria. The study further demonstrated moderate antimicrobial properties against *Proteus mirabilis* and *Staphylococcus aureus* as wells as to methicilin resistant strains. In southern Africa, verbascoside isolated from *Clerodendrum myricoides* proved to have antimicrobial properties (Cooper *et al*, 1980; Pardo *et al*, 1993) and the plant species was particularly used by Zulus for snakebites (Watt and Breyer-Brandwijk, 1962). Vasorelaxant (Hennebelle *et al*, 2008) and respiratory syncytial virus were amongst properties

verbascoside was known for (Chen *et al*, 1998, Kernan *et al*, 1998). However it has been recently established that verbascoside also bear antiviral activity against both HSV-1/2. Verbascoside was extracted from *Lepechinia speciosa* with ethanol and later fractionated with EtOAc: MeOH (6:1) yielding 212mg powder. The concentrations of the compound required to reduce HSV-1 and 2 by 50% were 58µg/mL and 8.9µg/mL respectively (Martins *et al*, 2009).

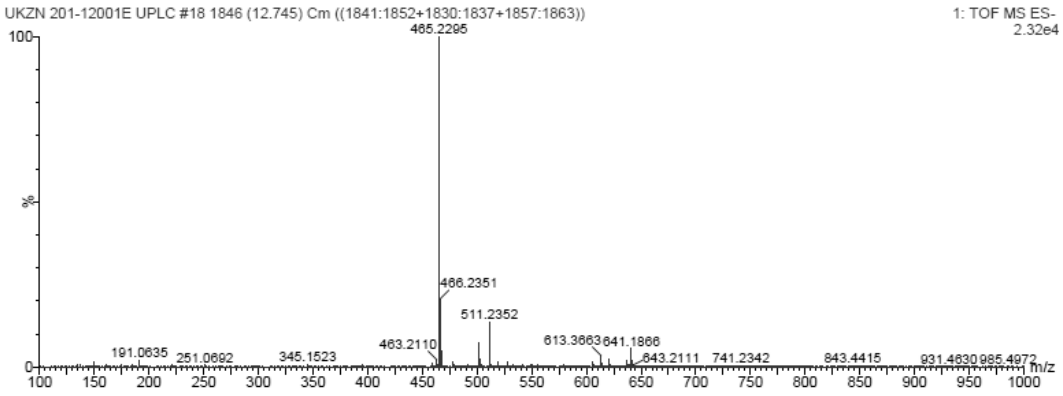

Figure 3.13: High resolution ESI-TOF-MS spectrum in negative mode

Compound 10

Name:	**Quercetin 3-*O*-β-D-glucopyranoside**
Molecular Formula:	$C_{21}H_{21}O_{12}$
Molecular Weight:	465.390
Accurate Mass:	465.103305
Percentage Composition:	C 54.20%; H 4.55%; O 41.25%
HRESIMS *m/z*	465.1033 $[M+H]^+$, calculated for $C_{21}H_{21}O_{12}$, 465. 1033

Compound 10: The high resolution mass spectrum HRESIMS in negative mode provided. The deduced molecular formula of $C_{21}H_{21}O_{12}$ corresponds to the pseudo-molecular ion of compound 10 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Quercetin 3-*O*-β-D-glucopyranoside, an amide that was isolated from *C. infortunatum* (Roy *et al*, 1996).

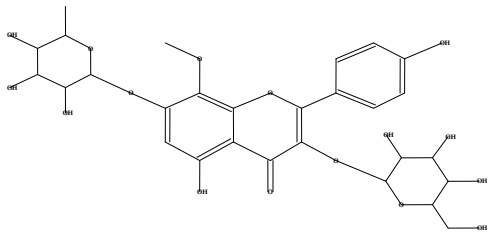

Figure 3.13.1: Proposed structure of Quecertin 3-O-β-D-Glucopyranoside

One compound in our tonic matched a flavanoid identified as **Quecertin 3-O-β-D-Glucopyranoside** or 7-O-β-L-Rhamnopyranoside according to an antifungal research conducted by Roy *et al*. (1996).The quercetin flavanoid was isolated from the root of *C.lerodendrum infortunatum* to which the bioassay displayed strong antifungal activity when tested against *Alternaria carthami* and *Helminthosporin oryzae* at concentrations 200, 500 and 1000mg/mL (Roy *et al*, 1996). Furthermore, the methanol extract of the root exudate of *Rhodolia rosea* proved to be active (MIC=50µg/mL) against *S. aureus*. The bioactivity-guided fractionation of the stem of *R. rosea* yielded gossypetin 7-O-**β**-L-rhamnopyranoside as on of the active compound. When antibacterial and anticancer (prostate cell line) evaluation were done, this compound displayed inhibitory activity at 50µg/mL and 100µg/mL concentrations respectively. 7-O-**β**-L-rhamnopyranoside exhibited cytotoxicity at 50µg/mL (Ming *et al*, 2005).

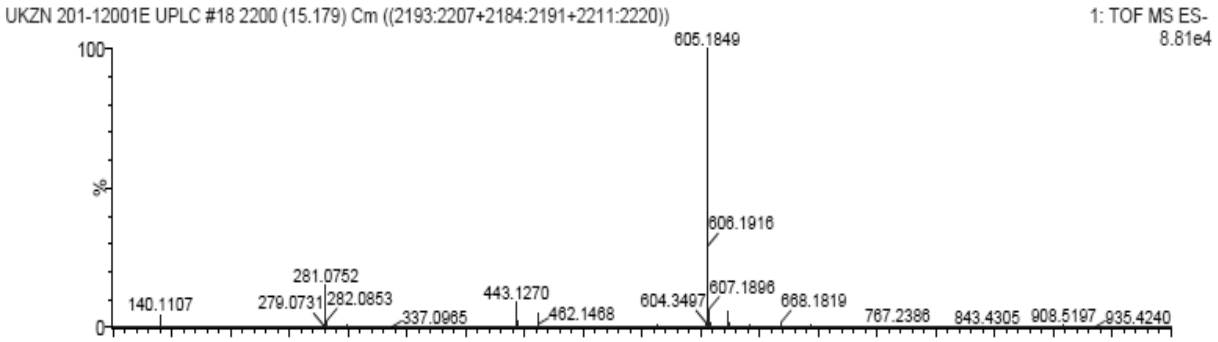

Figure 3.14: High resolution ESI-TOF-MS spectrum in negative mode

Compound 11

Name:	**Isoferuloyllupeol**
Synonym(s):	3-(3-Hydroxy-4-methoxy-E-cinnamoyl)
Molecular Formula:	$C_{40}H_{58}O_4$
Molecular Weight:	602.896
Accurate Mass:	602.43351
Percentage Composition:	C 79.69%; H 9.70%; O 10.62%
Physical Description:	Crystal.
HRESISM *m/z*	605.1849 $[M+3H]^+$, - (calculated for $C_{40}H_{61}O_4$, 605.1849)

Compound 11: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 605.1849 $[M-3H]^-$, calculated for $C_{40}H_{61}O_4$, 605.1849. The deduced molecular formula of $C_{40}H_{58}O_4$ corresponds to the pseudo-

molecular ion of compound 11 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Isoferuloyllupeol, a triterpenoid that was isolated from *Euclea natalensis* (Weigenand *et al*, 2004).

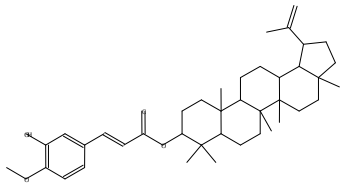

Figure 3.14.1: Proposed structure of Isoferuloyllpeol

Isoferuloyllpeol: *Euclea natalensis* revealed one of its active principles extracted with ethanol as isoferuloyllupeol together with other triterpenoids. Other compounds such as shinanolone, lupeol and betulin that were also extracted with ethanol displayed activity (MIC=100µg/mL) against *MTB* H37RV strain. This compound showed activity when tested against *Bacillus pumilus* at 100µg/mL in which streptomycin sulfate as a positive control displayed growth inhibition against bacterial strain at 10µg/mL with the exception of *Pseudomonas aeruginosa* when the radiometric Bactec system was used (Weigenand *et al*, 2004).

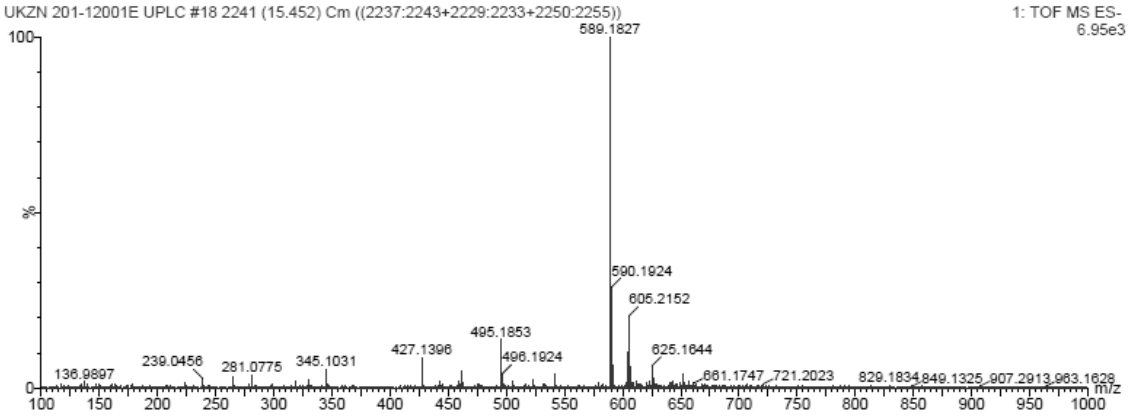

Figure 3.15: High resolution ESI-TOF-MS spectrum in negative mode

Compound 12

Name:	**Mauritine H**
Molecular Formula:	$C_{33}H_{43}N_5O_5$
Molecular Weight:	589.733
Accurate Mass:	589.32642
Percentage Composition:	C 67.21%; H 7.35%; N 11.88%; O 13.56%
Physical Description:	Cryst. (MeOH/petrol)
HRESIMS *m/z*	589.1827 $[M]^+$, -(calculated for $C_{33}H_{43}N_5O_5$, 589.1827)

Compound 12: The high resolution mass spectrum HRESIMS in negative mode provided a molecular ion at m/z 589.1827 calculated for $C_{33}H_{43}N_5O_5$, 589.1827. The deduced molecular formula of $C_{33}H_{43}N_5O_5$ corresponds to the pseudo-molecular ion of

compound 12 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Mauritine H, a cyclopeptide alkaloid that was isolated from *Zizyphus mauritiana* (Pandey *et al*, 1990; Tschesche *et al*, 1977).

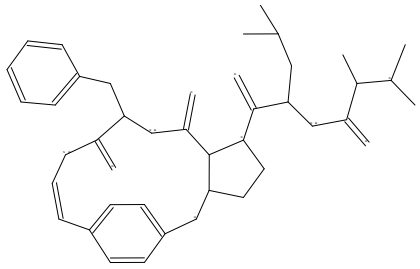

Figure 3.15.1: Proposed structure of Mauritine H

Mauritine H: Previous studies have revealed the presence of mauritine H that was isolated from *Zizyphus mauritiana*. Mauritine H possesses sedative, analgesic, anti-inflammatory, hypoglycaemic, antibacterial and antifungal properties (Pandey *et al*, 1990; Tschesche *et al*, 1977). Some of the active compounds isolated in *Z. mauritiana* included cyclopeptides alkaloids, mauritine A and B (Tschesche *et al*, 1977).

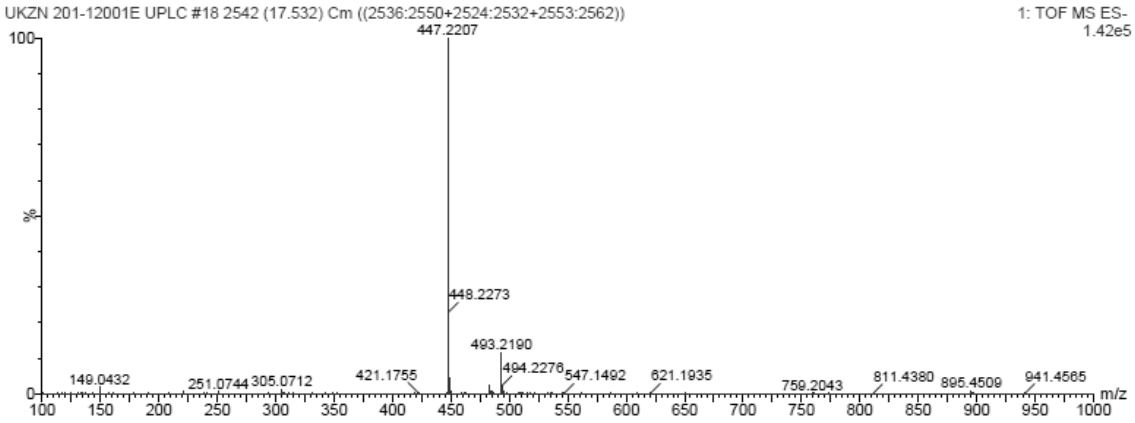

Figure 3.16: High resolution ESI-TOF-MS spectrum in negative mode

Compound 13

Name:	**Kaempferol 3-β-D-glucoside.**
Synonym:	**Astragalin**
Molecular Formula:	$C_{21}H_{20}O_{11}$
Molecular Weight:	448.382
Accurate Mass:	448.100565
Percentage Composition:	C 56.25%; H 4.50%; O 39.25%
HRESIMS *m/z*	447,0928 $[M-H]^-$, calculated for $C_{21}H_{19}O_{11,}$ 447.0928

Compound 13: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 447,0928 $[M-H]^-$, calculated for $C_{21}H_{19}O_{11,}$ 447.0928. The deduced molecular formula of $C_{24}H_{34}O_7$ corresponds to the pseudo-molecular ion of compound 13 in the UPLC/UV/ESIMS analysis of the aqueous extract.

According to the literature, this molecular formula corresponds to that of several **Kaempferol**, a flavanoid that was isolated from <u>*Clerodendrum*</u> *infortunatum* (Shrivastava and Patel, 2007).

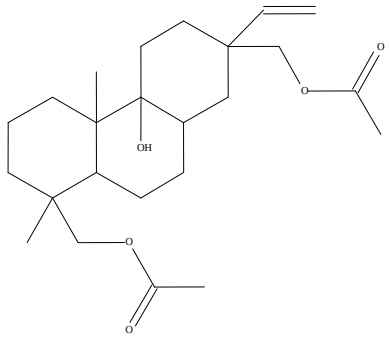

Figure 3.16.1: Proposed structure of Astragalin

Compound 13: Astragalin also known as Kaempferol-3-*O*-Glucoside is an astragalus flavanoid that is found in *Astragalus corniculatus* Bieb, *A. vesicarius* L, *A. ponticus* Pall all belong to *Fabaceae* family. Astraguls is particularly used by Bulgarian folk medicine as a diuretic for managing hypertension, renal disorder, nervous disease and rheumatism. Strong antioxidant activities and immune boosting qualities particularly the renewal of immune cells was also observed. Because of the plant's antimicrobial activities, it finds wide application especially to PLWA (Ivancheva *et al*, 2006). However, other feature such as diuretic effect makes it useful to individuals with high blood pressure. Mild antiviral activity has been attributed to astragalin however several prominent compound including quercetin, luteolin and rutin synergistically enhanced the anticipated health benefits (Krasteva *et al*, 2000). In another study kaempferol was isolated from Clerodendrum species where it is most prevalent as a flavanoid (Shrivastava and Patel, 2007).

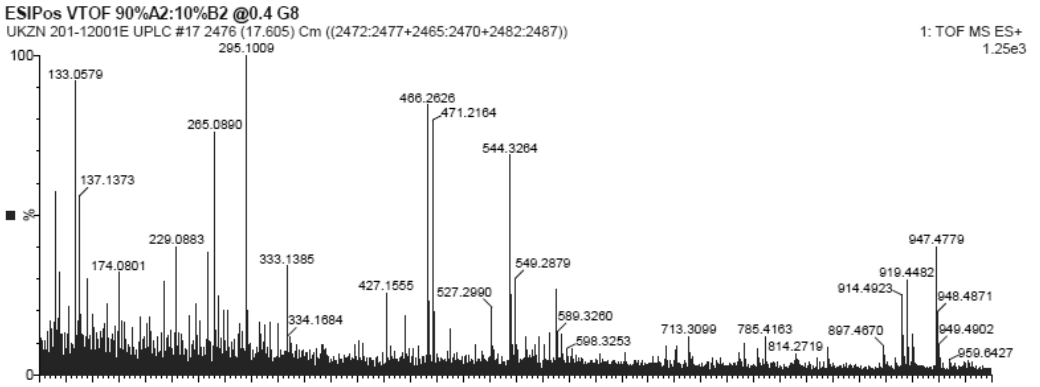

Figure 3.17: High resolution ESI-TOF-MS spectrum in positive mode

Compound 14

Name:	**Nuciferine**
Synonym(s):	1,2-Dimethoxyaporphine/Sanjoinine E.
Molecular Formula:	$C_{19}H_{21}NO_2$
Molecular Weight:	295.380
Accurate Mass:	295.157229
Percentage Composition:	C 77.26%; H 7.17%; N 4.74%; O 10.83%
HRESIMS *m/z*	295.1572 $[M]^+$, calculated for $C_{19}H_{21}NO_2$, 295.1572.

Compound 14: The high resolution mass spectrum HRESIMS in negative mode provided a pseudomolecular ion at m/z 295.1572 $[M]^+$, calculated for $C_{19}H_{21}NO_2$, 295.1572. The deduced molecular formula of $C_{19}H_{21}NO_2$ corresponds to the pseudo-

molecular ion of compound 14 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several **Nuciferine**, a alkaloid from *Nelumbo lutea* (Nelumbonaceae) and *Colubrina faralaotra* (Rhamnaceae), also from the Araceae, Berberidaceae, Lauraceae, Menispermaceae, Papaveraceae, Magnoliaceae and Annonaceae (Watt and Breyer-Brandwijk, 1962).

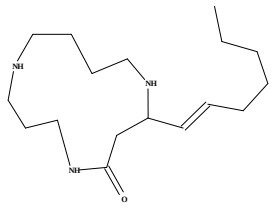

Figure 3.17.1: Proposed structure of Nuciferin

Nuciferin was isolated from *Croton gratissimus*. Burnt powder bark of *C. gratissimus* was used by Sothos for bleeding gums and leaves mixture for smoking rheumatism in patients (Watt and Breyer-Brandwijk, 1962). Nuciferin occurrence is widespread, it has also been isolated from (*Nelumbo nucifera* Gaertn.) leaves, a perennial species that is most prevalent in southern China where it is considered as foodstuff (Xu *et al*, 2011). Chinese traditional medicine has applied the species for heat resolution as well as to stop bleeding (Kashiwada *et al*, 2005). Previous studies revealed active alkaloids as some useful components with antioxidants (Cho *et al*, 2003) antimicrobial (Agnihotri *et al*, 2008) and anti-HIV (Kashiwada *et al*, 2005) properties.

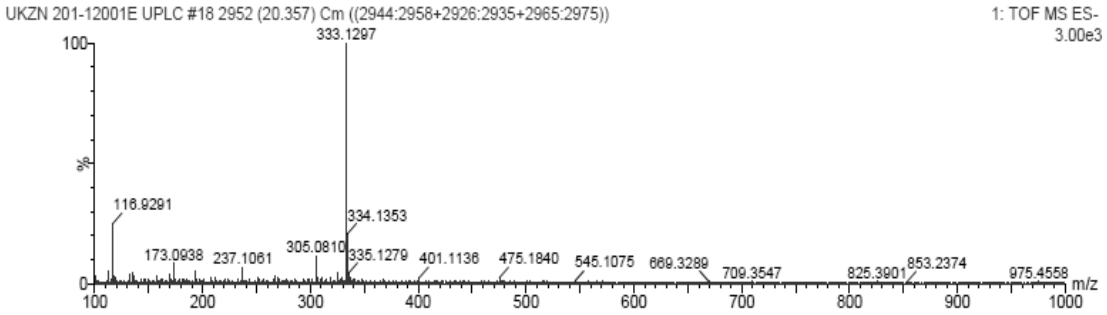

Figure 3.18: High resolution ESI-TOF-MS spectrum in negative mode

Compound 15

Name:	Narcissidine
Molecular Formula:	$C_{18}H_{23}NO_5$
Molecular Weight:	333.383
Accurate Mass:	333.157624
Percentage Composition:	C 64.85%; H 6.95%; N 4.20%; O 24.00%
HRESIMS *m/z*	333.0140 $[M]^+$, (calculated for $C_{18}H_{23}NO_5$, 333.0140)

Compound 15: The high resolution mass spectrum HRESIMS in positive mode provided a pseudomolecular ion at m/z 333.1297 $[M]^+$, calculated for $C_{18}H_{23}NO_5$, 333.0140. The deduced molecular formula of $C_{18}H_{23}NO_5$ corresponds to the pseudo-molecular ion of compound 15 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Narcissidine, an

amaryllidaceae alkaloid that was isolated from *Narcissus poeticus* (many other spp. in the Amaryllidaceae) (Martin, 1987).

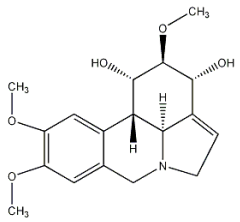

Figure 3.18.1: Proposed structure of Narcissidine

Narcissidine is an amaryllidaceae alkaloid that was isolated from *Narcissus* cultivar species (Martin, 1987). These types of alkaloids have biological function ranging from antiviral (Martin, 1987, Gabrielsen *et al*, 1999, Ghosal *et al*, 1988), immunostimulant (Ghosal *et al*, 1984), analgesic (Kametani *et al*, 1971), antimalarial (Likhitwitayawuid *et al*, 1993) and insect antifeedant (Martin, 1987, Ghosal *et al*, 1984). In another study, narcissidine that was isolated from *Narcissus tazetta* used to treat JE virus infected mice, displayed no difference between control and treated mice which experienced prolonged survival term (Furusawa *et al*, 1970).

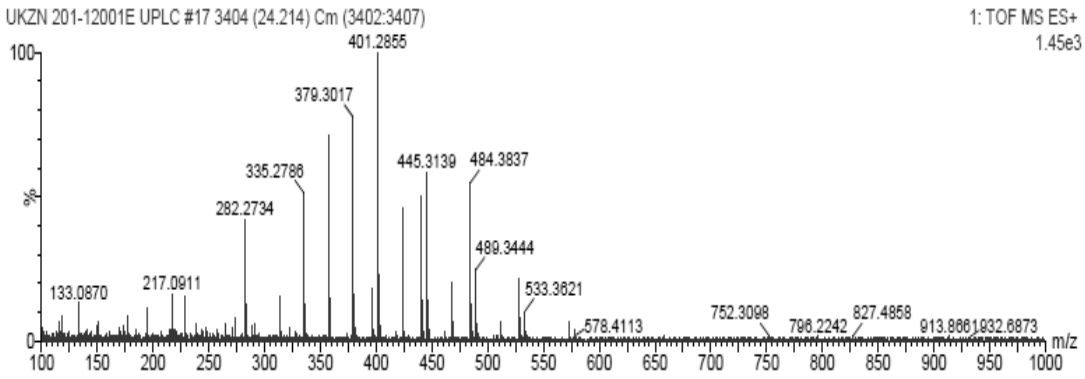

Figure 3.19: High resolution ESI-TOF-MS spectrum in positive mode

Compound 16

Name:	**Diosindigo A**
Molecular Formula:	$C_{24}H_{20}O_6$
Molecular Weight:	404.418
Accurate Mass:	404.12599
Percentage Composition:	C 71.28%; H 4.98%; O 23.74%
Physical Description:	Blue needles (petrol)
HRESIMS m/z	217.1027 $[M+H]^+$, (calculated for $C_{24}H_{20}O_6$, 217.1027)

Compound 16: The high resolution mass spectrum HRESIMS in positive mode provided a pseudomolecular ion at m/z 217.1027 $[M+3H]^+$, calculated for $C_{14}H_{27}NO$, 217.1027. The deduced molecular formula of $C_{14}H_{23}NO$ corresponds to the pseudo-molecular ion of

compound 16 in the UPLC/UV/ESIMS analysis of the aqueous extract. According to the literature, this molecular formula corresponds to that of several Diosindigo A, a naphthoquinone that was isolated from *Euclea natalensis* (Watt and Breyer-Brandwijk, 1962).

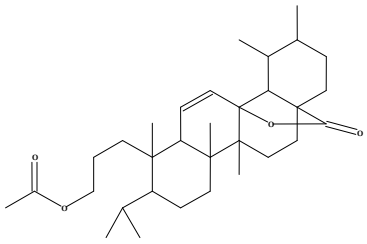

Figure 3.19.1: Proposed structure of Diosindigo A

Diosindigo A is one of the four naphthoquinones that was fractionated from root macerate of *Euclea natalensis* (van der Vijver, 1975). Furthermore, diosindigo A forms one of the isolated compounds from the root bark of *Diospyros usambarensis* that exhibited antifungal and molluscicidal properties (Marston *et al*, 1984). Death associated with the administration of *E. natalensis* is specifically confined to the utilization of the stem bark which is thought to posses strong cathartic effects (Cunningham, 1988).

Table 3.0: Summary of the16 proposed compounds that were identified with UPLC-MS**.**

Peak	Ret time (min)	M/Z $[M]^+$	Assignment
1.	1.19	217	Thalebanin B
2.	1.9	255	2-methyl-3-(piperidin-1-yl) naphthalene-1..
3.	3.4	479	Kuguacin B
4.	4.38	495	Kuguacin R
5.	4.58	233	Methylillukumbin A.
6.	5.6	333	3,5-Dihydroxy-4′,7….
7.	9.8	537	Anhydrocochlioquinone A
8.	10.8	477	Kuguacin J
9.	12.3	624	Verbascoside
10.	12.8	465	Quercetin
11.	15.2	602	Isoferuloyllupeol
12.	15.5	589	Mauritine H
13.	17.5	447	Kaempferol
14.	17.6	295	Nuciferine
15.	20.5	333	Narcissidine
16.	27.8	404	Diosindigo A

$[M]^+$denotes a molecular ion

The UPLC-MS system is an alternate method of choice for structural elucidation especially when co-eluting contaminants intervene. Although NMR and other chemical profiling techniques may suffice, an MS however remains the main tool geared for such purposes. Hence the fragmentation patterns or chemical fingerprints that can only be achieved in the presence of a high mass accuracy, MS^n fragmentation still remains very crucial (Technical Report vol 20). LCMS-TOF helps separate analytes from impurities. From the MS^n data acquired, structural composition and fragment patterns can be determined. Thus the UPLC-MS system has proven ideal for metabolites studies owing to

its utilization of several data analysis techniques that include peak picking, putative identification and alignment. Results confirmation was thereby made possible by variables such as retention times, mass and intensity that are derived from a full mass spectrum of a chromatogram (Negussie, 2009).

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Chapter 4

An investigation into the safety of *IHL*, *in vitro*

4.0 Introduction

Seasonal changes affect pharmacological profile of medicinal plants. However this is not the only factor affecting efficacy, several other conditions such as cultivation, manufacturing, marketing and distribution also contribute. Physiological, genetic and environmental factors can drastically alter plant's biochemical content. Secondary metabolites are said to be time dependant, storage, drying, extraction, processing, handling and managing plant medicine requires skill. Chemical consistency is important for ensuring efficacy and consumer safety (Sahoo *et al*, 2010).

In vitro cytotoxicity assays are useful in that they measure the effect of toxic chemicals on the basic functions of cells, and that the toxicity can be measured by assessing cellular damage. Chemosensitivity tests are commonly utilized in both research and clinical environments to determine the effect of compounds on tumour cells (Mossman, 1983). The cytotoxicity tests conducted seeks to establish the dosage that proves deleterious to cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and GSH-Glo which measures intracellular glutathione levels.

Methylthiazol tetrazolium assay is an important colorimetric assay used to assess activity of succinate dehydrogenase enzymes that reduce MTT salt. Essentially MTT or a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide is yellow tetrazolium salt that is reduced to purple formazan crystals in viable cells (Mosman, 1983). The MTT assay offers a quantitative, convenient method for evaluating cell population's response to external factors, whether it is increased in neither cell growth, nor effect or a decrease in

growth due to necrosis or apoptosis (Cooke and O'Kennedy, 1999). In order to assess cell viability, formazan measured in treated cells was compared to untreated cells or negative control and dose-response curve was produced.

Reduced glutathione (GSH) the most abundant non-proteinthiol, is an antioxidant found in eukaryotic cells. Oxidative stress and free radicals can cause a decrease in GSH levels either by oxidation or reaction with thiol group. A change in GSH levels is important for assessing toxicological responses and can promote oxidative stress, potentially leading to apotosis and cell death (Promega, 2007). The GSH-Glo™ is a luminescence-based assay for the detection and quantification of glutathione. The assay is based on the conversion of luciferin derivative into luciferin in the presence of GSH, catalysed glutathione transferase (GST). The signal generated in a couple reactions with firefly luciferase is proportional to the amount of GSH present in the sample. The assay generates stable luminescent signal and is simple, fast and easily adaptable to multiwall format such as 96-well plates. This assay can be used for detection and quantification of GSH in cultured cells or various biological samples. The GSH-Glo™ Glutathione assay can be applied for measuring glutathione levels as an indicator of cells viability or oxidative stress. It can also be applied for screening drugs and new chemical entities for their capacity to modulate glutathione levels in cells (Promega, 2007).

In this assay, cyclosporine A (CsA), a positive control was used. Cyclosporine A is an immunosuppressive drug that act by binding onto intracellular receptor and inhibit cytokines production. The medicinal concentration above that of positive control is taken

as a concentration in which cytotoxicity ensues, hence medicinal concentration dosage to be used especially in cell culture assay therefore must not exceed that marker.

4.1 Aim of these tests:

1. To determine cytotoxicity of *IHL* with regards to safe *in vitro* dosage and the effect of extended exposure.
2. To determine safe and effective medicinal concentration to be used throughout *in vitro* assays.

4.2 Materials and Methods

4.2.1 Reagents and Chemicals

4.2.1.1 Chemicals

MTT salt was purchased from Sigma Aldrich. RPMI 1640, EMEM and FCS were all purchased from LONZA, USA. GSH-Glo ™ Kit was acquired from Promerga.

4.2.2 Cells

Vero cells (African Green Monkey) were provided by the Department of Microbiology. These were maintained in Minimum Eagle's Medium supplemented with 10% fetal calf serum (FCS).

4.2.3 Plant extracts preparation

Traditional medicinal extract was filter-sterilized through 0.2µm filter and lyophilized. The lyophilized product was weighed out and 1000µg/mL of aqueous extract was made.

From the stock solution several working matrix-concentration in a range of 1000, 800, 400, 200, 100, 80, 50, 20, 10, 1 and 0.1µg/mL were made.

4.2.4 MTT stock preparations

A 5mg/mL MTT stock was made by dissolving MTT salt in RPMI-1640. The stock was filtered through 0.2µm filter and stored in 2-8°C. A working solution was further diluted ten-folds in RPMI without phenol red.

4.2.5 Cell Viability Assay

The assay was conducted following Wilson's MTT instructions. Vero cells were cultured in EMEM supplemented with 10% fetal calf serum and incubated in 5% CO_2 humidified incubator at 37°C. When cells became 90% confluent, they were washed three times in PBS and trypsinized using trypsin-versene solution. Trypan-blue exclusion was carried out in hemacytometer in order to determine viable cells. A 200µL volume cell suspension contained an average of 15000 cells and these were added in 96 well microtitre plates and incubated until they became confluent. Supernatant was removed and each well was treated with 100µL of medicinal extracts of each serial dilution and this was done in triplicates. For negative controls only 5% cell culture media was added and incubated. After 24 and 48 incubation, the supernatant was removed. Thereafter ten microliter of MTT working solution was added to each well followed by another addition of 5% EMEM and incubation at 37°C for 4 hours. After the incubation period, the supernatant was removed and 100µL dimethyl sulphoxide (DMSO) was added in order to dissolve formazan crystals and this was furthermore incubated for an hour. The plates were later

read on Beckman DU-600 Spectrophotometer at 570nm wavelength and background subtraction at 650nm.

4.2.6 GSH Assay

The GSH-Glo™ reagent was prepared by diluting the Luciferin-NT substrate and Glutathione-S-Transferase 1:50 in GSH-Glo™ reaction buffer. Each reaction (well) of a 96-well plate required 50µLof the GSH-Glo™ reagent; the total volume of the reagent prepared was adjusted according to the number of assay wells. The GSH-Glo-™ reagent needed to be prepared immediately before use and could not be stored for future use.

The luciferin detection reagent was prepared by transferring the contents of one bottle of luciferin detection buffer to the bottle of lyophilized luciferin detection reagent. The contents were mixed by inversion several times until the substrate was thoroughly dissolved. Each reaction (well) of a 96-well plate required 100µL of the luciferin detection reagent meaning the luciferin detection reagent needed to be 2:1 with the GSH-Glo™ reagent for the reaction to work properly. The luciferin detection reagent could be stored for up to 4 weeks at -20°C.

4.3 Results

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension was mixed with dye and it was visually examined to

determine whether cells would take up or exclude dye. The effect of the increasing concentration of *IHL* on cultured Vero cell line according to Trypan Blue dye test, showed that the extract to have no effect on cell viability following 7, 28 and 48 hour incubation with this extract (table 4.0). Phenotypically viable cell presented with a clear cytoplasm whereas a nonviable cell had a blue cytoplasm.

Table 4.0: Tabulated trypan blue exclusion test results showing time and dose effect of *IHL* on Vero cell lines.

#	Conc in µg/mL	% Cell viability in aqueous medium		
		7 Hrs	28 Hrs	48 Hrs
	control	100±0	100±0	100±0
1	1	98.9±0.15	99.1±0.49	98.3±0.50
2	50	99.0±0.31	98.4±1.27	98.6±0.70
3	100	98.7±0.84	99.3±0.15	98.2±0.50

With regards to cytotoxicity results of an aqueous extract of *IHL* as assessed by an MTT assay, it was established that there existed dose dependant activity. An aqueous extract as applied on uninfected Vero cell lines displayed an increasing trend of viable cells at lower concentration while high concentration decreased cell viability (figure 4.0). This trend was also proved to be time-dependant. The highest concentrations proved to be cytotoxic after 24hrs of incubation but this cytotoxicity was not shown over 48 hour incubation. The percentage cell viability was measured against untreated control. The highest concentration displaying cell viability above 80% after 24 hours was at 40µg/mL and the minimum concentration displaying cell viability that is less or equal to 50% was

obtained at 100µg/mL. After 48 hours the highest cell viability concentration was achieved at 10µg/mL (>90%).

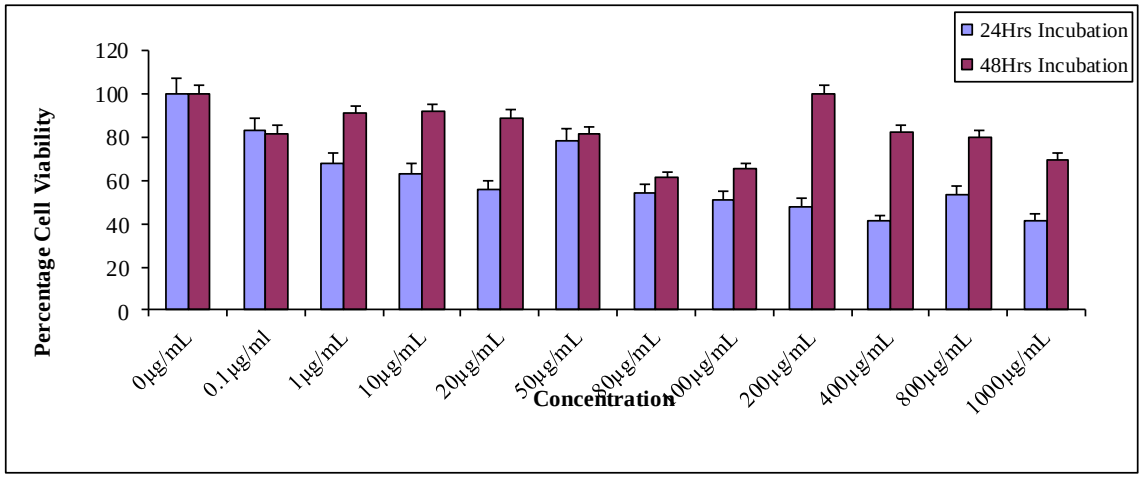

Figure 4.0: The effect of *IHL* on cell viability using MTT assay after 24 and 48hours incubation respectively.

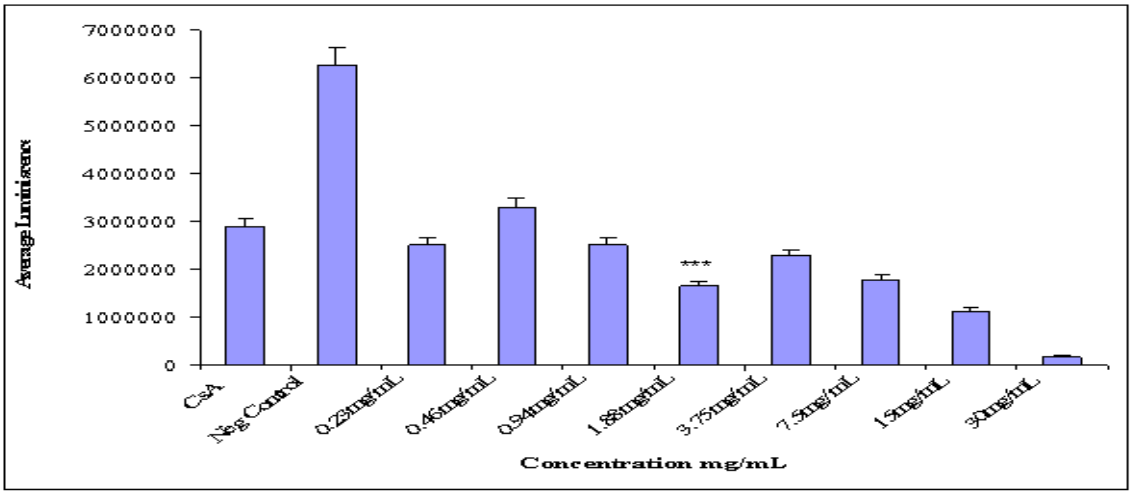

Figure 4.1: The effect of *IHL* aqueous extract's concentration on GSH-Glo™ level after 24 hours incubation period

The GSH assay using higher concentrations of traditional medicine was done to further verify the cytotoxicity of *IHL* on Vero cells. *IHL's* effect on antioxidants was shown to be dose dependent (figure 4.1) as seen in recordings taken 24 hours post treatment. Antioxidants levels were reduced in proportion relative to concentration levels. Luciferase was determined in terms of relative light unit (RLU) detected in the measured period. Luciferase assay is basically a cytotoxicity assay however its significance was made possible by positive control, cyclosporin whose dosage was used to eliminate interference without compromising on activity. Hence the presence of cyclosporin is to act as a biomarker that determines the extracts' concentration which once it is exceeded, cells start dying. This therefore makes luciferase assay such an important assay as applied to crude extract since it apparently addresses the fact that extract can cause non-specific reduction in luminescence (some as high as 98% interference) which could lead to erroneous interpretation (Shawar *et al*, 1997). Therefore in all forthcoming cell-based assays the safest dosage below that of a positive control is recommended to assess susceptibility of an organism (e.g. HSV) to a traditional medicine extract especially *IHL*.

4.4 Discussion

The aim of this study was to establish whether an aqueous extract of *IHL* would prove cytotoxic towards human epithelial cell lines as assessed by an MTT and GSH assays. The MTT assay tests for metabolic competence as well as assess mitochondrial performance (Fry and Hammond, 1993; Freshney, 2000). It is basically a colorimetric test that is based on the conversion of yellow tetrazolium bromide (MTT) to purple formazan derivative by mitochondrial succinate dehydrogenase in live cells (Mossman,

1983). Glutathione assay (GSH) assesses toxicological responses therefore GSH levels as expressed in luminescence indicate a number of live cells.

It would have been interesting had the assay been conducted on both infected and uninfected cell lines. This is particularly based on the effect of *IHL* on cell growth stimulation which was observed in the 48 hours post treatment assay particularly with the MTT assay. The inclusion of both infected and uninfected cells would establish whether *IHL* protects cells meanwhile killing the pathogen as well as to establish the safest dosage whereby cells would be preserved while pathogens would be killed. Nonetheless, we have established MTT activity on uninfected cell after 24 hours and what became apparent was the mitochondrial gradual retarded performance. This retardation was increased with an increase in traditional medicine concentration until a 50% decrease compared to the control was noted at 1000µg/mL. It is interesting to note that upon prolonged exposure of cells to *IHL* there is a derived health benefits accorded to cells that could probably be associated with the presence of flavonoids. In a study conducted by Singh *et al* (2005) on medicinal plants used to manage AIDS-related symptoms it was found to be constituted of this compound. Flavonoids contain antioxidants that scavenge free radical thus inhibiting tissue damage that is associated with the onset of AIDS. Furthermore, after 48 hours post treatment with *IHL*, the mitochondrial performance was significantly increased (30%) generally across all major concentrations i.e. from 80µg/mL to 1000µg/mL when compared to 24 hours exposure.

The most likely mechanism of action of extract includes its ability to scavenger reactive oxidative species or alternately, its ability to upregulate endogenous antioxidants levels. (Shtukmaster *et al*, 2010). Shtukmaster *et al* (2010) used an aqueous extract of *Teucrium polium* to display an ability to augment levels of important intracellular antioxidants. It is therefore safe to assume that the mechanism of extracts health benefit could be due to its ability to suppress oxidative stress thus strike a balance with endogenous antioxidant levels.

The study has proven that cellular integrity is not immensely compromised at low concentrations as it was maintained over prolonged periods of exposure of cells to the extract despite nonsignificant increase in GSH level that culminated at 0.94mg/mL concentration.

4.5 Conclusion

From data acquired, it was concluded that at low concentrations, an aqueous extract of *IHL* is not toxic. That may partly explain the elevated glutathione levels at such low concentrations.

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Chapter 5

ANTIVIRAL PROPERTIES OF A TRADITIONAL MEDICINE (*IHL*) USED FOR THE TREATMENT OF HERPES SIMPLEX VIRUS INFECTIONS

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ANTIVIRAL PROPERTIES OF A TRADITIONAL MEDICINE (*IHL*) USED FOR THE TREATMENT OF HERPES SIMPLEX VIRUS INFECTIONS

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5.0 Abstract

Introduction: *Ihlamvu laseAfrika* (*IHL)* is believed to have positive effects on HIV/AIDS patients however, this has not been proven in clinical or laboratory tests. Such effects can either be directly due to inhibition of the virus causing AIDS or indirectly by inhibition of organisms causing opportunistic infections.

Objective: To investigate antiviral properties of *IHL* against Herpes Simplex Virus (HSV) and its effect on GSH level.

Materials and Methods: A crude extract of *IHL* was lyophilised and reconstituted into stock standards that were prepared in either aqueous or solvents (methanol, ethanol, acetone, dichloromethane, cyclohexane and ethyl acetate) of differing polarities. Amongst the seven solvents used for extraction, the search for best solvent was conducted. The viral susceptibility of herpes simplex virus (HSV) towards solvent extract of *IHL* was assessed and quantified with real-time polymerase chain reaction (RT-PCR) and minimum inhibitory concentration (MIC) was then concluded.

Results: The methanol extract displayed the lowest viral yield (*Ct* = 19, p = 0.2473) as quantified with RT-PCR and an MIC of the extract proved to be 1.25mg/mL.

Conclusion: *IHL* has antiviral properties and RT-PCR is the best tool for quantifying viral yield.

Keywords: *IHL*, reduced glutathione (GSH), cytotoxicity

5.1 Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) present several clinical manifestations to mankind (Greco *et al*, 2007). The most common HSV-1 infections are cold sores and gingivostomatitis (Villareal, 2001). Usually symptoms wean after a week of viral shedding in immunocompetent individuals without taking antiviral drugs. Alternately, in immunocompromised individuals, life-threatening diseases may occur that might prompt infected host to take up antiviral therapy. Serious manifestations may present as encephalitis due to virus spreading to the central nervous system (Whitley *et al*, 1998).

Forty years have passed since the approval of a number of systemic licensed antiviral drugs for HSV treatment. These drugs primarily target the viral DNA polymerase. The examples of which feature guanosine analogues e.g acyclovir (ACV), famciclovir, valacyclovir VCV and ganciclovir, the acyclic nucleotide analogue cidofovir and finally the pyrophosphate analogue e.g. foscarnet (Villareal, 2001). The efficacy of these antivirals has been limited by development of drug resistance especially witnessed with immunocompromised patients if not the recurrence of latent viruses (Field, 2001). This therefore necessitates the development of new anti-HSV treatment drugs that will prove safe and effective.

Over the years traditional medicines have played a major role in a fight against infectious diseases essentially proving to be an alternate source of treatment meanwhile

displaying active leads for potent drug discovery (Kamatenesi-Mugisha *et al*, 2008). Traditional medicine use has become widespread (Thring *et al*, 2006). Treating human infectious diseases with traditional medicines (TMs/CAMs) is prevalent to most countries including South Africa, China, India and others. Screening tests done *in vitro* so far attest to presence of potent antiviral activities with no or limited adverse effects (McCutcheon *et al*, 1995; Vlietinck *et al*, 1995; Namba *et al*, 1998, Ma *et al*, 2002; Fernandez-Romero *et al*, 2003). Plants therapeutic activity can be attributed to the presence of secondary metabolites. Amongst secondary metabolites known to have shown antiviral activity *in vitro* include tannins, flavonoids, terpenoids, saponins and caffeic acid derivatives (Wyde *et al*, 1993; Namba *et al*, 1998, Kinjo *et al*, 2000, Chiang *et al*, 2002; Ma *et al*, 2002). Previous studies have revealed that *Punica granatum* had anti-HSV properties (Namba *et al*, 1998). Chemical analysis showed that some medicinal plants such as *Agrimonia pilosa*, *Pithecellobium clypearia* and *Punica granatum* had a high content of polyphenolics (Li *et al*, 2004). Some medicinal plants bearing anti-HSV properties attribute their activities to tannins (Chen, 1994; Zhao *et al*, 2000; El-Toumy *et al*, 2002).

Ihlamvu laseAfrika (IHL) is an indigenous medicinal extract that has been formulated such that it addresses the deleterious effect of HIV/AIDS and its secondary opportunistic infections. Opportunistic infections are regarded as a common complication in people living with HIV and AIDS (Jaffe *et al*, 1983; Selik *et al*, 1982). It was hypothesized that *IHL* has botanical constituents that have antiviral, antifungal and antimycobacterial properties. Moreover, some of its properties include immunomodulation according to traditional health practitioner (THP).

In this investigation we aimed at using several polar solvents that would compete for relative extraction activity. Studies have shown that the type of solvent used for the extraction determines the ultimate compound extracted (Hughes, 2002). The best solvents would present with highest threshold (*Ct*) value. *Ct* is inversely proportional to viral yield (Namvar *et al*, 2005). The best solvent extract would finally determine MIC.

5.2 Materials and Methods

5.2.1 Plant extract: *IHL* was received as a gift donated by the THP, Mr E.B Thabethe. The Intellectual Property/Confidentiality Agreement were reviewed and validated by the Research Legal Officer and University of KwaZulu-Natal (UKZN) Innovation. The study was approved by the ethics committee of the UKZN (ref. BF069/07).

5.2.2 Preparation of plant extract: Approximately 1kg of a mixture of plant material was mixed with about 6L of water and boiled for more than 6 hours. After cooling, it was filtered with muslin cloth and bottled. A 2L solution of this crude extract of *IHL* was further filtered, freeze-dried and lyophilized. An aqueous medium and solvents (90% v/v) of differing polarities were used to extract the product. The solutions were rigorously shaken and centrifuged at 3500rpm until the filtrate finally yielded a standard concentration of 30mg/mL for each extract.

5.2.3 Acyclovir preparation: Acyclovir (ACV) was purchased from GlaxoSmithKline. Stock solution of acyclovir (2mg/mL) was prepared by dissolving the drug in triple distilled water and storing it in the fridge until further use. ACV stock was further diluted

in solvents to obtain different working solution of 1mg/mL concentration. Diluted stocks were used as positive control, deionised water and drug-free solvents were used as vehicle controls.

5.2.4 Cells and Viruses: HSV was a donation received from Inkosi Albert Luthuli Central Hospital (IALCH). Human epithelial (HeLa) cells were provided by Medical Microbiology Department (UKZN). HeLa cells were cultured in minimum essential medium eagle (EMEM, LONZA, USA) supplemented with 10% fetal calf serum (FCS, Gibco BRL, USA) and 1% L Glutamine and 0.1% streptomycin. During transfections cells were grown in 5% FCS without drugs in a 6 well plate. Cell cultures were passaged regularly and maintained on 5% FCS without drugs. Cell cultures were incubated in 5% CO_2 humidified incubator at 37°C.

5.2.5 Viral Inoculation and treatment: HSV2 was propagated on HeLa epithelial cells. Virus stock was rapidly thawed and vortexed for 1-2 min. Virus suspension was inoculated onto confluent monolayer of HeLa cells at a volume of 500mL of tissue culture medium. Cultures were incubated at 37°C in a 5% CO_2 incubator overnight. Cytopathic effect (CPE) was observed after 1-2 days for HSV2. Cultures were treated with *IHL* 15hours post inoculation as cells started detaching from the surface, taking a round shape and lysis as observed under the microscope. Treatment with medicinal extracts enabled brief reattachment of cultures however, HSV infected cell lysate had to be harvested for viral extraction.

5.2.6 Cultivation of virus for extraction: HSV infected cell lysate was submitted for centrifugation. The 6 well plate was gently shaken several times and the supernatant including cell cultures were harvested in a sterile centrifuge tube. In order to release adenovirus from cells, supernatant with cells was thrice subjected to freeze/thaw cycle and centrifuged at 1500 X g for 10 min to pellet the cell debris. The supernatant was aliquoted into sterile tubes and stored at -80°C for further analysis.

5.2.7 Viral RNA Extraction with TriZol: The medium was transferred into a 2mL eppendorf and 400µL of TriZol was added into 1600µL sample. The sample mixture was vortexed for 10 s. Eighty microliter of chloroform was added to each sample and vortex for 30 s. The samples were then spun at 12000xg for 15 min @ 4°C to ensure phase separation. An aqueous phase containing RNA was transfer into fresh tube and 200µL of ice-cold isopropanol was added. All samples were allowed to precipitate at -20°C overnight. In order to pellet, RNA was centrifuged at 12000xg for 15 min @ RT. The supernatant was decanted and the pellet washed with 200µL of 70% ethanol and then centrifuged at 12000 for 10 min at RT. The supernatant was discarded and the RNA pellet dried. In order to reconstitute the pellet, 40µL of DNAse-free water was used. This was followed by incubation for 10 min for 60°C (in water bath). All RNA samples were then stored at -70°C until further use or temporarily put on ice. The quantity of the extracted RNA was read on the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA) at 260nm (A260).

5.2.8 DNAse Treatment: Based on the [RNA] readings, the calculated volume of 10X the reaction buffer was added (see appendix). This was followed by addition of equal volumes of DNase-1 and RNase free. A 30 min incubation at 37°C was followed by addition of 25mM EDTA which was used to stop the reaction and this was followed by a 10 min incubation at 65°C. The purified RNA was read on NanoDrop in order to verify its yield and it was then ready for cDNA synthesis or storage at -70°C.

5.2.9 cDNA synthesis: Reverse transcription was done with first strand High Capacity cDNA synthesis Kit by Applied Biosystems. The complementary DNA (cDNA) was reverse-transcribed from single strand RNA using random hexamer primers. Briefly, 10µL of RNA samples were placed in 96-well reaction plate. Ten microliter of 2XRT master mix was put onto each well and was homogenized by pipetting up and down. Plate was sealed and then briefly centrifuged to eliminate air bubbles. The plate was then loaded onto thermal cycler and cycling conditions set at four step temperature program and reaction volume set at 20µL. The DNA quantification was read on NanoDrop Spec reader 1000 (Thermo Fischer Scientific, USA) and then stored a -25°C until further use.

5.2.10 Real-time PCR Quantification: Quantitative real-time chain reaction (RT-PCR) was performed on transcribed cDNA product from HSV-2 treated with several extracts of *IHL*, acyclovir and negative controls. The test was run in triplicate on thermal cycler (ABI Prism 7000; Applied Biosystems, Foster City, CA) and each well contained cDNA, TaqMan 10X Universal PCR mix (Applied Biosystems) and target-specific TaqMan labeled dye primers (Applied Biosystems). The primers used for quantitative RT-PCR

were for the expression of the early HSV-2 gene UL30. Each sample was measured quantitatively by RT-PCR and known standards of both HSV-2 were used. Primers used for HSV-2:- primers were: - 5'-CTG CCG GAC ACC CAG GGG CG-3' (forward), 5'-CGA CCT CCT CGC GCT CGT CC-3'.

The cycling parameter were initial denaturing at 94°C for three minutes, followed by cycles of denaturing at 94°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 45 seconds and 10 minutes final extension at 72°C. The PCR reaction mixture contained 0.5µM of each primer, 2.5mM MgCl2, 8% Glycerol, 25µL Mastermix (Applied Biosystems, Courtaboeuf, France) and 10µL of cDNA product. Fluorescence measurements were recorded at each cycle, which enabled the detection of cycle threshold (*Ct*) value for every DNA sample. The detection threshold was set at 10 copies per run. IC_{50} was defined as the concentration of the antiviral agent that reduced the amount of DNA copies by 50% compared to the virus drug-free control (Thi *et al*, 2006).

5.2.11 Statistical analysis: The Student *T*-test was used to compare growth inhibition. All values were quoted as the mean ± SEM (standard error of the mean) and were considered significant at $p < 0.05$.

5.3 Results

PCR-based susceptibility: Four out of seven solvents were chosen as the less polar solvents namely dichloromethane, cyclohexane and ethyl acetate tended to "burn" the 6-well flat bottom plate (Becton Dickson). The RT-PCR-based HSV susceptibility was

done with the four most polar solvent extracts and the results were expressed in terms of virus quantity as given by the *Ct* value.

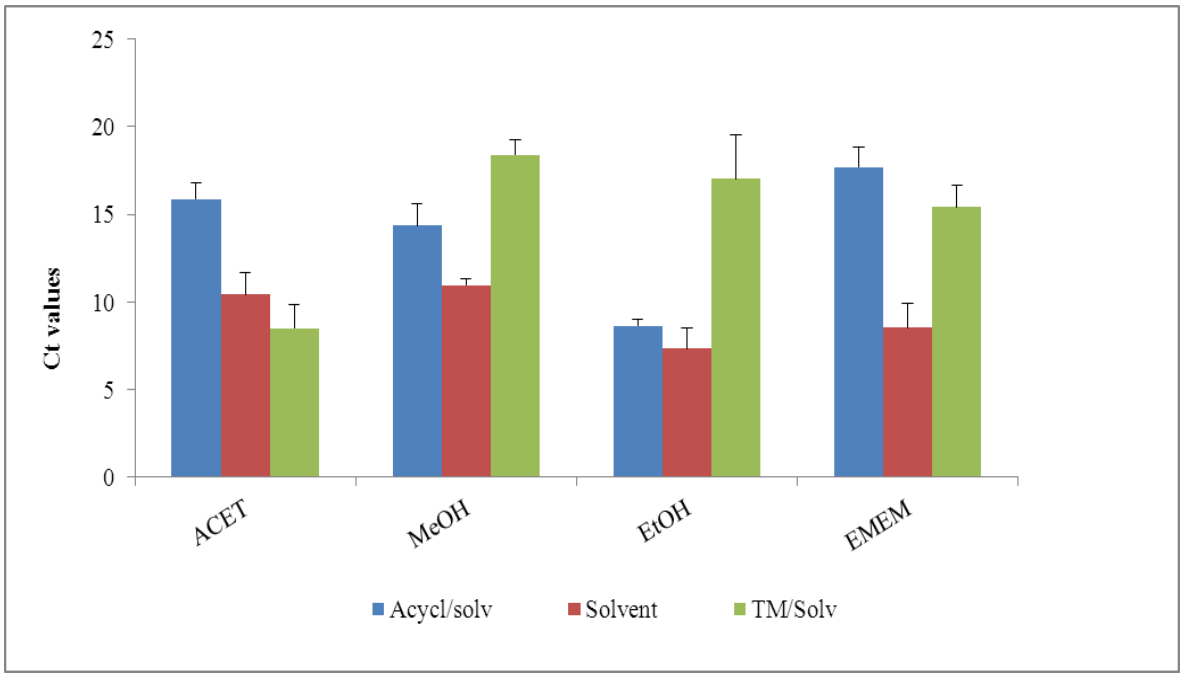

Figure 5.2: The graph display solvents' extraction potential.

An HSV growth inhibitory potential was tested on solvents (burgundy), acyclovir extracts (blue) and finally on traditional medicine extracts (green). The extraction potential was determined in terms of viral yield, the lesser the viral concentration the better the inhibitory potential. However, with regards to the *Ct* value, the higher the *Ct* value the lesser the viral yield, therefore the best inhibitor. Hence *Ct* value has an inverse relation to viral growth inhibition. In the case of a positive control, the fluconazole in EMEM achieved best HSV growth inhibition and fluconazole in ethanol recorded the least inhibition. In the case of *IHL* extracts, *IHL* in methanol yielded less virus quantity than the rest thus proving best activity, its *Ct* value was at its highest at (18.4± 0.86). The

least viral quantity as seen with the methanol extract of *IHL* serves to confirm that HSV proliferation was inhibited more than in any other solvent extracts. An *IHL* extract with the least activity was with *IHL* in acetone (8.50±1.33). Finally in the case of the four vehicle solvents, the one with the best and the least extraction potential, were methanol and ethanol respectively.

Minimum Inhibitory Concentration (MIC): Concentrations ranging from 2.00mg/mL to 0.1 mg/mL of the methanol extract of *IHL* were used to determine MIC (IC_{50}). The cytotoxicity assay helped to determine the safe dosage that had to be used during the investigation.

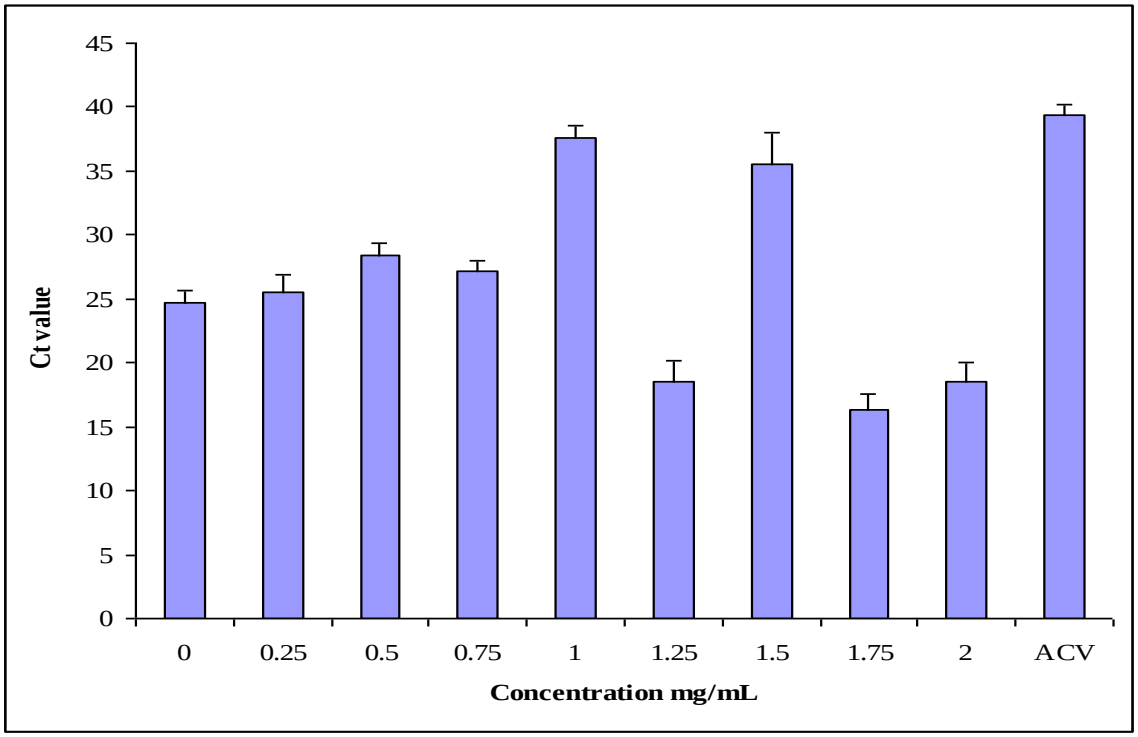

Figure 5.3: The above graph displays MIC (IC_{50}) of the methanol extract of *IHL*.

The *Ct* value rises as viral load decreases (inverse relation) therefore an increase in concentration corresponds to HSV growth inhibition increase, it is a dose dependant response. The IC_{50} of methanol extract is 1.25mg/mL. The trend followed in the graph shows an increase from left (0.25mg/mL) to right, the maximum point is reached at 1mg/mL (*Ct* = 36.7±0.78), (n=3) followed by a steep decline reaching the lowest (*Ct* = 18.9±1.14) at 1.25mg/mL, thus marking our IC_{50}. IC_{50} can be defined as an antiviral concentration that is required to reduce copies of DNA by 50% of the virus drug-free control. ACV concentration was 5.0mg/mL and the corresponding *Ct* values were (38.5±1.19).

5.4 Discussion

PCR-based susceptibility: In this investigation we explored quantitative real-time PCR technique as used for quantifying viral concentration taken from supernatants of infected and cell cultures that were treated with different solvent extracts of *IHL*. The procedure was comprised of DNA extraction with TriZol and detection using Taqman label probes in a 96-well format on an ABI 7000 real-time PCR instrument. The PCR data acquired was based on *Ct* value of each sample that was calculated by determining the point at which the fluorescence exceeded background limit of 0.04. Sample preparation in a quantitative PCR assay is an extremely cumbersome process that is prone to gross errors. During viral lytic cycle, some viruses were released from cells onto supernatant. However, some infected cell cultures after treating with test substance tended to adhere onto tissue culture flasks more than the others. That could probably be attributed to the

fact that *IHL* is concentration-dependent. Nevertheless, the PCR is still regarded as the most sensitive and efficient analytical technique.

The efficiency of all solvent extract of *IHL* were interpreted in terms of viral concentration that were expressed as threshold cycles (*Ct*). The *Ct* value is described as cycle when fluorescence has become detectable and is in exponential phase of amplification and the *Ct* value is inversely proportional to the log concentration of the target DNA (Namvar *et al*, 2005). With the $Ct < 20$ the corresponding viral copies should be above 108 copies/mL (Namvar *et al* (2005). As a guide standards were set which were used for quantitative purposes.

Reoviral infection establishes perturbation in host cell cycle progression. Their actions are known to activate cellular transcription factor (Connolly *et al*, 2000). For the purpose of this study, alteration in cell cycle was investigated in relation to gene expression by focusing on the UL30 gene. Primers and probes used targeted the DNA polymerase gene (UL30) that as mostly targeted by anti-HSV drugs (Greco *et al*, 2007).

The Taqman detection tool proved quite sensitive to all cultured specimen since it sufficiently identified differences between positive and negative controls as well as variations in different concentrations of standards. Based on the results it becomes obvious that the negative control should have a low *Ct* value which is indicative of high viral titre (Namvar *et al*, 2005). The efficiency of drug-free solvents and the respective solvent extracts of *IHL* were denoted by *Ct* values that ranged between 8 and 18 (figure 5.2) and also between 15 and 38 (figure 5.3). Furthermore, the results from previous

studies concur with our findings. In studies conducted by Espy *et al*, 2001 and van Doornum *et al,* 2003, the culture-positive samples had *Ct* values that were below 30 meanwhile negative-cultures that had also been proved positive by Taqman had their *Ct* values above 30. The MIC's of this study were determined within a tight margin, the medicinal concentration laid between 0.1 mg/mL and 2 mg/mL (figure 5.4). As a result, the *Ct* values for the negative and positive control lay between 35 and 38 respectively. The two samples in figure 5.3, the 1.75 and 2.00mg/mL are samples whose viruses were not wholly released into the supernatant. Infected viruses experience cytopathic effect detach and start floating. The introduction of an antiviral drug tend to reinforce cells attachment to the surface whether it is concentration dependant it is unproven, however we observed an unease of virus released onto the supernatant with the abovementioned concentrations. According to Namvar *et al* (2005), the viral concentration can be estimated based on assumption that the *Ct* value of 30 corresponds to approximately 100,000 copies/mL of DNA. In order to confirm a positive culture 20,000 virions are required.

Previous works recognizing RT-PCR for HSV analysis with Taqman includes Ryncarz *et al* (1999), whereby they targeted a conserved part of gB and gG for typing. Weidmann *et al* in 2003 used Taqman probes for the identification of different amplicons of HSV-1 and HSV-2.

The methanol, ethanol and aqueous extracts of *IHL* showed antiviral activity although methanol extract showed relatively best activity. A study by Nikomtat (2008) revealed that the antiviral effect of *Cissus repanda* Vahl plant extract on HSV had the best

activities for both methanol and dichloromethane extracts at various stages of HSV multiplication cycle i.e. attachment, penetration and multiplication. From that study the methanol extract of *C. repanda* showed highest inhibition.

5.5 Conclusion

HSV detection and quantification with Taqman after extraction with TriZol is quite sensitive and it has proven most susceptible to methanol-based extract of *IHL* which displayed anti-HSV properties. Caution should be taken however during harvesting of the infected lysate that all viral particles are released into the supernatant especially when viral quantification is incumbent. The active principle extracted with the methanol warrants further investigation into its chemical profile. Nonetheless, *IHL* has proven to have antiviral properties.

5.6 Acknowledgments:

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Chapter 6

ANTIFUNGAL PROPERTIES OF A TRADITIONAL MEDICINE (*IHL)* USED FOR THE TREATMENT OF *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS* INFECTIONS

Submitted for consideration for publication in the African Journal of Traditional, Complementary and Alternative medicines

ANTIFUNGAL PROPERTIES OF A TRADITIONAL MEDICINE (*IHL)* USED FOR THE TREATMENT OF *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS* INFECTIONS

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6.0 Abstract

Traditional medicines are anecdotally claimed to have positive effects on human immunodeficiency virus or Acquired Immunodeficiency syndrome (HIV/AIDS) patients though there has not been scientific validation of such claims. They could either act directly against the HIV or indirectly by inhibition of organisms causing opportunistic infections. To investigate antifungal properties of a traditional medicine, *Ihlamvu laseAfrika* (*IHL)*, used for treatment of AIDS related infections. A crude extract of *IHL* was lyophilised and reconstituted into stock standards that were prepared using seven solvents of differing polarities. The disk diffusion technique was used to assess growth inhibition of these extracts against *Candida albicans* and *Cryptococcus neoformans*. Using the extract displaying the greatest activity, several working matrix-equivalent concentrations made were used to determine minimum inhibitory concentration (MIC). Confirmatory test of MIC's was done with broth microdilution method. The minimum lethal concentration (MLC) was the subsequent test conducted after broth microdilution.

Results: *C. albicans* displayed extreme susceptibility towards an aqueous extract of *IHL* (30 mg/mL) than *C. neoformans* (p <0.001). The corresponding zone of inhibition for *C. albicans* was (10.5±1.64) mm and for *C. neoformans* was (21.3±2.34) mm on agar

culture. Both organisms had MIC's of 1mg/mL. The MIC's recorded for both *C. Albicans* and *C. neoformans* with broth microdilution technique was 2mg/mL, The MLC's recorded against *C. albicans* and *C. neoformans* were 32.0 and 8.00 mg/mL respectively. *IHL* proved fungicidal at higher concentrations and fungistatic at low concentrations.

Conclusion: *IHL* displayed antifungal activity and the broth microdilution method proved to be a sensitive tool for growth detection.

Keywords: *IHL*, *Cryptococcus neoformans*, *Candida Albicans*, broth microdilution

6.1 Introduction

Opportunistic fungal infections account for 58% to 81% prevalence and 10% to 20% morbidity and mortality rate (Drouhent *et al*, 1989) especially in immunocompromised hosts including organ transplant patients (Fischer-Hoch *et al*, 1995). Candidiasis is an example of nosocomial infections as a result of organ transplant (Cox *et al*, 1993; Holmstrup *et al*, 1990, Odds, 1988). Current antifungal treatment options are lacking in efficacy whilst showing high level of toxicity. Drug resistance has been greatly enhanced with AIDS epidemic highlighting the need to develop new classes of antifungal drugs (Sheehan *et al*, 1999). Both *Candida albicans* and *Cryptococcus neoformans* have recently displayed azole-resistant associated with AIDS epidemic (Poeta *et al*, 1998).

Antibiotics were discovered in 1940 as synthesized drugs either by man or microorganisms that act to inhibit growth of other microorganisms or kill them (Ritter *et al*, 1996). At present, Amphotericin B (AMB) is regarded as the "gold standard" for

treating fungal infection. Its efficacy however has been thwarted by its serious side effects that may be life-threatening including nephrotoxicity, chills and fever. Fluconazole and itraconazole are also two useful antifungal treatment drugs classified as azole (Graybill, 1989). It is such limitations that bring about the need for new antifungal agents (Salama *et al*, 2001).

Infectious diseases have been treated with plant extracts in Africa for centuries. Sexually transmitted diseases (STD) always responded well to such treatments. In 1999 there was an estimated 340 million new cases of STD reported (WHO, 2001). The prevalence of STD has been rife in South and East Asia followed by sub-Saharan Africa, Latin America, and the Caribbean (WHO, 2001). The organisms would prefer warm, moist and dark places such as the mouth, genitals and anus. The infecting organisms would vary from bacteria, viruses and fungi. There is growing evidence that prove that these pathogens facilitate acquisition and AIDS transmission (Wasserheit, 1992; Fleming *et al*, 1999). It is believed and thus proven that STD regulation may greatly control HIV infections (Grosskurt *et al*, 1995; Gilson *et al*, 1997; Mayaud *et al*, 1997).

IHL is the traditional medicine that is currently anecdotally used by people living with AIDS (PLWA). It is a polyherbal remedy consisting, among others, *Terminalia L* spp which has been investigated for antifungal activity (Baba-Moussa *et al*, 1999; Silva *et al*, 1996). According to Baba-Moussa *et al* in a study conducted in 1999, its antifungal activity was mainly attributed to tannins present. In another study by Masoko *et al*

(2005), on antifungal properties of *Combretaceae*, it displayed an MIC as low as 0.02mg/mL.

In this study, the disk diffusion method was used to determine both pathogens' susceptibility towards *IHL* and also establish MICs. The disk diffusion method had previously been used to determine antimicrobial activity (Salie *et al*, 1996; van Staden, 1997; Kelmason *et al*, 2000; McGaw *et al*, 2000; Madomombe *et al*, 2003; Samie *et al*, 2005). Further confirmatory test with the broth microdilution technique would also determine species susceptibility as well as its MIC. Broth microdilution has proved useful for antimicrobial screening (Espinell-Ingroff *et al*, 1995). As a spectroscopic technique, broth microdilution best mimics dynamics of fungus in a biological fluid (Thamburan *et al*, 2006).

6.2 Materials and Methods

6.2.1 Plant extract. The traditional medicine, *IHL*, was obtained from Mr E.B Thabethe, a local traditional health practitioner (THP). The materials transfer agreement was reviewed and validated by the Research Legal Officer and the University of KwaZulu-Natal (UKZN) Innovation. The study received ethical approval from the UKZN ethics committee (ref. BF069/07).

6.2.2 Preparation of plant extracts. Approximately 1kg of a mixture of herbs was mixed with about 6L of water and boiled for more than 6 hours. After cooling, it was filtered with muslin cloth and bottled. A 2L solution of this crude extract of *IHL* was filtered, freeze-dried and lyophilized. Different solvents such as methanol (EtOH),

ethanol (EtOH), acetone, ethyl acetate (EA), cyclohexane, dichloromethane (DCM) and water were used to extract the product. The solutions were rigorously shaken and centrifuged at 3500rpm until the filtrate finally yielded a standard concentration of 30mg/mL for each extract.

6.2.3 Micro-organisms. An isolate of *C. albicans* was obtained from Inkosi Albert Luthuli Central Hospital (IALCH) and *C. neoformans* was obtained from the Medical Microbiology Department, UKZN. The C. *neoformans* isolates were suspended in water and kept at ambient temperature until further use.

6.2.4 Antimicrobial susceptibility testing. The disk diffusion method was performed according to M44-P specifications. Overnight broth cultures were adjusted to match 1 and 0.5 McFarland standards for *C. neoformans* and *C. albicans* respectively. Molten Sabourad Dextrose Agar (SDA) was allowed to cool to 45°C. This was transferred into sterile Petri dishes and allowed to solidify. Three drops of the inoculum were put on plate's surface and these were streaked with sterile loop to ensure uniform growth of cultures. Susceptibility was carried out with 30mg/mL concentration of the six extracts per organism. Three disks were delivered onto each plate's surface and tests were done in triplicates. Disks impregnated with drug-free solvents were assayed along as negative controls and fluconazole (2mg/mL) (Hexal Pharma) served as positive control. Plates were incubated at 37°C for 24 and 48 hours for *C. albicans* and *C. neoformans* respectively. After incubation, zones of inhibition around disks measured and recorded in

millimeters. The defining criteria would be the measure of the zones diameter hence solvent extract displaying best activity would have largest zones of inhibition.

6.2.5 Minimum Inhibitory Concentration (MIC)

6.2.5.1. Disk Diffusion: The medicinal extract displaying largest zones of inhibition from the aforementioned test was used to determine MIC. That extract was serially diluted from 60mg/mL to 0.01mg/mL concentrations. Fluconazole was used as positive control and drug-free solvent served as a negative control. A loopful of fungal organisms previously diluted was used to inoculate each plate. These plates were incubated at 35°C for 24 and 48 hours for *C. albicans* and *C. neoformans* respectively. Zones of inhibition were recorded in millimeters. The MIC of an extract was defined as the lowest concentration that would not permit growth of test organisms.

6.2.5.2. Broth Microdilution: To mimic fungus dynamics within bodily fluids, MIC in broth was determined (Thamburan *et al*, 2006). Minimum inhibitory concentration was performed with broth microdilution assay as defined in NCCLS guidelines document M27-A12 however, with slight adjustments. In brief, RPMI 1640 (Sigma) was supplemented with 0.3g of glutamine/L buffered with 0.165M morpholinepropanesulfonic acid (MOPS, Gibco Laboratories) and its pH was adjusted to 7.0 and 2g of glucose was added. One hundred microliters of fluconazole and drug-free medium (controls) and serially diluted traditional medicine were dispensed into a 96 well microtitre plate (Falcon 3072; Becton Dickson). The concentration of traditional medicine extract after addition of inoculum ranged from 32mg/mL to 0mg/mL. Fungi

inocula (100µL) were adjusted spectrophotometrically. Briefly, an inoculum was prepared from each isolate taken from 24 hours old culture grown at 35°C and the turbidities of the resulting yeast suspension were adjusted spectrophotometrically from 1 $X10^6$ to 5 X 10^6 at 530nm. The adjustments were done such that *C. Albicans* had a percent transmission matching 0.5 McFarland and *C. neoformans* matched 1 McFarland. Plates were incubated for 24 to 48 hours for both species respectively. The drug-free and yeast-free controls were also added. Plates were briefly shaken at 250rpm at ambient temperature and absorbance read with microplate reader (Perkin Elmer) at 630nm followed by incubation for 24 and 48 hrs, *C. albicans* and *C. neoformans* respectively. After incubation, the species proliferation was reassessed spectrophotometrically and the second absorbance readings recorded. Hence, proliferation was determined by subtracting the absorbance values acquired before and after incubation (Das *et al*, 2010). The MIC was determined with reference to density of drug-free control and it was defined as the lowest antifungal concentration required to inhibit 50% or 80% of the control growth (Lozano-Chiu *et al*, 1999; Nguyen and Yu, 1999; Odds *et al*, 1995; Pfaller *et al*, 1995).

6.2.5.3 Minimum Lethal Concentration (MLC): The advantage with broth microdilution assay is its ability to further determine fungicidal/fungistatic activity. The minimum lethal concentration of each dilution was determined by plating 100µL extract sample displaying no observable growth onto SDA. According to definition, MLC is the lowest concentration of the drug at which no colonies displayed visible growth, hence at the established concentrations fungi is completely killed.

6.2.5.4 Statistical analyses: The Student T-test was used to compare zones of inhibition. All values were quoted as the mean ± SEM (standard error of the mean) and were considered significant at $p < 0.05$

6.3 Results

Figure 6.1 shows results of antifungal activities of seven solvents of differing polarities used to extract antifungal compounds from *IHL*, and tested against *C. neoformans* using the disk diffusion assay compared with fluconazole as a positive control. In the case of fluconazole, triple distilled H_2O (tdH2O) was the best solvent to yielding a zone of inhibition of about (39.7±0.94) mm, followed by ethanol (29.7±3.68) mm, and the least was cyclohexane which results in no inhibition. All the solvents used to extract *IHL* resulted in an inhibition zone of less than 10mm, with the exception of tdH2O with (21.1±2.40) mm. Acetone, dichloromethane and ethanol resulted in equivalent zones of inhibition of *C. neoformans* making them not suitable solvents to use. Furthermore, the zones of inhibition by *IHL* extracted with these solvents may not reflect its efficacy against *C. neoformans* but rather that of the solvents. On the one hand, tdH2O, methanol, ethyl acetate and cyclohexane supported antifungal activities of *IHL*. However, tdH2O resulted in significantly high inhibitions zones than the rest ($p < 0.005$).

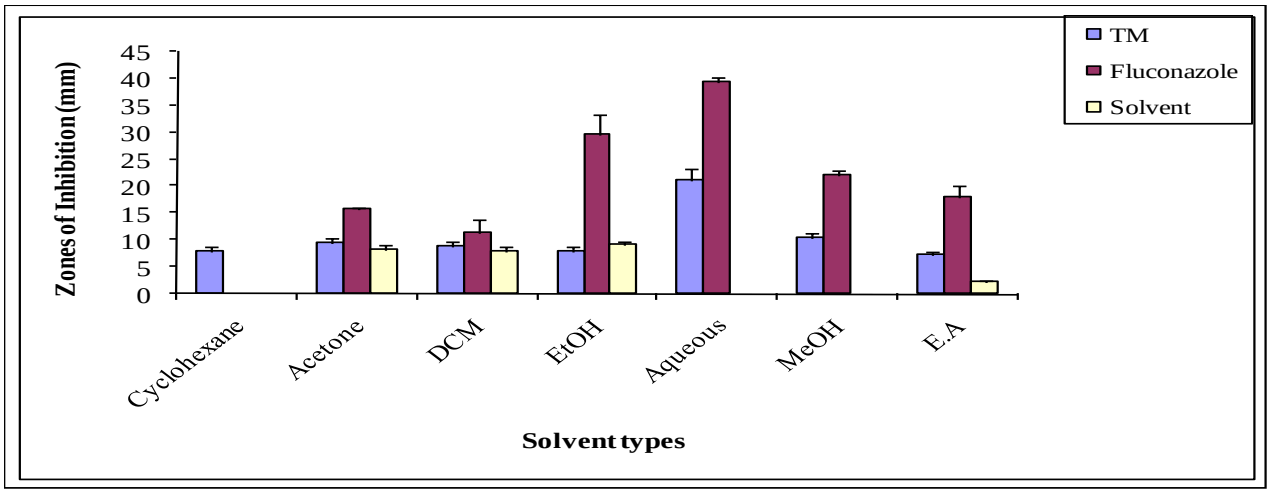

Figure 6.1: Results of antifungal screening of extracts of *IHL* against *Cryptococcus neoformans* using the disk diffusion assay and fluconazole as the positive control. Tests were done fifteen times for statistical purposes.

The seven extracts of *IHL* traditional medicine extracts were also tested against *C. albicans* as indicated in figure 6.2 using fluconazole as a positive control. The results were similar to those of *C. neorformans* in that, triple distilled H_2O (tdH2O) was the best solvent to use with fluconazole yielding a zone of inhibition of about (30.67±0.57) mm, followed by ethanol (26.0±1.00) mm, and the least was cyclohexane which resulted in no inhibition. All the solvents used to extract *IHL* resulted in an inhibition zone of less than 10mm, with the exception of cyclohexane and ethanol where *IHL* was not efficacious making them not suitable solvents to use.

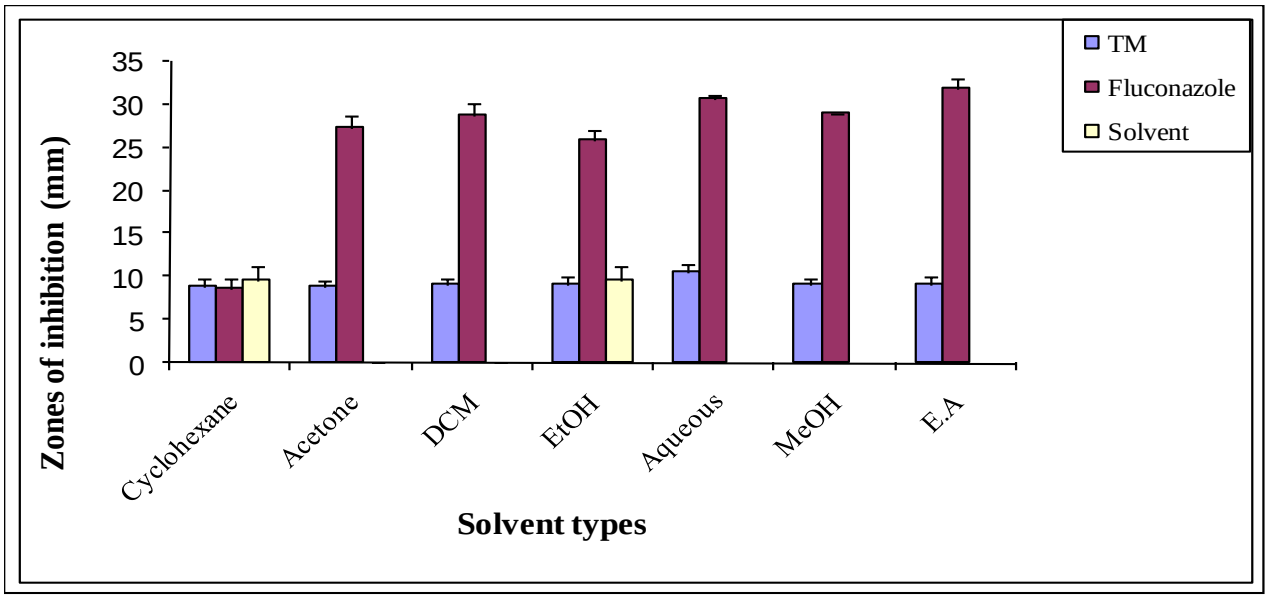

Figure 6.2: Results of antifungal screening of several extracts of *IHL* tested against *Candida albicans* with disk diffusion assay are displayed. Fluconazole was used as a positive control and tests were also done in replicates of fifteen. In the case of fluconazole extracts, it displayed largest zones of inhibition than most *IHL* extracts and controls alike. In this instance the positive control with the largest zone (32.0±1.00) mm was achieved with ethyl acetate (E.A) followed by an aqueous extract (30.7±0.58) mm and the least was the cyclohexane extract (8.67±1.15) mm. The two negative controls, cyclohexane (9.67±1.53) mm and ethanol (9.67±1.53) mm displayed antimicrobial properties with similar inhibitory potential making their contributions towards growth inhibition impossible to ignore (p<0.05). Finally, amongst *IHL* extracts, it was noticed that an aqueous extract displayed the largest zones of inhibition (10.5±1.64) mm and the least zone of inhibition produced by DCM was (9.00 ±0.00) mm.

Having established the aqueous extract as the most active, it was therefore used to determine MIC of *IHL* for both organisms. The disk diffusion results in figure 6.3 depict the MIC of *IHL* against *C. neoformans* and *C albicans* to be both 1mg/ml.

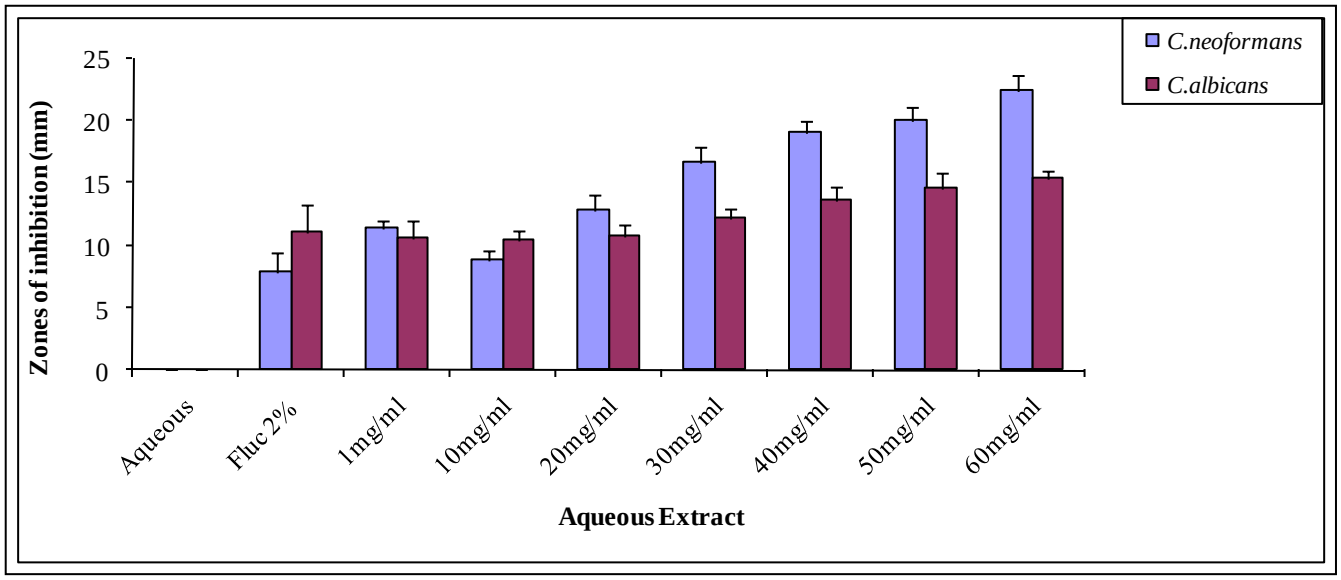

Figure 6.3: The dose dependant response curve of *C. neoformans* and *C. albicans* after treating with an aqueous extracts of *IHL* in which the MICs were concluded using the disk diffusion assay. Fluconazole was used as a positive indicator and tests were also carried out in replicates of fifteen in each organism. *C. neoformans* proved to be the most susceptible towards *IHL* against *C. albicans*. However, both organisms achieved similar MIC at 1mg/ml however, *C. neoformans* had the largest zones of inhibition (11.4±0.55) mm confirming its sensibility whereas (10.6±1.34) mm for *C. albicans* was produced (*- significant $p<0.05$ and ***- extremely significant $p<0.0001$). All results compare to a negative control.

The broth microdilution results are graphically as displayed in the figure 6.4.

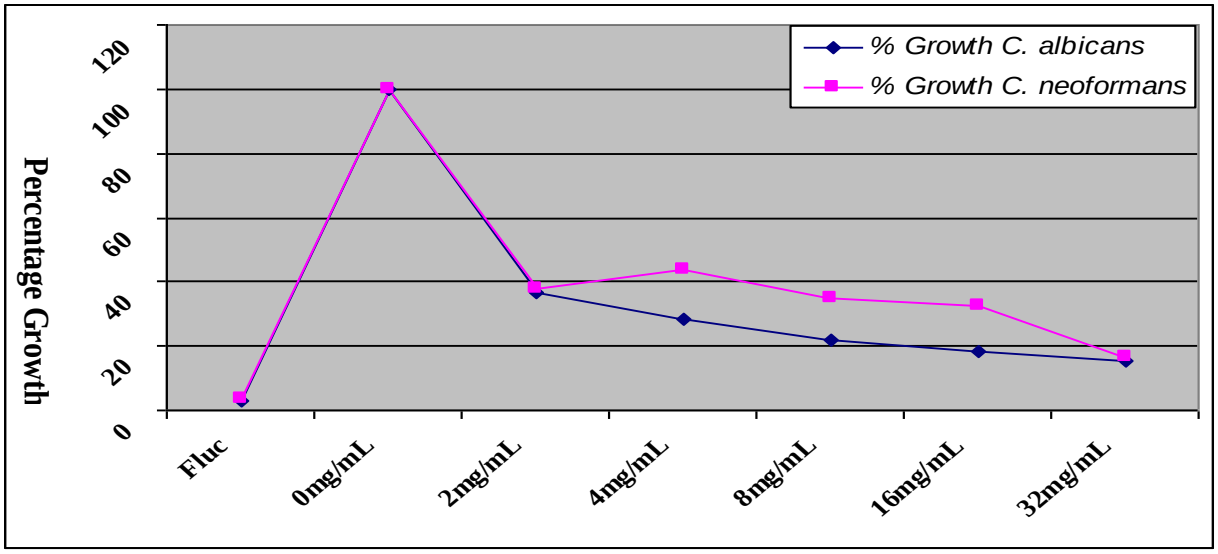

Figure 6.4: The above graph displays MIC results obtained with broth microdilution method. In this graph an apparent proliferation decrease in both *C. neoformans* and *C. albicans* that is dose-dependant was depicted. Both *C. albicans* and *C. neoformans* shared a common MIC at 2mg/mL. The corresponding percentage growth at 2mg/mL was (36.7±2.924)% and tests were done in triplicates.

The broth microdilution test gave way to the determination of the minimum lethal concentration (MLC) of the neat extract of *IHL* which helped ascertain the extract's fungicidal or fungistatic nature.

Table 6.1: In the above table the MLC results of both *C. albicans* and *C. neoformans* against neat extract of *IHL* are illustrated. Y represented a "yes" and N represented a "no" to growth. The MLC of *IHL* acquired when tested against *C. albicans* and *C. neoformans* was 32 mg/mL and 8 mg/mL respectivley. Tests were done in triplicates.

	Species Growth (Y/N)	
Medicinal Concentration	*Candida albicans*	*Cryptococcus neoformans*
32mg/mL	N	N
16mg/mL	Y	N
8mg/mL	Y	N
4mg/mL	Y	Y
2mg/mL	Y	Y
-ve control	Y	Y
+ve control (0.1mg/mL)	N	N

6.4 Discussion

Disk Diffusion: *IHL* is hypothesized to act as a non-specific repressor of pathogens a person living with HIV and AIDS presents with. The antifungal properties of *IHL* has been confirmed thus further justifies its utilization by people living with AIDS (PLWA) who present with opportunistic infections due to *C. albicans* and *C. neoformans*, depending on severity of damage to the immune system.

The agar disk diffusion method has gained a wide appreciation and reputed for best determining plant's antimicrobial properties (Freixa *et al*, 1996; Salie *et al*, 1996; Ergene

et al, 2006) and most particularly, antifungal properties. The agar disk technique has proven most appropriate for preliminary screening of trace substances or small sample (Rios *et al*, 1988). It was also established that antifungal properties of *IHL* varies with solvent utilized. Previous studies also concur with the notion that in order to extract biological active principle from plant material, the choice of solvent used for extraction plays a crucial (Parekh *et al*, 2005). Water represents a universal solvent that can extract active compounds having antimicrobial properties that most THP's use. Complementary to the work done by THP's, research into medicinal plants have revealed even the most effective means of extraction involving solvents (Parekh *et al*, 2005). The choice of solvent utilized should be such that it should not partake in the bioassay (Ncube *et al*, 2007). An aqueous control displayed no growth inhibition hence, the result output was zero. However, solvents such as acetone*, dichloromethane*** cyclohexane and ethanol* with growth inhibition recordings that are above 5mm, they play a significant role (*-significant, $p<0.05$, ***- extremely significant, $p<0.001$ respectively) to be ignored.

Ethanol, isopropyl and propanol proved to possess broad-spectrum antimicrobial activities against vegetative bacteria, viruses and non-sporicidal fungi (Morton, 1983). The results due to an ethanol extract of *IHL* seem to concur with the said statement. For instance in figure 6.1, there is significant and obvious contributions attributed to ethanol, DCM, acetone and cyclohexane controls. It has also been established that *IHL* possess antimicrobial properties hence, it is incumbent that potentiation or synergism of antimicrobial properties should be anticipated. However, the ethanol extract output seems to contradict these expectations. There is strong probability however that the given set of

ethanol extract results might be as a result of denaturation of certain proteins (Larson *et al*, 1991, Morton, 1983) thus lessening antimicrobial effect of *IHL* as it was observed during plant extraction which resulted in the formation of two layers.

In a study done by Thamburan *et al* (2006), the susceptibility of *C. albicans, Candida glabrata, Candida Krusei* and *C. neoformans* towards *Tulbaghia alliacae, Allium sativum* and *Tulbaghia violacea* that was extracted with an aqueous, methanol and chloroform solvents on an agar plate method was established. From the study it was concluded that the chloroform extract of *T. alliacea* was the most potent than *A sativum* and *A violacea* in inhibiting growth of *C. albicans.* It therefore seems that *C. albicans* proved most sensitive to test substances than most organisms. According to the THP, *IHL* is composed of several plants. The active components whether working singularly or in unison in inhibiting fungal growth, only the fractionated components could confirm, however the results seemed to have displayed synergism as observed with both strains in which *C. albicans* proved most sensitive (figure 6.3).

Broth microdilution: The microtitre plate or broth microdilution technique has proven most useful in determining MIC in large scales. The presentation of *IHL* posed a serious challenge especially when proliferation had to be visually assessed. In general, species proliferation after incubation has been assessed with changes in turbidity, a marker of species growth. The wells that seem clear would denote an MIC of an agent (Das *et al*, 2010). However, some researchers chose indicators such as tetrazolium salts or resazurin dye (Umeh *et al*, 2005), or spectrophotometric techniques to gauge proliferation

(Devienne *et al*, 2002; Matsumoto *et al*, 2001). Spectrophotometric assessment was chosen because *IHL* presented as a dark and viscous substance which upon serially dilutions introduced colour variance thus giving 'phenotypic' inconsistencies. Subsequently, to determine organisms' proliferation the absorbance values were subtracted from those acquired before incubation. That was done to eliminate the interference of the test substance (Das *et al*, 2010). MIC's were thus spectrophotometrically determined by comparing against density of the drug-free control since MIC's is defined as the lowest antifungal concentration that inhibit 50 or 80% of the drug-free control (Lozano-Chiu *et al*, 1999; Nguyen and Yu, 1999; Odds *et al*, 1995; Pfaller *et al*, 1995).

It was incumbent that the negative control displayed 100% proliferation of species with broth technique in order to enable distinguish the activity of the test substance. *IHL* markedly decreased species proliferation in a dose dependant manner. A sharp decline that resulted in 80% growth inhibition of *C. albicans* when compared to the negative control, befitting definition of an MIC was observed.

Susceptibility testing using broth microdilution method proved quite sensitive and reproducible as well reputed as best tool since the test displayed good correlation *in vitro-in vivo* clinical findings in oropharyngeal candidiasis patients infected with HIV (Revankar *et al*, 1998). The MIC's established with both broth microdilution (2mg/mL) and agar plate disk assay (1mg/mL) on *C. albicans* showed slight difference. The study has managed to show that the solid medium (disk assay) when compare to broth assay, it

is the most sensitive. However, according to Revankar *et al* (1998) in a similar study they felt that such deduction was most unlikely and they subsequently dubbed their results as inconclusive.

Minimum lethal concentration: The advantage of performing broth microdilution assay is that it gives way to the agar dilution method which helps determine agent's lethal activity. The minimum lethal concentration (MLC) of *IHL* was determined on SDA plates. The MLC can be defined as an antifungal concentration of an agent at which no colonies displayed visible growth after 24hrs and 48hrs incubations of *C. albicans* and *C. neoformans* respectively. The MLC assay was performed by aliquoting 100µL of each sample and plated that onto agar followed by species incubation. Normally samples would be taken where no growth displayed on microtitre plate. The results as seen in table 6.1 proved *C. neoformans* to be the most sensitive of the two organisms. Apparently with the two disks assays (MIC and MLC), a trend was noticed whereby *C. neoformans* again proved relatively sensitive towards *IHL*. *C. neoformans* and *C. albicans* were both killed at 8mg/mL and 32mg/mL respectively. Resilience displayed by *C. albicans* lead to an observation that it doesn't readily respond to medication be it orthodox drugs or traditional medicines as witnessed in both figures 6.1 and 6.2 (aqueous extracts).

When comparing the effect of *IHL* against *T. alliacae*, in a study by Thamburan *et al* (2006) it was shown that *T. alliacae* extract was capable of reducing cell densities (colony forming units) 5 hours post-treatment and an additional 1000 fold reduction after 24 hours exposure, virtually displaying its fungicidal activity. *IHL* displayed fungicidal

activity towards *C. albicans* and *C. neoformans* at 32 mg/mL and 8mg/mL, respectively. Both fungicidal concentrations (8mg/mL and 32mg/mL) as sampled on the microtitre plate fall below the percentage growth rate (20%) (figure 6.4) which confers fungicidal characteristics to *IHL*. Furthermore the agar disk diffusion MIC's should thus be also considered to be fungistatic as well since these concentrations rather inhibit proliferation as opposed to killing these pathogens. It can be argued that the MLC of *IHL* could not have been realized had only concentrations displaying no growth were considered. Such a hypothesis is viewed to be limited to test substances that introduce no colour changes upon utilization. The MLC exercise had previously been limited to concentration that prohibited growth (Falahati *et al*, 2006). However, in this investigation what became apparent is that at higher concentrations *IHL* proved fungicidal and fungistatic at lower concentrations.

6.5 Conclusion

IHL displayed positive activity with regards to growth inhibition towards *C. neoformans* and *C. albicans*, they both proved susceptible towards it. Further fractionation and identification of active components is warranted.

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Chapter 7

An investigation into antimycobacterium properties of *IHL*, *in vitro*

7.0 Abstract

Introduction: *Ihlamvu laseAfrika (IHL)* is traditional medicine (TM) that is currently used for human immunodeficiency virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) related infections in KwaZulu-Natal. Several properties characterize *IHL* since immunocompetent and patients co-infected with *Mycobacterium tuberculosis* draw benefit. Research revealed immense antimycobacterial properties *Euclea natalensis* plant species had displayed irrespective of portions of the plant used (Lall *et al*, 2000). These may singularly or together with other plants species constituting *IHL* treat tuberculosis.

Objective: To investigate antimycobacterial properties of *IHL* used for control tubercular infection.

Materials and Methods: A crude extract of *IHL* was prepared as previously stated. 1% proportion method and the agar dilution technique were used to measured growth inhibition of H37Rv against several extracts of *IHL*. The plant extract capable of displaying the strongest activity was used to determine MIC.

Results: H37RV strain proved susceptible towards *IHL* since visible growth less than 1% of the control was observed. Similarly, the agar dilution results concurred with 1% proportion results. The acetone extract of *IHL* displayed best activity, the MIC of 16mg/mL was common in both methods.

Conclusion: The acetone extract of *IHL* displayed antimycobacterial properties despite method utilized.

7.1 Introduction

The Southern African region is enriched with a wide array of plant species (Arnold and de Wit, 1993). A multitude of these plants-bearing-health-benefits have been utilized for centuries by indigenous people (Watt and Breyer-Brandwijk, 1962; Iwu, 1993; Hutchings *et al*, 1996; Eldeen *et al*, 2005). It is only after lab-based research ensued that diseases treated with medicinal plants were identified and classified according to their symptoms. For instance, investigation into the following plant species viz:- *Acacia nilotica*, *Cassine papillosa, Chenopodium ambrosioides, Combretum molle, Croton sylaticus, Cryptocarya latifolia, Ekebergia capensis, Protasasparagus africanus, Rapanea melanophloeos* etc, used to treat symptoms such as coughs, fever and blood in sputum of which these symptoms were later identified as classic Tb-related symptoms (Watt *et al*, 1932, Pujol, 1990; Hutchings, 1996).

Several health benefits may be found in one plant species however, these active compounds can be evenly spread throughout. In the Eastern Cape (South Africa), *Pelargonium reniforme* Curtis is one indigenous multipurpose plant that is a classical example (Latte *et al*, 2004). Investigation into *P. reniforme* aerial part displayed presence of benzoic, cinnamic acid derivatives, flavonoids and tannins as its phenolic content. Tannins play an active role in wound healing (Kolodziej, 2007). Tannins have been also been featured by most healers in their regimen used to treat gastrointestinal disorders for example, diarrhea. The root infusion of *P. reniforme* in Southern Africa features quite prominently because of its curative and palliative effects in respiratory tract infections, gastrointestinal disorders, hepatic disorders and menstrual complaints (Watt and Breyer-

Brandwijk, 1962; Hutchings 1996). Also found in the root portion also are proanthacyanidins that are included in the treatment of several ailments (Scholz, 1994; Hor *et al*, 1995). Furthermore in the root are phenolic compounds, phenolics have scavenging effect on hepatic disorders (Latte and Kolodziej, 2004; Kolodziej, 2007). Phenolic compounds as well as coumarins have moderate antibacterial effect and strong immunomodulatory effects (Kayser and Kolodziej, 1997). Other studies revealed its antibacterial, antifungal and antitubercular properties based on the root part which explains its utilization in tuberculosis (TB) and coughs treatment (Mativandlela *et al*, 2006).

It has been reported elsewhere that traditional medicine usage is widespread. In Rwanda, mycobacterium showed susceptibility towards *Tetradenia riparia* and *Bidens pilosa, in vitro*. When the active ingredient was isolated, it inhibited *Mycobacterium tuberculosis* (*MTB)* growth at 100mg/mL (van Puyvelde *et al*, 1994). In China, a traditional drug known as A-ji-ba-mo used for *MTB* treatment is derived from the root of *Dipsacus asperiodes* (Zhou *et al*, 1994).

The properties of an *MTB* bacillus may include anaerobic, non-motile bacillus with an estimated 21 to 28 days growth period *in vitro* as well as *in vivo*. It is characterized by its thick waxy cell wall that enables it survival within harsh conditions including toxic conditions with macrophages (Bowler and Chou, 2004; Weatherrall *et al*, 1988). Its ability to lie dormant makes it hard to eradicate, hence it latent effect especially amongst undeveloped countries population (Bowler and Chou, 2004).

Reliance on traditional medicine is inevitable since it spans long history of use and the emergence of resistant strains further justifies its use.

Effective TB treatment is required however not only due to co-infection in the case of AIDS patients however just so that TB is effectively controlled as a health concern affecting immunocompromised and immunocompetent alike (WHO: IUATLD Working Group, 1989).

The emergence of drug resistance calls for development and search for new therapeutic agent that effectively control *MTB* infection (Suksamram *et al*, 2003; Lee *et al*, 2006; Jimenez-Arellanes *et al*, 2007). Treating drug-resistance TB is difficult and very expensive. Worst TB treatment would require surgery to remove some infected portion of the lung drugs couldn't access (National Jewish Medical and Research Centre, 1994). For some case treated in the USA and other resourceful region, the cost attached to drug-resistance can amount to US$ 250 000 per case involving a long course of rather toxic drugs (WHO, 1997).

Currently South Africa is faced with massive multi-drug resistance and extreme-drug resistance case of TB (Gandhi *et al*, 2006). Antimicrobials taken from natural sources according to research have huge impact on human health as it has been observed in undeveloped countries. These interventions have been greatly improved in developed countries as a result a number of drugs have been synthesized and developed from natural plants (Irobi *et al*, 1994).

7.2 Aim of the study

1. To investigate antimycobacterial activity of extracts of *IHL,* in vitro.
2. To validate results obtained with 1% proportion versus agar dilution's regarding activity of the choice of solvent.
3. To determine MIC with best extracting solvent.

7.3 Materials and Methods

7.3.0 Chemicals and Reagents

7.3.1 Chemicals

All chemicals (acetone, methanol, ethanol, ethyl acetate, hexane and dichloromethane) were of AR grade and were all purchased from Sigma Aldrich Company. Isoniazid (INH) and rifampicin (RIF) were purchased from Sigma Aldrich Company.

7.3.2 Microorganisms

The H37Rv strain was donated by the Medical Microbiology department, TB laboratory. The *mycobacterium tuberculosis* strain was cultured in Middlebrook 7H10 supplemented with casitone and (oleic acid + albumen + dextrose + catalase) (OADC).

7.3.3 Anti-*MTB* drugs

Two anti-*MTB* drugs of masses 0.1605g of INH and 0.100g of RIF were extracted with 20mL of each of the six solvents and distilled water. They constituted a positive control and drug-free solvents made up a vehicle control whilst water made up negative controls.

7.3.4 Plant Extraction

IHL (coutersy of E.B Thabethe) was assessed for its antimycobacterial properties. Approximately 9.00mg of lyophilized medicinal product was dissolved in 300mL deionized water and extracted with both aqueous and solvent media.

7.3.5 Plates Preparation

For susceptibility testing Middlebrook 7H10 (Difco, Becton, Dickson & Company, Sparks, USA) agar was used to prepare plates. Approximately 7.6 g of 7H10 agar base, 2mL of glycerol, 0.4 g of casitone and 360 mL of deionised water were homogenously mixed in 500mL Schott bottles. The mixture was autoclaved at 121°C for 15 min and allowed to cool to 52-56°C. Forty milliliters of OADC was added followed by further addition of controls and 10mg/mL of the seven different medicinal extracts at a ratio of (1: 4 v/v, plant extract: culture medium). Quadrant plates to be used for susceptibility testing were configured such that there was drug-free quadrant and quadrants 2, 3 and 4 contained 5mL of INH, RIF and traditional medicine respectively.

7.3.6 Susceptibility testing

An H37Rv suspension was prepared and adjusted into liquid suspension that matches 1 McFarland standard. One McFarland made from H37Rv suspension was reported to be equivalent to 1mg/mL (Kent *at al,* 1985). The suspension was diluted ten-fold and was streaked onto plate's surface with and without plant extracts in order to determine colony forming units. After 21 days incubation, CFU were determined with the aid of Quebec colony count device. Susceptibility was determined by comparing number of colony

counts on extracts containing quadrant versus extract-free quadrant. The second determinant was the solvent extractant displaying relatively best colony inhibitory activity.

7.3.7 Minimum Inhibitory Concentration (MIC)

One thousand milligrams of lyophilized product was made up into 100mL of distilled water. This was shaken and centrifuged at 3500rpm. The filtrate was used to constitute a stock. From the stock, several working matrix-equivalent concentrations were made and used for MIC exercise. Plates were prepared in similar fashion to susceptibility assay except that various concentrations were incorporated onto each of the plates. A plant extract exhibiting strong CFU inhibitory activity was used to determine MIC and the concentrations used ranged from 25mg/mL to 2.5µg/mL. Quadrants displaying 1% growth or more to growth in the control were considered resistant to that concentration. The MIC was regarded as that concentration that inhibited 100% colony growth.

7.3.8 MIC (Agar Dilution method)

Several concentrations of the test substance were serially diluted to match concentration ranges of 0 to 64 mg/mL and these were incorporated onto agar and left to dry. The dilutions made such that the concentration was made in 10mL of the medium and 5mL of the medium at each concentration was then poured into quadrant corresponding to concentration in petri dishes. There was also a growth control or drug-free medium.

The standard inoculum size matching 1 McFarland standard were inoculated onto plate surfaces and incubated for 21 days. After incubation the effect of the tests substance was measured in terms of growth inhibition. The MIC was defined as the lowest concentration of the extract inhibiting visible growth of each microorganism on the agar plate (Nostro *et al*, 2000; Hammer *et al*, 1999). Alternately, the number of colonies in drug containing quadrant were counted and compared to those in drug-free quadrants and expressed in percentages.

7.4 Results

Isolates are considered resistant if colonies grown in drug presence are greater or equal to 1% of colonies growing in drug-free control quadrant. The results of an aqueous extract (113%) as depicted in figure 7.0, the number of colonies counted in drug containing quadrant was well above 50 counts which far exceeded 1% of colony growth in drug-free control, thus proving existence of resistant.

The second set in graph represent ethyl acetate solvent (negative control), rifampicin solvent extract (positive control) and a medicinal ethyl acetate extract, the test substance. From this set of results, it becomes clear that the H37Rv displays resistance towards *IHL*, since growth of 5% was observed.

The third set of results as depicted in the bar graph shows acetone solvent (negative control), the acetone extract (positive control) and the traditional medicine extract (test substance). According to tabulated results featured on the left hand side table, it becomes

clear that acetone extract had the greatest inhibitory activity. This is so because colonies counted on the acetone extract quadrant were 0.2% less than colonies counted on the control quadrant, essentially rendering acetone extract when compared to ethyl acetate extract (5%), the best. The methanol extract results also proved resistance (50%). In the case of DCM extracts (100%), resistance was again experienced and the positive control displayed alarming H37Rv growth. Ethanol extracts (110%) also displayed strong resistance since growth experienced in all quadrants could not fulfill the definition of the 1% proportion method.

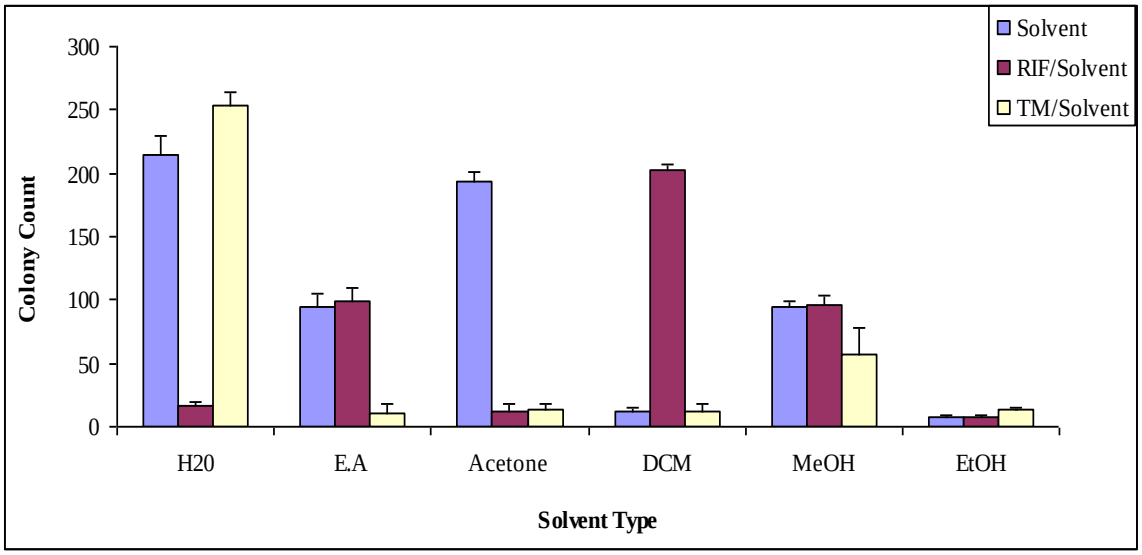

Figure 7.0: The effect of different *IHL* solvents' extracts on *Mycobacterium tuberculosis* growth inhibition using 1% proportion method (n=9).

The concentration of *IHL* remained at 30mg/mL for comparative reasons. [MeOH= methanol; E.A= ethyl acetate; Acet= acetone; DCM= dichloromethane; EtOH= ethanol; solv =solvent type; RIF/Solv= rifampicin in solvent and TM/Solv=*IHL* in extraction

solvent]. All negative controls are depicted in blue, the positive control are coloured in burgundy and all *IHL* extracts are given in white.

Table 7.0: The effect of solvent type used to extract *IHL* using an agar dilution method.

	MIC H37Rv (30mg/mL)					
	DCM	Acet	MeOH	EtOH	E.A	H2O
-ve contrl	*	R	R	S	*	R
+ve contrl	*	S	R	S	*	S
T/M	*	S	R	S	*	R

(-ve contrl = negative control of a given solvent used, +ve contrl = fluconazole used as a positive control, T/M = traditional medicine extract and * denotes no results as plates were damaged by the solvent, R = resistant and S = susceptible. Above are results of an agar dilution assay. Similarly, the *IHL* concentration remained at 30mg/mL for comparative reasons, (n=3).

Solvents such as dichloromethane and ethyl acetate and their relative *IHL* extracts and RIF extracts when brought onto a plate surface they virtually destroyed the plate, hence the results were declared null and void. The H37Rv strain once more proved to be susceptible towards all acetone extracts (table 7.0), no growth was observed with *IHL* extract in as much as it was observed in the negative control. In the case of all methanol extracts, the *MTB* strain proved resistant towards methanol extract of *IHL* and its positive control, growth was observed. However, with regards to ethanol extracts, growth was

observed with *IHL* extract as well as in its positive control. In the case of ethyl acetate, the results were also declared null and void, all plates reacted with the solvent. Finally, an aqueous extract, recorded an alarming growth in both *IHL* extract and the negative control. From the above findings, it was concluded that the acetone-based extract of *IHL* achieved the best *MTB* growth inhibitory activity than other solvent-based extracts. The growth inhibition activity of the acetone extract of *IHL* when agar dilution method was used to determine MIC, proved that *MTB* could not grow at MIC of 32 mg/mL (table 7.1).

Table 7.1: MIC results for an acetone-based extract of *IHL* using Agar dilution method.

MIC Medicinal Concentration (acetone extracts)	***MTB* Growth (Y/N)**
64 mg/mL	**S**
32 mg/mL	**S**
16 mg/mL	**R**
8 mg/mL	**R**
4 mg/mL	**R**
2 mg/mL	**R**
1 mg/mL	**R**
0 mg/mL (neg control)	**R**
RIF (pos control)	**N**

(*R represents resistant strains which therefore means there was positive growth and *S means susceptible strains therefore no growth observed). The results were done in triplicates to ensure statistical significance, (n=3).

7.5 Discussion

The susceptibility test performed on *Mycobacterium tuberculosis* H37Rv was done according to a 1% proportion method in which the search for best extracting solvent was also carried out. The results as depicted in figure 7.1 portrayed acetone-based extract of *IHL* as the one with relatively best growth inhibitory activity. The set of results displayed in tables 7.2 and 7.3, seek to establish why acetone-based extract of *IHL* and its negative control both qualify the definition of a 1% proportion method. The vehicle control showed colony counts in excess of 200 whereas no growth was observed in a quadrant bearing acetone extract. In a study done by Manjunathan *et al* (2010) on solvent based effectiveness of antibacterial activity of edible mushroom *Lentinus tuberregium,* it was observed that the antibacterial activity of a mushroom sample varied according to solvent used. According to Moore *et al*, (1999) an isolate is considered susceptible to an anti-*MTB* agent if the number of colonies counted in the presence of a drug are less than 1% of the colonies grown in drug-free control quadrant.

Table 7.2 (left) **and 7.3** (right) displays rounded off figures of results for an acetone-based extract of *IHL* using 1% proportion method.

T/M in Medium	**+ve control**		**-ve control**
Acetone	**INH**	**Rif**	**Acetone**
<20	<20	<20	2+
<20	<20	<20	3+
<20	<20	<20	3+

Ratings	**Colony Count**
Scanty	<20
1+	20-100
2+	100-200
3+	>200

Water and methanol are highly recommended for this type of research (Akihisa *et al*, 2005; Jimenez-Arellanes *et al*, 2003; Rai and Acharya, 1999). Other research has been carried out using non-polar solvents a choice necessitated by structural feature of *MTB* mycolic cell wall (Gomez-Flores *et al*, 2008). However, in other areas of research low polar solvents such as hexane and acetone based extracts have proved quite effective *MTB* inhibitors (Torrado-Truiti *et al*, 2003; Jimenez-Arellanes *et al*, 2003; Molina Salinas *et al*, 2006) these findings seems to concur.

An argument was raised related to 1% percent proportion method's application especially when one has to differentiate between growing 1% of a strain on a certain drug concentration versus an MIC test whereby a small quantity of inoculum is used. Apart from pyrazinamide which is an exception, there is no evidence suggesting that using smallest inoculum concentration would enhance the ability to differentiate between sensitive and resistant strain (Zhang *et al*, 2003). It was recommended that if one wants to establish the proportion of susceptible and resistant organisms in a heterogenous mixture, one should therefore setup a decreasing order of drug concentrations to be incorporated onto an agar as opposed to calculating inaccurate from dilutions on negative controls or drug-free medium (Matchison, 2005). We experienced similar argument hence we took to the suggestion and validated the 1% proportion results with an agar dilution method and results in table 7.1 show concurrency.

Because of the concurrency shown, an acetone-based extract therefore had to be used to determine MIC. The agar dilution is not particularly geared for small a sized product

which makes it disadvantageous to use however the results obtained are easy to interpret and are quite repeatable.

The Rifampicin used as a positive control gave results that matched those of an acetone extract of *IHL* proving that our extraction technique worked efficiently, they both displayed no growth. The aqueous extract results are disconcerting though for a traditional medicine that is supposed to benefit Tuberculosis patients particularly given that the results are based from 30mg/mL concentration which is supposedly high. It is generally known that to culture *MTB* it takes more than 3 weeks, treating TB takes even longer. In HIV positive patient's reemergence of TB as an opportunistic disease is most likely to happen. Treatment default by patients is one of the leading courses to MDR-TB and XDR-TB development (Morozova *et al*, 2003). The fact that an aqueous extract did not display growth inhibition meanwhile an acetone extract did, still makes traditional medicine an important intervention not only for its therapeutic benefits, but also as a strong lead for new drug discovery (Dimayuga and Garcia, 1991).

In a study done by Eloff (1998a) in which several solvents were examined for their extraction potential by way of a) solubilizing antimicrobials from plants, b) rate of extraction, c) ease of removal and d) toxicity in bioassays, acetone was overall ranked high on that solvent list.

In another study by Lall and Meyer (2000) that seek to establish antibacterial activity of water and acetone extracts of the root of *E. natalensis*, it was concluded that acetone

extract best yield compounds that are active against gram positive bacteria at 5.0 mg/mL concentration.

The traditional medicine activity may sometimes appear inactive *in vitro* however become active when applied *in vivo* such as prodrugs. Prodrugs are initially metabolized prior to becoming active *in vivo* (Tangyuenyongwatana *et al*, 2009). Thus there is a possibility that an aqueous extract of *IHL* may have acted like prodrugs.

High medicinal concentrations may be required to bring about activity. An extract isolated of *T. riparia* showed antimycobacterium activity at 100 mg/mL (van Puyvelde *et al*, 1994).

7.6 Conclusion

IHL has displayed antimycobacterial activity an activity only realized with acetone-based extract as opposed to what should readily be displayed by an aqueous extract. The results acquired with an agar dilution method compare to those obtained with 1% proportion method. The acetone-based results of *IHL* give an indication that it may possibly give an active lead with antimycobacterial properties and possibly a novel compound for treatment of MDR/XDR-TB. Nevertheless, further investigations are warranted to isolate and identify an active principle of this extract.

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Chapter 8

An investigation into anti-HIV properties of *IHL*, *in vitro*

AN INVESTIGATION INTO AN ANTI-HIV PROPERTIES OF *IHL, in vitro*

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8.0 Abstract

Introduction: Plants products are rapidly gaining recognition as possible anti-HIV agents (van Wyk and Gericke, 2000; Scott *et al*, 2004). Clinical cases of patients displaying signs of improvement after taking traditional medicines are amongst some of reasons that generated interest into their properties.

Objective: We conducted an *in vitro* investigation into the anti-HIV properties of *Ihlamvu laseAfrika* (*IHL*) that is currently used by people living with AIDS (PLWA) for managing viral replication, *in vivo*.

Materials and Methods: A "neat" aqueous extract of *IHL* was diluted 5 folds until -8th. An XTT technique was used to assess cell viability of viral infected MT-4 cells cultures after exposing to several concentration of *IHL*, azidovidine (AZT) served as a positive control.

Results: Results showed that at high doses, the *IHL* increased proliferation of uninfected and infected cells, cell protection reached maximum. At approximately -3.29 dilution, *IHL* proved to be toxic to both uninfected and infected cells, below -3.29, most cells became non-viable.

Conclusion: *IHL* did not demonstrate an antiviral effect at these concentrations. However, what become apparent was the toxic effect of *IHL* displayed at (IC_{50}=-3.29).

8.1 Introduction

Plants products are rapidly gaining recognition as possible anti-HIV agents (van Wyk and Gericke, 2000; Scott *et al*, 2004). Clinical cases of patients displaying signs of improvement after taking traditional medicines are amongst some of reasons that generated interest into their medicinal properties. Resolution of HIV/AIDS symptoms may include improvement in appetite, exercise tolerance, mood, sense of well-being, and weight gain and upregulated CD^4 T-cell count after taking traditional medicine for six weeks (Gericke *et al*, 2001). Moreover, there are also serious claims made by some traditional health practitioners (THPs) that they cure HIV. Hence in order to validate these claims and regulate the informal and booming traditional medicine industry, research into these products is therefore necessitated. Subsequently traditional medicines that often get selected for scientific research are based on anecdotal use by AIDS patients hence it had to show relative anti-HIV activity *in vivo* (Farnsworth, 1994; Fabricant and Farnsworth, 2001). Most often *in vitro* research is usually superseded by the *in vivo* application of medicines within public domain, therefore no scientific proof or conclusion could be made regarding the efficacies of these medicines making for what could be termed reverse pharmacology.

Some plants have proven useful against infectious organism e.g. tuberculosis (TB) which indicate that they may have antibacterial, or immunomodulatory properties (van Wyk *et al*, 2000; Bouic, 1996; Yang *et al*, 2004; Malini and Vanithakumari, 1990). *Hypoxis hemerocallidea* is a typical example of plant species that has proved beneficial to HIV and AIDS patients (Malini and Vanithakumari, 1990). *H. hemerecallidea* antimicrobial

activity is attributed to its macerated rhizome that was used for wound healing and multiple AIDS related opportunistic infections (Bouic, 1996; Ojewole, 2002).

In another study done by Klos *et al* (2009) on South African medicinal plants, the ethanol extract of *Leonotis leonurus* significantly inhibited HIV-1 by 33% while both aqueous and ethanol extracts of *Bulbine alooides* displayed greater than 50% inhibition. Some traditional medicinal products have displayed activity at different points of drug intervention in HIV's life cycle (Masutse *et al*, 1999). A methanolic extract of the root of *Bridelia micrantha* (Hochst) Baill (Euphorbiaceae), an RNA-dependant-DNA (RDDP) also displayed activity of HIV-1 RT with an IC_{50} of 7.3µg/mL (Bessong *et al*, 2006).

The possibility of inhibiting reverse transcription catalytic activity can result in failure of the virus to transcribe its genetic information that regulates translation of viral protein into provirus formation. This is a key step since the viral DNA is copied into cell's DNA hence, the cellular machinery manufacture new viral DNA as programmed in the form of RNA. T-cell could only get infected once viral RNA has been transcribed into DNA and that is enabled by reverse transcriptase enzymes (AIDSMED, 2001). Nucleoside analogues are that class of drugs responsible for inhibiting HIV active replication. However, challenges often faced with highly active antiretrovirals therapy (HAART) regimen include toxicity, development of resistance, limited availability, requires strict adherence to therapy, high cost and serious lack of curative effect (Friedland, 1997; Romanelli, 2005). South Africa is incredibly rich in biodiversity and hence medicinal plants with promising anti-HIV activity (van Wyk and Gericke, 2000; Scott *et a*l, 2004).

It is plants' lowcost that enable possible production of biomedicine and vaccines (Karasev *et al*, 2005). These activities could be based on phenylcoumarins and plant proteins that displayed best anti-HIV activity with regards to NF-αB and Tat protein inhibition (Akesson *et al*, 2003; Reddy *et al*, 2004; Marquez *et al*, 2005).

IHL is hypothesized to act as a non-specific inhibitor of opportunistic pathogens in persons infected with HIV/AIDS. In the previous chapters, *IHL* was screened for anti-*MTB*, anti-fungal and anti-HSV activities and for this section it would be screened for its anti-HIV property.

8.2 Materials and methods

8.2.1 Reagents and Chemicals

8.2.2 Chemicals

Colourless RPMI-1640

In vitro toxicological assay kit XTT based (Sigma-Aldrich, Missouri, USA)

Phosphate buffer saline (PBS)

8.2.3 Viruses

MT4 cell line used was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. HIV Viral load assay was performed with the Nuclisens EasyQ® HIV-1 V1.2, Product number: 285036, Lot number: 080812

Virus isolate HTLVIIIB was used (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). The XTT Assay was performed with the *In vitro* toxicology kit, XTT Based (Sigma), Lot #. 126K8418.

8.2.4 Cytotoxicity Testing

Viral propagation was carried out on MT-4 cells. Cells were maintained on phenol red RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 1% L-glutamine and antibiotics. A 96 well flat bottom plate (Costar, Cambridge, MA) was used for susceptibility testing. In preparation for susceptibility, cells were spun at 1000rpm and at RT for 5min, supernatant discarded and the colourless RPMI was added. Fifty microliters of MT-4 cells at $6X10^5$ cells/mL concentration was added into each well. Furthermore, 50µL of colourless RPMI was also added onto row B-D, columns 2-10 (depending on number of dilutions). It is important to note that these rows contained mock infected (uninfected) cultures. Virus was thawed, diluted (100-300 $TCID_{50}$) and added on the rows E-G, columns 2-10 (depending on the number of dilutions). These rows contained virus stock that was tested against traditional medicine that was five-fold diluted from neat, 100% (5^0 to 5^8) and a blank. In order to assess possibility of cytotoxicity, the virus-free control wells contained dilutions well mentioned above for medicinal extract. Drug susceptibility plate was incubated at 37°C in a humidified 5% CO_2. After 5 days plates were read in spectrophotometer (Molecular Devices, Menlo Park, CA). Fifty microliters of XTT was added to each well and plate was gently agitated in a rotator for an even distribution and then incubated for 4 hours. After incubation plates were read in

spectrophotometer at 450nm wavelength and reference wavelength of 650nm. Results were tabulated on excel template and both IC_{50}, EC_{50} and selectivity index calculated.

Wells were taken as positive for HIV if the optical density was less than OD of the control wells. Effect of medicinal dilutions on cell viability was assessed. Its effect was expressed as the amount of traditional medicine required to prevent a reduction OD compared to mock infected cultures. The concentration of *IHL* required to prevent 50% reduction OD was defined as IC_{50}.

8.3 Results

The results displayed in figure 8.1 and 8.2 given below were acquired through an antiviral screening protocol that was derived from cytotoxicity assay with tetrazolium salt, the XTT.

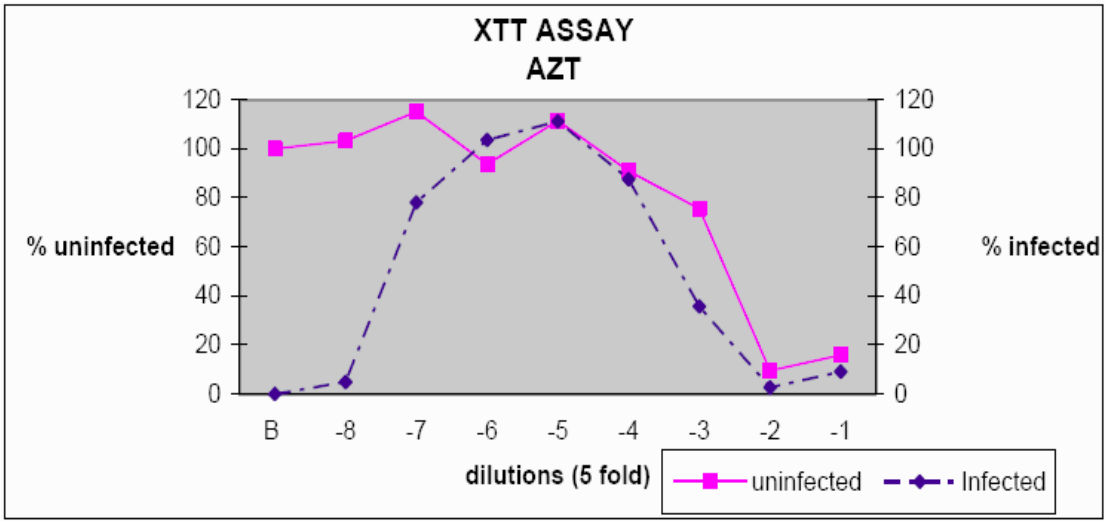

Figure 8.1: HIV susceptibility results with Azidothymidine (AZT) applied to both infected and uninfected cells.

(Dilutions expressed in $\log_5$ i.e. 5-fold dilutions. B represents the 'Blank' or a negative control i.e. the culture medium (RPMI) without tests substance. 0 represents 'neat' substance. -1 to -8 represents the successive 5-fold dilutions on a logarithmic scale i.e. 1/5, 1/25, 1/125, 1/625, 1/3125, 1/15625, 1/78125)

The positive control, AZT (figure 8.1) tested demonstrated the expected antiviral activity towards uninfected cell, high cell viability at low dilutions and less cell viability at high dilutions. The reported selectivity index approximates the published selectivity index of 1027. As AZT become diluted cell protection is enhanced and so is cell viability. Meanwhile the infected cells lose their viability as AZT becomes more diluted thus becoming weak.

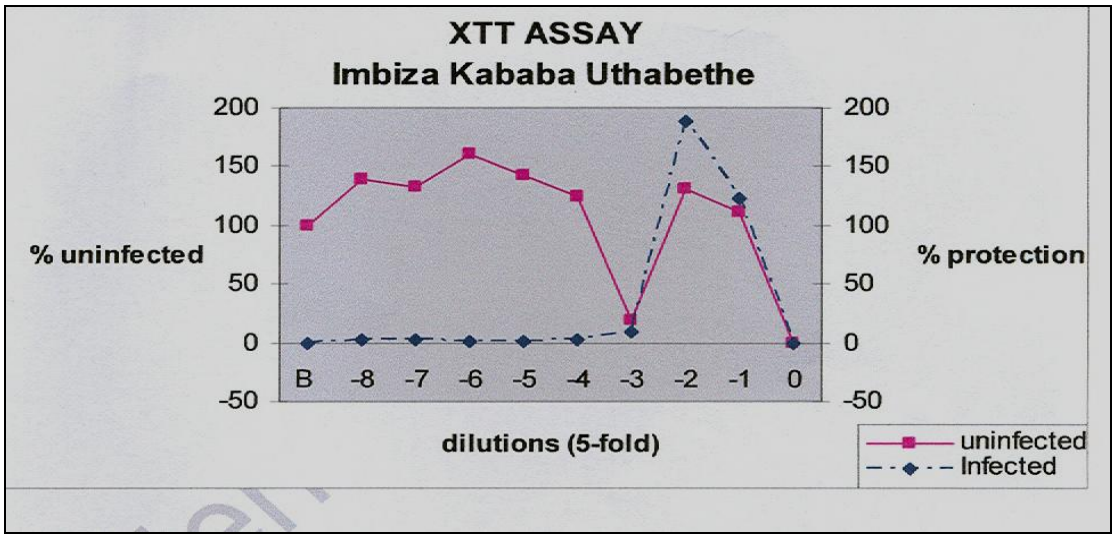

Figure 8.2: HIV susceptibility results of an aqueous extract of *IHL* also known as *Imbiza kababa uThabethe.*

According to results displayed in figure 8.2, it can be noticed that at high doses of *IHL* there is an increased number of uninfected and infected cells. At approximately -3.29 concentrations (based on fig 8.2 above it is represented by -3) it has proven to be toxic to both uninfected and infected cells. Below a -3 concentration, most cells were killed. As per explanation given previously, what became apparent is that lower doses did not kill uninfected cells but killed infected cells only. Hence, the test substance therefore demonstrates greater or equal to 50% protective effect.

8.4 Discussion

In the previous chapters tests conducted to establish antimicrobial susceptibility towards *IHL* as well as MIC, the *IHL* was initially lyophilized, weighed and reconstituted and extracted with several solvents of varying polarities. That may or may not present a great possibility that *IHL* could either be overly concentrated or diluted, hence give a misguided impression on *IHL*'s efficacy. Hence in the test for anti-HIV properties, the *IHL* was presented as a "neat substance" thus in its original formulation as presented to us by our THP, suffice to say that it was destined for public consumption despite laboratory tests and clinical trials being conducted. Nevertheless, it had been hypothesized that *IHL* is a non-specific repressor of opportunistic infections in people living with HIV and AIDS related infections. Traditional medicines are known to have a positive effect on AIDS patients. They could either exert their activity by suppressing virus causing AIDS or microorganisms causing opportunistic infections.

The antitumour XTT protocol as designed by Weislow *et al* (1989) ensured preliminary evaluation of cells growth characteristics in microtiter trays, and drug dilutions. Firstly, it was made certain that there would be sufficient time during the test, for the virus to target cells or modulate cell growth. Secondly, it had to be ensured that time was sufficient for antiviral activity of the test substance and finally, it had to be determined that cell culture growth and hence XTT formazan production would not exceed the limit set for its measurement with the spectrophotometer used.

The XTT protocol makes use of chronically infected H9 cells as a virus source. In theory mock-infected cells (uninfected controls) would normally give out a soluble orange XTT formazan colour and therefore yield high optical densities (Weislow *et al*, 1989) and these are drug protected cells. On the contrary, those cells not protected by drugs are killed by virus hence not allowed to proliferate thus presents a low optical density. The 50% cytotoxicity concentration (CC_{50}) is defined as that concentration of the test substance that reduces the absorbance of uninfected control by 50%. Meanwhile, the effective concentration at 50% (EC_{50}) is defined as that concentration of test substance that achieves 50% protection in infected cultures (Weislow *et al*, 1989).

It has been established that AZT is toxic at higher concentrations (Mir & Costello, 1988; Duesberg, 1992; Freiman *et al.*, 1993; Tokars *et al.*, 1993; Bacellar *et al.*, 1994; Goodert *et al.*, 1994; Seligmann *et al.*, 1994) which subsequently explained cell viability that was below 20% for both uninfected and infected

XTT is a thus a relatively simple and reliable assay used for measuring of *in vitro* antiviral activity and cytotoxicty. Hence the technique determines both infection and subsequent viral replication in target cells that is correlated to formazan produced (Weislow *et al*, 1989). Although the technique may have proved useful it is however not without limitations. False positive results have been said to be due to the following: a) non-specific reduction of XTT by tests compound, b) poorly understood drug-cell-XTT interaction and c) human error.

8.5 Conclusion

IHL did not demonstrate an antiviral effect at the concentrations tested. However, what become apparent with these tests is the toxic effect the substance seems to have (IC_{50}=-3.29). This warrants further studies that are necessary to give explanation to the apparent increase in percentage mockinfected and infected cells between dilutions -2 and -1. The EC_{50} cannot be calculated since the 1% protection is always below 50%.

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Chapter 9

Overall Discussion and Conclusion

Chapter 9

9.1 Overall Discussion and Conclusion

The incessant challenge of antimicrobial drug resistance presents a serious challenge to current drug regiment hence the continuous search for novel entities from medicinal plant is important (Kuete *et al*, 2010). Medicinal products play a crucial role in drug discovery and new products development (Newman *et al*, 2007). Some regard plants as a significant source of highly active antimicrobial metabolites (Gibbons, 2005, Pauli *et al*, 2005). The continuous scientific search for antimicrobial entities from traditional medicine, led us to investigate the antifungal, antiviral and the antimycobacterial properties of *IHL*. *IHL* is botanically constituted of plants mostly found in the southern African region both coastal and inland, according to our THP. *IHL* is currently used by immunocompromised patients as a non-specific repressor of pathogens AIDS patients usually present with.

The preliminary cytotoxicity assays (MTT) revealed *IHL* as not toxic to mock-infected cells despite prolonged exposure to the traditional medicine. This is crucial because of the duration it takes for some cultures to grow such as *MTB* is prolonged. Moreover, AIDS bestows lifelong sentence to treatment, cells should therefore be protected from death. In retrospect HeLa cell line that was exposed to *IHL* experienced superb cell growth enhancement. Cell viability that was done according to trypan blue test on HeLa cells displayed insignificant cell death (table 4.0) after exposing cells to several concentrations of *IHL* for 24hours. When considering damaged caused in the event of toxic medicinal substance ingested, cell numbers become drastically reduced. For HIV assay, chronically infected H9 cell lines were used. It had to be ascertained whether cell proliferation would

persist despite being infected with HIV or die. Alternately the virus would be killed whilst cells remain viable. It was noticed however, that *IHL* could not demonstrate anti-HIV effect at the concentrations tested. Instead the toxic effect of *IHL* particularly at (IC_{50}=-3.29) was noticed. In essence, HIV conferred deleterious effect onto cells since acute viral infected cells had undergone apoptosis. A similar phenomenon could have been experienced with HSV infected cells since HSV would also confer cytopathic effect.

Necrotic cell damage can be intrinsically linked to damage in genetic material due to mutagens found in man as well as organisms. Development of most cancers is attributed to these mutations including degenerative disorders and genetic defects in offsprings (Carino-Cortes *et al*, 2007). South African plants have been subjected to mutagenic studies (Elgorashi *et al*, 2003, Verschaeve *et al*, 2004, Reid *et al*, 2006, Verschaeve *et al*, 2008). Plant poisoning on the other hand, has not been well recorded due to serious lack of report by the consumers (Steenkamp *et al*, 2006).

IHL has indeed proved to have positive effects on HIV/AIDS. However, we had to establish whether such effects could either be directly due to inhibition of HIV causing AIDS or indirectly due to its inhibition of organisms causing secondary opportunistic diseases.

Previously it was mentioned that HIV susceptibility assay was carried out on 'neat' concentration of *IHl*, in order to preempt and compare the would-be *in vivo* clinical trials results to *in vitro* results. The working concentration of the 'neat' substance remained unknown since was donated in aqueous form for research purposes. We formulated our

working concentrations relative to the amount of medicinal product we could freeze-dry and lyophilize hence we could not possibly achieve the original concentration since small aliquots were used. Our aim was to standardize the traditional medicine in order to achieve certainty in our experiments. It was realized that our standardized concentrations were relatively weaker however they proved effective against microorganisms it was tested against. For example, an MIC of *IHL* as tested against *Candida albicans* and *Cryptococcus neoformans* was 1.0mg/L on solid media (with the disk diffusion method) but on liquid media (with broth microdilution assay) MIC's were 1.0 mg/mL and 2.0 mg/mL, respectively. Furthermore, the MIC of the test substance as tested against *MTB* cultured on Middlebrook agar was 16mg/mL. The last MIC acquired with PCR technique obtained on HSV susceptibility assay was 1.25mg/mL. All concentrations were compared against the activity of the 'neat' substance which was tested against HIV. When considering activities of the above MIC's they reveal that 16mg/mL becomes the effective dosage required to formulate *IHL* that might circumvent cytotoxocity and possibly mutagenicity assuming that treatment is a lifelong process as witnessed with the HAART program.

The solvents used to extract the bioactive compounds from *IHL* varied from one microorganism to the next. An active principle or the target compound in an extract that bears antimicrobial properties is highly influenced by the solvent used for its extraction (Ncube *et al*, 2006). It was established that both *C. albicans* and *C. neoformans* were strongly inhibited with an aqueous extract of *IHL*. Hence it is safe to deduce that water exhibited relatively best extraction potential than other most solvents used. In a previous

study done by Steenkamp *et al*. (2007) it was revealed that a crude methanol and water extracts of *Combretum molle* (root), *Piper capense* (bark), *Solanum aculeastrum* (fruit), *Syzygium cordatum* (bark) and *Zanthoxylum davyi* (bark) and aqueous bark of *Afzelia quanzensis* and root extract of *Tabernaemontana elegans* contained antifungal compounds that inhibited growth of *C. albicans* at < 1mg/mL. Furthermore, bioassays resulted in the identification of active principles with antifungal properties from these extracts for example, tannins and saponins were isolated from *Terminalia* species (Baba-Mousa *et al*, 1999), tannins were also isolated from *Combretum* species (Kolodziej *et al*, 1999) and ecodysteroids was isolated from *Asparagus* species (Dinan *et al*, 2001).

In our study the main constituents of the aqueous extracts identified with an UPLC-MS comprised 16 compounds that were identified as steroid, alkaloids, phenols, binaphthoquinone, homoisoflavonoids, terpenoids, triterpenoids, coumarins and saponins. This study was however limited to antimicrobial properties of *IHL*, but there exist secondary metabolites with other properties such as immunomodulation, antioxidants, analgesics etc found within this tonic.

Schmourlo and co-workers (2005) conducted a study in which antifungal agents precipitated with ethanol according to bioautographic method, resulted in an aqueous extract of *Mormodica charantia* totally losing its antifungal activity against *Candida albicans* and *Cryptococcus neoformans* after fractionation. Lost of activity was attributed to some proteins that possessed hyperglycaemic, antitumour, antileukemic and antiviral properties such as lectins (MAP30), charantins, trypsin-inhibitors and elastase inhibitors

(Yeung *et al*, 1988; Hara *et al*, 1989; Arazi *et al*, 2002; Parkash *et al*, 2002) found in *M. charantia* that may have been lost during precipitation. It was therefore deduced that antifungal activity may have been attributed to the aforementioned proteins. Such an observation also explains the reduction in antifungal activity of an aqueous extract of *IHL* which was upon its extraction with several organic solvents of differing polarities compromised activity. The water-soluble compounds for example fabatin, polysaccharides, proteins and some lectins have displayed favourable inhibitory activity on viruses that would adsorb to their host (Zhang and Lewis, 1997). This also concurs with 70% methanol extract which practically dissolved a lyophilsed product of *IHL* when less polar solvents couldn't. Herpes simplex virus II was strongly inhibited by methanol extract of our traditional medicine (figure 5.2) than other solvents' extracts which coincide with the above findings.

The test for antimycobacterial properties of *IHL* yielded peculiar findings especially since an aqueous extract did not show activity. The aqueous extract represent 'neat' sample that is available to the public. However, upon its extraction with solvents of differing polarities, some activity was noted in which an acetone extract gave the best *MTB* growth inhibition than the rest (Figure 7.1). It can be then be concluded that acetone best extract naphthoquinones as it was the case with diosindigo A, an active principle isolated from *Euclea natalensis* (van der Vijver, 1974).This was further confirmed in study conducted by Lall and Meyer. (2000), that water and acetone showed relatively best antimycobacterial results when used to extract an active principle in *Euclea natalensis*. Furthermore the presence of compounds such as isoferuloyllpeol that was isolated with

EtOH justifies activity of the ethanol extract of *IHL*, but acetone extract remained unsurpassed.

Research has established that anti-HSV properties of *IHL* are attributed to verbascoside. In previous studies done by Martins *et al* (2009), verbascoside extracted with ethanol displayed the best anti-HSV-1 and 2 growth inhibition results which concur with our findings. In our anti-HSV assay, we observed an insignificant variation ($p = 0.2473$) between methanol and ethanol extracts of *IHL* (figure 5.2).

Amides such as methylillukumbin A that were isolated from *Mauritine* species displayed antifungal activity. Moreover, compounds such as thalebanin B (Greger *et al*, 1996), kuguacin J (Kimura *et al*, 2005) and quecertin (Roy *et al*, 1996) also possess antifungal activity, despite several solvents used for extraction. The presence of the aforementioned compound presented a significant fungal growth inhibition when compared to viral and mycobacterial organisms with regards to MICs.

It has been established that during chemical profiling of *IHL* that it would provide with specific secondary metabolites responsible for antimicrobial activities plus further useful properties not intended for this study such are immunostimulation, anti-inflammatory, analgesics and antioxidants. The established secondary metabolites this tonic presents ranged from amides, flavanoids, terpenoids, triterpenoids, sterols, quinones, alkaloids and naphthoquinones (table 3.0). The compounds that do not possess antimicrobial properties however remain useful to PLWA such as may have immunostimulating, analgesic and

antioxidants properties. Some compounds were unintentionally unidentified as it can be seen from the chromatogram.

Table 9.0: Summary of isolated principles with their respective antimicrobial properties

Secondary Metabolite	Compound	Biological function	Reference:
phenethyl/styrylamine-derived amides	Thalebanin B	Antifungal	Greger *et al*, 1996
Naphthoquinone	2-methyl-3-(piperidin-1-yl)..	Anti-*MTB*	Mital *et al*, 2010
Cucurbitacins	Kuguacin B	Anti-HIV	Chen *et al*, 2008
Cucurbitane-type triterpenoid	Kaguacin R	Antiviral	Chen *et al*, 2009
Amides	Methylillukumbin A	Antifungal	Greger *et al*, 1996
Homoisoflavonoids	3,5-Dihydroxy-4,7-dimethoxyhomoisoflavonone	Antimicrobial	Jiang *et al*, 2007
Quinone	Anhydrocochlioquinone A	Antitumour	Jung *et al*, 2003
Cucurbitane-type triterpenoid	Kuguacin J	Antibacterial, antifungal	Kimura *et al*, 2005
Phenolic antioxidant	Verbascoside	Anti-HSV	Martins *et al*, 2009
Flavonoid	Quecertin	Antifungal	Roy *et al*, 1996
Triterpenoids	Isoferuloyllpeol	Anti-*MTB*	Weigenand *et al*, 2004
Cyclopeptides alkaloids	Mauritine H	Antifungal, antibacterial	Pandey *et al*, 1990; Tschesche *et al*, 1977
Flavonoid	Kaempferol	Anti-oxidant, immunostimulant	Shrivastava and Patel, 2007
Alkaloid	Nuciferine	Anti-HIV, antimicrobial	Cho *et al*, 2003, Kashiwada *et al*, 2005
Amaryllidaceae alkaloid	Narcissidine	Antiviral	Furusawa *et al*, 1970
Naphthoquinone	Diosindigo A	Antimycobacterial	van der Vijver, 1974

9.2 Conclusion

It has been scientifically validated and proven that the pharmacological properties present in *IHL* warranted its use against HIV/AIDS and its secondary opportunistic diseases. However, the results do not agree with the hypothesis initially presented. It was hypothesized that *IHL* might exert its activity through its inhibition of the HIV causing

AIDS or via inhibiting microorganisms causing opportunistic diseases. According to results, it was established that *IHL* is capable of inhibiting growth of microorganism causing opportunistic infections. This overrides the presence of secondary metabolites such as kuguacin B and nuciferin with anti-HIV activities established. Such inability to initiate anti-HIV activity might probably be dose-dependent since in literature their anti-HIV activities were established.

The study further revealed the important role played by THP not only to their respective community but the scientific community at large regarding their contributions. Traditional medicine is undoubtedly infested with some charlatans who exploit the poor in a bid to get rich creating "AIDS opportunism" (Richter, 2003). These findings further add to the call already made to respective governments to streamline traditional health practices into the mainstream. The efficacy displayed by *IHL* offers a steppingstone towards the construction and development of pharmacopeia of TM's and monographs. Published clinical results of traditional medicines are currently minimal in South Africa despite strides being taken to validate most TMs currently marketed. Such information would assist in the development of sound policies that can pave a way for the regulation of production, quality testing, safety standards, efficacy as well as preservation of natural resources. The need for quality and safety assessment ranks high in Africa and Latin America that have approximately 80% depending on traditional medicines (WHO Fact Sheet No. 134, 2008). The research output that has been offered by world renowned scientist such as van Staden, Afolayan, Lall, Dilika, Meyer, Eloff and others on South African traditional medicine has laid grounds for smooth transition to modern medicine

that can eventually afford status enjoyed by western medicine. However, the aim is not competition but to increase affordable healthcare and social benefits to respective communities.

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Appendix 1

Chemical Preparations:

Trypsin :

- 25g trypsin dissolved in 1litre distilled water (2.5% solution)
- Filter sterilize using 0.1um filter
- 20ml of 2.5% soln made up to 200ml with PBS (0.25% solution)
- Aliquoted 1ml into each vial and stored at -20◦C

EDTA: 0.5M

- Weigh 2g NaOH pellets, add 80ml water
- Place on magnetic stirrer, dissolve
- Add 18.6g of EDTA to dissolved NaOH soln
- Soln must look clear
- pH soln to pH 8. this will help clear up the soln
- Once dissolved, make up to 100ml.
- Autoclave. Dilute 1:5 with PBS/ autoclaved water
- Alternatively make up initial soln to 500ml and autoclave
- Aliquote 1ml into each vial and store at -20◦C

Trypan Blue:

- 4g of trypan blue powder is dissolved in 100ml PBS

Freezing Fluid:

- Add 20ml foetal calf serum, 60ml of media used for cell line and 20ml of DMSO in the above order
- Filter through a 0.22um filter and store in 20ml at -20◦ C
-

McFarland Standard

- A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4). Store in dark or cover glass bottle with aluminiumfoil.

Preparation of 10% FCS

- Aliquote 500µL of FCS into 4.5mL culture medium