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A STUDY OF THE ANTIHERPES SIMPLEX VIRUS TYPE 1 PROPERTIES OF BARRINGTONIA RACEMOSA

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DECLARATION

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ABSTRACT

There are limited safe, effective and inexpensive treatment options against the herpes simplex virus. The local biodiversity has made available a host of potential drug leads for the treatment and management of viral skin infections. The herpes simplex virus inhibitory effect of twenty-two South African medicinal plants was studied using an *in vitro* Vero cell system. The crude aqueous extract and succeeding fractions of *Barringtonia racemosa* derived from reverse phase high performance liquid chromatographic separation of the crude extract showed antiviral activity against herpes simplex type 1 virus. Virus induced cytopathic effects and plaque reduction assays were used to evaluate antiviral activity. The concentration for 50% inhibition of viral replication in Vero cells was 23 µg/ml for studies in which the virus and extract were added to the cell system concurrently; 28 µg/ml whereby the extract was added to the cell system one hour before the addition of the virus and 75 µg/ml when the virus was added to the cell system one hour before the addition of the test material. The crude sample was fractionated using reverse phase HPLC and sample aliquots were collected at 2-minute intervals. The bioassays revealed that fraction 3 collected at a retention time of between 4 – 6 minutes had the greatest activity. The concentration for 50% inhibition of viral replication by fraction 3 was 26.2 µg/ml.

Time-of-addition studies showed that the active antiviral agent inhibited the viral absorption and penetration through possible binding with viral glycoproteins resulting in viral inactivation and/or competing with the virus for binding sites on the Vero cells. Cell viability and MTT studies showed that the active fractions were not cytotoxic.

Phytochemical investigations of *Barringtonia racemosa* revealed the presence of galactose, polyphenolics and traces of isoleucine, glycine and aspartic acid. Antiviral activity decreased following the removal of either the polyphenolic or saccharide groups. The 50% effective dose for extracts that contained polyphenolics, in the absence of saccharides, ranged between 50 – 58 µg/ml. Extracts in which the saccharide was present and the tannins had been removed showed an ED_{50} of between 48 – 54 µg/ml.

The study of the aqueous extract of *Barringtonia racemosa* has produced strong indication of antiviral effect against herpes simplex virus type 1 *in vitro*. Although *in vivo* testing against an animal model of an HSV-1 system did not fall within the scope of this thesis that would be the next and final step required to establish proof of principle to antiviral HSV-1 infection of *Barringtonia racemosa*. Once such proof of principle has been established, assuming confirmation of activity *in vivo*, *Barringtonia racemosa* and notably, fraction 29AQC would be candidate for study of their potential as an antiviral agent.

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ABBREVIATIONS

Abbreviation	Full Term
ACN	Acetonitrile
ACV	Acyclovir
29AQC/ 29C	Cold Aqueous Extract of *Barringtonia reacemosa (Plant 29)*
29AQH/ 29H	Hot Aqueous Extract of *Barringtonia reacemosa (Plant 29)*
CD	Cell Destruction
CPE	Cytopathic Effects
DCM	Dichloromethane Extract
EC	Eastern Cape Province
ED50	50% Effective Dose
GC	Gas Chromatography
GP	Gauteng Province
HPLC	High Performance Liquid Chromatography
HSV	Herpes Simplex Virus
IEC	Ion Exchange Chromatography
IEHPLC	Ion Exchange High Performance Liquid Chromatography
IF	Immunofluorescence
IKS	Indigenous Knowledge Systems
IR	Infra Red
KZN	KwaZulu Natal
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - Time of Flight
MEH	Hot Methanol Extract
MEC	Cold Methanol Extract
MeOH	Methanol
MP	Mpumalanga Province
MS	Mass Spectroscopy
NP	Northern Province
NC	Northern Cape Province
NMR	Nuclear Magnetic Resonance
PRA	Plaque Reduction Assay
SD	Swaziland
SEC	Size Exclusion Chromatography
SPE	Solid Phase Extraction
$TCID_{50}$	50% Tissue Culture Infectious Dose
TK	Traditional Knowledge
TLC	Thin Layer Chromatography
UV	Ultra Violet
VIS	Visible
WC	Western Cape

CHAPTER I

1. INTRODUCTION

It is acknowledged that traditional medical practice plays a significant role in the discovery and subsequent development of new drug products (Matthe 1989, Vlietinck & Vanden Berghe 1991, Sofowora 1993). Numerous medicines in current use have originated from ethnomedical practices (Unander, Webster & Blumberg 1995, Elisabetsky 1991). A variety of plant-derived compounds are presently undergoing clinical evaluation as agents against viral infections (Kurokawa et al. 1997a Kurokawa et al. 1997b, Kurokawa et al. 1999, Vanden Berghe, Vlietinck & Van Hoof 1986, Floch & Werner, 1976, Vlietinck & Vanden Berghe, 1991). Current treatment options for viral infections show limited potency, hence there is a critical need to investigate new medicinal agents that will improve the antiviral activity of drugs in present employ. Of particular interest in this study is *Barringtonia racemosa*, which is utilised by traditional healers and has potential use in the treatment of cutaneous lesions resulting from herpes simplex virus infections. At present, several natural plants have shown potential to act as antiviral agents and a selection of these plants are currently being investigated for antiviral activity (Vlietinck et al. 1995, Elanchezhiyan et al. 1993). Certain plant agents are believed to possess extracellular virucidal activity and the development of such agents is important for the elimination of airborne pathogenic viruses. In addition to direct viral inhibition, it is speculated that extracts from these plants may possess an interferon-inducing capacity. However, such activity requires further investigation for use in the treatment of herpes simplex infections (Beuscher et al. 1994). The isolation and subsequent characterization of the biologically active compounds in the above-mentioned plant is likely to yield several novel compounds whose pharmacological and toxicological effects may play a significant role in drug development.

There is a high incidence of herpes virus infections worldwide and of particular concern is the increasing recurrence of the illnesses associated with the infection. In addition, the high susceptibility of immunodeficient patients to viral infections together with the gravity of neonatal herpes has necessitated the investigation for more effective and safer antiviral agents capable of limiting morbidity and mortality. It is estimated that 50% to 90% of adults have experienced a herpes simplex virus type 1 infection. The type 1 infection is easily recognisable by the characteristic vesicular lesions that appear on mucous membranes of the mouth and nose (Figure 1). Type 2 herpes, on the other hand, is the most common infectious cause of genital ulceration in the developing world.

Figure 1: An illustration of labial herpes simplex infection (http://www.herpes-coldsores-treatment-pictures.com/)

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Studies have shown that the extent of infection is dependent upon the age, ethnic origin and socioeconomic status of the individual. Manifestations of type 2 herpes are more prevalent among individuals of lower socioeconomic status and lower education attainment (Shaikh 1998). Statistical data obtained from the Department of Epidemiology and Biostatistics of the Ministry of Health, shows that there are approximately 2100 new episodes of sexually transmitted genital herpes discovered in males in the Western Cape each year (Shaikh 1998). In comparison, there are about 815 new episodes of sexually transmitted genital herpes recorded for women within the same period. The reason for such low data for women can be attributed to the fact that herpes lesions, in the female genital region, are often difficult to recognise. Hence, in some cases, women are not aware of having contracted genital herpes. The population distribution of individuals infected with genital herpes virus indicates that 86% of male and 66% of female sufferers are from the Cape metropole area. This region of the Western Cape, when compared to the Boland Overberg, West Coast and South Cape areas, has a high proportion of socially and economically disadvantaged people. The incidence of herpes simplex 2 virus infection continues to rise and of great concern is the role that herpes plays as a cofactor for HIV transmission. The fact that 80% of all genital herpes transmissions are acquired from asymptomatic sexual partners makes the management and control of the infection difficult. In addition, various socioeconomic factors have restrained the effective treatment of herpes viral infections. The high cost of currently available antiviral agents has limited the treatment options for countless individuals suffering from herpes simplex virus. Related to the issue of high medical expenses is the incapacity of ill-funded health service providers to supply, distribute and provide antiviral drugs to patients. Due to irregular provision of effective medication and high treatment costs, patient compliance to therapy is likely to decline. The combined effect resulting from inadequate treatment management is the inability to control recurrent infections and to restrict the emergence of resistant viral strains. Ethnopharmaceuticals are an important source of effective, safe and inexpensive phytopharmaceuticals. Reports from traditional practitioners recount that *Barringtonia racemosa* is commonly prepared as an aqueous mixture taken orally or swabbed in the genital region. The dosage and frequency of dosage varies among practitioners.

The aim of this study is to examine the antiviral properties of selected traditional medicinal plants for specific use in the treatment of skin lesions associated with herpes simplex viral infections.

1.1. AIMS:

- To investigate the antiviral properties of traditional medicinal plants used by traditional healers for the treatment of herpes simplex virus.
- To extract and isolate the active antiviral component(s) from the medicinal plants selected for the study and to elucidate the chemical structure,

mechanism of action, therapeutic index and cytotoxicity of the active substance(s).

- To analyse and develop an effective and safe antiviral agent for the treatment of skin lesions arising from herpes simplex virus.
- To make a significant and valuable contribution to scientific research concerned with the development and advancement of herpes antiviral treatment and in doing so, to improve the long-term outlook for the management of herpes simplex virus.
- To provide scientific evidence supporting the belief of traditional healers in the therapeutic efficacy of medicinal plants used in the treatment of viral infection, hence acknowledging the significance of ethnomedicine in drug development. Results obtained from the study shall improve knowledge on the use, activity and availability of traditional medicinal plants.

1.2. OBJECTIVES:

- To develop a simple and efficient assay method for the extraction, isolation and identification of the active components of the medicinal plants under investigation. Furthermore, to establish whether activity is due to single or combined effects of the plant extracts.
- To develop a suitable cell line for the inoculation of herpes simplex virus and for the analysis of the antiviral properties of the plant extracts to be tested.
- To develop a suitable technique for the identification, evaluation and monitoring of *in vitro* antiviral activity.

1.3. HYPOTHESIS:

Traditional medicinal plants possess lead compounds that exhibit anti-herpes simplex type 1 activity.

CHAPTER II

2. BACKGROUND

2.1. HERPES SIMPLEX VIRUS

2.1.1. Viral Replication

Viruses are the smallest self-replicating structures known that contain nucleic acid and viral encoded and/or host encoded proteins. A virus is an obligate intracellular parasite and therefore lacks its own metabolic machinery required for translation and replication (Knight 1974, Luria et al. 1978, Pelczar, Chan & Kriep 1986, Foye 1989, Hudson 1994). To initiate replication, the herpes simplex virus genome must enter the cell causing infection. Within the cell, viral protein components self-assemble into complex structures capable of infecting other cells. The herpes simplex virus envelope contains 8 glycoproteins. The parasite replicates by providing the genetic blueprint for viral protein synthesized by the host. Through the formation of virus encoded regulatory proteins, the virus is then able to take over a host. The herpes simplex virus binds to specific receptors on the host cell and fuses with the host cell plasma membrane. Fusion with the cellular membranes requires the action of several viral glycoproteins. After fusion with the plasma membrane, the nucleocaspid that covers the DNA viral genome delivers the viral genome to the nuclear membrane of the host cell. This transfer is thought to result in the viral DNA being injected through the pore while the capsid remains in the cytoplasm. The transcription of the HSV genome during productive infection occurs with cellular transcriptional machinery and viral promoters utilising cellular transcription factor binding sites. During the three phases of viral transcription, the host cell machinery is invaded resulting in viral replication:

i. Alpha: immediate early – the DNA binding proteins promote the invasion of the host cell. Immediate early transcripts are transported to the cytoplasm, translated and the immediate-early proteins migrate back to the nucleus. All further transcription requires the action of these proteins

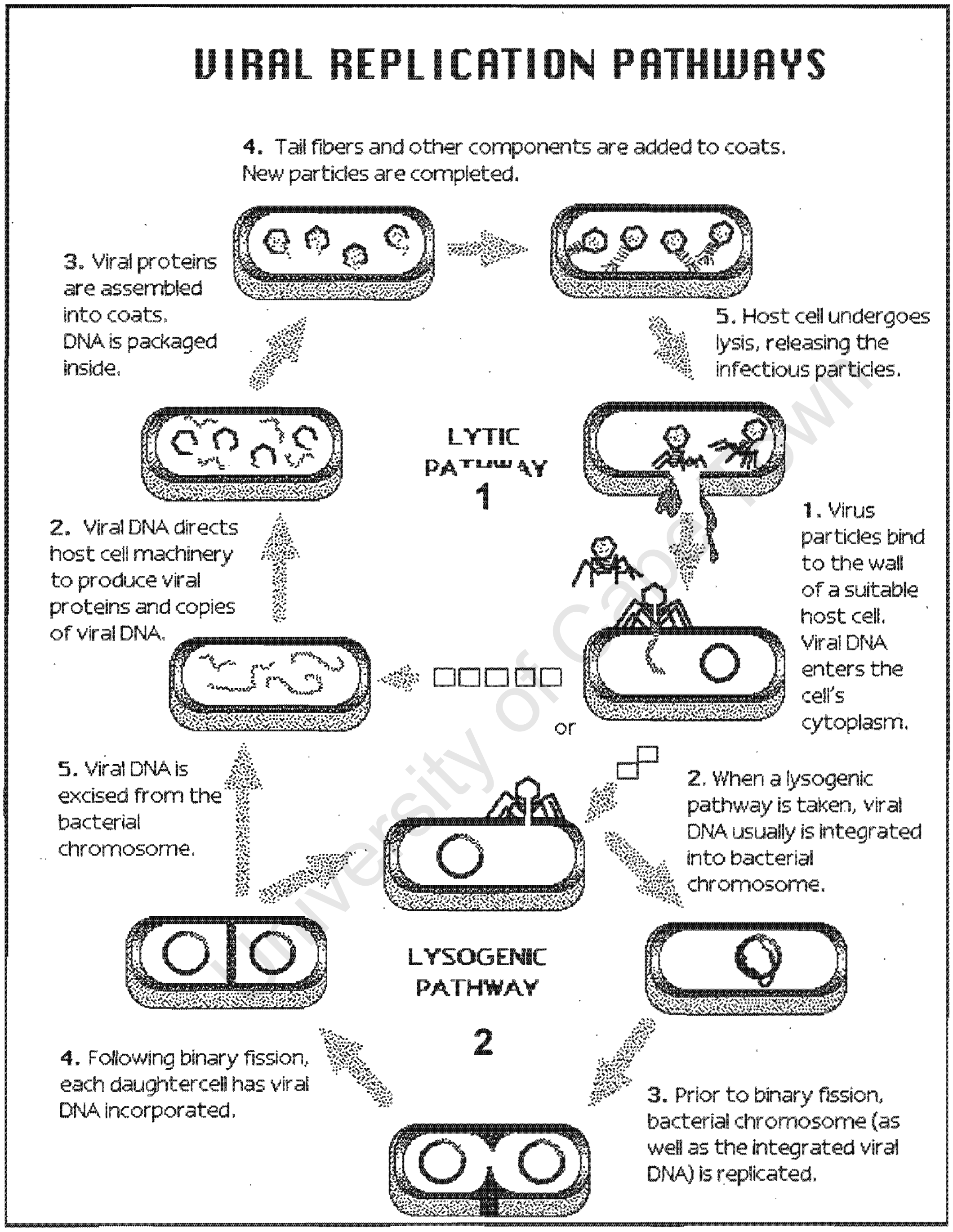
ii. Beta: delayed early – the proteins and enzymes, including viral DNA-dependent DNA polymerase required for replication are formed. As the genome replicates, it concentrates in discrete compartments. Late transcription takes place within these compartments.

iii. Gamma: late – major structural proteins, viral capsomers and glycoproteins, required to package the DNA are formed.

Viral replication occurs via two pathways (Figure 2, p22). The lytic pathway involves the lysis of the host cell's membrane resulting in cytoplasm leakage and subsequent cell death. Virus particles are released during cell death. The cytologic changes that bring about cell death are specific to the virus. It is this replication specificity that allows for the detection of cytopathic effects in in-vitro cell systems. The lysogenic path is one of several temperate pathways in which the host cell is not killed, but instead, is occupied by the virus and used as a factory for replicating the viral genes. The viral infection enters a latent period during this process.

The various steps of the viral replication cycle at which the virus deviates from normal host processes are potential targets for chemotherapeutic intervention. The main targets for antiviral agents are viral adsorption, penetration, uncoating and viral nucleic acid and protein synthesis. Specificity for infected cells may occur when virus-specified enzymes activate drugs.

Figure 2: Viral replication pathway
(http://www.accessexcellence.com/AB/GG/Sxamples of viral.html 2000a)



1. Lytic pathway: during lysis of the host cell membrane, cytoplasmic contents are leaked out resulting in cell death and the resultant release of viral particles.
2. Lysogenic pathway: the virus occupies host cells without causing cell damage. The virus uses the cell to generate viral genes.

2.1.2. *Pathogenesis*

The herpes simplex virus requires a moist environment for survival. Primary infection occurs through a break in the mucus membranes of the mouth or throat. Because of the universal distribution of the virus, most individuals are infected by one to two years of age. Virus transmission occurs by direct contact, during which the virus is transmitted onto a susceptible mucosal surface or through breaks in the skin. Local multiplication ensues, followed by viraemia and systemic infection. Thereafter, life-long latent infection follows and it is characterised by periodic reactivation (Corey & Spear 1986, Baringer & Swoveland 1972, Online 2000b).

During primary infection, the virus enters peripheral sensory nerves and migrates along axons to sensory nerve ganglia in the central nervous system. There, the virus becomes permanently latent in the nerve root ganglia that correspond to the cutaneous or mucous membrane site of inoculation. Once the acute infection resolves, small amounts of viral DNA and specific types of antisense messenger RNA can be detected in ganglion cells. During this time, *in situ* hybridisation techniques demonstrate viral DNA but not viral antigens or complete viral particles. The delicate balance of latency may be upset by various disturbances that may be physical, such as injury and U.V. exposure, or psychological such as stress and emotional upset. During reactivation, virus replication occurs within the ganglia and progeny virions travel peripherally along sensory nerves to the mucosal or epithelial surface innervated by the reactivated ganglion. Active virus replication at the cutaneous surface then produces clinical symptoms and lesions that are typical of recurrent HSV infection.

The severity of infections is dependent upon:

- The cytopathic effect of the virus. The cytopathic effect includes toxic viral products and/or the accumulation of viral products.
- The virus inoculum size.
- The healing capacity of the host.
- Host genetics.
- Age

- Viral encoded virulence factors that include replication capabilities, and immune response evasion.
- Attenuated strains resulting in diminished virulence.

2.1.3. Viral Immune Defences

Infection with HSV induces cell-mediated immunity and the production of type-common and type-specific antibodies (Doherty 1998, Biron 1994). Although these immune mechanisms apparently do not affect the development of latent infections or the frequency of recurrences, they may modulate the severity of clinical recurrences and reduce HSV replication once reactivation occurs. The immune response to infection appears to provide partial protection against subsequent infection with HSV.

Cell mediated immunity refers to:

i. The recognition and/or killing of the virus and virus infected cells by leukocytes.
ii. The production of cytokines by infected cells when stimulated by the virus or virus-infected cells (Herberman & Ortaldo 1981).

Cytotoxic T lymphocytes, natural killer cells and antiviral macrophages recognise and kill virus-infected cells. Helper T-cells recognise virus-infected cells and produce several important cytokines. Cytokines produced by monocytes, T-cells and lymphokines play an important role in regulating immune functions and developing antiviral immune functions. The early non-specific responses limit virus multiplications during the acute phase of infections. The function of later specific immune responses is to help eliminate the virus at the end of the acute phase, and subsequently to maintain specific resistance to reinfection. Table 1, p25 gives a summary of immune responses to viral infection.

Table 1: A summary of immune responses to viral infection
(http://www.md.huji.ac.il/microbiology/book/ch050.html)

Host defence	Time of first detection	Effector	Effector target
Early non-specific response	Hours	• Fever • Phagocytosis • Inflammation • Natural killer cell activity • Interferon	• Virus replication • Virus • Virus-infected cells
Immunomodulation and cell mediated immune responses	Days	• Cytotoxic T-Lymphocytes • Activated macrophages • Lymphokines	• Virus-infected cells • Virus • Immunomodulation
Humoral immune responses	Days	• Antibody-dependent cell mediated cytotoxicity • Antibody • Antibody and complement	• Virus-infected cells • Virus

In addition to the above-mentioned cellular responses, newly induced host cell proteins stimulate interferon expression. Secreted interferon binds to cells and induces them to block various stages of viral replication. This process results in uninfected cells being resistant to the same or other super-infecting viruses (Pelczar, Chan & Krieg 1986, Bowman & Rand 1990). The activities of interferon include the inhibition of the growth of some normal and tumour cells and several intracellular parasites; modulation of the immune response and manipulation of cell differentiation. There are three main types of interferon, alpha, beta and gamma. Alpha interferon is produced mainly by certain leukocytes; beta interferon by epithelial cells and fibroblasts, and gamma interferon by T and natural killer cells. Three types of observations identify the important role played by interferon as a defence mechanism. First, for many viral infections, a strong correlation has been established between interferon production and natural recovery. Second, inhibition of interferon production or action enhances the severity of infection; and third, interferon offers protection against infection.

2.1.4. Anti-Viral Chemotherapy

The successful treatment of viral conditions is dependent upon the selective toxicity of the principle agent. Ideally, an antiviral drug is considered effective if it is able to interfere with viral replication processes without affecting the normal cellular metabolic processes, nucleic acid and protein synthesis of the host (Foye 1989, Bean 1992). Unfortunately, several of the early antiviral agents were shown to be too toxic as they inhibited cellular nucleic acid or protein synthesis (Vanden Berghe, Vlietinck & Van Hoof 1986). More recently, antiviral agents that target specific viral functions have been developed thus protecting host cellular functions. Despite considerable research into antiviral agents coupled with improved understanding of viral molecular biology and the complexity of virus-host interactions, few antiviral agents are available. Antiviral agents can be divided into two categories (Crumpacker 1989, De Clercq 1993).

i. Those that interact directly with their target and;
ii. Those that require initial intracellular activation by phosphorylation giving rise to the active forms.

Most antiviral agents are nucleoside analogues which inhibit viral polymerase and therefore, reduce viral replication.

Vidarabine, idoxuridine, trifluorothymidine and acyclovir are the approved antiviral agents for the systemic and local treatment of herpes simplex virus (Fields, Knipe & Howley 1990). Idoxuridine and trifluorothymidine exhibit myelosuppressive, mutagenic and teratogenic properties and therefore are limited to topical applications for the treatment of cutaneous and mucocutaneous herpes simplex lesions. Vidarabine is used for both topical and systemic treatment of HSV infections.

Few antiviral agents have been discovered through rational drug design whereby molecular targets are first identified and drugs are then tailored on the basis of the molecular configuration and action of the protein (Vanden Berghe, Vlietinck & Van Hoof 1986, Vanden Berghe et al. 1978, Hudson 1994, McCutcheon et al. 1995, Sindambiwe 1990, Vlietinck & Vanden Berghe, 1991, Garcia 1999, Kott 1999, Kudi & Myint 1999). In most situations, the antiviral activity of agents is first detected, often by chance, and their molecular targets determined later (Vanden Berghe, Vlietinck & Van Hoof 1986, Hudson 1994, Sauter & Wolfensberger 1989). Due to the intracellular activities of viruses, they present with more difficult targets than do more autonomous microorganisms.

Idoxuridine and Trifluridine

These agents are limited to local administration due to their myelosuppressive, mutagenic and teratogenic effects. Both Idoxuridine and trifluridine interrupt the DNA replication process resulting in impaired viral DNA. To achieve this, the prodrug of each is converted to a 5-monophosphate which becomes incorporated into viral DNA in place of thymine. Viral death is accomplished through the formation of incomplete virus particles that are unstable to support viral replication (Foye, 1989).

Vidarabine

The activity of vidarabine is believed to be brought about by the inhibition of virus-specified DNA polymerase by the active triphosphate (Bowman and Rand 1990, Foye 1989). Vidarabine is used topically and systemically for the treatment of herpes simplex virus infections. The agent is poorly soluble and therefore requires large volumes of solvent when administered intravenously. Due to the various toxic side effects such as tremors, seizures, myalgia and ataxia, vidarabine is not the treatment of choice for HSV infections.

Acyclovir, Valaciclovir, Famciclovir

These agents are phosphorylated to the monophosphate by HSV-specific thymidine kinase followed by di- and triphosphorylation by cellular enzymes (Alrabiah & Sacks 1996, Fields, Knipe & Howley 1990). The active triphosphate selectively inhibits viral DNA polymerase and causes the termination of viral DNA synthesis (Whitley 1992). The main indications for the use of acyclovir are primary genital herpes, herpetic encephalitis, and HSV infections in immunosuppressed patients (Fields, Knipe & Howley 1990, De Clercq 1995). Acyclovir can be used topically, intravenously, or perorally. Valaciclovir and famciclovir act as produgs of acyclovir and penciclovir respectively. The activity of penciclovir is similar to acyclovir, although the former generates higher intracellular levels of the active triphosphate form.

Interferon and interferon-inducers

Interferons are proteins that are formed in almost all kinds of animal cells infected by viruses. When they are released from infected cells, they protect uninfected cells from subsequent viral-induced damage by inhibiting viral replication in those cells. Interferons are species-specific, but not virus-specific. As a result, interferon prepared for human use must be purified from human sources or perhaps from other primates. This process is costly and laborious since large sample quantities are required due to the short plasma half-life of interferon. The short half-life is due to the rapid distribution of interferon throughout the body and its rapid activation and excretion (Bowman & Rand 1990, Foye, 1989, Pelczar, Chan & Krieg 1986).

2.1.5. Anti-Viral Drug Resistance

Several publications have reported an increased incidence of severe mucocutaneous disease due to increased drug-resistant HSV infections in recent years (Online 2000c). This increase has been largely attributed to the HIV epidemic, as the majority of patients with drug-resistant HSV infection are HIV infected and have CDC (Centre for Disease Control)-defined AIDS diagnosis. A deficiency of thymidine kinase activity in the resistant HSV strains accounts for the majority of resistant mutants. In the absence of thymidine kinase, acyclovir and penciclovir are not phosphorylated to triphosphate active agents (Steinstrasser & Merkle, 1995, Vogel 1993). Thymidine kinase deficient strains have been shown to be pathogenic in patients with advanced HIV disease, as demonstrated by the high incidence of severe mucocutaneous disease and occasional visceral infection in this population (Online 2000c). Alternative mechanisms of drug resistance in HSV include mutants with altered thymidine kinase substrate specificity and altered DNA polymerase (Steinstrasser & Merkle, 1995, Unander, Webster & Blumberg 1995, Online 2000c).

2.1.6. Management Of Herpes Simplex Virus In Immunocompromised Patients

The incidence of HIV/AIDS in sub-Sahara Africa is a major concern. While effective retro-virals are being investigated, work directed at reducing the occurrence of opportunistic infections is equally important for the improvement and maintenance of a patient's health status. Phytomedicine research can provide viable solutions to the problem of drug resistance by making available novel active compounds. Such investigations are likely to increase the understanding of the mechanisms by which viral organisms effect drug resistant activity while simultaneously introducing lead antivirals with more effective mechanisms of action.

Local care of mucocutaneous lesions is important for comfort and the prevention of secondary infection in HIV and organ transplant patients. Treatment with acyclovir or valacyclovir decreases local symptoms, accelerates lesion healing, decreases virus shedding, prevents new lesion formation and decreases the risk of lesion progression or dissemination (Steinstrasser & Merkle, 1995, Wickberg 1993, Online 2000c). Suppressive oral acyclovir therapy is utilised in patients with frequently recurring or severe HSV recurrences.

Viruses are responsible for up to 33% of deaths in organ transplant populations (Herfindel, Gourley & Hart 1992). The mortality from viral infection is high amongst patients receiving organ transplants due to severe immunosuppression to prevent rejection of the transplanted organ. Viral infection can be either a primary infection or a reactivated infection where the virus is harboured in a nerve. The main goal of treatment of such viral infections is to prevent the spread of the virus systemically. The treatment of choice is acyclovir, which has been shown to be highly effective in several different studies (Herfindal, Gourley & Hart 1992, Steinstrasser & Merkle, 1995, Vanden Berghe et al. 1978, De Clercq 1984).

2.1.7. Limitations Of Antiviral Drugs

The clinical use of many existing anti-viral agents is limited by their toxic side effects. These side effects are specific for each compound. There are also general limitations inherent in antiviral chemotherapy. Firstly, the more selective the antiviral agent, the narrower its antiviral activity spectrum and therefore, in the absence of specific symptoms of viral infection it is difficult to determine the efficacy of an antiviral drug (Foye 1989). Secondly, since antiviral drugs target steps in viral replication, the latent phases characteristic of herpes viral infections are not amenable to chemotherapy. In this situation, problems in the administration of the antiviral drug are experienced as intervention often occurs much too late to inhibit the early stages of viral replication. Therefore, the eradication of latent viral infections is not yet feasible. Thirdly, antiviral drug treatment should begin early, before irreversible tissue damage has occurred. Such early diagnosis is difficult to make due to a lack of effective early and accurate diagnosis techniques. Fourthly, the problem of drug resistant human immunodeficiency virus strains isolated from AIDS patients has presented concern due to resistance to virtually all specific virus inhibitors.

2.1.8. Anti-Viral Drug Discovery And Development

It is believed that one of the most promising approaches that can be used for the screening of new antiviral agents from plants is to screen plant extracts for antiviral activity followed by bioassay fractionation of active plants leading to the isolation of the pure constituent (Vanden Berghe, Vlietinck & Van Hoof 1986, Semple et al.

1998, McCutcheon 1995, Vlietinck et al. 1995, Vanden Berghe et al. 1978, Pinilla & Luu 1999). It is estimated that 900 plant species from about 150 different plant families have been analysed for antiviral activity (Balandrin et al. 2001 , Vanden Berghe, Vlietinck & Van Hoof 1986). The active component of only 37 species has been partly or completely characterised. The reason for this low figure is that in several situations, the original proposed antiviral activity was too low, non-specific or only detectable in toxic concentrations for the host. Studies have shown that antivirals having significant chemotherapy based on the viability of cells were mainly 3-methoxyflavone derivatives. Other substances, including polysaccharides, alkaloids, phenolics, and triterpenes exhibited extracellular virucidal activities or interfered with the host replication process at specific concentrations. The spectrum of activity of these agents has ranged from adsorption of virus to the host cells to their release. In addition to direct antiviral action, these agents can potentially be used to complement existing antiviral drugs. The difficulty of finding active therapeutics has accelerated the search for new agents (McCutcheon 1995, Vanden Berghe et al. 1978). Ethnopharmacology provides an alternative approach to drug discovery and deviates from the present situation of attempting to design narrow spectrum drugs for specific molecules.

2.1.9. Laboratory Diagnosis

Laboratory diagnostic studies are commonly used to confirm a suspected diagnosis of HSV infection. Of all laboratory diagnostic techniques available, the preferred procedure of choice is direct virus culture of material from suspected herpes lesions. Diagnosis by viral culture is more sensitive and specific than demonstration of multinucleated giant cells or inclusions by Tzanck smear; direct staining of infected cells for virus antigen; antibody detection and identification of virus particles by electron microscopy (Online 2000c). Microscopic examinations of scrapings from lesions using staining techniques show the characteristic cell morphology of HSV infection, however, this method can result in a false negative. Serologic tests for detecting antibodies to HSV are helpful in primary infections since antibody titres remain virtually unchanged during recurrent disease.

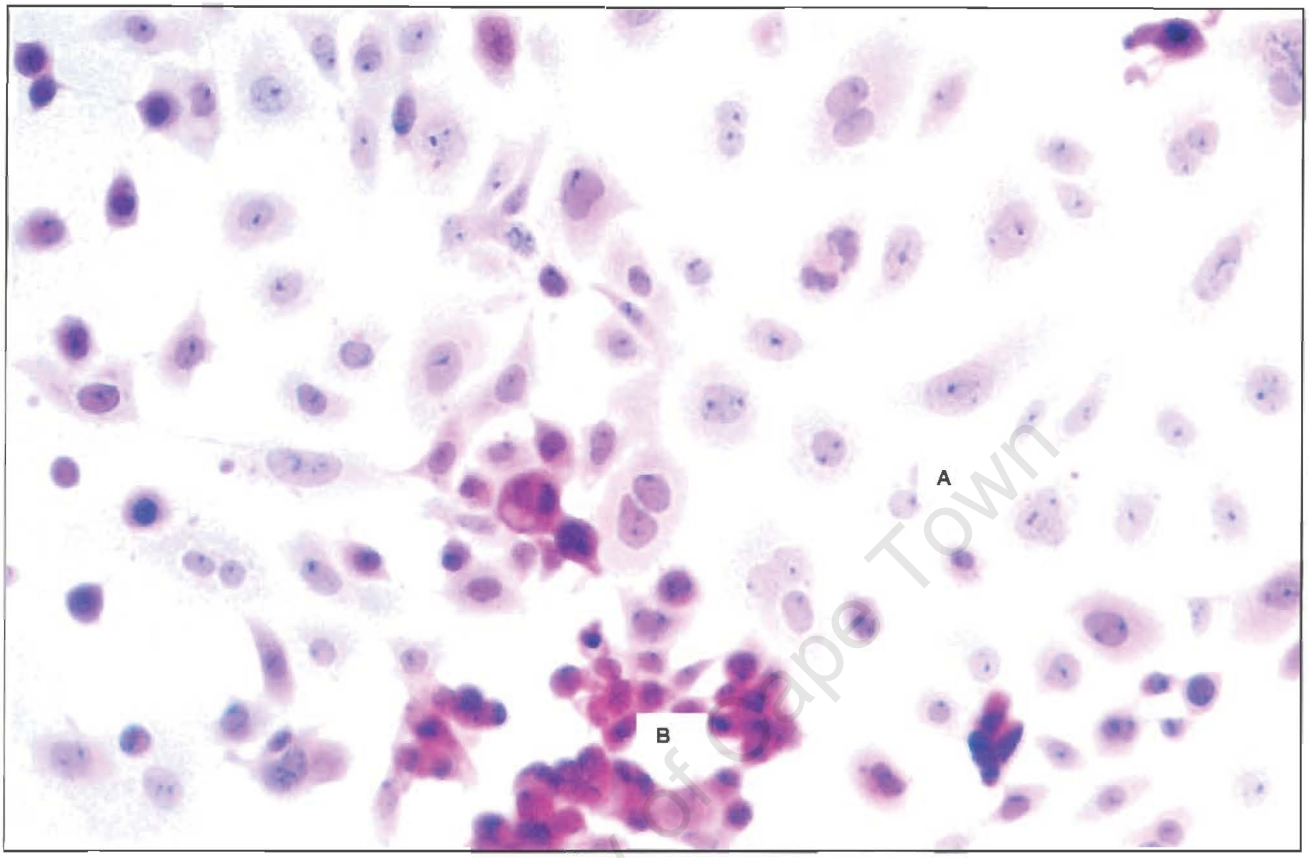
Antibodies may also cross-react with both HSV-1 and HSV-2 due to antigenic similarities (Herfindal, Gourley and Hart 1992, Doane 1980). Laboratory diagnoses of viral samples used during *in vitro* experimentation were determined using serologic tests.

2.1.10. Cytology

There are various ways to monitor viral growth:

i. Cytopathic effects, which include cell lysis, inclusion body formation, giant cell formation, and cytopathic vacuolisation (Figure 3, p33), (Ballew 1975).
ii. The appearance of a virus-specific protein, for example hemagglutination of influenza virus. The agents formed are generally detected by specific antisera to the protein of interest.
iii. The adsorption of erythrocytes to virally infected cells resulting in a positive reaction before any visible cytopathic changes.
iv. Interference during which a non-cytopathic virus interferes with the replication of a cytopathic virus in the infected cell.
v. Morphologic transformation which is generally associated with infection of cells by oncogenic viruses. Lack of contact inhibition or piling up of cells may occur (Online 2000c)

Figure 3: An illustration of cytopathic effects by HSV on Vero cells



Above figure shows the typical cell morphology of Vero cells viewed microscopically. A refers to cells uninfected with HSV. B shows herpes simplex virus infected cells chracterised by clumping and cell rounding. Cell staining technique is explained in appendix 3, p219.

Cytopathological effects (CPE) may be indicative of the type of virus present. To obtain CP effects, host cells are grown directly from an infected patient. Alternatively, cell lines are exposed to virus-containing patient serum. These virus-containing cells are then grown for observation. The damage caused by the virus to the host cells in which they grow results in a change in cell morphology that appear microscopically different to normal uninfected cells. This change in cell morphology can be observed as infection progresses and it is characteristic for a given virus-cell combination. The observed changes to a viral infected cell can be vaculolation and/or cell lysis resulting in the disintegration of the cell layer. Viruses with cell fusing properties undergo syncytial formation resulting in giant cells with characteristic forms. Although CPE are typical for a virus, variations may occur due to differences in cell strains and conditions (Catalogue of strains 1981). Characteristic CP effects were noted during experiments in which HSV was inoculated onto a Vero cell line.

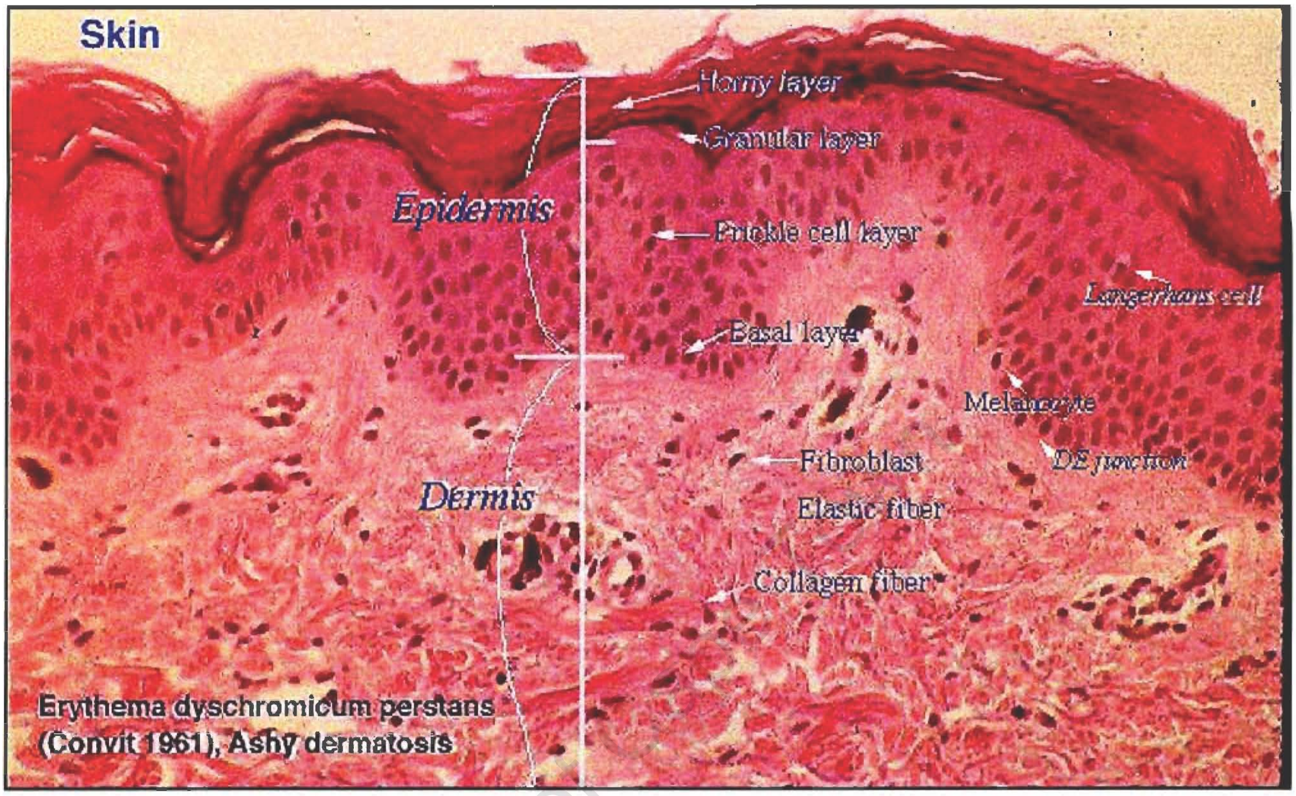
2.1.11. The Skin

Herpes Simplex Virus infections mainly inflict the skin and mucosal surfaces. The physiological changes that take place on the skin surface during infection will impact the choice of treatment and the efficacy of topical applications. It is therefore necessary to gain some understanding of the physiology of normal skin and the changes that occur to it during herpes infection. Skin tissue consists of aggregates of closely packed cells that have both lipid and aqueous regions. The regulatory functions of the skin include the maintenance of temperature and blood pressure while its protectional function offers defence against physical and chemical damage and invasion by microorganisms (Michel et al 1997). The skin is a heterogeneous structure consisting of stratified levels which can be divided into three distinct tissue layers, the epidermis, dermis and subcutaneous fat. Adding to the complex nature of skin are blood vessels, hair follicles, glands and nerves which traverse the layers of this organ (Figure 4, p36), (Barry 1983, Chien 1987, Danckwerts 1991, Guy & Hadgraft 1985, Hadgraft 1993, Zatz 1987).

The epidermis, which is in contact with the external environment, is a non-vascular layer pierced by hair follicles and sebaceous glands that allow for the passage of nutrients and waste products from the dermis to the skin surface (Doherty 1988). Anatomically, the epidermis varies in thickness, with the palms and soles being the thickest regions while the eyelids are the thinnest area of the skin (Knight 1974, Biron 1994). The outermost layer of the epidermis comprises stratified epithelial squamous cells called the stratum corneum (Herberman & Ortaldo 1981). It is this external layer which forms the physical barrier of the skin to exogenous material and infection. Immediately below this keratinised epithelial tissue lies a monolayer of constantly dividing cells called the stratum basale. The stratum basale produces keratinocytes which migrate towards the skin surface where they ultimately form the cells of the stratum corneum. Additionally, the stratum basale contains melanocytes that produce the pigment melanin. The melanin produced is transferred to cells of the hair and epidermis. There, they are ingested by the keratinocytes forming melanin granules around the nucleus of each keratinocyte. Changes in the activity of melanin by melanocytes influence the degree of skin pigmentation (Boukamp et al. 1988). The greater the amount melanin entering keratinocyte cells, the darker the skin observed. Oxygen and nutrient deprivation of mitosing cells from the stratum basale causes shrinking and a loss in the metabolic activity in these cells. As the cells reach the skin surface, they become flattened keratinised laminates which are eventually removed from the surface by sloughing. It takes approximately 4 weeks for a keratinocyte to migrate from the basal region to the stratum corneum and eventually to the outer layer where it is removed by abrasion. Although the stratum corneum is highly impervious to the ingress and egress of water, when it is immersed, it is capable of absorbing large quantities of water. Various intercellular lipid processes such as cholesterol, fatty acid and sphingolipid synthesis are known to occur within the epidermis. The barrier function of the stratum corneum is influenced by the presence of these intercellular lipids and inhibition of the synthesis of these components can result in delayed barrier formation, abnormal lamellar bodies and progressive barrier perturbation. Enzymes are also involved in skin barrier homeostasis (Steinstrasser & Merkle 1995). Diminished enzyme production is said to arrest hydrolysis and the resultant water loss from the skin surface may lead to diminished barrier efficacy.

The pathogenic potential of microorganisms, including viruses, is dependent, amongst other things, on the physiological condition of the stratum corneum. Damage to the stratum corneum decreases its barrier efficiency. The barrier properties of skin can be compromised by various conditions and situations ranging from water loss to reduced immunity and genetics.

Figure 4: An illustration of skin histology showing the epidermal, dermal layers (http://www.medic.mie-u.ac.jp/derma/anatomy.html)



2.2. FIELD WORK

2.2.1. Introduction

Southern Africa contains one of the richest diversity of plant life, most of which is concentrated in a relatively small geographical area. Approximately 20 000 species are known to be endemic and occur within the country's borders, of which 80% are unique to South Africa (White paper on Biological Diveristy 1997). In addition, South Africa has a rich heritage of indigenous knowledge relating to the medicinal use of its plants. In a recent study, it was shown that about 80% of South Africans consult the approximately 250 000 traditional healers who form a significant part of the primary health care system (Kale 1995, Edinburg 1998, Abdool-Karim, Ziqubu-Page & Arendse 1994). Therefore, opportunities of utilising the country's indigenous flora and knowledge as means of enhancing the health and quality of life of local people were recognised.

The discovery of new drug leads or the introduction of cheaper, safer and more effective botanicals can contribute significantly to easing the burden of disease and alleviating pressure on the provision of medicines. An important aspect of these ethnomedical initiatives is that researchers continually add value to traditional knowledge through scientific investigation of local plant species (Aquino et al., 1889, Bohlin, 1993, Borris, 1996, Abdool-Karim, Ziqubu-Page & Arendse 1994). It has long been established through history that scientists, in their attempts to gain an understanding of traditional systems of medicine, once profited by obtaining crucial knowledge from indigenous communities. The socio-political and ethical issues that have arisen as a result of such exploitation has forced researchers of natural plant products to redress the manner in which they work with traditional healers (Richter & Carlson 1998, Burger & Crabbe 1984, Hess 1998, Banister & Barrett 1998). Therefore, the work performed here has taken place in collaboration with traditional healers, subject to agreements that define confidentiality, intellectual property rights and reciprocity (Mshana 1999, Posey & Dutfield 1996, Chand et al. 1996, Online 1999b). This section discusses the research strategy adopted to collaborate and engage with traditional practitioners regarding practices and uses of traditional medicines.

2.2.2. Defining Indigenous Knowledge Systems (IKS)

In order to understand and respect traditional medical knowledge, it is essential that the researcher is aware of the factors that govern and influence this information base (Brouwer 2000). This exercise serves to enhance one's sensitivity towards a previously disenfranchised group of influential community health providers. Many attempts have been made to define an indigenous knowledge health system, however, little, if any, success has been achieved (WIPO Report, 2000). Often, definitions of indigenous knowledge (IK) have tended to be controversial or partial and have thus been misguiding and distortive. The reason for this is that traditional knowledge, embedded in the culture of communities, has become tacit and uncodified knowledge that, for example, exists in linguistic nuances or long established codes of living. In other words, the knowledge does not conform to a standardized structure that enables easy access (WIPO Report, 2000). Hence, it is easier to characterise traditional health knowledge rather than seek to define it. Nine key factors that help to characterise this knowledge system are as follows:

1. Historical locality – traditional health services usually exist within communities that share a specific and unique culture that has existed for centuries.
2. It is predominantly rural – urbanisation has resulted in the erosion of many traditional knowledge systems and customs due to the adoption of Western living practices resulting in increased utilisation of Western medicine.
3. It is both tangible and intangible – the process of preparing medicinals is an intangible form of traditional knowledge whilst the actual plant part(s) used is the tangible form of this knowledge.
4. It involves bio-diversity, which is the interplay between human nature and the local ecosystem.
5. It involves the use of technology that has been handed down through demonstration, repetition and imitation – that is; it is an integral part of customs and practices that have existed in communities since time immemorial.

6. It is deeply embedded in a culture, traditions, customs, myths and folklore that have become an important source of traditional knowledge.
7. It may be communal and therefore, it becomes shared knowledge, which is then known as "collective consciousness". This feature has caused controversy between healers and ethnoresearchers with respect to the ownership of such knowledge as it refers to information in the public domain.
8. It is an identity by which members of a community identify themselves and in turn, are able to identify with a community.
9. It serves cognitive interests that include:
   - Technical interests which yield technological knowledge
   - Moral interests which yield values
   - Critical interests which yield culture.

2.2.3. Value Added

Until recently, Western scientific culture disregarded the importance of traditional systems and practices mainly because the knowledge is not presented in conventional forms. Modern science's failure to appreciate traditional culture has resulted in the misconception that local practices are primitive and the knowledge is limited. Despite the negative undertones that plague traditional medical systems, natural products research has benefited substantially from traditional practitioners' knowledge of indigenous biodiversity (Brouwer, 2000, Burger & Crabbe, 1984, Cordell & Shin, 1999, Van den Berghe, Vlietinck & Van Hoof, 1995, Vleitnck et al., 1995). Ethnomedical information has provided researchers with a tremendous variety of lead structures that can be used as templates for the development of new drugs (Borris 1996). It is estimated that 15% of known plant species has been analysed for therapeutic potential and only 1% has been examined extensively (Scott 1993). The plant kingdom possesses an array of elements that possess unique activities. Knowledge gleaned from the local plant kingdom can be used to generate valuable botanical information for science and medicine. Furthermore, bioassay technology and chemical methodology has progressed considerably thus making natural products development a cost effective and viable source for new leads (Borris 1996).

Recent study reports and investigations have increased the attention on local medicinal plant markets (Online 1999a, Hamilton 1999 , Macilwain 1998, Williams, Balkwill & Witkowski 1997, Glasser 1999). The traditional medicines market is an already thriving industry in South Africa, which has been allowed to operate without monitoring and regulation. Scientific plant inventory research into the safety and efficacy of local medicinal plants shall provide valuable information for regulatory bodies that require some control over the industry. Furthermore, consumers who expect assurance on the safety, efficacy and authenticity of medicines shall have ready access to research findings (Williams, Balwill & Witkowski 1997).

For the poor majority who cannot afford medical fees for conventional health care, traditional health then becomes the more economical option. Health provision in South Africa, like in many other developing nations, is constrained by the high costs of pharmaceuticals. Alternative health systems approaches need to be considered to reduce escalating health costs and control the burden of disease caused by parasitic agents. Investigation into the efficacy of plant-derived medicines is one such health systems approach which has the potential to give rise to readily accessible pharmaceuticals. Scientific studies can be used to validate medicinal plants and researched herbal preparations can be recommended for use.

2.2.4. Planning of Field Trip

Increased research amongst ethnic communities has resulted in the development of new research strategies designed to optimise the interaction between communities and scientists whilst ensuring that ethical codes of conduct are adhered to (Mshana 1999, Chand et al. 1996, Doms 1998). Current studies show that inter- and multi-disciplinary approaches to ethnobotanical research can lead to more meaningful results for ethnoscientists. To optimise the outcomes of the field trips organised, local participatory rural appraisal models were investigated to understand the primary health care needs of local societies and the role that traditional healers play in health service delivery (Chun 1998, Cocks & Dold 1998, King 1998). Participatory rural appraisal describes a system of approaches, methods, behaviours and conditions that make people, seeking treatment, to plan themselves, take action, monitor and evaluate their health. The use of the participatory rural appraisal techniques provides a thorough and systematic understanding of the interactions between indigenous peoples and the plants they use (Cock & Dold 1998). An awareness of the factors that influence healer relations and therapeutic management, improves the competence of a researcher in obtaining the desired information without causing offence to the interviewee. Lastly, knowledge of participatory rural appraisal enables the researcher to develop the skills to make sense of the significance of certain practices and to be able to interpret these cultural issues in the correct manner. This improves the efficiency of collecting and recording information.

The planning of the fieldwork began with identifying national regions in which viral conditions are common. In this instance, viral skin disorders are a common affliction amongst all South African communities. However, the incidence of genitally related herpes viral infection is likely to be higher in areas where sexually transmitted diseases (STDs), including HIV/AIDS, are more prevalent. The Kwazulu-Natal province is such a region and therefore its inclusion in the trip was significant. To broaden the consultancy base, an attempt was made to visit an equal number of healers residing in rural and urban areas of the different provinces.

2.2.4.1. Discussions With Healers

Discussions with healers were held in informal settings at a venue chosen by the healer. All conversations were recorded using note taking and audio equipment for future reference and visits took place within the working environment of the practitioner. This made it possible to view facilities used by practitioners, examine the storage conditions and preparations of plant remedies and, in certain cases, discuss with local communities and apprentices aspects of traditional healing. With each healer, the natural history of the disease and its perceived cause and transmission amongst local people was discussed. An understanding by the traditional healer of the disease in question was important as any misinterpretation could lead to incorrect diagnosis. To ensure complete comprehension, the healer was questioned about the signs and symptoms of viral skin conditions, how the patient feels, the physical presentation and consequent examination of the patient, the duration of the disease, disease stages, morbidity, gender, age, lifestyle, incidence and prevalence. This line of questioning encouraged the healer to expand on the issue thus discussing it at length. Where necessary, a case presentation was used to stimulate discussion. In cases where medicinal plants were within the locality of the meeting venue transect walks were taken with the traditional practitioner. This enabled the researcher to visit and view the plant collection area. During the walks, the value and importance of the plant parts, collection area and time of collection were discussed at length. The conditions under which dialogues with healers were conducted varied and after numerous meetings of this nature, it was found that a semi-structured approach with limited numbers of participants resulted in the expression of important qualitative and quantitative information. Through such communication between researchers and healers, it was possible to promote intercultural dialogue, improve ethnomedical research methodologies, learn from and with healers and test the effectiveness and relevance of ethnomedical explorations.

2.2.4.2. Determining Informed Consent

It is still not uncommon to find that research of traditional knowledge systems, practices and indigenous communities takes place without sanction or prior consent from the peoples concerned. This has resulted in the wrongful expropriation of the cultural and intellectual knowledge of the affected peoples (ISE Code of Ethics 1998, Macilwain 1998, Masood 1998, Dove 1998, Mshana 1999, Posey & Dutfield 1996). To avoid causing harm to and violating the rights of indigenous communities a set of guidelines and principles were formulated ahead of the field trips (Appendix 2, p218). To optimise understanding, the purpose of the research; relevant affiliations; the use of the information given; possible outcomes of studies and the potential benefits to indigenous communities were explained in simple, non-scientific terms. The guidelines were verbally translated into the local language and discussions were conducted with healers to determine their understanding and suitability of the principles stated. An attempt was made to attain wide consultation with the traditional healing fraternity and obtain approval for the research undertaking. Factors adopted to ensure that informed consent was reached using ethical approaches included:

- ensuring that adequate information was rendered such that the traditional healer gained sufficient understanding of the information and issues regarding just compensation.
- ensuring that the healer was allowed sufficient time to assimilate information given and formulate a response.
- providing information in a manner that conformed to local consultative customs and procedures.
- recording conversations with traditional practitioners.
- ensuring that the informant(s) were not subjected to any undue pressures.
- ensuring that consent was given freely and willingly.
- ensuring that the customs and traditional practices of the individual(s) were not compromised in obtaining consent.
- ensuring that traditional healers were aware of all stages of the project from inception to completion. This was achieved through regularly updating and informing healers about the progress of the research and application of results.

Not all healers consented to participating in the research and their right to decline disclosure was respected in these cases.

2.2.4.3. Collection of Plant Material

A representative list of plants used in the treatment of viral conditions by ethnic communities in Eastern Cape, Mpumalanga, Northern Province, Gauteng and KwaZulu-Natal was compiled. The acquisition of the plants was carried out in June 1998 and they were predominantly collected from the KwaZulu-Natal region with additional plants obtained in the Eastern Cape (December 1998) and Western Cape (February to March 1999). From the forty-six plant species listed on the ethnobotanical list compiled following a literature survey and interviews with medicinal plant practitioners, twenty-two plant specimens were collected in total and tested. The remaining plants could not be obtained due to traders being unable to recognise the ethnic name given by a healer. Attempts to search for the vernacular names in literature sources proved unsuccessful too Table 2, p45. provides a listing of the ethnobotanical list compiled. Plants showing potential antiviral activity were later identified and verified by a botanist of the National Botanical Institute's Kirstenbosch Gardens. Samples from these identified species were taken and used in subsequent experimentation.

The trading of medicinal plants is a growing business in South Africa and provides the public with easy access to phytomedicines. Some plant material was sourced from formal herb traders occupying fixed licence stores. In these instances, most shop proprietors were traditional healers who were able to verify information and confirm the plant material purchased.

Transport packaging of plant material collected was carried out by placing the plant in plastic bags that allowed free airflow. Attached to each bag was a labelling tag on which information on the Latin and Vernacular names of the plant, collection date and time, plant part collected and site of collection were recorded. Where possible, plant material was obtained directly from healers. Plant material that had to be purchased was stored and labelled likewise. However, where plants were obtained from formal and informal traders, one was reliant on them for information on the date and site of collection.

Table 2: Ethnobotanical list of plants with potential antiviral activity

FAMILY	GENERA	AFRICAN NAME	REGION	PLANT AREA USED
ACANTHACEAE	*Hypoestes aristata*		EC/KZN/SEC	root/ bark
AMARYLLIDACEAE	*Clivia cyrtanthiflora*			
AMARYLLIDACEAE	*Clivia miniata* Regel		KZN/MP/SD	rhizome/ roots/ leaves
	Hymenocallis littoralis			
AMARYLLIDACEAE	*Crinum bulbispermum*	umduze (inembe)	KZN/MP	bulbs (whole plant)
APIACEAE	*Alepidea amatymbica*	ikhatazo	MP/KZN	rhizome/ roots
APIACEAE	*Centella asiatica*	icukudwane	SD/MP/GP	leaves
APIACEAE	*Foeniculum vulgare*	imbozisa	MP/KZN	leaves/ stems
APIACEAE	*Lichtensteinia interrupta*	imbozisa	MP/KZN	roots
APOCYNACEAE	*Rauvolfia caffra*	umhlambamanzi	KZN/MP/SD	bark/ root
ARECACEAE	*Acorus calamus* L.	ikalamuzi	KZN/MP	rhizomes
ASCLEPIADACEAE	*Xysmalobium undulatum*	ishongwe	KZN/MP	tubers
ASPHODELACEAE	*Bulbine natalensis*	ibuchu	KZN/MP/SD	stem/ roots
COLCHICACEAE	*Gloriosa superba* L.	ihlamvulomfana	EC/KZN	fruit/ leaves/ root/ corm
ASTERACEAE	*Artemisia afra*	umhlonyane	MP/KZN/WC	leaves
ASTERACEAE	*Helichrysum aureonitens*		MP/KZN/WC/EC	leaves/ stem
ASTERACEAE	*Senecio serratuloides*	insukumbili	KZN/MP	leaves
BIGNONIACEAE	*Kigelia africana*	umfongothi	KZN/MP/SD	fruit/ bark
CANELLACEAE	*Warburgia salutaris*	isibaha	KZN	stem bark
EUPHORBIACEAE	*Bridelia cathartica*		MP/KZN/SD	stem/ root/ bark
EUPHORBIACEAE	*Clutia abyssinica*		EC/WC	leaves
EUPHORBIACEAE	*Clutia robusta*		EC/WC	leaves
EUPHORBIACEAE	*Croton pseudopulchellus*		KZN/MP/SD	roots/ leaves
FABACEAE	*Elephantorrhiza elephantina*	umdabu	KZN/MP	roots
GENTIANACEAE	*Chironia krebsii*		KZN/MP/EC/WC	root

FAMILY	**GENERA**	**AFRICAN NAME**	**REGION**	**PLANT AREA USED**
HYACINTHACEAE	*Scilla natalensis*	imbiza	KZN/MP	bark
HYACINTHACEAE	*Eucomis autumnalis*	umathunga	KZN/MP	bulbs
LAMIACEAE	*Tetradenia riparia*	iboza	KZN/MP	leaves
LECYTHIDACEAE	*Barringtonia racemosa*	iboqo	KZN/MP	root/ bark
MYRSINACEAE	*Embelia ruminata*	ibinini	KZN/MP	roots/ bark
OLEACEAE	*Jasminum fluminense*		MP/KZN	stem/ twigs
OLEACEAE	*Olea europaea*	umquma	WC/KZN/NC/EC/GP	leaves
POLYGALACEAE	*Polygala virgata*	ithethe	WC/KZN/EC	arial plant parts
RHIZOPHORACEAE	*Cassipourea gummiflua*	isinukani	KZN/MP	bark
THYMELAEACEAE	*Gnidia anthylloides*	indolo	KZN/MP	leaves/ roots
THYMELAEACEAE	*Gnidia burchellii*	isidikile	KZN/MP	leaves/ roots
THYMELAEACEAE	*Gnidia cuneata*	isidikile	KZN/MP	leaves/ roots
THYMELAEACEAE	*Gnidia kraussiana*	isidikile/ imfuzana/ umsilawengwe	KZN/MP	roots
VERBENACEAE	*Lippia javanica*	umsuzwane	KZN/MP	leaves
VITACEAE	*Cissus quadrangularis*		KZN/MP/SD	leaves
VITACEAE	*Cyphostemma lanigerum*	umthambiso	KZN/MP	roots
VITACEAE	*Rhoicissus tomentosa*	unungwane	MP/KZN	roots
ZINGIBERACEAE	*Siphonochilus aethiopicus* *S. natalensis*	isiphephetho (wild ginger)	MP/KZN	roots
FLACOURTIACEAE	*Rawsonia lucida* Harv. & Sond.	upentshisi	MP/KZN	rhizome
ASTERACEAE	*Calendula officinalis*	marigold/ ikhaleniula	MP/KZN	fresh flower

2.2.4.4. *Ethnopharmaceutical Use of Barringtonia racemosa*

Barringtonia racemosa is the name given to the medicinal plant studied that showed potential antiviral activity. *Barringtonia racemosa* is mainly found in the Kwazulu-Natal region with some distribution in Mpumalanga province and Swaziland. Its bark and fruit are used for medicinal purposes, which include malaria, stomach ache and skin diseases (Hutchings et al. 1996). The bark, which was used in antiviral analyses, is reported to possess insecticidal effects. None of the literature sources surveyed, including a Napralert enquiry, made mention of any antiviral properties of *Barringtonia racemosa* (Quinn 1998). The reported chemical constituents and biological properties of *Barringtonia racemosa* mainly refer to those isolated from the ripe fruit rather than the bark. However, it is mentioned that the bark is rich in tannins. The limitations noted with current anti-viral agents have prompted the search for new agents. This search has focused on compounds that are active against herpes viruses, retroviruses and rhinoviruses (Vanden Berghe, Vlietinck & Van Hoof 1986, Hudson 1994, Vanden Berghe et al. 1978, Yamada et al. 1991, Abou-Karim & Sher 1990, Bedows & Hatfield 1982, Berge et al. 1999, Hayashi, Hayashi & Kojima 1996, Collins et al. 1997, Garcia et al. 1999, Jun-Bum et al. 1999, Hudson, Graham & Towers 1994).

CHAPTER III

3. PREPARATION AND EXTRACTION OF PLANT MATERIAL

3.1. INTRODUCTION

There are four basic methods for selecting plant material for screening purposes against viral conditions. The first is random collection of plants which is followed by high throughput screening; the second is selection based on ethnomedical information; the third is substantiating literature leads and the fourth is based on chemotaxonomic approaches (Vlietinck & Vanden Berghe 1991, Harvey 1999). Though random plant collection provides more novel leads, this approach requires a high-throughput screening unit. Such a unit is costly and cannot be afforded by most university-based research groups. The research strategy adopted during this project was a combination of gathering ethnomedical data from traditional healers and pursuing ethnomedical and phytochemical information from literature sources. This approach, by far, is the most popular and has been shown to yield the most accurate therapeutic targets (McCutcheon et al. 1995, Holland 1994, Cox & Balick 1994, Kyerematen & Ogunlana 1987, Yue-Zhong 1998, Bohlin 1993, Farnsworth 1993, Edinburg 1998, Malone & Robichaud 1962, Harvey 1999, Turner 1996). Natural plant products provide the pharmaceutical industry with an immeasurable source of molecular diversity to drug discovery that complements combinatorial approaches to drug molecular research (Malone 1983, Yue-Zhong 1998, Montanha et al. 1995, Turner 1996, Jaroszewski 1984). The standardisation of bioassay guided fractionation, isolation and structure elucidation procedures has accelerated the lead generation time. Natural product extracts often exhibit potent activity and they especially benefit therapeutic areas that lack valid synthetic leads (Yue-Zhong 1998). This work not only seeks to provide potential drug leads with antiviral activity, but also contributes to the creation of new research strategies directed at accelerating the analysis time. Collaboration with ethnobotanists and herbarium staff specialised in the plants of interest was essential for correct identification of plants collected (Bohlin 1993, Malone 1983).

3.2. PLANT EXTRACTION

After collection, the plant material was air-dried in a darkened area with sufficient air passage. Roots, stem and bark material were ground to a crude powder using a contemporary food grinder. Bulbs were cut into small pieces and large leaves were powdered by hand. Approximately, 20g of the size-reduced material was weighed into two preweighed 1000ml conical flasks. The first flask was filled with 750ml distilled water and the second with an equal amount of analytical grade methanol. Both flasks were placed on a Labcon shaker (5741, Labdesign Engineering, Maraisburg) oscillating at approximately 180rpm for twenty-four hours at room temperature. After shaking, the liquid from both flasks was filtered through a Wattman 32cm filter paper. Each flask was refilled with either water or methanol and the extraction process was repeated for a further twenty-four hours. After forty-eight hours the filtrates of similar solvent from the successive extractions were combined. The cold water extraction was thereafter followed by a hot water extraction of the same plant material. The plant material remaining from the cold methanol extraction was air-dried under a fume hood and the flask used for extractions was reweighed before the start of forty-eight hours of cold dichloromethane extraction. Fresh size reduced plant material was used to carry out the hot methanol extraction. The extraction was conducted using a soxhlet.

The hot and cold water extracts were placed into round-bottomed flasks and cooled down to 4°C before being placed into a –20° C freezer until frozen. The solidified sample was attached to an Alpha I-5 (01954, Medizincher Apparatebau, 336 Osterode, Germany) model freeze dryer for twenty-four hours or until a dry powder was obtained. The dry extract was placed into preweighed vials and the percentage yield was determined. The vials were airtight sealed and stored at room temperature.

The hot and cold methanol extracts were placed in separate round-bottomed flasks that were attached to a Büchi Rotavapor R114 (10035882, Büchi Laborthechnik, Switzerland) rotary vacuum evaporator and placed in a Büchi Waterbath B-480 (10035859, Büchi Laborthechnik, Switzerland). The rotary evaporator was connected to a Büchi Vacuum controller B-720 (1398199, Büchi Laborthechnik, Switzerland). The flasks were rotated at approximately 30rpm whilst a vacuum of between 150 – 550 psi and a water bath temperature of 65°C facilitated the evaporation of the organic liquid. Once the remaining liquid appeared oily and thick, the rotary evaporator was turned off and the liquid was aspirated into preweighed vials. The open vials were placed under a fume hood to accelerate the evaporation of the remaining organic solvent. When complete drying was achieved, the percentage yield was determined and the vials were sealed and stored at room temperature.

The evaporation procedure of the dichloromethane was performed in the same manner as that of the methanol extracts. The water bath temperature used for the dichloromethane extract was 50°C. Likewise, the percentage yield was determined following complete evaporation of the organic liquid and the sealed vials were stored at room temperature until required for use. The extracts were labelled according to the plant and extraction solvent used. The plants listed in the plant list were categorised in alphabetical order according to the family name (Table 3, p51). A number was allocated to each plant on the list and the following abbreviations were used to identify the solvent used:

AQC	Cold aqueous extract
AQH	Hot aqueous extract
MEH	Hot methanol extract
MEC	Cold methanol extract
DCM	Dichloromethane extract

Table 3:Medicinal plants with potential antiviral activity

GENERA	VERNACULAR	AQC	AQH	MEC	MEH	DCM
Clivia miniata Regel		—	—	CD	CD	—
Crinum bulbispermum	umduze (inembe)	CD	—	CD	—	CD
Alepidea amatymbica	ikhatazo	CD	—	—	CD	
Rauvolfia caffra	umhlambamanzi	—	—	—	CD	—
Acorus calamus L	ikalamuzi	—	—	—	CD	
Xysmalobium undulatum	ishongwe	CD	CD	CD	CD	—
Bulbine natalensis	ibuchu	—	—	—	—	CD
Artemisia afra	umhlonyane	CD	—	—	—	CD
Senecio serratuloides	insukumbili	—	CD	CD	CD	
Kigelia africana	umfongothi	—	—	—	CD	CD
Warburgia salutaris	isibaha	CD	—	CD	CD	—
Chironia krebsii		—	—	—	—	—
Eucomis autumnalis	umathunga	CD	CD	CD	CD	CD
Tetradenia riparia	iboza	CD	CD	CD	CD	CD
Barringtonia racemosa		+	—	CD	CD	—
Cassipourea gummiflua	isinukani	—	—	—	—	—
Lippia javanica	umsuzwane	—	—	—	CD	—
Siphonochilus aethiopicus *S. natalensis*	isiphephetho (wild ginger)	CD	CD	CD	CD	CD

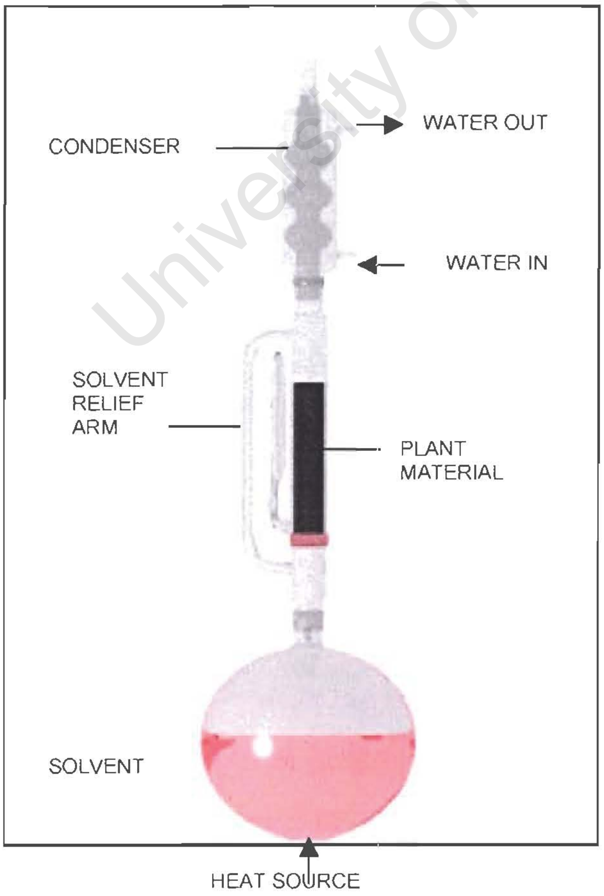
'—' denotes no antiviral activity; CD refers to cell destruction and '+' denotes positive antiviral activity.

Plants listed in Table 3 were selected through collating ethnobotanical information derived from literature surveys and interviews with traditional practitioners.

3.2.1. Soxhlet Operation

The soxhlet extractor was used to extract heat stable organic compounds from plant material. To improve the efficiency of the extraction, the material was size reduced to pieces of not more than 2cm in size using a contemporary food grinder. The smaller the particles, the greater the surface area and therefore, the more efficient the extraction. The round bottom flask attached to the condenser contained the solvent of choice. The round bottom flask was heated such that sufficient vapour pressure was created to produce a steady flow of liquid drops from the condenser at the top of the soxhlet apparatus. Once the solvent in the upper chamber rose above the relief arm, the solvent returned to the round bottom flask and the process repeated itself. Most extractions were run for at least four hours and some extractions were run from twelve to twenty-four hours. Timing experiments can be run to determine the best analysis time for the best extraction results.

Figure 5: An illustration of a soxhlet
(http://www.instrumentalchemistry.com/sampleprep/pages/soxhlet.htm),



3.2.2. Percentage Yield Calculations

When performing an extraction, the amount of a product obtained will be a fraction of the starting material. To determine the efficiency of the extraction procedures performed, the percentage yield was calculated. This is often expressed in terms of the yield of an extract, which is the ratio of the amount of substance actually obtained to the amount of material originally used. The mass of the substance was used to obtain the actual amount and the ratio of mass obtained to the starting mass. The yield is expressed as the per cent yield or percentage yield, which is simply the actual yield expressed as a percentage.

Equation 1

$$\boxed{\text{percentage yield} = \frac{\text{actual mass}}{\text{expected mass} + \text{actual mass}} \times 100}$$

The percentage yield of each dry extract was calculated using the above equation.

3.2.3. Preparation of Sample

The solubility of the dried crude extracts were first tested in water. In situations whereby there was limited or no solubility in water, then solubility was determined in methanol or acetonitrile. The organic solvents were used as co-solvents to aid dissolution of the test material in an aqueous environment. To prevent interference of the solvent to the cellular system the organic liquid concentration did not exceed 1% during all co-solvency investigations. Pursuing dissolution in an aqueous solvent was essential because cell media is water based and it was therefore necessary to ensure compatibility of the test sample with the test system. Water-soluble extracts were dissolved in 1ml of media prior to testing. Water insoluble agents were first diluted in the least amount of organic solvent possible. Co-solvency with the culture media decreased the amount of organic solvent present in the final solution. These dilutions were carried out such that the final concentration of drug in all stock solutions was equal. To determine the effective concentration of the extract, serial dilutions of the extract were prepared and inoculated onto a cell system. The effect of different concentrations of organic solvent on the cell system was determined to ensure that no undesirable reactions occurred due to the presence of minute quantities of organic solvent.

3.3. SUMMARY

There is no existing standard protocol for the extraction of plant materials. Various publications suggest different fractionation methods that have been successful at a particular laboratory under certain conditions (Bakana et al. 1987, Locher et al. 1995, Hudson 1994, Mitscher et al. 1971, Xu et al. 1999, Kraus 1991, Claeson et al. 1998, Chen et al. 1999, Ngan et al. 1988, Verpoorte 1989). Natural product chemists who have used their experience and expertise to produce solvent and fractionation techniques that optimise the yield of the desired compound have derived most methods in current use (Samuelsson, Kyerematen & Farah 1985, Mizuno et al. 1997). Investigations in to the optimisation of extraction techniques cannot take place independent of a traditional healer or herbalist who has knowledge on the preparation and application of the material of interest. A disregard of this knowledge can lead to the loss of potentially important lead compounds.

CHAPTER IV

4. THEORY OF ANTIVIRAL ANALYSIS

4.1. INTRODUCTION: VIRUSES AND CELL LINES

4.1.1. Introduction: Growth Of Cell Lines

Cell culture studies were performed on a simple, culture model that comprises monkey kidney (Vero) cells grown on either treated plastic or glass vessels.

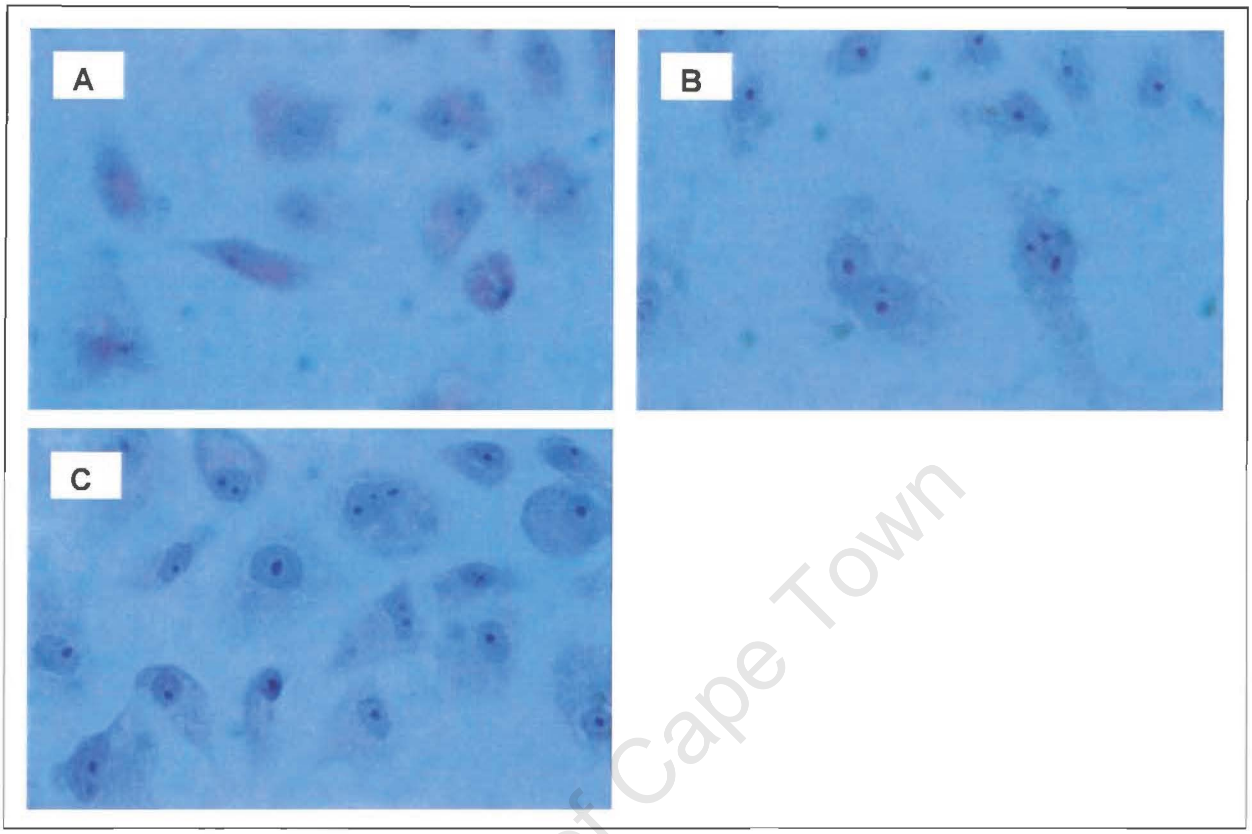
There are three basic types of tissue culture cells (Online 2000c):

i) Primary cell lines: these are cells that have been freshly dispersed (by trypsin treatment) from a piece of freshly removed intact tissue. Primary cell lines are only able to grow for more than a few passages as diploid cells (Freshney 1990a, Freshney 1990b).

ii) Secondary cell lines: these are diploid cells that have picked up a mutation that allows them to be passaged for about 50 generations. These cells are able to retain their normal chromosome structure. The problem encountered with these cells is that they are programmed to die after about 50 cell divisions resulting in limited use.

iii) Continuous cell culture: these are cells that are capable of growing for prolonged, perhaps indefinite, lengths of time. Vero cells belong to this category of cell lines. Continuous cell lines are attachment dependent and readily form a confluent monolayer. Once a monolayer is formed the cells enter a resting stage where they stop dividing. Eventually, the cells age and the monolayer disintegrates and dies. The images in figure 6 give a visual illustration of this process (Figure 6, p56). The different stages of cell growth and decline can be observed microscopically. The technique is powerful enough to observe changes in confluency and cell shape (Freshney 1990a).

Figure 6: An illustration of the growth process of Vero cells



The above figure shows Vero cells at different confluencies as viewed micropscopically. A shows approximately 25% confluency, B approximately 50% confluency and C approximately 80% confluency.

Prior to commencing infectivity studies, it is necessary to validate the cell system selected using quantitative methods. Standard indicators used in cell culture techniques are:

- Efficiency of cell cloning.
- Cell viability.
- Cell density in a given time period.
- Cell suspension at a specified concentration.

i. Efficiency of Cell Cloning

The cloning efficiency of a cell system is known as the percentage of inoculum that attaches to the substrate. This therefore implies viability but not necessarily, proliferative ability. Cloning efficiency is dependent upon the nutritional quality of the cell culture and the density of cells present. At high cell densities, cells secrete messengers required for survival and growth. Using nutritionally rich media, optimising the carbon dioxide levels and adding substrates that support certain cell types can improve the cell cloning ability of a system.

ii. Cell Viability

Cell viability assays measure the ability of a cell system to live, grow and develop. The Dye Exclusion Test differentiates viable cells from non-viable cells. Trypan blue, a staining dye was used. During the breakdown of the cell wall, non-viable cells absorb the dye resulting in the dark blue stain. Viable cells have an intact cell wall that prevents the dye from entering the cell and therefore, these remain unstained. Viral agents reduce the integrity of membranes by interfering with the basic cellular functions (Koelle et al. 2001, McNeil & Steinhardt, 1997). Binding of the virus to a cell receptor causes confirmational changes in virion proteins. This change sets in motion events that destabilise the host membrane allowing the virus or its nucleic acid to enter the cell. A known volume of cell suspension was loaded on to a cell counting device called a haemocytometer. With the aid of a microscope, the number of viable and non-viable cells was counted. The cell viability was determined using the equation below:

Equation 2

$$\boxed{\text{Percent Viability} = \frac{\text{viable cells}}{\text{viable cells} + \text{non - viable cells}} \times 100}$$

iii. Cell Density

Cell density influences cell division and therefore the regenerative capability of a cell culture system. A haemocytometer is used to count the number of viable cells within each large grid square of the device. The number of cells per millimetre of a cell suspension is first calculated using the following equation:

Equation 3

$$\boxed{C = N \times 10^4}$$

where C = cells per millimetre

N = the average cells counted

10^4 = the volume conversion factor for 1mm^2

The total yield of cells was then calculated using:

Equation 4

$$\boxed{\text{Total yield} = C \times V}$$

where V = the total volume of cells in the suspension.

In order to obtain a specific volume of a specific number of cells, the following formula was used to calculate the dilution required to obtain the specified suspension:

Equation 5

$$\boxed{V_i \times C_i = V_f \times C_f}$$

Where V and C refer to the volume and concentration respectively and *i* and *f* refer to the initial and final volume and concentration of the suspension.

The ideal plating concentration used in experiments was 2.5×10^5 cells/ml.

Vero cells were convenient to use in studies because they exhibited specific cytopathic effects during infection with herpes simplex virus and their cultivation technique was uncomplicated and time-effective. The system therefore, allowed for the study of the anti-viral effects of plant extracts in a controlled environment that could be easily manipulated. The choice of cell lines available for use in plant research is dependent upon the disease being studied and the conditions required to determine toxic effects, the function and mechanism of action of the anti-viral agent (Koyama Uchida 1987). Vero cells fulfilled these criteria and were therefore used to test the performance and feasibility of the proposed model system developed. It was important to verify the suitability of the cell-virus combination because some viruses have no effect on certain cell cultures or establish persistent infection resulting in unspecific cytopathic effects. Once a suitable cell model was developed for the quantitative analysis of viral activity in the absence and presence of an antiviral agent, then studies could be developed using a more complex system of skin cells such as keratinocytes. Keratinocytes are a major cell type of the epidermis and are fundamental to the structure and mechanical functions of the skin (Weller 2001). A keratinocyte in vitro system would provide structural and functional similarities in the clinical human situation. It is acknowledged that a model can only offer an approximation and is never the same as the prototype. Studies that determine the suitability of cell culture lines have shown that basal or stem keratinocytes obtained from a punch biopsy exhibit satisfactory proliferative activity provided that this delicate system is sustained by a specific combination of maintenance media and growth factors (Hay 1998a, Hay 1992, Hay et al. 1996, Hay 1998b, Hay 1996). The procedure requires that skin samples from the same body site be incubated in trypsin, an enzyme that digests skin fragments. This digestion procedure results in the isolation of the keratinocytes. Thereafter, the cells are seeded using irradiated 3T3 cells (Rheinwald & Green 1977, Navsaria 1995). Seeding allows for the attachment and growth of keratinocytes on cultivation plates while inhibiting the growth of fibroblasts. Fibroblasts interfere with the attachment and growth of keratinocytes and are therefore, removed from the system. The necessary nutrients and growth factors are added prior to incubation of the cell cultures. Satisfactorily cultivated cell cultures can then be utilised in subsequent experimentation.

4.1.2. Propagation of Keratinocytes

Keratinocytes are the most abundant cell type of the epidermis and were therefore identified as a viable source for skin cells for antiviral efficacy determination studies. To gain an understanding of the *in-vitro* experimental procedures required to establish such a cell system, an extensive literature survey on the propagation techniques used to grow keratinocytes was conducted (Vogel 1993, Steinstrasser & Merkle, 1995, Audus et al. 1990, Daniels, Kearney & Ingham 1996, Parenteau et al. 1991, Michel at al. 1997, Andreassi 1992, Garlick & Taichman 1994, Goulet et al. 1996, Watt 1988, Germain et al 1993, Navsaria et al. 1995, Candal et al. 1989, Shipley & Pittelkow 1987, Normand & Karasek 1995, Hukkanen 1996). In addition, discussions were held with the Red Cross Children's Hospital cell physiology unit who are pioneering work to simplify the propagation of keratinocyte cell lines from skin biopsies. These research seminars were attended by various cell physiology researchers and they revealed that studies in this area are novel and until simple and affordable cell lines are available for use, this part of the work would require investigations that are beyond the scope of this project. Within the context of this project, it is however useful to present the general approach to keratinocyte cell growth. Keratinocytes are particularly useful because they undergo normal processes of differentiation in vitro resulting in confluent colonies that retain their proliferative activity (Parenteau 1991). Various propagation techniques are suggested, however, the method described by Norman and Karasek was investigated further due to its perceived simplicity (Normand & Karasek 1995, Daniels, Kearney & Ingham 1996, Tencheni, Ranzati & Malcovati 1992, Allan-Hoffmann & Rheinwald 1984, Boukamp et al. 1988, Steinstrasser, Koopman & Merkle 1997). The skin sample was collected in growth medium to which streptomycin, penicillin and amphotericin B was added. The tissue was first washed with calcium- and magnesium-free Hanks' balanced salt solution (HBSS) and thereafter, immersed in dispase solution for 12 hours at 37°C. The dispase aided the separation of cell layers. Following incubation, the epidermal layer was detached from the dermal layer. The epidermal layer was washed with HBSS and was subsequently incubated for 10 minutes in 0.3% trypsin and 1% EDTA. The isolated cells were centrifuged into pellets and the supernatant was withdrawn. The cells were resuspended in equal volumes of serum free medium prior to plating them on tissue culture vessels previously coated with an acid solution of type 1 collagen. Various combinations of cell media were investigated to obtain a system that would support cell attachment to the culture vessel and promote cell proliferation.

4.1.3. *Viral Inoculation of Cell Cultures*

A confluent vessel of healthy Vero cells was used for bioassays. Prior to use, undesirable growth by-products and media substances with potential antiviral activity were removed by cleansing with essential media. The plates were inoculated with serial dilutions of herpes virus simplex obtained from pre-existing virus stocks stored at the Department of Virology. Incubation of the inoculated plates allowed for the absorption and subsequent replication of the virus. Each plate was examined daily after inoculation for signs of cytopathic effects. Following the collection of cytopathic effect results over a five-day period, the tissue culture infectious dose 50 ($TCID_{50}$) was calculated by determining the viral quantity required for 50% of the inoculated culture to be infected (Dayan et al. 1984). Identification and typing of the virus was determined by utilizing specific staining techniques outlined in section 4.2.5, p69 and appendix 3, p219.

The Reed and Muench method, which was used in these experiments, is widely used for calculating the endpoint of viral activity (Kalter 1963, Cunningham 1990). Cunningham, in his Laboratory Guide in Virology best defines the endpoint as an interpolation of the cumulative frequencies of the positive and negative responses occurring in a dilution in which there would be 50 per cent positive responses and 50 per cent negative responses (Cunningham 1990). The formula used to calculate the Reed and Muench endpoint is:

Equation 6

$$\boxed{I = \frac{50\% - \%\text{ of wells infected at a dilution below } 50\%}{\%\text{ of wells infected at a dilution above } 50\% - \%\text{ of wells infected at dilution below } 50\%}}$$

The 50% endpoint titre = $10^{\log \text{total dilution above } 50\% - (I \times \log h)}$

Where I = the interpolated value of the 50% endpoint h = the dilution factor.

Table 4 p62 provides a simplified example of this calculation method:

Table 4: Reed and Muench calculation technique

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Positive wells	24	22	15	8	3	0
Wells inoculated	24	24	24	24	24	24
% infected	100	91.6	62.5	33.3	12.5	0

The calculation is as follows:

$$I = \frac{(62.5 - 50)}{(62.5 - 33.3)}$$

$$I = \frac{(12.50)}{(29.2)}$$

$$I = 0.42$$

50% endpoint $= 10^{-3 - (0.42 \times 1)}$

$= 10^{-3.42}$ (this dilution contains $1TCID_{50}$)

According to the above illustration, to ensure 100% infectivity, the minimum dilution that can be used is 10^{-1}. Alternatively, it is possible to calculate the Multiplicity of Infection (MOI) which enables one to infect a given proportion of cells within a culture. Multiplicity of infection determinations are performed using plaque assays that rely upon (i) a confluent monolayer of cells that are susceptible to the virus, (ii) the existence of visible cytopathic effect by the virus and (iii) the use of agar to prevent virus diffusion to neighbouring cells (Schmidt & Emmons 1989, Online 2001c). Small distinct plaques form on the agar as cell damage takes place. The plaques can be visualized using a staining dye such as crystal violet. Equation 7 is used to determine the multiplicity of infection.

Equation 7

$$\boxed{\begin{array}{l} P(0) = e^{-m} \\ m = -\ln P(0) \end{array}}$$

where P (n) is the fraction of cells infected by n amount of virus particles
m is the multiplicity of infection
P(0) is the proportion of uninfected cells.
Therefore, to obtain 95% infectivity then P(0) = 5% = 0.05
m = -ln(0.05) = 3.0 plaque forming units per cell.

Normally, a cell culture is infected at a high multiplicity of infection (MOI) of 10. This is done so that virtually all of the cells are infected at the same time and the growth cycle is more or less synchronous (Online 2001c). Viral infection of cells is random and normally follows a Poison distribution. At various times during post-infection, both the culture supernatant and the cells can be removed and analysed for the presence of infectious virus. Immediately after infection there is a period of time in which no infectious virus is detected in either the cell or the supernatant. This period is termed the eclipse phase and lasts 10 – 12 hours. The eclipse phase corresponds to the uncoating period of the virus. At some point hereafter, new viruses can be detected intracellularly, but not extracellularly. The eclipse phase ends when these viral particles begin to be detected. This second phase is known as the synthetic phase and is associated with the assembly of the virus particle. The synthetic phase begins approximately 12 hours post-infection. As the viruses begin to be released, they can then be detected extracellularly. Extracellular detection of virus takes place approximately 18 hours postinfection. The time from the point of infection to the detection of extracellular virus is the latent phase. The burst size of the virus can be calculated based on the number of viral particles in the supernatant and cells, divided by the number of cells. This gives an approximation the degree of viral multiplication inside the cell. Some viruses have a burst size of around 1,000, whereas other viruses may have burst sizes in the 10-100 range. In addition, viruses vary widely in the amount of time that they take to produce maximum infection. Figure 3, p33 illustrates the typical changes that occur to the morphology of Vero cells during infection with HSV. Contamination of the culture environment can alter virus-cell interactions thus affecting the characteristic development of the cells within the system. There are two main categories of contamination: chemical and biological contaminants (Online 2001a). Sources of chemical contaminants can include cell culture media, storage vessels, reagents, water or the gas mixtures used in incubators. Bacteria, molds and yeast are the most common forms of biological contamination. Less frequent contaminants include protozoa, mycoplasmas and other cell lines. Unlike chemical contamination, bacterial contaminants can escape detection over several cell passages. As a result, it is essential to ensure that preparation protocols are validated and strictly adhered to to avoid compromising the quality of the research.

4.1.4. Antiviral Bioassays

After demonstrating the satisfactory growth of herpes simplex virus in Vero cell lines, it was then possible to test the antiviral action of the medicinal plant extracts obtained. Following successful inoculation of the culture media with herpes simplex virus, the plant extract was added to the culture plates. Each plate was incubated for a specific period to allow for sufficient absorption of the inoculate. Plates were examined daily for changes in cell growth and appearance. The difference in biological activity of treated and control viruses was determined by the cytopathic effects assay using the Reed and Muench method. For comparative purposes, cell plates with and without the antiviral agents were prepared and analysed. From this study, the following parameters were determined:

- *Antiviral activity* - a ratio of the virus titre in the absence and in the presence of extract. This parameter provides a measure of the antiviral activity taking place within a given cell system inoculated with virus.
- *Cytotoxicity* - a measure of cellular DNA synthesis derived by determining cell viability and cell growth. Therefore, the destruction of cell morphology can be determined microscopically using staining techniques to distinguish viable cells from non-viable cells.
- *Therapeutic index* - a ratio of the maximum drug concentration at which 50% of the growth of normal cells is inhibited to the minimum drug concentration at which 50%-90% of the virus is inhibited. The therapeutic index provides a crude measure of the safety of the extract. The measurement is calculated by determining the ratio of the minimum toxic dose and the minimum effective dose.
- *Dose-response analysis* - a measure determined by assessing the appearance of cytopathic effects in plates in which serial concentrations of extract have been added. The dose-response analysis provides an indication of the efficacy of the extract at different concentrations.
- *Colony efficiency* - the percentage of counted colonies to the total number of plated cells. Colony efficiency is measurable in cell systems capable of forming distinct colonies of virus. The emergence of the colonies is dependent on the strength of antiviral activity.

4.2. METHODOLOGY: CELL CULTURING AND ANTIVIRAL ASSAYS

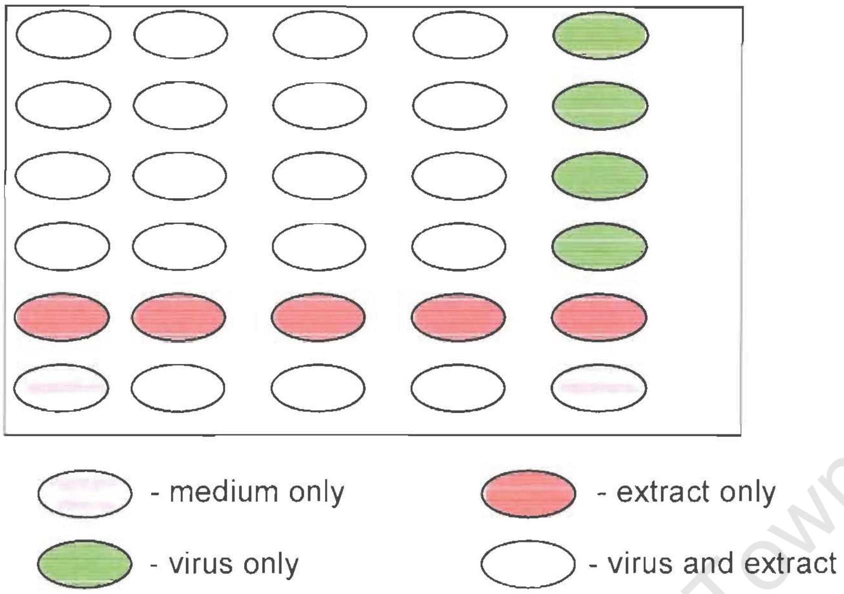
4.2.1. Cell Culture Techniques

All cell culture techniques and manipulations were carried out in a sterile environment using good laboratory standard practices. Permanent cell lines from African green monkey kidney (Vero) were cultivated in Eagle's minimal essential medium (MEM), which was supplemented with 10% foetal bovine serum (FBS). Adding to the solution, 4% sodium bicarbonate and carbon dioxide to the atmospheric environment of the incubator buffered the medium. The ideal pH range at which the system was maintained was between 7.2 and 7.4. It was possible to monitor buffer activity visually through a change of colour in the medium. To prevent bacterial interference, in the cell system, benzylpenicillin (100IU/ml) and streptomycin (100μg/ml) were added to the medium. Cell cultures were stored in a 37°C incubator for several days. After every 7 to 10 days confluency was reached and it was possible to subculture the flask of cells. The medium inside the culture vessel was removed and the vessel was cleaned twice with sterile phosphate buffered saline (PBS). Thorough rinsing of the flask was essential because the presence of media interfered with the dissociation and detachment of the cells during tryptinisation. Approximately 2.5 ml of Trypsin, a digestive enzyme, was added to the culture flask and the vessel was placed in a 37° C incubator for approximately 15 minutes to promote cellular dissociation. The cell suspension was transferred to a centrifuge tube and the culture vessel was washed with PBS, which was added to the centrifuge tube. The cells were centrifuged at 1500 *g* for 5 minutes. The liquid PBS mixture was removed and the cells were washed with a further 5 ml PBS. Prior to centrifugation, the cells were resuspended in the centrifuge tube. After a second centrifugation process, the PBS was removed and 2.5 ml of medium was added to the tube. The cells were re-suspended. A 0.1 ml aliquot of the suspension was removed from the sample and deposited in a Wasserman tube containing 0.1 ml of 0.4% Trypan blue. The Trypan blue dye mixture was thoroughly mixed and 50μl was extracted and carefully pipeted under the cover slip of the haemocytometer. Caution was taken not to over- or under-fill the counting chamber. Using a light microscope, the number of stained and unstained cells in the four corner and centre squares were counted. The number of viable cells per millimetre was calculated using equations 3 and 4, pp57 and 58, respectively. After calculating the optimal cell density, a determined concentration of cell suspension was added to a new culture vessel and a calculated quantity of medium was added prior to incubation. Care was taken to maintain the original cell growth conditions because altering the cell system environment modifies the cell proliferation rate.

4.2.2. Plaque Reduction Assay

During the screening process of the different plant extracts, antiviral activity was monitored using cytopathic effects, thus providing qualitative data (Elanchezhiyan et al. 1993, Meyer et al. 1997). To obtain quantitative data of viral infectivity and antiviral action, plaque reduction assays were used. A confluent layer of Vero cells were grown on a sterilised 24 well plate using techniques previously described. The growth media was removed from the culture vessel. Appropriate dilutions of virus were calculated and prepared. A 0.2 ml aliquot of each virus dilution was added to four wells in the order illustrated in Figure 7, p68. The cells were incubated for 1 hr at 37° C. The plates were then filled with 2 ml agar prepared as described in the formula on p68. The temperature of the agar was maintained at no more than 40°C. The plates containing agar were allowed to cool to room temperature and then inverted before transferring them to the incubator. The incubation period and conditions used were as previously described. Inoculated plates were monitored daily for plaque formation. After 5 days, the plates were removed from the incubator and the agar overlay was carefully lifted off. The cells were stained and fixed using an ethanol-formalin-acetic acid solution comprising 1% crystal violet in 20 parts 70% ethanol, 2 parts formalin and 1 part acetic acid. Excess fixative solution was removed and each well was rinsed with water prior to counting the number of plaques. Vero cells used in plaque reduction assays produced clear plaques resulting in their easy detection.

Figure 7: An illustration of the inoculation design using a 24 well plate



4.2.3. *Agar Overlay Medium*

The following formula was used to prepare the agar overlay in plaque reduction assays:

Agar (Agar-Noble)	1.8 g
MEM	98 ml
Sodium bicarbonate	2 ml

Agar noble was specifically selected because of its compatibility with the cell system and the absence viral inhibition effects. Furthermore, the agent is such that it is liquid at temperatures of above 40°C and remains solid at 37°C. The agar was dissolved in 80 ml of boiling water then cooled to approximately 42°C. The 10% minimum essential medium containing sodium bicarbonate was warmed in a water bath before being added to the agar. The mixture of agar and medium was maintained at 42°C until required for use.

4.2.4. Propagation of Viruses

The herpes virus was obtained from a storage bank containing clinically isolated samples. A 0.1 ml aliquot of virus was extracted from this bank and inoculated into a vessel containing healthy confluent Vero cells. The inoculated cells containing the virus were placed in an incubator using conditions previously mentioned. The spread of the virus and subsequent destruction of the cell surface was monitored daily until no further change was observed in the cell system. The end point was determined by the presence of viral syncti suspended in the culture medium and the absence of attached cells. This was normally achieved by day four of incubation and from day five onwards, no further developments were observed in the cell system. Convenient aliquots of virus were measured from the virus suspension and frozen. Aliquoted viruses were stored at –4°C and thawed prior to subsequent use. Prior to use in antiviral assays, the infectious titre of the stock solution was determined to ensure that a $100TCID_{50}$ is achieved during experimentation.

4.2.5. Serotyping of HSV

Immunofluorescence is a rapid diagnostic method which was used to confirm the presence and type of herpes simplex virus in clinical specimen and infected cell cultures (Duff & Rapp 1975, Schmidt & Emmons 1989). Immunofluorescence involves the detection and identification of antigenic material by observing microscopically the fluorescence of known, specific fluorescein linked antibodies that have become attached to the specimen (Youngson 1992). A direct or indirect immunofluorescence method can be used. The preferred method was the direct method which is quicker, simpler and more specific (Lyerla & Forrester 1979). Test-tubes containing a confluent layer of Vero cells grown onto glass slides were inoculated with herpes simplex virus. Slides on which infected cells were fixed were stained and labelled with monoclonal antibodies that differentiate herpes simplex type 1 from type 2. The stained slides were viewed under fluorescent light.

Three test slides were prepared. The first was a negative control slide; the second, a positive HSV 1 infected slide and the third was the test slide. The controls were obtained from the diagnostic kit used. Virus inoculated slides were placed in acetone maintained at -4°C for 10 minutes. The slides were then removed from the acetone and air-dried. A HSV-1 antibody test solution was added to each slide and the slides were incubated in a moist chamber maintained at 37°C for 45 minutes. Following incubation, the slides were rinsed in phosphated buffer saline followed by water. After air-drying each slide was mounted and viewed for fluorescence. Typical staining reactions of both HSV-1 and HSV-2 are presented in Table 5. The intensity of the fluorescent light is influenced by the staining reaction of the fluorescein isothiocyanate (FITC)-conjugate viral immunoglobulins. Test kits contain both HSV-1 and HSV-2 conjugates which react with the test substance to give a characteristic staining intensity.

Table 5: Grading intensities for immunofluorescence tests for HSV

FLUORESCENT APPEARANCE	*GRADING*
Glaring yellow-green fluorescence	4+
Bright yellow-green fluorescence	3+
Dull yellow-green fluorescence	2+
Very dim yellow-green fluorescence	1+

4.2.6. Cytotoxicity Evaluations

Cytotoxicity evaluations were carried out on all crude extracts identified with activity during the screening process to ensure that the anti-viral activity detected was not due to toxic effects, but rather direct or indirect anti-viral activity. Two techniques were used to determine cell viability. The first measurement was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. The MTT assay is a colorimetric test in which viable cells reduce the water-soluble yellow coloured MTT to a water insoluble purple formazen product via a process of mitochondrial reductase activity. Vero cells were seeded onto a 96 well plate using procedures previously described. When a confluent cell layer was obtained each well was inoculated with 0.1 ml serial dilutions of the test agent and an equal amount of virus with an infectious concentration of $100TCID_{50}$. The culture system was incubated at 37°C for 5 days. After the incubation period, 20 μl of a 1.0 mg/ml MTT solution was added to each well. The vessels were incubated at 37°C for a further 4 hours. After 4 hours, the medium was carefully aspirated and 100 μl dimethylsulphoxide was added to each well resulting in the dissolution of the formazan crystals. The plate was gently shaken for 10 minutes to complete the dissolution of formazan. Each well was then read using a computer-controlled microplate reader at 540nm and 620nm wavelengths.

A second system used to determine cell viability, utilises cell proliferation properties as a measure of toxicity (Kyerematen & Ogumlana 1987). To determine the effect of the extract on uninfected Vero cells, dilutions of the crude extract were prepared at a concentration range of 50μg/ml to 5000μg/ml (final concentration range being 1.25μg/ml to 125μg/ml). After an incubation period of 5 days, cytotoxicity was determined by microscopic examination of cell morphology and counting the cell number of treated and untreated cultures. The trypan blue dye exclusion assay method was used after tryptinisation of the system to distinguish living cells from dead cells. Calculations of cell survival were accomplished using equation 2, p57 for the cell proliferation determination and equation 8, p71 for the MTT assay.

Equation 8

$$\boxed{\text{Percent cell control} = \frac{(OD_T)_{extract} - (OD_c)_{hsv}}{(OD_c)_{mock} - (OD_c)_{hsv}}}$$

Where $(OD_t)_{extract}$ is the absorbance of the test substance

$(OD_c)_{hsv}$ is the absorbance of the virus-infected control in the absence of extract

$(OD_c)_{mock}$ is the absorbance of the uninfected control

The optical density of the plates was determined using a microplate reader. The negative control baseline reading was subtracted from the readings of the test substance. The 50% toxic concentration was the concentration required to reduce the optical density by 50%

4.2.7. Antiviral Assays

The herpes simplex virus was selected for the antiviral bioassay because of its specific virus-cell reactions following inoculation in Vero cells (Elanchezhiyan et al. 1993, Buthod, Schindler & Rogers 1987). The antiviral activity was determined by the degree of suppression of viral activity in treated cultures. The morphological changes in cells treated with extract and virus were compared with untreated cells that contained medium only; cells containing just the extract and cells containing the virus alone. The effect of time of addition experiments were performed to elucidate the possible mechanism of action of extracts. In the first group of experiments, the virus was inoculated 0, 1, 3, 6, 12, 18, 24, 36 and 48 hours after the inoculation of the extract. The second group of experiments involved inoculating the cell system with the virus first, using the same absorption time intervals mentioned, then later followed by the addition of the extract. The third set of experiments involved inoculating the cells with the virus and crude extract together. The last experiments varied slightly from previous studies in that the extract and virus were mixed together prior to inoculation in the cell system. This procedure ensured concurrent inoculation of the extract and virus. All systems were analysed over a five-day period. Results showed that the most effective anti-viral activity was obtained when the virus was added to the cell system together with or 0 to 1hr after the inoculation of the extract. In subsequent antiviral assays, the virus was added 1 hour following the inoculation of the antiviral test substance.

4.3. SUMMARY

Statistical evaluations of the bioassay systems developed and validated give an indication of the level of virological acceptance. The utility of the bioassay methodologies for quantitative and qualitative analyses will be influenced by the problems of variation common in biological assay systems. Nonetheless, assessments performed on the accuracy of virus titrations and viral reduction capacities reflect the reliability of the study.

CHAPTER V

5. PHYTOCHEMICAL ANALYSIS

Ethnopharmacology researchers are required to develop skills in a range of disciplines including chemistry (Wickberg 1993). Knowledge of various analytical techniques is essential when deciding on analysis and purification processes. The analysis of plant material is divided into two categories. The first is 'wet' chemistry which focuses primarily on the extraction and isolation of the active agent and the second category is termed 'dry' chemistry (Wickberg 1993). Chemists have provided rapid analytical tests to determine the physical and chemical properties of an unknown entity (Tyler 1988). Advancement in chromatography and spectroscopy has shortened the time of analysis and in most cases, increased the specificity of the techniques. Basic phytochemical methods for determining biologically active substances include liquid chromatography, in particular, high performance liquid chromatography (HPLC), ultra violet detection (UV), infra red detection (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) (Tyler 1988). The use of other techniques is dependent upon the nature of the compound being isolated and the resources that a given laboratory can afford.

5.1. PHYSICO-CHEMICAL CHARACTERISATION

According to Samuelsson et al., before initiating purification and identification analysis, it was necessary to determine the physico-chemical properties of the active crude extract (Samuelsson, Kyerematen & Farah 1985). This was accomplished by determining its dissolution, solubility and distribution properties. Knowledge on these physical parameters is important for selecting the best solvent medium. The type of solvent medium used has implications on the purification and detection techniques used.

The solubility of the crude extract is dependent upon the physical and chemical properties of the solvent and solute and is influenced by factors such as temperature and the pH of the solution. Polar solvents dissolve ionic solutes and other polar substances and therefore an extract that is freely soluble in water has few non-polar groups. The solvent action of non-polar liquids differs from that of polar groups. Due to the strong presence of non-polar groups in non-polar solvents, they are unable to form ionic linkages with other agents. Non-polar solvents therefore dissolve non-polar solutes. Semi-polar solvents such as alcohols can induce a certain degree of polarity in a non-polar solvent thus promoting the solubility of a non-polar agent in a polar solvent. Table 6 gives terms used to determine the degree of solubility of the active agent in different solvents.

Table 6: Solubility profiles.

Term	**Parts of Solvent Required for 1 Part of Solute**
Very soluble	Less than 1 part
Freely soluble	1 to 10 parts
Soluble	10 to 30 parts
Sparingly soluble	30 to 100 parts
Slightly soluble	100 to 1000 parts
Very slightly soluble	1000 to 10,000 parts
Insoluble	More than 10,000 parts

(Martin, Swarbrick & Cammarata 1983, p.273)

The majority of medicinal plant mixtures are prepared from either hot water by boiling or cold water by soaking (Samuelsson, Kyerematen & Farah 1985). It is therefore reasonable to believe that the pharmacologically active constituent is extractable by water, making water the preferred extraction solvent. However, in plant research, aqueous extracts are often avoided due to their complexity and difficulty in developing a working strategy to purify and characterise them (Hudson, 1994, Hudson, Graham & Towers, 1994 and Vanden Berghe et al., 1978) .

5.1.1. Experimentation

Aliquots of the crude extract, prepared as mentioned in section 3.2, were measured and dissolved in water, methanol, ethanol, acetonitrile, dichloromethane and dimethylsulphoxide. The solubility determinants listed in Table 6, p75 provided guidance on the degree of solubility of the extract in the different solvents. The aqueous sample was lypholised and the organic solvent in the remaining samples was evaporated. The dry sample was tested for anti-viral activity.

5.2. COLORIMETRIC PHYTOCHEMISTRY

Qualitative chemical analysis involves the identification of classes of compounds in a sample. Plant extracts are complex mixtures of compounds and a systematic approach to analysis is required in order to identify the constituents. Colorimetry was used early in the process to determine the major chemical groups present in the extract. The formation of a coloured compound by the addition of an appropriate reagent is the basic principle applied during the colorimetric identification of major chemical groups present in the crude aqueous extract of *Barringtonia racemosa*. *Barringtonia racemosa* showed the most promising antiviral activity and therefore subsequent phytochemical analysis was focused on this plant. The quantitative results obtained from these investigations can provide information on the nature of constituents in the extract and their complexity. After identifying the major chemical groups present, it is easier to plan the analytical methods for the separation and resolution of constituents.

Colorimetry was used to identify saccharides, saponins, quinones, tannins, anthraquinones and flavonoids present in the extract. The colorimetric determinations were rapid and simple to execute and due to their specificity and sensitivity, they provided valuable information, in a relatively short time, about constituents in an extract.

5.2.1. Colorimetric Procedures

The colorimetric assays described below were utilised to obtain a chemical profile of *Barringtonia racemosa*.

5.2.1.1. Saponins

Saponins are steroidal glycosides that contain twenty-seven carbon atoms. The glycosidic units, which usually consist of one to six monosaccharides are attached at the carbon three site. The formation of persistent foaming of an extract in water signifies the presence of saponins (Harborne 1998). A pre-weighed sample of plant extract was gently boiled in 10ml of water in a test-tube. Following heating, the test-tube was vigorously shaken for 30 seconds and thereafter, the mixture was allowed to settle. The presence of foaming was observed and the height of persistent foaming was measured.

5.2.1.2. Quinones

Quinones may be present in the bark, root or leaves of plant material (Harborne 1998). These agents are coloured and are divided into four groups; benzoquinones, naphthaquinones, anthraquinones and terpenoid quinones. The first three groups are related in that they are generally hydrolysed and posses phenolic properties. They may exist either as a combined sugar as in glycoside, or in a colourless quinol form. The latter group of quinines are involved in cellular respiration and photosynthesis.

A pre-weighed sample of the plant extract was moistened with approximately 1ml of 10% hydrochloric acid solution. The mixture was allowed to stand in 40ml of a diethyl ether/chloroform mixture (3:1) for 5 minutes. The mixture was filtered and 1ml of the filtrate was mixed with 1ml of 10% sodium hydroxide. The presence of quinones was detected by the formation of a red colour in the mixture.

5.2.1.3. *Anthraquinones*

To a pre-weighed sample of the crude extract, 5ml of 15% ferric chloride solution and 5ml hydrochloric acid was added. The mixture was immersed in a water bath maintained at between 55°C and 60°C for 10 minutes then filtered. The filtrate was extracted with 10ml chloroform. The chloroform layer was then washed with 5ml water and 5ml ammonia solution was added to the remaining solvent. The formation of a pink/ red colour in the ammoniacal layer indicated the presence of anthraquinones.

5.2.1.4. *Tannins*

Tannins are chemically complex substances that normally exist as mixtures of polyphenols that are difficult to separate and crystallize (African Pharmacopoeia 1986). These agents are characterized by their high affinity for proteins which results in a variety of biochemical reactions (Collins et al. 1998). The prevalence of these reactions in polyphenolics makes the isolation and characterisation of these agents problematic.

A pre-weighed sample of the crude plant extract was boiled gently in 10ml of water in a test-tube. The mixture was cooled and filtered. A few drops of 5% ferric chloride were added to the filtrate and the presence of tannins was determined by the formation of a blue-black precipitate.

5.2.1.5. *Flavonoids*

Flavonoids are mainly water-soluble compounds that can be extracted with 70% ethanol (Harborne 1998). They are present in varying classes in vascular plants and exist as mixtures comprising different classes. Due to their phenolic properties, flavonoids undergo a characteristic colour change in the presence of an acidic or basic solution. In plants, these agents are commonly found bound to a glycoside and may occur in a single plant as different glycosidic combinations.

A pre-weighed sample of the plant extract was dissolved in 10ml water and warmed in a water bath for 5 minutes. The mixture was filtered. To 3ml of the filtrate, 3ml of acid alcohol, solid magnesium and a few drops of *t*-amyl alcohol were added. The appearance of an orange to violet colour indicated the presence of flavonoids.

5.2.1.6. Sugars

Carbohydrates are a central constituent in plant metabolism and therefore, their identification and estimation is important. Furthermore, carbohydrates are an essential component of several other chemical components and as a result, may influence the physical and chemical properties of other agents (Kennedy 1974). Low molecular weight sugars such as monosaccharides and oligosaccharides are aliphatic polyhydroxy compounds with good water solubility and show optical activity (El Rassi 1995, Bailey 1962). Sugars are unstable in the presence of extreme heat and pH and undergo hydrolysis under such extreme conditions.

To a 1ml solution of the sugar sample, 3ml sulphuric acid was added. The mixture was vortexed and then placed in an ice bath for 2 minutes. To the cooled solution, 0.05ml of 80% phenol was added and mixed. The appearance of an orange solution indicated the presence of sugars. The higher the content of sugar in the sample, the greater the intensity of colour obtained. Results to all the above tests are presented in Chapter VI.

5.3. THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) techniques are widely used in medicinal plant analyses to provide quantitative information of high precision and accuracy over a relatively short time span. TLC techniques were applied at the early stages of chemical characterisation of the crude extract in order to determine the major constituents of the samples (Adachi 1965, Shimi & Imam 1969). Aluminium plates pre-coated with silica gel were used to separate the compounds present in the crude aqueous extract. Each plate measured approximately 3cm by 10 cm. An origin line, on which a sample spot was placed, was drawn 1cm from the bottom of the plate. Different assay procedures were used for the detection of different chemical groups. The assay methods used are described in detail below. The chromatograms were developed using the ascending technique whereby, the plate was immersed in the developing solvent to a depth of about 0.5cm. The chromatographic chamber was lined with filter paper saturated with the solvent. The presence of saturated filter paper promotes the progression of volatile solvent up the chromatographic paper. The chromatographic chamber was closed to prevent evaporation of the solvent. The solvent front was developed to 1cm from the top of the plate, after which the plate was removed from the chamber and air-dried. The solvent front was marked on the chromatogram prior to evaporation of the organic solvent. The plates were examined using spray reagents as described below and eluted compounds were detected by visible light or UV light set at 366 and 254nm. Developed spots on the chromatogram were marked and the R_f value for the given chromatographic system was calculated using the equation:

Equation 9

$$\boxed{R_f = \frac{\text{Distance compound has moved from origin}}{\text{Distance of solvent front from origin}}}$$

5.3.1. TLC Experimental Procedure

On each plate, three test samples were used. The first sample was the extract alone, the second of the standard alone and the third was an equal mixture of the standard and test agent. Two solvent systems were used for the development of chromatograms. The first was an ethyl acetate-methanol-water system in the ratio 100:13.5:10 and the second was a toluene:ethyl acetate system in the ratio 93:7. The former solvent system was for the analysis of polar compounds and the latter for the detection of non-polar compounds. Two TLC sheets were therefore prepared for each test using the procedure previously described. Various spray reagents were used to develop specific chromatograms. The formulae of the spray reagents are provided in appendix 4 p221.

5.3.1.1. Polyphenolics

A 0.1 g quantity of the crude extract was weighed and extracted with 10 ml of 50% methanol for 10 minutes using a reflux system. After cooling, 15 ml water saturated n-butanol was added. After shaking, the sample was filtered and approximately 20 µl of solution was used for TLC. Spraying with vanillin-sulphuric acid reagent developed the chromatogram. After gently warming the sheet, the plate was examined visually and under UV. The appearance of rose coloured or blue fluorescent spots indicated the presence of polyphenols. The standard used was vanillin.

5.3.1.2. Alkaloids

A 0.1 g quantity of the crude extract was weighed and moistened with approximately 1 ml of 10% ammonia solution. To the mixture, 5 ml methanol was added and the preparation was placed in a water bath for 10 minutes. The sample was filtered and approximately 20 µl was used for TLC. The chromatogram was developed using Dragendorffs spray reagent. The appearance of brown/orange visible spots indicated the presence of alkaloids. The standard used was 0.1 g quinine.

5.3.1.3. Saponins

The sample was prepared in the same manner as the quinone sample. After the methanolic extraction, the solvent was evaporated to about 1 ml. A 0.5 ml aliquot of water was added followed by 3 ml of water-saturated n-butanol. The mixture was filtered and approximately 20 µl was used for TLC. The chromatogram was developed using vanillin-sulphuric acid spray reagent. The appearance of blue visible spots indicated the presence of saponins. The standard used was glycyrrhizin.

5.3.1.4. Cardiac Glycosides

To a 0.1 g quantity of crude extract, 5 ml of 50% methanol and 10 ml of 10% lead acetate was added. The mixture was placed in a water bath for 10 minutes. Thereafter, the preparation was cooled and then extracted twice with 10 ml dichloromethane. The dichloromethane fractions were combined and the solvent evaporated. The remaining residue was dissolved in 1:1 dichloromethane-methanol. Approximately 20 μl of this solution was used for TLC. The chromatogram was developed using chloramine-trichloroacetic acid spray reagent. After spraying the reagent, the plate was heated at 80° C for 5 minutes, taking care not to blacken the surface. The plate was examined under UV. The appearance of blue spots with a yellow-green fluorescence indicated the presence of cardiac glycosides. The standard used was digitoxin.

5.4. REMOVAL OF TANNINS AND POLYPHENOLICS FROM AQUEOUS PLANT EXTRACTS

5.4.1. Background

Tannins are secondary metabolites which occur widely amongst vascular plants. In the presence of proteins, tannins form stable water insoluble co-polymers brought about by strong cross linkages (Harborne 1998). Tannins and other polyphenolic compounds interfere with detection and bioassay techniques. Therefore, their presence in samples makes the isolation and characterisation of other compounds difficult (Collins et al. 1998). Tannins exist as two main chemical categories. The first, condensed tannins are commonly found in ferns, gymnosperms and angiosperms and are comprised of oligomers of catechins. The second group of tannins, known as hydrolysable tannins, exist primarily in dicotyledonous plants and are further divided into two groups. This second group of tannins may either be a group of galloyl esters linked to a glucose core or hexahydroxydiphenic acids also linked to a glucose core.

5.4.2. Methodology

A 250mg quantity of the crude extract was weighed. A 5g quantity of polyamide S was weighed and placed in a dry conical flask. A 100 ml volume of water was added to the conical flask resulting in the formation of a slurry. A Buchner funnel linked to a vacuum pump was connected and the polyamide S suspension was filtered through the system resulting in the formation of a firmly packed column bed. Thereafter 100ml 80% ethanol was added to the extract and the suspension was thoroughly mixed. The sample of extract was placed on the column bed and drawn through the column using a vacuum. The eluent from each chromatographic run was collected in separate vials. The polyamide bed was washed with an additional 100ml of 80% ethanol. Each ethanol fraction was lyophilised prior to anti-viral and tannin content testing. The brown coloured material bound to the column was removed from the polyamide bed using 0.1M NaOH. The colour intensity of the eluent increased with increased NaOH washes of the polyamide bed. The NaOH samples were pooled and lyophilised prior to anti-viral and tannin testing (Collins at al. 1998).

5.5. LIQUID CHROMATOGRAPHY

Rapid expansions in analytical technology have enhanced the reliability, utilisation and applicability of HPLC analysis resulting in the availability of an indispensable separation, detection and quantitation technique (Gill 2000; Doonan 1996). Factors that have made HPLC a popular analytical method among researchers working in diverse fields include its high selectivity and specificity. For initial phytochemistry analysis, approximately 90% of assays rely on HPLC methodology. HPLC techniques are widespread due to the relatively small amounts of sample material required, which is useful when analysing extracts with initially low levels of activity. Active samples are serially purified and separated by preparative TLC, semi-preparative and analytical column chromatography.

Chromatograms produced from HPLC are graphic representations of a given separation. To be able to interpret chromatograms, it is important to have an understanding of the underlying principles of HPLC. Chromatography is the separation of a complex mixture through partitioning between a liquid and solid phase. The mobile phase carries the complex mixture through the column to which compounds of the mixture bind in differing degrees according to their physico-chemical properties. After the compounds have been separated in the column, they pass through a detector which interprets the electronic signals received into a chromatogram. Each compound has a characteristic retention time for the sample mixture in a specific chromatographic system. The retention time represents the time from injection of the sample mixture into the system to the time of detection at the apex of the resulting peak. The suitability of a chromatographic system for a given mixture will influence the asymmetry, resolution, selectivity and efficiency of the peaks separated. A well-researched chromatography system, will result in more specific and selective separations.

The utility of liquid chromatography can be significantly improved by linking the system to a mass spectroscopy unit which then allows for the concurrent identification of the masses of the chemical moieties that correlate to a specific peak. Factors that influence detection and are characteristic of a specific detector are sensitivity, the linear response over a given concentration range and the type of response. Universal response enables the detection of all constituents in a sample whereas a selective response is the identification of specific components. The cold aqueous extract of *Barringtonia racemosa* is a novel compound and therefore a new HPLC method had to be developed and validated for its quantitative analyses. High performance liquid chromatography and other liquid chromatographic techniques were used to quantitatively determine the components present in the cold aqueous extract of *Barringtonia racemosa*. The three main liquid chromatographic separation techniques used were size exclusion chromatography which separated constituents on the basis of their molecular weight; reverse phase and normal phase chromatography which separated components according to their hydrophobic/hydrophilic properties and ion-exchange chromatography which makes use of the ionic characteristics of the molecule. Plant extraction and fractionation procedures were continually linked to biological assays to ensure that anti-viral activity was not lost in the process.

5.5.1. Size Exclusion Chromatography

Size Exclusion Chromatography was used to desalt samples and perform size exclusion studies on the crude extract. Size exclusion chromatography is a simple and reliable technique for the separation of molecules on the basis of their size. The media used in size exclusion chromatography comprises cross-linked dextrans that produce beads containing an extensive network of pores. These cross-linked dextrans are carbohydrate polymers containing a large number of hydroxyl groups thus rendering the gel extremely hydrophilic (Le Maire, Viel & Moller 1989). The hydrophilic environment acts as a denaturing environment for most proteins. Due to its ready availability and widespread use, Sephadex gel medium was selected over xerogels for use in these experiments (Tomoda et al. 1990, Chandresekaran, Bemiller & Lee 1978, Barth, Boyes & Jackson 1994, Claeson et al. 1998, Andrews 1964, Awasthi 1981, Chen et al. 1999, Gonda & Tomoda 1991, Granath & Kvist 1967, Tabba, Chang & Smith 1989, Vogel). Furthermore, Sephadex is the standard medium of choice for fractionating mixtures of proteins, peptides, smaller nucleic acids and polysaccharides (Squire 1964, Granath & Kvist 1967, Turan, Nagat & Kuru 1996, Redgwell 1980). Size exclusion chromatography is also frequently used in applications requiring molecular weight estimation and desalting. Different Sephadex gel media are commercially available and they differ in their degree of cross-linking and therefore, fractionation range (Le Maire, Viel & Moller 1989). Table 7, p86 lists the molecular weight range separated by each type of gel agent available commercially. To select the most appropriate gel column material, some knowledge of the physico-chemical characteristics of the sample is necessary. Size exclusion chromatography columns contain semi-solid beads of a polymeric gel that will admit ions and small molecules into their interior pores and exclude larger molecules from entering the pores. Compounds small enough to enter the pores have a much longer potential path through the column and therefore travel slower than the larger molecules. The rate of passage of these smaller molecules is dependent on their shape and size. Molecules are therefore eluted in order of decreasing molecular size. The different flow rates of molecules through the column therefore facilitate the non-destructive fractionation of the components in a mixture.

Table 7: Fractionation ranges of Sephadex gel agents

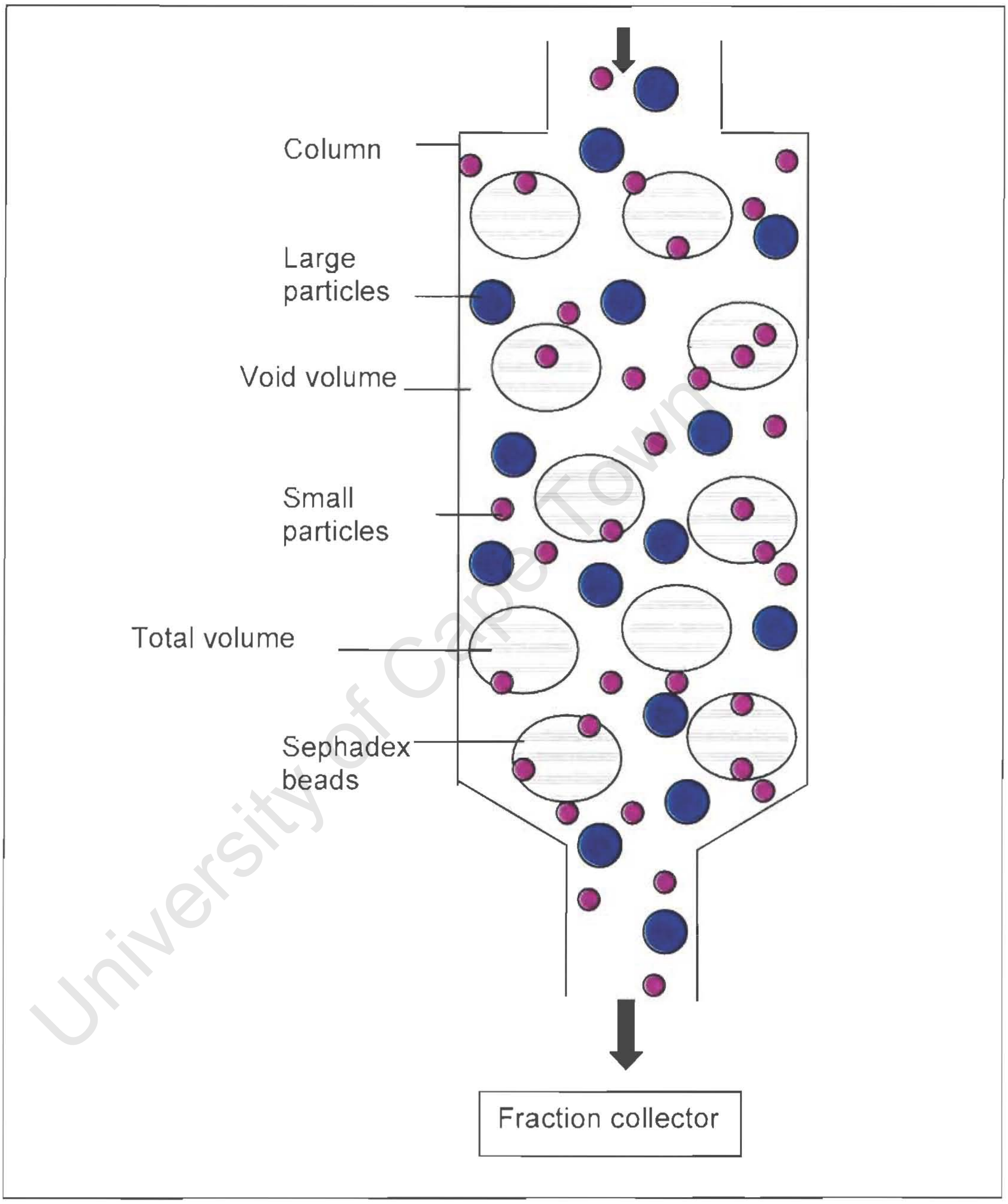
SEPHADEX	*[1]MW FRACTION RANGE
G-10	>700
G-15	>1500
G-25	1000 – 5000
G-50	1500 – 30 000
G-75	3 000 – 80 000
G-100	4 000 – 150 000
G150	5 000 – 300 000
G-200	5 000 – 600 000

*[1]Sigma chemical company. Molecular weight determinations calibrated using globular protein.

5.5.1.1. Size Exclusion Chromatography Theory

During the passage of a sample through a size exclusion chromatography column, molecules that flow past the beads without entering the pores are said to be *Excluded* and such molecules travel through the column rapidly. This early elute is found in the mobile phase and the volume at which it elutes is called the *Void Volume* (V_o), (Figure 8, p87). Small molecules that are able to enter the pores of the gel matrix beads are said to be *Included*. As these molecules spend more time in the stationary phase, they travel at a slower rate and are therefore eluted at a later stage. The volume of solvent required to elute all molecules included in the matrix is called the *Total Volume* (V_t).

Figure 8: An illustration of the behaviour of molecules during size exclusion chromatography



Intermediate sized molecules may either enter or remain outside the gel pores depending on the shape and size of the molecule. The elution of intermediate molecules is based on their molecular weights and the volume at which they elute is called the *Elution Volume (V_e)*. The terms V_e, V_o and V_t can be used to calculate the distribution coefficient K_d. K_d gives an indication of the size of the molecules eluted. The bigger the K_d value, the smaller the molecule and therefore the more likely that the molecule is *Included*. The inverse can be said for a small K_d value. The K_d value can be calculated using the following equation:

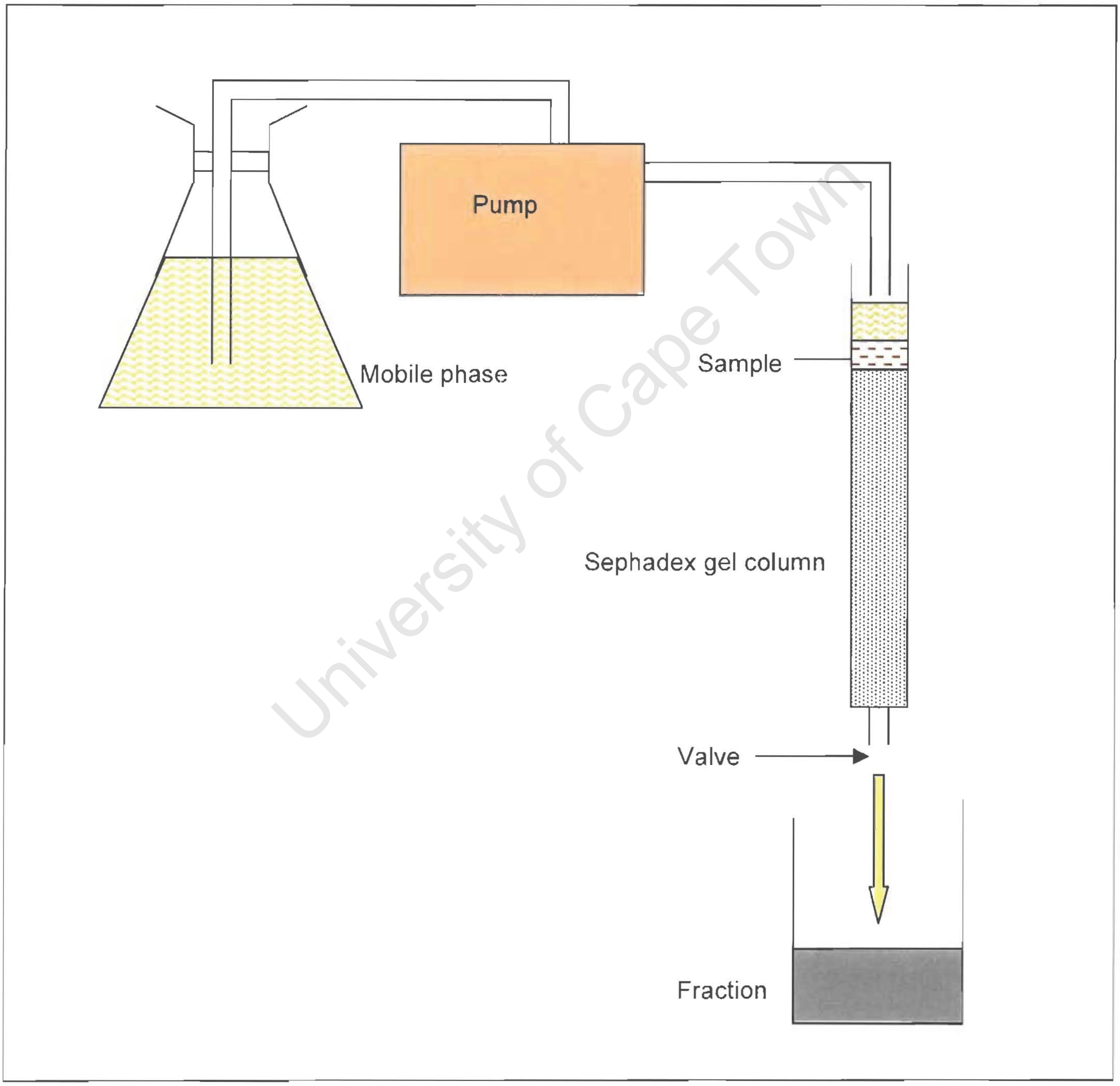
Equation 10

$$Kd = \frac{Ve - Vo}{Vt - Vo}$$

5.5.1.2. Column Preparation

A column, tubing and connectors were assembled according to the manner illustrated in Figure 9, p89. Approximately 15g of column material was mixed with small aliquots of water until a moist slurry was formed. The column was filled to a quarter of its size with purified water. The column material was suspended in the water and the column was filled with the filling material. The bottom valve of the column was opened the excess water was allowed to run out. Care was taken not to allow the column to run dry. The top of the column was sealed and the column material was allowed to settle overnight. After the column material had settled, two column volumes of the eluent were allowed to run through the column prior to using the column. The sample material dissolved in water was added to the top of the column, ensuring that the column bed was not disturbed. The bottom valve was opened to allow the sample to run through. The valve was then closed and the eluent was added to the column. As the sample passed through the column, fractions were collected. The eluting solvent was continually added to the column to prevent the packing material from running dry. The size exclusion chromatography standard used in experiments was blue dextran, which is completely excluded from the column and elutes in the void volume. During initial screening tests, superfine G50 medium was used. To increase the specificity and resolution of the separation, superfine G25 medium was used, replacing the G50 material.

Figure 9: A size exclusion chromatography configuration



The analysis system assembled was simple and not attached to any spectrometric equipment. Therefore, antiviral assays had to be conducted to determine active fractions.

5.5.1.3. Size Exclusion Chromatography Experimental Procedure

A 10 mg/ml preparation of the test sample was constituted in water. In the first column run, 0.5 M sodium chloride was prepared and 0.5 ml was added to the test sample. The sample was sonicated then centrifuged at 3000 *g* for 10 minutes. The supernatant was withdrawn and added to the top of the chromatography column. The bottom valve of the column was opened and the test sample was allowed to run through the column. Water was used as the eluting solvent. Fractions of the eluent were collected in 5 ml aliquots. The elution of fractions of different molecular size was monitored using the silver nitrate test. It was therefore possible to determine the number of low molecular weight and high molecular weight fractions. Fractions collected before the detection of the chloride ion were categorised as high molecular weight samples while fractions collected after the detection of the chloride ion were categorised as low molecular weight samples. Subsequent runs were performed without the addition of the sodium chloride salt. The fractions collected were lyophilised and tested for anti-viral activity.

5.5.2. Ion Exchange Chromatography

5.5.2.1. Theory

Ion exchange chromatography (IEC) has been successfully used in phytochemistry research for separating different classes of sugars, amino acids, organic acids and phosphate esters (Vanecek & Regnier 1980, Haq et al. 1990, Gonda et al. 1992, Cheng & Kindel 1995, Redgwell 1980, Yamada et al. 1990, El Rassi 1995). Ion exchange chromatography is a rapid and convenient technique and was used to analyse the chemical groups present in *Barringtonia racemosa*. The basic principle of IEC is that charged biological moieties will bind to oppositely charged groups on an ion exchange matrix. There are two basic classes of ion exchange media; anion-exchange media which comprises positively charged groups to which negatively charged agents bind; and cation-exchange media which have negatively charged groups that bind to positive moieties on the column. The separation of bound substances occurs when the matrix is eluted with a displacing salt causing the strength of binding to decrease. Consequently, the strongly bound sample molecules are displaced from the column and subsequently pass through the detector. Molecules with small differences in charge can be separated using this procedure. Ion exchange chromatography is therefore a high-resolution technique that enables one to use large sample volumes to give high yields.

DEAE Sephacel was selected for this procedure because it was found that the extract material bound to DEAE Sephacel at neutral pH and could be eluted by 0.5M NaCl. Other factors that were considered when selecting the gel medium were sample capacity, the stability of the sample components, buffer pH and molecular size of the sample constituents. Sephacel is a cellulose ion exchanger formulated as rigid beads. The rigid beads prevent the formation of fines, and therefore, the system exhibits better flow rates, resolution, recovery and column life. Furthermore, the gel medium is able to maintain a constant separation capacity over a wide pH range of 2-10. DEAE Sephacel is a weak cationic exchange medium that has a sodium counter ion bound to its surface. Elution of the bound molecules was achieved by establishing a salt gradient resulting in gradual displacement of molecules. The salt competes with the bound protein for the charged groups on the column, and at a salt concentration for each desired agent, the active is eluted.

5.5.2.2. Ion Exchange Experimental Procedure

The same procedure was used to prepare the ion exchange column as was earlier described for the preparation of the size exclusion column. The eluting solvent used was a phosphate buffer and DEAE Sephacel packing material was chosen. A 10 mg/ml mixture of the crude extract was prepared in water. The sample was sonicated and centrifuged at 3000 *g* for 10 minutes. The supernatant was removed and carefully placed on the column surface. The bottom valve was opened and the buffer solution carrying the sample was allowed to run through. The column was then rinsed twice with the buffer solution. Sodium chloride was prepared in concentrations of 0.05 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 2.5 M. Solvents of different sodium chloride concentrations were passed through the column and the eluent was collected in regular fractions. The samples collected were lyophilised then desalted using the procedure mentioned in section 5.5.1.3. The presence of sodium chloride in fractions collected was monitored using silver nitrate. Fractions collected before the detection of the salt were pooled together and so were fractions collected after the salt was no longer detected. The samples were lyophilised and tested for anti-viral activity.

5.5.3. Ion Exchange High Performance Liquid Chromatography Conditions

Carbohydrates are major constituents of plant material. The colorimetry studies indicated the presence of sugars in the sample material tested and therefore ion exchange high performance liquid chromatography was employed to identify and isolate the sugar moieties. Ion exchange chromatography involves the attraction of charged ionic groups to the anionic or cationic groups on the surface of the solid stationary phase. The separation of compounds is achieved through the use of a displacing salt whose concentration can be manipulated to allow for the selective dislocation of chemical groups from the column surface. Therefore, selective chromatographic separation is highly dependent upon establishing the correct HPLC conditions for the separation of the extract in question (Duncan, Chen & Siebert 1987, Gill 2000). Lengthy investigations were carried out into developing a solvent system that generated chromatograms with good resolution and selectivity. An HPLC solvent system that was compatible with the detector and system components and above all, in which the extract exhibited good solubility was required (Doonan 1996). It is possible to obtain complete peak resolution within a short analytical period through the manipulation of pH in buffer solutions. Changes in the resolution of the chromatographic traces generated when the composition of the mobile phase was altered have been studied in other laboratories and were explored in this investigation (De Smet et al. 1988). In addition, variations in the chromatographic profile, brought about by substituting the displacing salt were also examined. Of the three salt systems tested; sodium chloride, sodium acetate and sodium sulphate, the chromatograms produced using a sodium acetate buffer solution gave the best resolution. Following an extensive study into solvent system optimisation, it was found that a mobile phase of phosphate buffer as solution A and phosphate/Na acetate buffer as solution B resulted in the formation of chromatograms that exhibited adequate selectivity, separation and resolution.

A strong anionic exchange (SAX) column packed with Partisil was used for separations. The strong anion-exchange column surface enabled negatively charged groups of the sample to bind to the positive surface charge. A literature survey on IEHPLC columns revealed that strongly charged surface materials resulted in better-resolved separations than when weak ionic exchange materials were used (Mazsaroff 1988, Kopaciewicz & Regnier 1983, Regnier 1982, Duncan, Chen & Siebert 1987). Furthermore, the life expectancy of strongly charged ion exchange are longer due to their capacity to manage solvents with wide ranging pH values. In addition to mobile phase investigations, effect of sample load, mobile phase velocity, pH and gradient elution studies were performed.

5.5.3.1. Materials And Methods

All chemicals used were of HPLC grade obtained from BDH (Poole, England). Milli-Q purified water (Millipore, Watford, UK) was used in all experiments. Pre-weighed samples (5mg) of the extract residue were prepared and dissolved in 1.0 ml water. The mixture was sonicated for 5 minutes using a UMC 5 sonicator (Ultrasonic Manufacturing, Krugersdorp, South Africa) and centrifuged at 1500 *g* for 5 minutes using an Abbot centrifuge (Abbot, Germany). The resulting supernatant was aspirated and used in HPLC fractionation assays. To obtain a comprehensive chromatographic profile of the cold aqueous extract analysed, a linear gradient beginning at 100% sodium acetate buffer (ionic strength, I = 0.5) and 0% phosphate buffer over 5 minutes was established and followed by an increase in buffer composition to 100% over the next 15 minutes. A constant flow rate of 1ml/min was maintained throughout analysis. The absorption spectra of the eluent was monitored at 205, 240 and 300nm using a Shimadzu diode array detector (SPD-M10A, Mitsubishi, Japan). The initial separation yielded two fractions, which were collected manually at the time intervals 0-10 minutes for fraction 1 and 10 – 20 minutes for fraction 2. Following the removal of the salt and solvent from each fraction collected, the samples were then tested for antiviral activity using methods previously described. Desalting was achieved through the use of size exclusion chromatography.

5.5.3.2. Apparatus

All HPLC analyses were conducted using a Shimadzu instrument equipped with a Shimadzu SPD-M10A diode array detector (Shimadzu, Mitsubishi, Japan), a Shimadzu SIL-10A auto injector (Shimadzu, Japan) and Shimadzu LC-10AS pumps (Shimadzu, Mitsubishi Japan). IEHPLC samples were separated on a strong anionic analytical exchange column. A Grom manufactured column of 250 by 4 mm and filled with 10 µm Partisil SAX was used.

5.5.4. Reversed Phase Chromatography

5.5.4.1. Theory

The majority of plant extracts are fractionated using reverse phase mode. This is by far, is the most established chromatographic technique in medicinal plant analysis (Jung-Bum et al. 1999, Mahoney & Hermodson 1980, Cairnes, Kingston & Rao 1982, Heyraud & Rinaud 1980, Rajakyla 1986, Verhaar & Kuster 1984, Welss et al. 1972, Blanken et al. 1985, McNeil et al. 2000). Reverse phase chromatography is useful for the analysis of compounds that have dual polar and non-polar characteristics (Gazdag & Szepesi 1989). A C_{18} Octadecylsilane column was utilised, which when combined with a appropriate mobile phase, provided a suitable system in which constituents could be easily separated (De Smet et al. 1988, Gazdag, Szepesi & Szelecki 1988, Snyder & Glajch 1982, Glajch & Snyder 1981, Heyraud & Rinaudo 1980). The non-polar surface of a reverse-phase column facilitates the separation of compounds carried by a polar mobile phase. Retention of a compound increases with increasing molecular size or hydrophobicity. The mobile phase component is of equal importance in reverse phase HPLC (De Smet et al. 1988, Gazdag, Szepesi & Szelecki 1988, Snyder & Glajch 1982, Glajch & Snyder 1981, Snyder, Glajch & Kirkland 1980, Doonan 1996). Buffers are frequently used, however, greater column care is required due to possible precipitation of the buffer salt within the system. Acetonitrile, methanol, isopropanol and tetrahydrofuran are mobile phases commonly used singly, in varying combinations with water or together in various proportions (Gazdag, Szepesi & Szelecki 1988, Glajch & Snyder 1981, Snyder, Glajch & Kirkland 1980). A UV-VIS photodiode array detector is a useful instrument for the detection of eluting compounds.

5.5.4.2. System Validation

The precision, limit of detection and sensitivity assays were used to determine the suitability of the HPLC method selected for the analysis of plant extracts. The validation results demonstrate the selectivity and specificity of an analytical technique. Through these tests, it is possible to determine the reliability and reproducibility of the method developed (Szepesi, Gazdag & Mihalyfi 1989).

Precision Studies

Precision studies are concerned with the repeatability of a given analytical method (Renke 1996, Krull & Swartz 1997). The precision of the system was validated through monitoring intra- and inter-day HPLC variations using an internal standard (Szepesi, Gazdag & Mihalyfi 1989). Precision is often expressed as the percent relative standard deviation and the data obtained from these studies reflects the distribution of test results around their mean value. Most reported chromatographic methods have percent relative standard deviations of between 5% and 10%.

Limit of Detection

Limit of detection studies are used to determine the lowest concentration of standard that may be accurately detected by the chromatographic system in a given sample (Renke 1996, Krull and Swartz 1997). Results for this test were obtained by investigating instrumental response to progressively lower concentrations of the standard.

Sensitivity Studies

Sensitivity studies are dependent upon the degree of response, by the system, to given concentrations of analyte (Hamilton and Sewell 1982). The standard deviation about the mean of each point that represents a given concentration is an indication of the accuracy with which the system is able to respond to decreasing concentrations of standard.

Specificity Studies

An investigation of specificity needs to be conducted during the validation of identification tests. During the tests, the ability of the chromatographic system to distinguish between chemical constituents that are structurally similar and are closely related was investigated. Mannose was used as the internal standard and D-glucose was used as the test analyte. A 50:50 solution of each compound was prepared at a concentration of 0.05mg/ml and was injected into the HPLC system. The resolution of the resulting peaks was examined and presented in Figure 16, p158.

Calculations

The percent relative standard deviation gives the percentage variation of the standard deviation of the peak area ratios calculated about the sample mean while the percent relative error is the degree of correlation of the test results to a theoretical value.

Equation 11

$$\text{Percent Relative Standard Deviation} = \frac{\text{Standard Deviation}}{\text{Mean of Sample Population}} \times 100$$

5.5.4.3. Experimental Conditions

Initial studies performed investigated the suitability of various mobile phases, flow rates and mobile phase compositions to develop the most suitable chromatographic system for the test material. A mobile phase of HPLC grade acetonitrile obtained from BDH (Poole, England) and Millipore purified water was eventually selected for subsequent studies. Prior to use, the mobile phase was filtered and degassed using helium gas. The flow rate was maintained at 1.0 ml/min over a gradient starting from 0% acetonitrile to 50% acetonitrile/water during a period of 20 minutes. The system was validated using mannose as the internal standard. Mannose was selected because it elutes as a single peak in an acetonitrile/ water system that does not interfere with the elution of extract peaks. Parameters investigated were precision, limit of detection and sensitivity. To prolong the column life, a C_{18} guard column was used in all experiments. Both the analytical and semi-preparative column used were packed with C_{18} octadecylsilane. An automated injector withdrew 50 μl aliquots from the injection vial containing the test sample. The chromatographic apparatus used was as previously described. During fractionation studies fractions of the eluent were collected at predetermined intervals using a Pharmacia Frac-100 fraction collector. The fractions collected were lyophilised and the dry residue was tested for anti-viral activity.

5.5.4.4. Sample Preparation

For the investigation of compounds using an analytical column, 0.05 g of the test sample was weighed and in studies where a semi-preparative column was used, 0.1 g of test material was measured. The sample was dissolved in 1 ml Millipore water and placed in a sonicator for 5 minutes. The sample was then centrifuged at 3000 *g* for 10 minutes. The supernatant was withdrawn and placed in a vial. Sample not used at the end of a chromatography session was placed in a 4°C refrigerator until required for use.

5.5.5. Solid Phase Extraction

Solid phase extraction (SPE) is a rapid procedure for the fractionation and purification of natural plant material. This technique allows for the purification of large volumes of sample material through the use of different sized cartridges with varying sample capacity. SPE techniques were used after having determined the suitable chromatographic technique for the fractionation of the crude extract. As the chromatographic system of choice was reversed phase chromatography, a C_{18} SPE column was selected for the purification of large amounts of extract. Due to the opportunity of performing rapid isolations using SPE, it was possible to determine the suitability of other column systems for the analysis of the crude extract. The advantages of SPE are that it enables one to achieve high recoveries of the desired analyte at high concentrations and the compound obtained is highly purified (International Sorbent Technology 1999).

5.5.5.1. Sample Preparation

A 10g/60ml C_{18} reversed phase SPE column was used during purification processes of the test sample. A 0.3 g measure of the extract was weighed and dissolved in 10 ml water. The mixture was sonicated for 5 minutes and centrifuged at 3000 *g* for 10 minutes. The supernatant was withdrawn and applied to the column surface. A vacuum was used to draw the sample through the column.

5.5.5.2. Column System Preparation

Prior to initiating chromatographic fractionation, it was necessary to condition the column. The column was first rinsed twice with water. Thereafter, the sample was eluted through the column. Elution of the test material was followed by washes of acetonitrile ranging in concentration from 0% to 100% in 10% increments. Each elution was collected. The samples collected were lyophilised and tested for anti-viral activity.

5.5.6. Normal Phase Chromatography

5.5.6.1. Theory

Normal phase chromatography involves the separation of compounds on a hydrophilic stationary phase using a non-polar agent such as hexane and methylene chloride. The polar charge of the stationary phase is attributed to the presence of hydroxyl groups on the surface. The hydroxyl group can be modified through the formation of bonded-phase packing such as amino, octyl, phenyl, octadecyl and cyano groups. The mechanism of retention in normal phase chromatography is the interaction of polar compounds in a sample with the polar groups of the column (Jandra, Kucerova & Holikova 1997). The more polar the mobile phase, the shorter the retention time due to displacement of polar compounds by the solvent. Within phytochemistry, the use of normal phase chromatography is less wide spread than reverse phase chromatography. This is mainly attributed to the fact that most phytochemicals investigated are non-polar and therefore, reverse phase HPLC is more applicable. Normal phase chromatography was investigated because of the nature of the active agent and the fact that useful information can be derived to assist in further chromatographic characterisation of the aqueous active extract. Properties that are important during characterisation include the lipophilic and hydrophilic character of the compounds, the polarity of the compounds and the basicity of the functional groups. (Gazdag, Szepesi & Farbian-Varga 1988, Gazdag & Szepesi 1989)

5.5.6.2. Experimental Conditions

The test sample was prepared using the same procedure as that used for reversed phase chromatography. A Waters analytical normal phase column with dimensions 4 mm by 250 mm and packed with amino-modified silica facilitated the separation of the sample constituents. A mobile phase of acetonitrile and water flowing at 1.0 ml/min, eluting at a gradient of 100% to 0% acetonitrile over 30 minutes was used during investigations. HPLC grade reagents were used and the mobile phase was filtered and degassed prior to use. The chromatographic apparatus used has been previously described. During fractionation studies, fractions of the test material were collected at predetermined intervals. The collected samples were lyophilised and tested for anti-viral activity.

5.6. GAS CHROMATOGRAPHY

5.6.1. Theory

Gas chromatography (GC) involves the use of a volatile inert gas which serves as the mobile phase to carry a volatile substance through a liquid or solid stationary phase (Biermann & Mcginnis 1989, Fox, Morgan & Gilbart 1989, Dutton 1974, Bishop 1964). The vaporised sample partitions between the carrier gas and the stationary phase. Factors such as the nature of the solute, the nature and amount of stationary phase, the temperature and the carrier gas flow influence the partitioning of the test sample. For quantitative determinations, gas chromatography systems are normally attached to mass spectrometers.

Due to GC being limited to volatile substances, its use in plant analysis is restricted. This technique was utilised to analyse the alditol acetate samples prepared from HPLC fractions of *Barringtonia racemosa*. Due to the unavailability of a GC apparatus within the department, dry alditol acetate samples were sent for GC analysis to the University of the Western Cape, chemistry laboratories.

A gas-liquid chromatographic system was utilised to analyse the hydrolysed crude sample. This form of chromatography involved the vaporisation of the sample injected onto the head of the chromatographic column. The sample was transported through the column by the flow of an inert, gaseous mobile phase. The column itself contained a liquid stationary phase which was adsorbed onto the surface of an inert solid. Eluting products were analysed using a selective detector that responded to a range of compounds with common physical or chemical property. Results derived from GC analysis are presented.

5.7. CARBOHYDRATE CHEMISTRY: A BRIEF INTRODUCTION

Carbohydrate derivatives are important constituents of the plant kingdom that exist as various chemical derivatives possessing valuable pharmacological properties. A significant number of reports have been published on the anti-viral properties of oligosaccharides and polysaccharides isolated from plants (Vanden Berghe, Vlietinck & Van Hoof 1986, Hudson 1994, Vanden Berghe et al. 1978, Eo et al. 1999, Garcia-Villalon & Gil-Fernandez 1991, Garcia et al. 1999, Gonzalez, Alarcon & Carrasco 1987, Hayashi, Hayashi & Kojima 1996, Jung-Bum 1999, Xu et al. 1999, Witvrouw & De Clercq 1997, Marchetti et al. 1996, Gonda & Tomoda 1991, Lin, Kuo & Chou 2000). In view of this and given the results obtained in this study, it was essential to study the physicochemical properties of carbohydrate compounds (Biermann & McGinnis 1989, Kennedy 1974, Dutton 1974, Bishop 1964).

Carbohydrates are defined as polyhydroxy aldehydes or ketones or as substances that yield one of these compounds on hydrolysis. Carbohydrates are classified according to the number of sugar units they contain (El Rassi 1995). Monosaccharides are known as simple sugars that cannot be hydrolysed to smaller units under mild hydrolysis conditions. Monosaccharides exist in stereoisomeric forms and exhibit optical activity. Disaccharides and oligosaccharides are hydrolysable polymers of monosaccharides and contain two to six molecules of simple sugars (Bailey 1962). They are therefore formed by the condensation of monosaccharides. Disaccharides contain two sugar residues and are most abundant in nature. Oligosaccharides, on the other hand, contain three or more subunits and are often found bound as side chains in glycoproteins. Oligosaccharides exhibit similar chemical properties as monosaccharides. These low molecular weight sugars are aliphatic polyhydroxy compounds that normally exhibit good solubility in water. Aliphatic hydroxy constituents are difficult to crystallize and therefore are frequently isolated as derivatives.

Polysaccharides are polymers of hundreds of covalently linked monosaccharides. They are usually water soluble and may either be branched or linear in structure. Structurally, polysaccharides can exist as complex molecules due to the different configurations that can exist in a single molecule. Sugar units within a polysaccharide can be linked to one another via glycosidic linkages in several different ways. Furthermore, the ether linkage can have either a α- or β- configuration depending on the stereochemistry of constituent sugars.

The structural conformation of sugars allows them to easily undergo isomerisation and/or ring opening during extraction and concentration procedures carried out at high temperatures or pH (Harborne 1998). Micro amounts of saccharides can be detected using the phenol-sulphuric acid reaction.

5.8. HYDROLYSIS AND ACETYLATION

The analysis of alditol acetate derivates by gas chromatography (GC) spectrometry is a useful technique for the identification of sugar constituents of polysaccharides (Gonda & Tomoda 1991, Gonda 1990, Gonda 1993, Gonda 1992, Biermann & McGinnis 1989, Fox, Morgan & Gilbart 1989, Lonngren & Svensson 1974, Courtin, Vanden Broeck & Delcour, McNeil et al. 1980). In brief, the process involves acid hydrolysis of the polysaccharide into its constituent monosaccharides followed by reduction of the monosaccharides into their corresponding alditols. Acetylation of the alditol groups resulted in the formation of a volatile derivative that was suitable for GC analysis (Fox, Morgan & Gilbart 1989, Biermann & McGinnis 1989, Linberg, Lonngren & Svensson 1975, Bailey 1962, Yamada, Otsuka & Omura 1986). Alditol acetates of test samples were produced using the procedure reported below.

A 100 mg quantity of the saccharide-containing extract was placed in a round bottomed flask to which 20 ml of 4M trifluoroacetic acid (TFA) was added. The mixture was gently stirred to aid dissolution. The flask was connected to a condenser and placed in silicon oil heated to 125° C. The mixture was hydrolysed for 1 hour. After cooling of the hydrolysed compound, 10 ml methanol was added to the vessel. The methanol was thereafter evaporated using a rotary evaporator. A further 5 ml of methanol was added and again, evaporated. This procedure was repeated three times. The resulting precipitant was dissolved in approximately 5 ml water and excess sodium borohydrate ($NaBH_4$) was added to the preparation. The mixture was left to stand for 12 hours. After 12 hours, glacial acetic acid was added to the vessel drop-wise until the resulting effervescence ceased. A solution of 10% acetic acid in methanol was added to the mixture and evaporated. The remaining solute was rinsed with 10ml methanol which was then evaporated to dryness. A 1 ml measure of pyridine and 5 ml of acetic acid was added to the dry compound. The mixture was refluxed for 1 hour. The solvent was thereafter evaporated to dryness. The dry mixture was dissolved in chloroform and the solution was placed in a centrifuge tube. The chloroform containing preparation was first washed with water followed by acidified water, water again, alkalinised water and finally plain water. After each wash, the mixture was lightly centrifuged and the aqueous layer removed. After the final wash, sodium sulphate was added to the tube. The chloroform layer was removed using cotton wool. Additional chloroform was used to wash the wool. The solution from each wash was placed in pre-weighed vials. The organic liquid was evaporated and the remaining residue was sent for GC analysis.

5.9. *PROTEIN/ AMINO ACID ANALYSIS*

Plant proteins comprise of high molecular weight polymers of amino acids. The amino acids act as building blocks that are arranged in a specific linear order, as determined by the triplet base code of the DNA in the nucleus (Harborne 1998). Proteins are divided into two main categories known as simple and conjugated proteins. Simple proteins may consist of a single polypeptide chain while conjugated proteins contain other structural elements other than amino acids. Plant proteins can be further classified according to their degree of solubility in water, aqueous acid and alkali and 70% alcohol. A third system of classification is based on the molecular weight of the protein using size exclusion chromatography techniques on a Sephadex column. The mobility of the protein through the column is dependent upon its molecular weight and partly influenced by its net charge (Squire 1964, Granath & Kvist 1967, Lumley, Patel & Cohen 1987).

A 0.1 mg quantity of purified sample of the crude extract was weighed and sent for amino acid analysis to a laboratory that possesses the necessary apparatus. The purpose of the protein analysis is to obtain a measure of protein present in the extract and predict probable secondary structures and folding classes for the amino sequence of the plant protein.

Samples under going amino acid analysis were hydrolysed in 5 M hydrochloric acid at 110°C continuously for 24 hours. A custom designed amino-acid analyser was used to measure amino-acids in hydrolysed protein samples. The equipment used is based on modular ionic exchange HPLC components and has been designed to give maximum versatility in methods of separation and detection.

5.10. MALDI-TOF/MS

Matrix-assisted laser/desorption ionisation, time of flight (MALDI-TOF) mass spectrometry enables the rapid analysis of proteins, peptides, oligonucleotides, carbohydrates and glycoproteins (El Rassi 1995, Kochetkov & Chizhov 1966, Gorin 1981, Lonngren & Svensson 1974, McNeil et al. 2000). Depending on the sensitivity of the instrument, this technique is able to identify unknown proteins, their masses and amino acid sequencing of peptides. The basic principle behind MALDI analysis is that the sample is imbedded in an excess of a solid matrix that upon laser irradiation, assists in the volatilisation and ionisation of the analyte. The analyte does not absorb the laser energy directly, allowing for the analysis of complex bio-molecules. The ions produced during the MALDI process are analysed using time-of-flight (TOF) mass spectrometry (Online 2001b).

A 0.1 mg amount of purified sample from the crude extract was weighed and sent for MALDI-TOF analysis to the analytical laboratory of the Department of Biochemistry, University of Cape Town, which possesses the required apparatus.

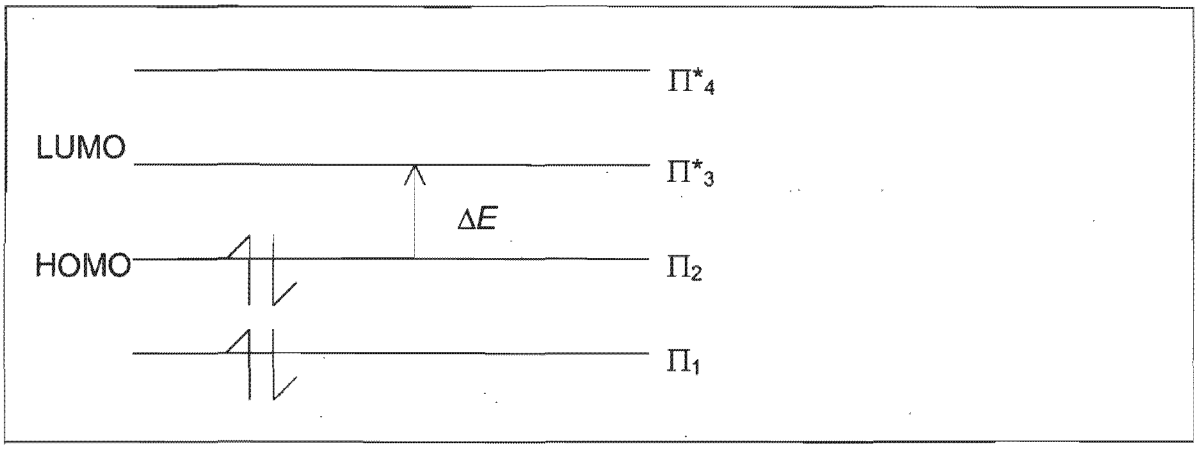
5.11. METHODS OF IDENTIFICATION

Following the extraction and isolation of an active plant component it is necessary to determine the chemical structure of the compound of interest. Initially the physico-chemical characteristic of the component can be determined by establishing the solubility, melting point, pH, optical rotation and ionic charge. Elucidation of the structure is achieved by utilising spectral techniques such as infrared (IR) spectroscopy, ultra violet (UV) spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Cordell & Shin 1999, Harborne 1998). Each technique proves unique structural information and it is important to apply all four in identifiying the structures of the active compounds.

5.11.1. Ultra-violet/ Visible (UV-VIS) Spectroscopy

UV-VIS provides basic information on the number and nature of conjugated multiple bonds in a molecule. UV-VIS spectroscopy involves the irradiation of chemical constituents in a sample. Absorption of UV-VIS radiation takes place when an electron is promoted from a bonding pi- molecular (Π) orbit to an anti-bonding (Π^*) molecular orbit or form a non-bonding molecular orbital to a Π^* orbital. For two or more conjugated bonds, the transition is always from the Highest Occupied Molecular Orbit (HOMO) to the Lowest Unoccupied Molecular Orbital (LUMO), (Figure 10).

Figure 10: Electromagnetic energy conversions



Equation 12

Where $\boxed{\Delta E = h\nu = h\,\frac{c}{\lambda}}$

h is Planck's constant

To undergo an energy change, molecules need to be conjugated, possessing double or triple bonds. Different functional groups absorb electromagnetic energy at characteristic wavelengths. The absorption of light at a fixed wavelength can be determined using Beer-Lambert's Law, which is expressed as follows:

Equation 13

$$\boxed{A = \log_{10}\frac{Io}{I} = \varepsilon c l}$$

Where A is the absorbance
I_o is the intensity of the incident radiation
I is the intensity of the transmitted radiation
c is the concentration of the solution in mol/dm^3

ε is the molar extinction coefficient

l is the path length

Dilute solutions of organic compounds dissolved in solvents such as ethanol, methanol, cyclohexane, chloroform, acetonitrile and water are used. The UV-VIS spectrometer plots the wavelength of absoption as a function of the absorbance. During analysis the instrument automatically subtracts absorption due to the solvent from the absorption of the sample plus solvent. The ultraviolet range is between 100 – 400 nm while the visible light range is between 400 – 800 nm. Each absorption band for a compound is then characterised by the wavelength of asorption (λ_{max}) and the molar extinction coefficient (ε_{max}), (Harborne 1998, African Pharmacopoeia 1986, Campbell 1998, Martin, Swarbrick & Cammarata 1983).

5.11.2. Infrared Spectroscopy

Infrared (IR) spectroscopy provides information on the types of functional groups present in a molecule. The energy of most molecular vibrations corresponds to that of the infrared region of the electromagnetic spectrum. Functional groups vibrate within a specific region of the spctrum (2.5 – 16 µm). The position of absorption of a specific functional group is usually expressed as the reciprocal of the wavelength, cm^{-1} and thus the usual rane is 4000 – 625 cm^{-1} This is referred to as the wave number. The frequency of vibration of the functional group ($v = \frac{1}{\lambda}$) is related to the strength of the bond (k) and the mass of the atoms in the bond (m_1 and m_2).

Equation 14

$$\boxed{v = \frac{1}{2\pi c}\sqrt{\frac{k}{\mu}}}$$

Where c is the speed of light

μ is the reduced mass = $\frac{m_1 \times m_2}{m_1 + m_2}$

Because the vibrations of the whole molecule influence the vibrational frequency of functional groups, absoprtions take place over a range (cm^{-1}). Chracteristic wave number ranges can be found listed in reference tables (Harborne, African Pharmacopoeia 1986, Campbell 1998, Martin, Swabrick & Cammarata).

5.11.3. Nuclear Magnetic Resonance (NMR)

NMR has become one of the most valuable identification techniques available in phytochemistry (De Tommasi et al. 1991, Gonda & Tomoda 1991, Hall 1965). It is a method of structure determination whereby a chemical shift distribution of the carbon-hydrogen configuration of a molecule is given. The chemical shift is derived from the interaction of electromagnetic radiation from the radio wave region of the spectrum with the spin of nuclei in a magnetic field. The resonance absorption is specific for a particular atomic isotope. From this technique, it is possible to determine each type of carbon and hydrogen atom in a molecule and the immediate environment of each group. The principle behind NMR is that the test sample is dissolved in a deuterated solvent and placed between the poles of a powerful magnet (Harborne 1998, Hall 1965, Campbell 1998). Upon irradiation with a radio frequency signal, the protons within the structure resonate at different positions on a chemical shift scale that is specific to that environment in the molecule. These chemical shifts are measured relative to a standard. NMR active isotopes include ^{1}H, which is the most abundant and ^{13}C, which gives good spectra under special conditions that enhance sensitivity. The complexity of NMR spectra reflects the number of protons present in the sample. The development of additional 1D and 2D procedures, such as COSY, DEPT, HSQC, HMBC and NOESY has made NMR a more powerful tool.

NMR is a useful fingerprinting technique for determining the types of atoms that are present in a molecule, how the atoms are connected and the relative number of each type of atom in that molecule.

5.11.4. *Mass Spectroscopy (MS)*

MS provides a measure of the mass of the molecule of interest. High resolution mass spectrometry (HRMS) enables one to determine the molecular mass of compounds to six decimal places. Using a computer programme this can be matched to the calculated masses of a number of combinations of carbons, hydrogens, nitrogens and oxygens and thus a molecular formula can be generated. In addition to molecular weight determination, this technique provides useful structural information of unknown components by measuring the masses of fragmentations produced from the splitting of the high-energy molecular ion (Kochetkov & Chizhov 1966, Gorin 1981, Lonngren & Svensson 1974). The basis of MS is that a stream of high-energy electrons bombard a small amount of sample introduced to the spectrometer. The rate at which the stream of electrons bombards the sample can be controlled. When a high energy electron strikes an organic molecule, a valence electron is dislodged from the molecule resulting in the formation of a radical cation. Due to the amount of energy transferred from the electron, these radical cations fragment to produce fragmentations. The fragmentations are passed through a strong magnetic field where they are deflected according to their mass-to-charge ratio *m/z*. These ions are recorded by a detector, which converts them into *m/z* peaks. Normally the charge (z) on the fragmentations is 1 and therefore the peak of ratio (m/z) is the mass (*m*) of the ions. The mass spectrum of a given compound is usually presented on a bar graph with unit values (m/z values) on the x-axis and the relative abundance on the y-axis. The highest peak on the chromatogram is called the base peak and is arbitrarily assigned a relative abundance of 100%.

CHAPTER VI

6. RESULTS

6.1. SCREENING OF PLANT EXTRACTS

A total of twenty-two plant extracts were tested for antiviral activity. The results obtained for tested extracts of each plant are presented in Table 15, p127. This chapter presents results obtained for the various assay procedures performed on the cold aqueous extract of *Barringtonia racemosa* which showed notable antiviral activity.

Any screening system developed is required to meet good scientific practice standards of validity, reproducibility, simplicity and to be cost effective. The screening procedure used to test the antiviral properties of the 108 plant extracts prepared was designed to meet these criteria. The method developed had to be selective, specific and sensitive to handle the analysis of crude extracts containing a range of different compounds in varying concentrations. The virus control started showing CPE 24 hours post-inoculation and extensive CPE was evident within 48 hours post-infection. Extracts that failed to inhibit antiviral activity or showed extensive cell damage, even at low viral inoculation concentrations were excluded from further study. Extracts that exhibited virucidal or virustatic activity were investigated further and subjected to the biofractionation process. Virucidal and viruidal activity was confirmed by inoculating new Vero cells with the test media. The resurgence of viral activity indicated virustatic activity and virucidal activity was identified by the lack of viral activity in the second cell system. Table 15, on p127 presents the screening results of extracts tested. It is evident from the table that the cold aqueous extract of *Barringtonia racemosa* showed significant antiviral activity to warrant further study.

Initial antiviral investigations of extract 29AQC involved testing activity using different inoculation procedures. Graph 7, p129 represents the plaque reduction assay results of 29AQC as presented in Table 16, p129. The scoring technique was also used to determine the rate of inhibition brought about by the extract. Graph 8, p130 represents test results obtained when the extract was inoculated an hour before the virus. Graph 9, p131 shows the inhibition profile obtained following simultaneous inoculation of the extract and virus. Finally, Graph 10, p132 shows the degree of viral inhibition when the virus was inoculated an hour before inoculating the extract. The hot aqueous extract of *Barringtonia racemosa* was also tested for activity and the results as presented in appendix 9, p234 show an absence of antiviral activity. The ED_{50} concentrations for the cold aqueous sample when added to the cell system before the virus was $10^{-1.45}$; when added after the cell system had been inoculated with the virus was $10^{-1.88}$ and when inoculated together with the virus was $10^{-1.36}$. These values refer to Graphs 8, 9 and 10 and translate to effective concentrations of 28.1 μg/ml, 23 μg/ml and 75 μg/ml, respectively[1].

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[1] Refer to section 4.1.3 p.61 for explaination on computation of figures.

Table 8: A listing of plants tested for activity

GENERA	**VERNACULAR**	**AQC**	**AQH**	**MEC**	**MEH**	**DCM**
Clivia miniata Regel		—	—	CD	CD	—
Crinum bulbispermum	umduze (inembe)	CD	—	CD	—	CD
Alepidea	ikhatazo	CD	—	—	CD	
Rauvolfia caffra	umhlambamanzi	—	—	—	CD	—
Acorus calamus L.	ikalamuzi	—	—	—	CD	
Xysmalobium undulatum	ishongwe	CD	CD	CD	CD	—
Bulbine natalensis	ibuchu	—	—	—	—	CD
Artemisia afra	umhlonyane	CD	—	—	—	CD
Senecio serratuloides	insukumbili	—	CD	CD	CD	
Kigelia africana	umfongothi	—	—	—	CD	CD
Warburgia salutaris	isibaha	CD	—	CD	CD	—
Chironia krebsii		—	—	—	—	—
Eucomis autumnalis	umathunga	CD	CD	CD	CD	CD
Tetradenia riparia	iboza	CD	CD	CD	CD	CD
Barringtonia racemosa		+	—	CD	CD	—
Cassipourea gummiflua	isinukani	—	—	—	—	—
Lippia javanica	umsuzwane	—	—	—	CD	—
Siphonochilus aethiopicus *S. natalensis*	isiphephetho (wild ginger)	CD	CD	CD	CD	CD
Polygala virgata	ithethe	—	—	—	—	—
Olea europaea	umquma	—	—	—	—	

Key: '—' indicates negative antiviral activity, CD indicates destruction of the cell system by the agent and '+' indicates positive antiviral activity.

6.2. SOLUBILITY STUDIES

Solubility studies were carried out using solvents of different polarity commonly used in liquid chromatographic separations, including thin layer chromatography. Solubility determinations of the active agent are significant because they influence the choice of solvents used in biofractionation assays during the isolation and purification of the active analyte.

Table 9: Solubility determinations of *Barringtonia racemosa* in different solvents

	COLD AQUEOUS EXTRACT	
SOLVENT	SOLUBILITY	PARTS OF SOLVENT FOR 1 PART OF SOLUTE[*1]
Water	1g/100g of solvent at 25°C pH 6.8[*2]	1 part: freely soluble
Acetonitrile	0.005g/100g of solvent at 25°C	200 parts: slightly soluble
Methanol	0.001g/100g of solvent at 25°C	1000 parts: very slightly soluble
Dimethyl-sulphoxide	0.05g/100g of solvent at 25°C	20 parts: soluble
Dicholoro-methane	Insoluble	
Ethyl acetate	Insoluble	

The above table summarises the solubility profiles of the cold aqueous extract of *Barringtonia racemosa* in different solvents.
[*1]Refer to table 6 p75. [*2] All other pH determinations were not made

The pH of the water solvent of the cold aqueous extract was determined and found to be 6.8 Other pH estimations were not performed.

The results presented in Table 8 p112 show that the cold aqueous extract of *Barringtonia racemosa* has limited solubility in organic solvents and only achieves complete dissolution in water. The extract is freely soluble in water, soluble in dimethylsulphoxide, slightly soluble in acetonitrile and very slightly soluble in methanol.

6.3. PERCENTAGE YIELD

The percentage yield value of the biofractions of *Barringtonia Racemosa* were determined.

Table 10: Percentage yield values of biofractions of *Barringtonia racemosa*

BIOFRACTIONATION TECHNIQUE	COLD AQUEOUS EXTRACT (% Yield)	
Crude	25.86	
SEC: G50		
	[*1]F1: 45 ml	23.77
	F2: 35 ml	81.52
	F3: 45 ml	20.02
SEC:G25		
	[*2]F1: 25 ml	0.646
	F2: 30 ml	6.50
	F3: 30 ml	46.67
	F4: 35ml	5.08
IEHPLC	1.13	
C_{18} SPE COLUMN		
WATER	0.36	
5% ACN	0.28	
10%ACN	0.80	
20%ACN	0.46	
RP-HPLC		
F1: 0-2 minutes	0.38	
F2: 2-4 minutes	0.56	
F3: 4-6 minutes	0.64	
F4: 6-8 minutes	1.66	
F5: 8-10 minutes	0.94	

[*1] F1 – high molecular weight fraction, F2 – intermediate molecular weight fraction, F3 – low molecular weight fraction.
[*2] F1 and F2 – high molecular weight fractions, F3 – intermediate molecular weight fraction, F4 – low molecular weight fraction.

Presented in table 10 above are the w/w yields of *Barringtonia racemosa* starting with the crude material and includes the various fractionations generated during purification steps.

A significant quantity of yield was obtained during extraction from the natural plant material. Due to the loss of material during subsequent isolation and purification procedures, the quantity of available material decreased. Therefore, the quantity of starting material decreased resulting in the decreased yields observed in Table 10. The percentage yield results calculated during initial fractionation assays, made it possible to evaluate the amount of extract material that is required for further fractionation studies. Furthermore, it was essential to ensure that the quantity of material available was sufficient for the anti-viral assays.

6.4. COLORIMETRIC TESTS

6.4.1. Phenol-sulphuric acid assay

An aqueous solution of extract containing micro-molar quantities of sugar moieties produces an orange colour when treated with 98% sulphuric acid followed by 80% phenol. The basis of this test is that the heat produced when the strong acid is added to an aqueous solution hydrolyses the sugar moiety. The mechanism by which phenol produces the colour formed is not known (Thibert & Mazzachin 1982, Rao & Pattabiraman 1989). However, the intensity of the colour increases with increasing quantities of simple sugars, oligosaccharides, polysaccharides and their derivatives. Colour intensity is also influenced by the quantity of phenol added and therefore, during experimentation, a fixed volume of phenol was used (Djurdjic & Mandic 1990, Rao & Pattabiraman 1989, Boratynski & Zal 1990, Dubois et al. 1947). Extracts of *Barringtonia racemosa* and subsequent fractionation products were tested for the presence of sugar moieties.

Table 11: Phenol-sulphuric acid test results

FRACTION	OUTCOME
29AQC	Orange coloration obtained
[*1]IEHPLC: 29AQC	Intense orange coloration
[*2]RPHPLC: 29AQC	
[*3]F3: 3-4 minutes	Orange coloration obtained
F4: 4-6 minutes	Orange coloration obtained
Control: glucose	Intense coloration obtained

[*1] IEHPLC – ion exchange high performance liquid chromatography.
[*2] RPHPLC – reverse phase high performance chromatography.
[*3]F3 and F4 refer to reverse phase HPLC fractions collected during the retention times of 3 – 4 and 4 – 6 minutes, respectively.

The above results indicate that *Barringtonia racemosa* contains sugar moieties. It is important to note, however, that the crude extract of *Barringtonia racemosa* and the reverse-phase chromatography fractions had a light brown coloration prior to the addition of reagents. This coloration is likely to influence the intensity of the orange colour obtained. In contrast, the fraction obtained from the ion exchange high performance chromatography analysis was colourless. This fraction was derived from the cold aqueous crude extract. The sample was crystal-like in appearance, resembling glucose, and it produced an intense orange colour upon the addition of phenol to the acidified aqueous solution.

In summary, the above results confirm the presence of sugar moieties in the cold aqueous extract of *Barringtonia racemosa*.

6.4.2. Saponins

The formation of persistent foams during the extraction of the crude samples with an aqueous alcohol signifies the presence of saponins. The test for the presence of saponins in the cold aqueous extract of *Barringtonia racemosa* was negative.

6.4.3. Quinones

Quinone containing compounds turn a characteristic red colour upon the addition of a diethyl-ether chloroform mixture to the acidified powdered extract. Upon the testing of the cold aqueous plant extract, a clear yellow liquid was obtained thus indicating that the compound does not possess quinone moieties.

6.4.4. Tannins

The presence of tannins is widespread within the plant kingdom. These weakly acidic compounds react with ferric chloride to produce a dark blue colour. This typical reaction for tannins was observed after adding 5% ferric chloride to the cold aqueous solution of *Barringtonia racemosa*.

6.4.5. Anthraquinones

A positive result for anthraquinones is characterised by a rose-pink to cherry-red coloration in the ammonia layer of a test substance extracted with ferrous chloride and hydrochloric acid followed by chloroform. No colour formation was observed when testing the aqueous extract of *Barringtonia racemosa*. This suggests that the extracts does not contain anthraquinones.

6.4.6. Flavonoids

Flavonoids are mainly water-soluble compounds that can be extracted using acid alcohol and t-amyl alcohol. The appearance of a rose-orange or violet colour signifies the presence of flavonoids. No colour change was evident when testing the crude extract of *Barringtonia racemosa*.

6.4.7. Summary

The colorimetric studies indicate that sugar and tannin moieties are among the major chemical species present in *Barringtonia racemosa*. As the tests performed were not quantitative, it was not possible to determine the relative quantity of each chemical constituent present.

6.5. VIRAL ANALYSIS

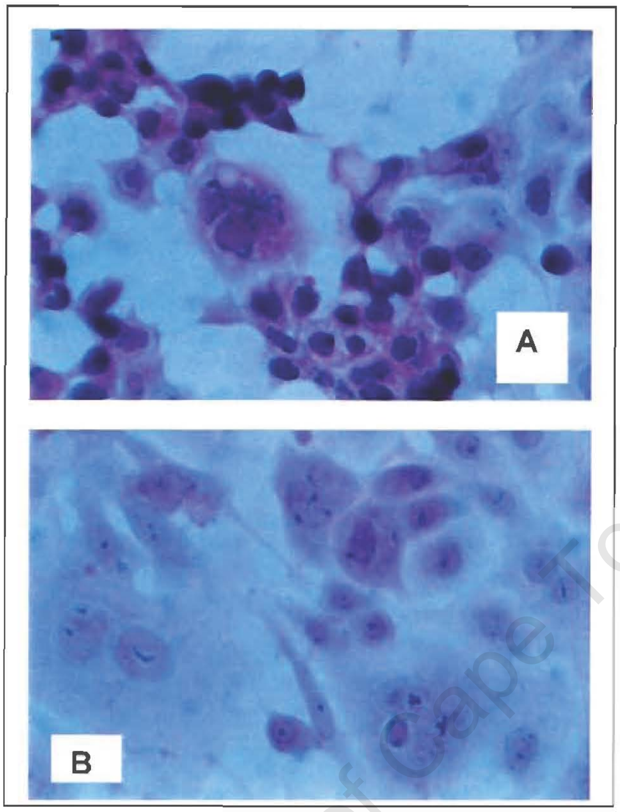
6.5.1. Immunofluorescence Analysis

The immunofluorescence (IF) assay method was used to distinguish herpes simplex virus type 1 from type 2. The staining reaction when the test sample was spotted with an HSV 1 conjugate was a distinct glowing yellow-green fluorescence. In contrast, a less clear and dull staining intensity was obtained when the test virus was stained with the HSV-2 conjugate. Figure 11, p119 shows the staining differences between a negative and positive test for herpes simplex viruse type 1. The staining pattern of the test virus confirmed that the test virus was herpes simplex type 1.

In addition to IF, cytopathic effects were used to distinguish HSV-1 from HSV-2. Cell rounding, clumping, syncytial formation and complete cellular degeneration typify HSV type 1 cytopathic effects. In contrast, cytopathic effects caused by HSV-2 result in syncytial formation and piling of cells. Observations of the cell systems developed during this study show the appearance of cell rounding, syncytial formation and cellular degeneration (Figure 3, p33). This further confirms that the virus used was HSV-1.

It is known that certain commercially available serologic techniques do not reliably differentiate between HSV-1 and HSV-2 (Schmidt & Emmons 1989). Therefore, to confirm initial laboratory findings, immunofluorescence analyses were repeated and replicated IF tests verified that the virus used was HSV-1.

Figure 11: An illustration of immunofluorescence staining patterns of HSV



[1] A shows the positive HSV 1 control which is a bright immunofluoresence and B shows the HSV 1 negative control which is dull. The above illustration shows the bright immunofluorescence characterising a positive IF test and the dull stain charactising a negative stain.

6.5.2. Cell System Validation

Biological assay systems are prone to problems of variation. Hence, to ensure the reliability of a study, it is necessary to verify the accuracy of virus titrations with validity studies. Tissue culture infectious dose (TCID) assays were performed to provide qualitative measurements of infectivity. Plaque reduction assays provided quantitative evaluations for infectivity. Both qualitative and quantitative assays were subjected to statistical evaluation to provide an indication of the suitability of the selected test system for the investigation of *in vitro* bioassays.

Different single assay runs were compared to determine the accuracy of standard methods used. The robustness of the bioassay method was determined by comparing assay runs performed on different days. The latter studies determined the between-assay variations of the study method. The accepted 95% confidence limits for both assessments should be in the order of ≤ 5 percent of the mean estimate, however, limits in the order of 10 percent have been accepted (Therapeutics Products Programme 1994, Therapeutics Products Programme 2000).

Table 12 below provides the data of a single plate inoculated with serial dilutions of virus. During a single assay run four microtitre plates were inoculated with serial dilutions of virus; each set of dilutions was prepared on the same day. The percent infectivity values of each dilution tested were translated onto Graph 1 p121. The relative standard deviations, as represented by the error bars, indicate that reasonable accuracy was achieved.

[*1] Table 12: Virus titre determinations using Reed and Muench method

	VIRAL DILUTIONS						
Virus dilution	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Uninfected wells	0	0	0	0	3	6	7
Infected wells	7	7	7	7	4	1	0
Accumulation uninfected	0	0	0	0	3	9	16
Accumulation infected	33	26	19	12	5	1	0
Infectivity ratio	33/33	26/26	19/19	12/12	5/8	1/10	0/16
% infected	100	100	100	100	62.5	10	0

[1] Refer to table 4, p62 for illustration of the Reed and Muench method. Graphic representation is shown in Graph 1, p121. Readings taken on day 5 post-infection. Raw data presented in appendix 6, p223. n = 3 of an average of 16 readings per plate.

The percent infectivity rate was obtained by using a scoring system to grade the degree of viral infectivity in a cell system. The scoring system used was as follows:

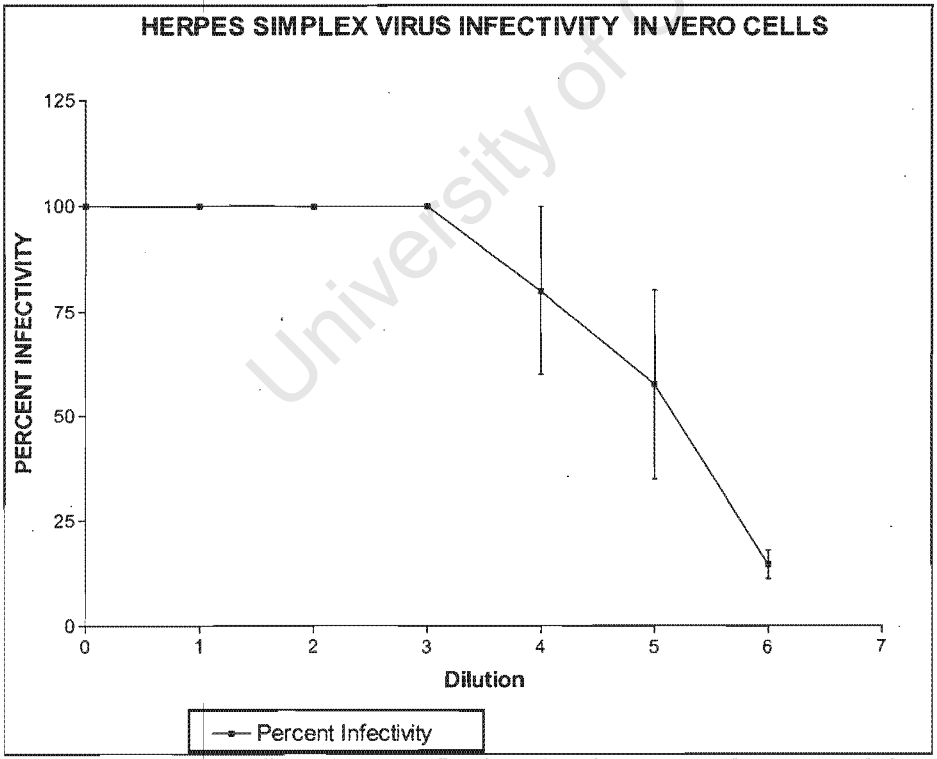
Table 13: Grading systems used for cytopathic effect readings

Allocated Grading	A	A⁺	B⁻	B	B⁺	C⁻	C	C⁺	D⁻	D
Percent infectivity rate	0	12.5	25	37.5	50	50	62.5	75	87.5	100

Scoring system used to determine the degree of infection in cell growth vessels. Degrees of scoring were adapted from Bathod (1987).

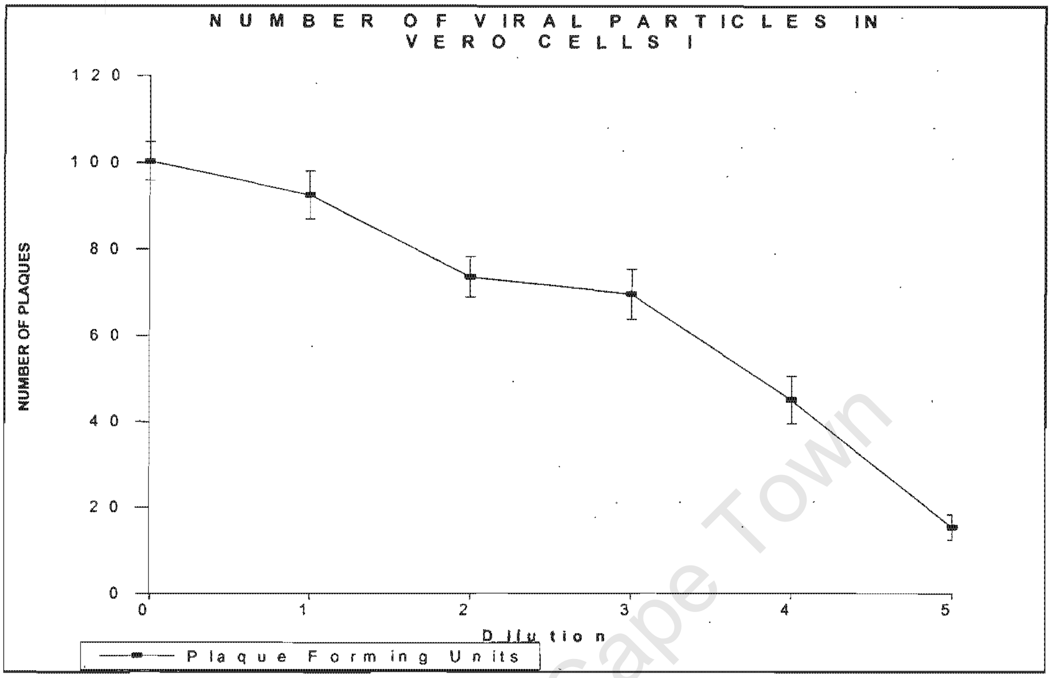
Graph 1 shows the infectivity rate of virus dilutions after a 5-day period using the scoring method. Graphs 2 and 3 p119 are interpretations of the plaque reduction assays; Graph 2 represents plaque reduction assay results produced from experiments performed on the same day and Graph 3 presents values obtained on different assay days.

Graph 1: Herpes simplex virus titration in Vero cells



A profile of viral growth at different dilutions on Day 5 post-inoculation. n = 3. of an average of 12 readings. $TCID_{50} = 10^{-5.179}$.

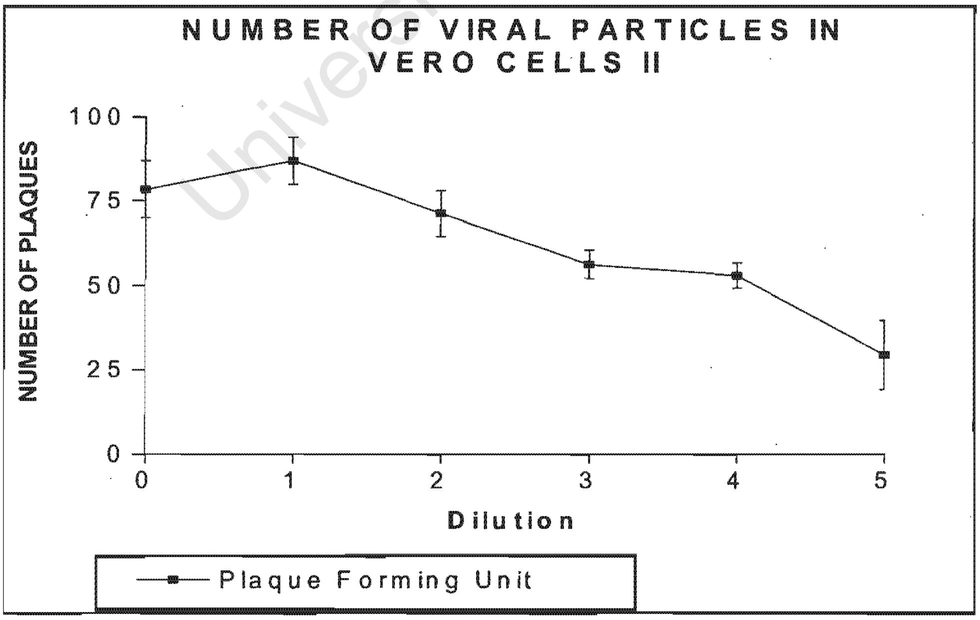
Graph 2: Plaque reduction assay plot for HSV 1 [*1](single assay)



A profile of viral plaques produced at each dilution on day 5 post-viral inoculation for assay performed on the same day. n = 3 of an average of 3 readings.

[*1] Single assay runs were performed on the same day whereby three plates were inoculated simultaneously.

Graph 3: Plaque reduction assay plot for HSV I [*1](between assay)



A profile of plaques produced at each dilution on day 5 post-viral inoculation for assays performed on different days. n = 3 of an average of 3 readings.

[*1] Between assay runs refer to runs performed on different days.

A study of the $TCID_{50}$ profiles indicates that there is slight variation about the mean value of each point. An interesting observation is an apparent increase in variation close to the $TCID_{50}$ value. This is reflected in the raw results (Appendix 6, p223) where the calculated coefficient of variations for the 10^{-3} and 10^{-4} dilutions were 34.6% and 39.0% respectively. At the higher dilutions at which one uses a concentration of $100TCID_{50}$ during regular assays, the coefficient of variation was 0%. The corresponding coefficients of variation values for the single- and between-assay plaque reduction studies at a $100TCID_{50}$ viral inoculation were 12.8% and 17.4% respectively. The error bars represent the standard deviation about the mean value. The systems validation studies data presented show that the *in vitro* anti-viral assay method selected is sufficiently accurate for the quantitative and qualitative analysis of plant-derived extracts. The validation results demonstrate that the bioassay method is selective and specific and is reliable and reproducible. Therefore, the method developed can be used to determine the antiviral activity of plant extracts.

6.5.3. Anti-Viral Assays

During replication, a virus undergoes limited intrinsic enzymatic system and growth process stages and therefore, there are fewer sites that may serve as specific target areas for antiviral agents (Vanden Berghe, Vlietinck & Van Hoof 1986). As a result of this feature, antiviral agents are required to show less specificity in their antiviral action. Of importance is that the agent needs to irreversibly block viral synthesis to prevent cell death and therefore, restore normal cellular functions. The antiviral results presented for *Barringtonia racemosa* will show that this agent is capable of blocking the replication of herpes simplex virus in a Vero cell system and furthermore, the agent does not interfere with normal cellular functions.

6.5.3.1. *$TCID_{50}$*

The Tissue Culture Infectious Dose 50 ($TCID_{50}$) was determined by titrating the virus into Vero cells grown in microtitre plates (Kalter 1963). The $TCID_{50}$ endpoint was calculated from the distribution of cultures showing typical virus cytopathic effects. The results in table 14, p126 and graph 4, p126 show the daily infectivity rate for each viral dilution from day 1 post infection to a maximum of 5 days. At day 5 the end point infectivity was 10^{-5} and the calculated $TCID_{50}$ was $10^{-5.18}$, therefore, the $TCID_{50}$ per millilitre was $10^{-5.18}$. From this calculation, to achieve complete viral infection of a cell system or 100 $TCID_{50}$, the minimum amount required during viral inoculation is a 10^{-3} viral dilution. The $TCID_{50}$ was calculated using the Reed and Muench method previously described. $TCID_{50}$ determinations standardise viral inoculum use during subsequent analyses. The concentration of the original viral sample inoculated is correctly referred to as $10^{5.18}TCID_{50}$ per 1ml. $TCID_{50}$ evaluations were performed regularly during the course of analyses to ensure that the virus used maintained its virulence (Appendix 6, p223).

6.5.3.2. *ED_{50} Determinations*

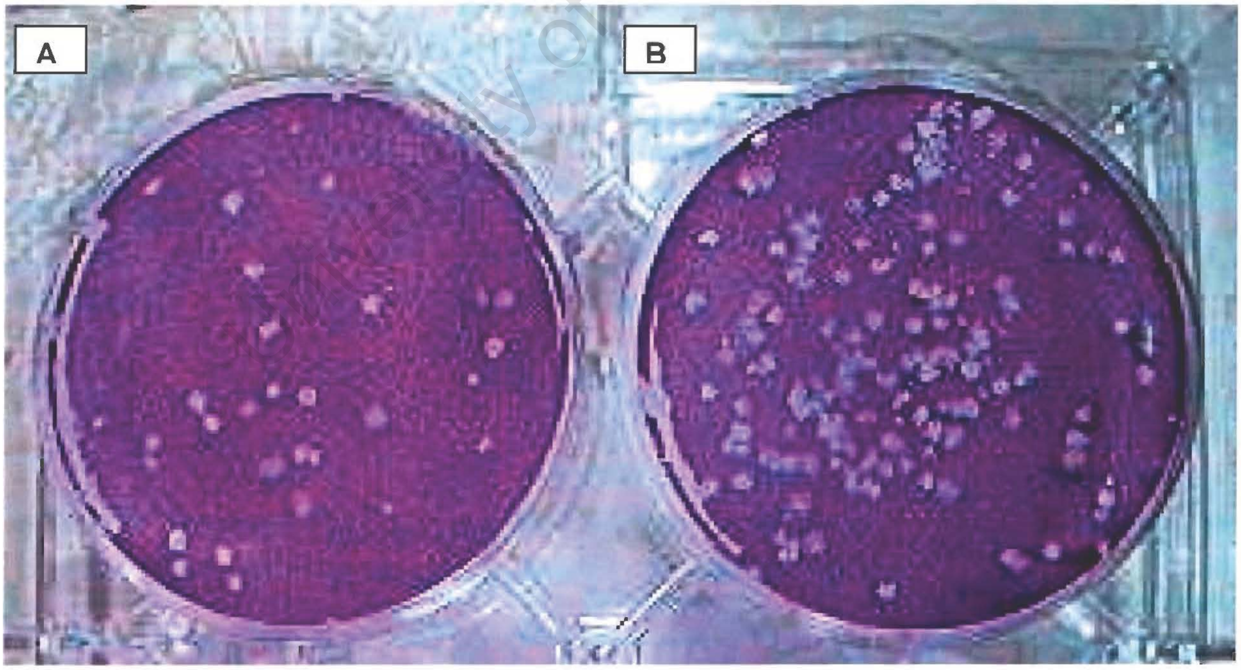
Statistical techniques were used to compute the amount of extract required to produce 50% viral inhibition. This effective dose was determined by interpolation of the dose-response curve and this value can be used to standardise the degree of viral inhibition by means of which the potencies of biofractions can be compared.

Cells cultivated in a microtitre plate were infected with a fixed concentration of virus and increasing concentrations of the active extract and positive control, acyclovir. After 5 days, the cell layer was fixed, stained and the viral plaques were counted. The ED_{50} was calculated as the extract concentration that decreased the number of plaque forming units per well to 50% with respect to the plaque forming units of the viral control in the absence of the extract. Plaque reduction assays were used to standardise quantitative viral assay methods.

6.5.3.3. Acyclovir Analysis

Figure 12 shows the presence of viral plaques following the inoculation of acyclovir (ACV) to a biosystem of a fixed concentration of herpes simplex virus of 100TCID$_{50}$. The concentration of agent required to inhibit viral growth by 50% can be estimated from the inhibition curve using linear regression analyses. The PRA results for acyclovir are presented in Graph 5, p127. Graph 5 illustrates that when the cells were inoculated with 100TCID$_{50}$ concentration of HSV, the concentration at which ACV inhibited 50% of the virus at day 5 post-infection was 0.033 μg/ml. The specificity of activity at lower concentrations is reduced and therefore Graph 5 shows better antiviral activity at the concentration 0.01 μg/ml than at 0.1 μg/ml. Graph 6 p128 presents results obtained from acyclovir inhibition tests using the scoring technique. In comparison, the ED$_{50}$ of acyclovir using this method was calculated as 0.026 μg/ml.

Figure 12: A photographic image of viral plaques



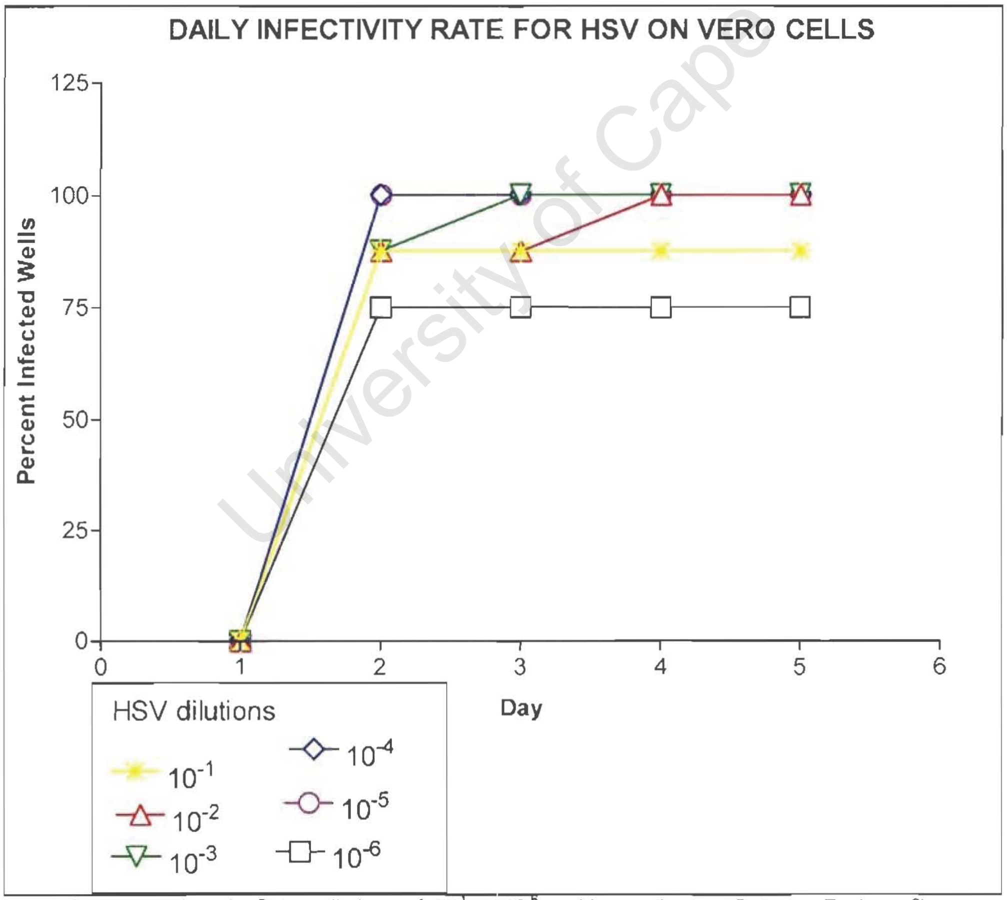
A – acyclovir concentration of 1.0 μg/ml. B – acyclovir concentration of 0.01 μg/ml. The above figure shows the formation of viral plaques that can be quantified on the cell medium.

Table 14: Daily cell infection profile for HSV over a 5 Day period

	PERCENTAGE OF WELLS INFECTED				
DILUTION	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
-1	0	87.5	87.5	87.5	87.5
-2	0	87.5	87.5	100	100
-3	0	87.5	100	100	100
-4	0	100	100	100	100
-5	0	100	100	100	100
-6	0	75	75	75	75

The table shows the rate of infectivity of a Vero cell system by different dilutions of HSV 1 over a 5-day period. Graphic representation shown in Graph 4, p126.

Graph 4: Infection rate for HSV on Vero cells



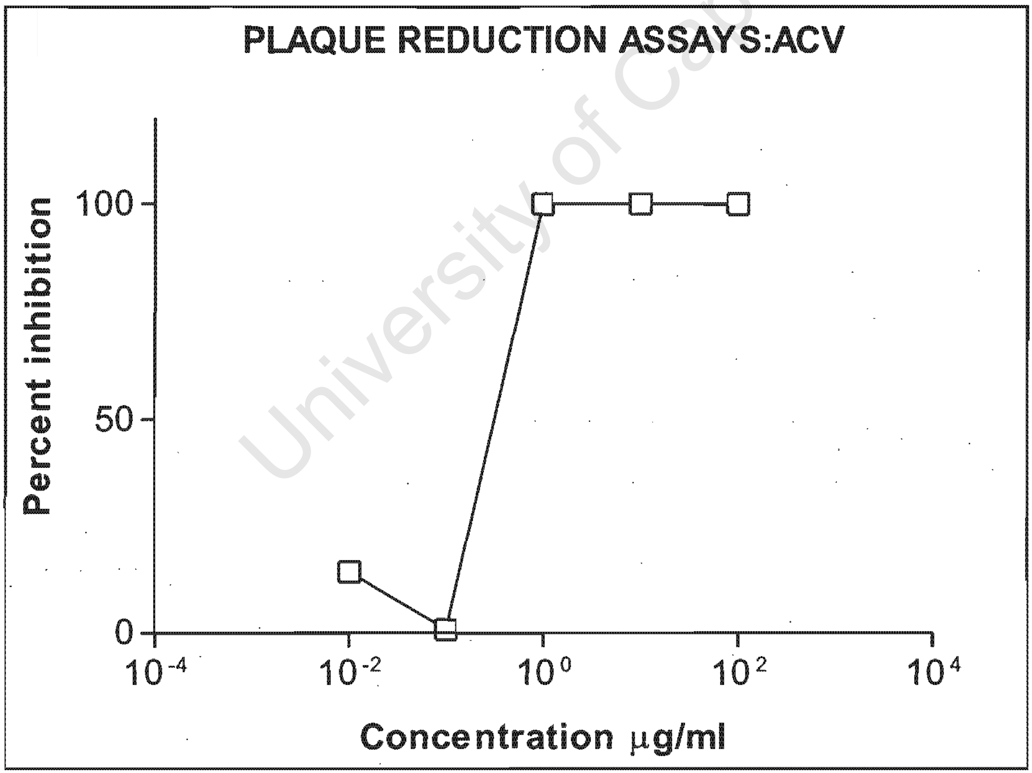
Viral infectivity profile of HSV at dilutions of 10^{-1} to 10^{-6} on Vero cells over 5 days. Each profile represents different dilutions of virus. n = 3 of an average of 12 readings per plate.

Table 15: ED$_{50}$ determinations for acyclovir using the plaque reduction assay method

ACV concentration µg/ml	**100**	**10**	**1**	**0.1**	**0.01**	**Virus control**
No of plaques formed	0	0	0	78	98	84
	0	0	0	82	73	67
	0	0	0	61	84	72
Average no of plaques	0	0	0	73.7	85	74.3
Percent plaque inhibition	100	100	100	0.81	14.4	

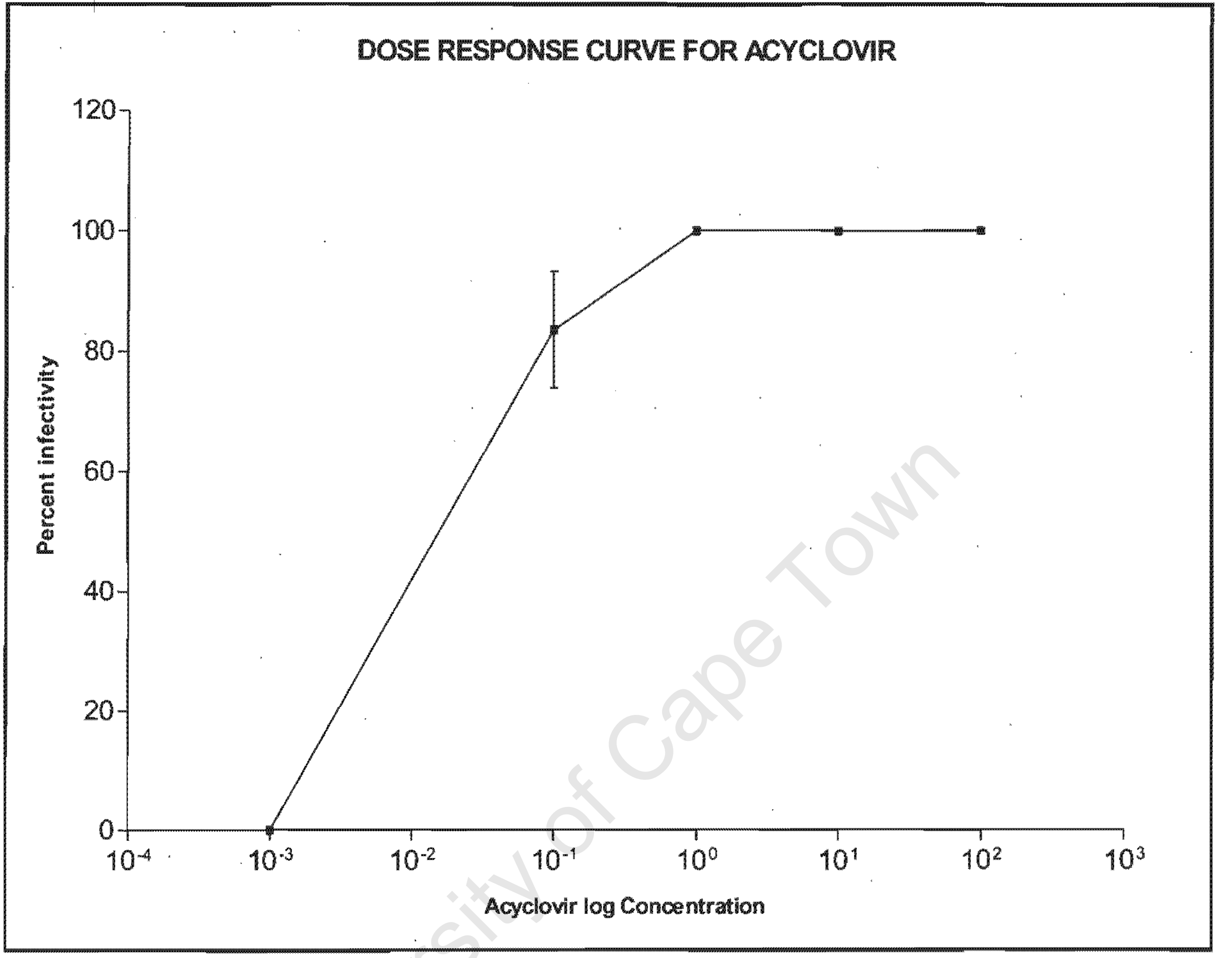
Percentage inhibition growth of HSV in the presence of acyclovir, the positive control. The percent plaque inhibition = [(Ave no plaques in extract – ave. no. plaques of virus control)/ ave. no. plaques of virus control]X 100. Graphic representation shown on Graph 5, p127.

Graph 5: Plaque reduction assay profile for acyclovir



HSV inhibition by different concentrations of acyclovir over a 5 day period. n = 4 of an average of 6 readings. ED$_{50}$ = $10^{-1.48}$ or 0.033µg/ml

Graph 6: Dose response curve for acyclovir against herpes simplex virus



Log dose response profile for different concentrations of acyclovir using the scoring method. Refer to table 13, p121 for scoring system. n = 4 of an average of 6 readings. $ED_{50} = 10^{-1.587}$ or 0.026 µg/ml

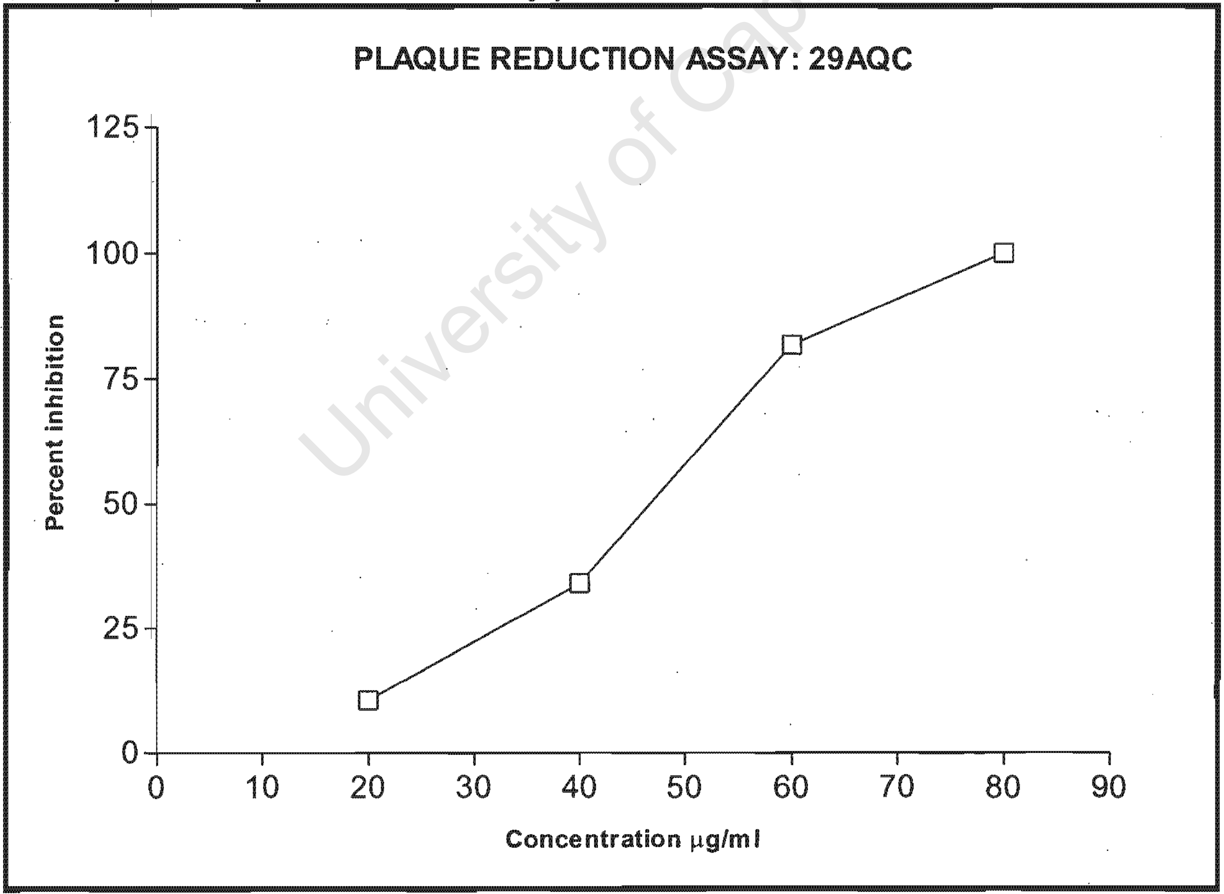
In summary, the concentrations at which acyclovir inhibited 50% viral growth were 0.033 µg/ml and 0.026 µg/ml for the plaque reduction assay and scoring method, respectively.

Table 16: ED$_{50}$ determination for 29AQC using the plaque reduction assay method

Extract concentration μg/ml	**80**	**60**	**40**	**20**	**Virus control**
No of plaques formed	0	15	83	108	102
	0	26	74	102	141
	0	18	104	115	125
	0	32	69	122	132
Average no of plaques	0	22.75	82.5	111.75	125
Percent plaque inhibition	100	81.80	34.0	10.6	▨

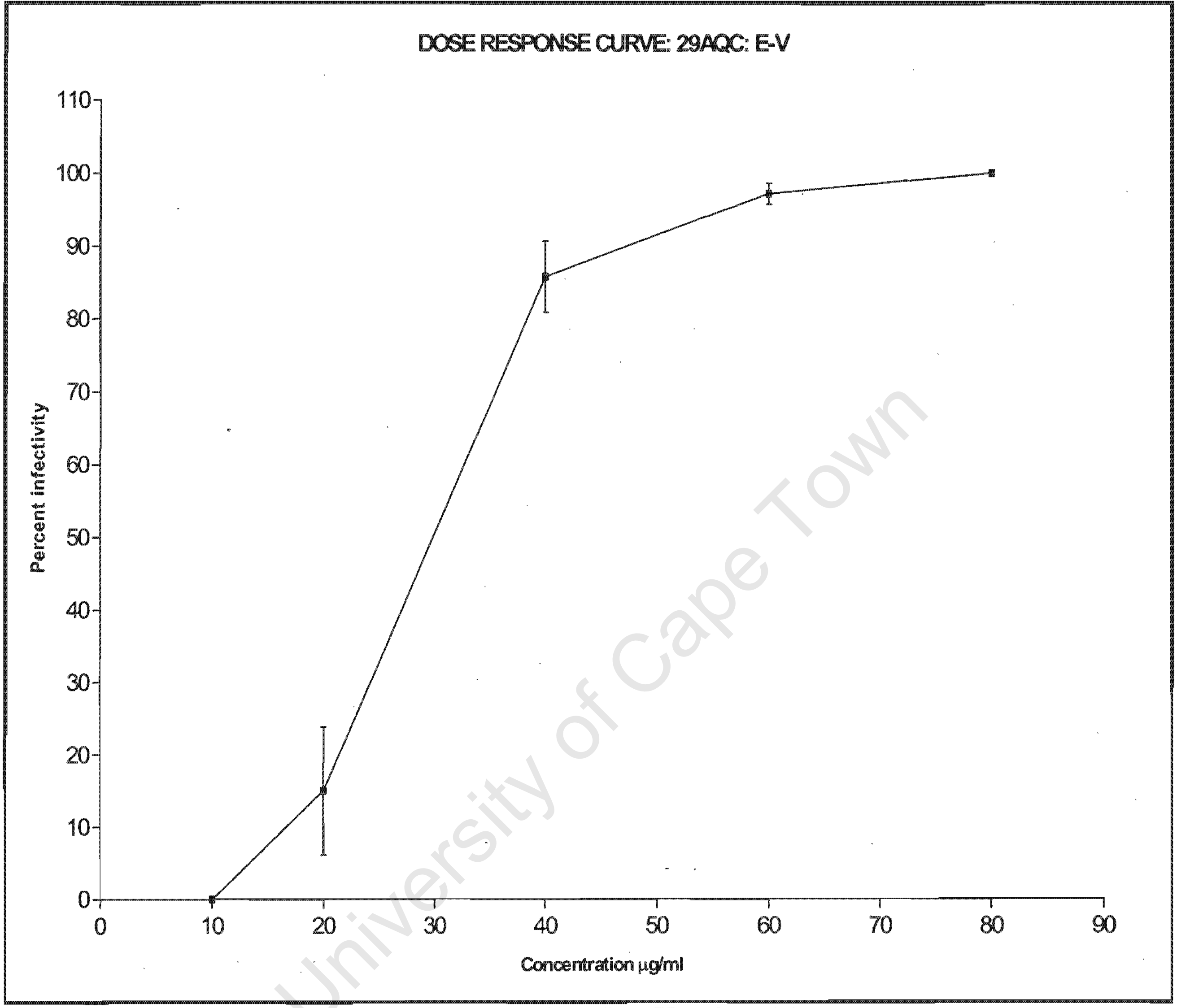
Plaque reduction assay results for the aqueous extract of *Barringtonia racemosa*AQC on day 5 after inoculation. Graphic illustration shown in Graph 7, p129.

Graph 7: Plaque reduction assay profile for 29AQC



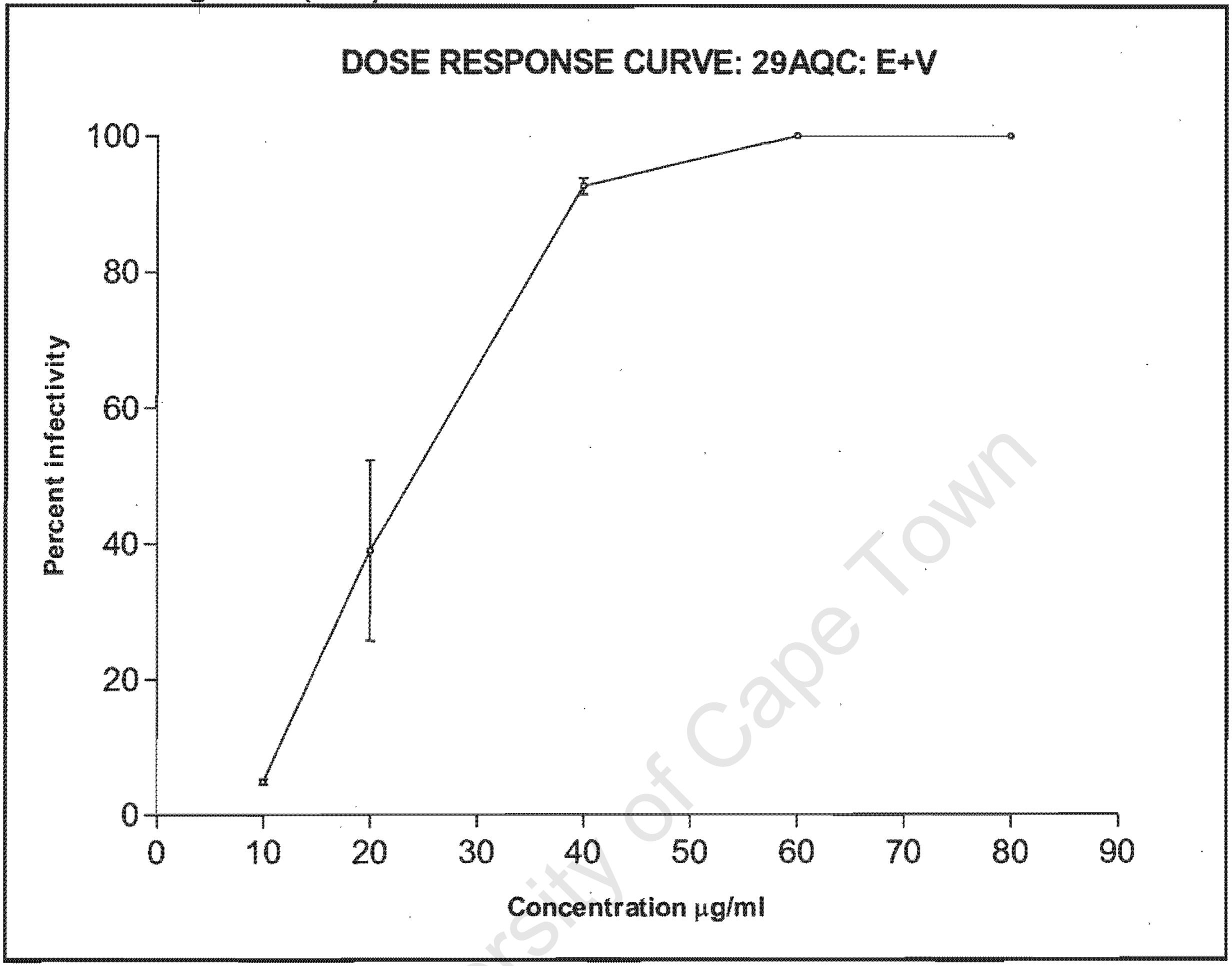
Graphical representation of plaque reduction assay results for the cold aqueous extract of *Barringtonia racemosa*. ED$_{50}$ = 45 μg/ml.

Graph 8: Dose response curve for 29AQC when adding the extract 1 hour before the virus (E-V)



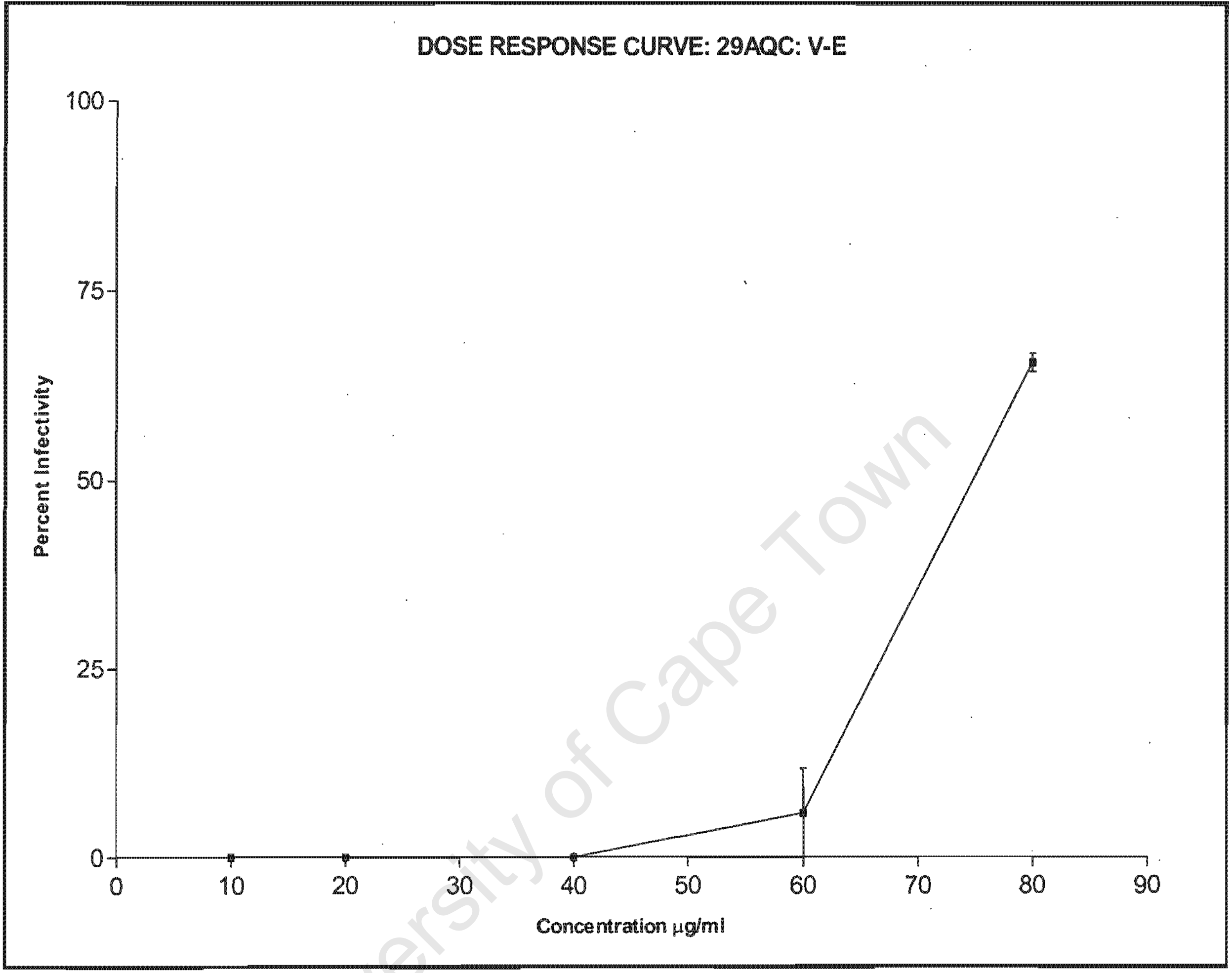
Results obtained using the scoring method. n = 3 of an average of 6 readings. ED_{50} = 28.1μg/ml.

Graph 9: Dose response curve for 29AQC when adding the extract and virus together. (E+V)



Results obtained using scoring method. n = 3 of an average of 6 readings. ED_{50} = 23.0 μg/ml.

Graph 10: Dose response curve for 29AQC when adding the virus 1 hour before the extract. (V-E)



Results obtained using scoring method. n = 3 of an average of 6 readings. ED_{50} = 75 μg/ml.

In total, twenty-two plants underwent extensive and thorough antiviral testing. From the results, it can be shown that out of the twenty two plants, 108 extracts were generated of which 37.9% showed cell toxicity, 0.9% showed significant antiviral activity and 61.2% were inactive, including the hot aqueous extract of *Barringtonia racemosa* (Table 8, p.112).

In summary, the antiviral assays showed that the ED_{50} value obtained when the extract was inoculated an hour before the virus was 28.1 μg/ml. The ED_{50} value obtained when the analyte and virus were added to the cell system simultaneously was 23.0 μg/ml and lastly, the ED_{50} value obtained when the virus was added an hour before the extract was 75 μg/ml. In comparison, the ED_{50} result calculated using the plaque reduction assay method illustrated in Graph 7, p129 was 45.8 μg/ml. No antiviral activity was observed during the antiviral analysis of extract 29AQH.

6.5.3.4. Time Of Addition Assays

Time of addition studies were conducted to determine the possible mechanism of action of the antiviral agent. The active agent was added at predetermined intervals that targeted at different stages of viral replication. During the experiments performed, the time intervals used were 0, 1, 3, 6, 12, 18, 24, 36 and 48 hours. After 48 hours there was significant cellular infectivity in cases where the virus was added before the extract. Based on the time of inclusion, it is possible to establish the mechanism by which the active agent inhibits viral replication in the infected host. Tests in which the virus and extract were mixed together and inoculated concurrently assisted in determining the virucidal ability of the compound by direct inactivation[2] (Vanden Berghe, Vlietinck & Van Hoof 1986, Hudson 1984). Studies that involved adding the active compound before the virus were performed to establish the antiviral state in cells that is

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[2] According to Hudson (1994), when the extract is mixed directly with the virus and the mixture is added to cell cultures, the test mode evaluates the virucidal ability of the compound (that is, its capacity to inactivate virions directly). Vanden Berghe et al. reports that polyphenolics and polysaccharides cause irreversible denaturation of virus proteins resulting in a complete loss of infectivity. This effect is called virucidal activity since the virus is killed.

capable of inducing the production of interferons[3]. The results obtained and publications analysed, confirm that *Barringtonia racemosa* possesses comparable virucidal and indirect antiviral activity. The third study performed involved adding the active extract after the viral agent was absorbed and had penetrated into the cell system. This mode is used to determine the ability of the compound to inhibit one or more stages of replication.

Through the interpretation of graphs 11 and 12, p134, the time of addition assay results indicate that the most effective antiviral activity takes place when the active agent is added together with or prior to the viral agent. The graphs presented show that a 1 hour incubation period of the extract with cells is sufficient to optimise activity. Graphs 11 and 12, respectively reveal that *Barringtonia racemosa* has antiviral activity. However, this activity diminishes significantly after one-hour incubation periods of either the virus or the extract.

Investigations of the cold aqueous extract show that significant viral inhibition was achieved when the extract was added to the cell system an hour before the addition of the virus. Activity was also achieved when the virus and extract are added together and the comparative ED_{50} values for both systems being 28 μg/ml and 23 μg/ml, respectively. There is significantly reduced antiviral activity when the virus is added before the extract. Antiviral activity diminished significantly after a three hour incubation of either the extract or virus.

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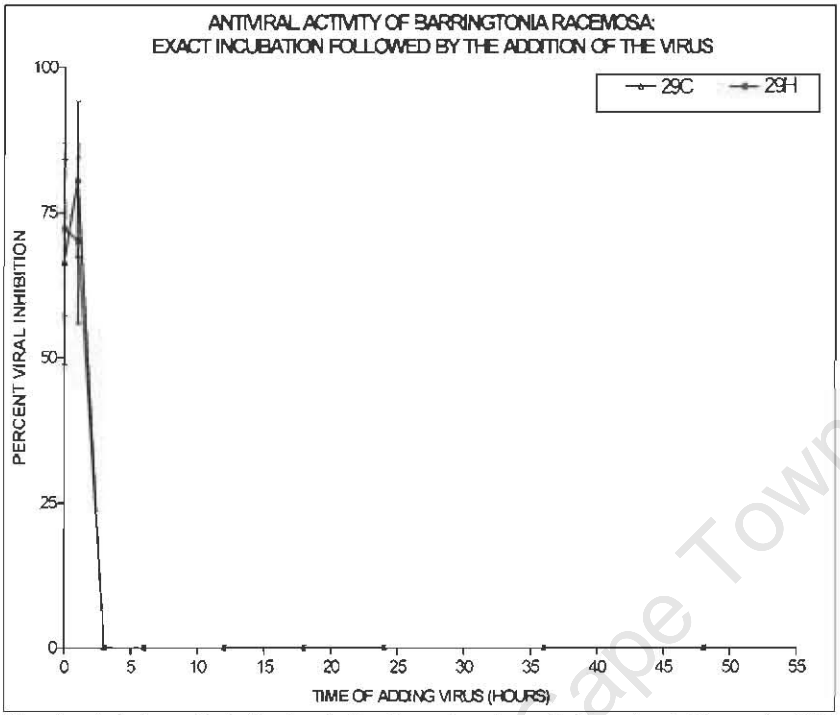
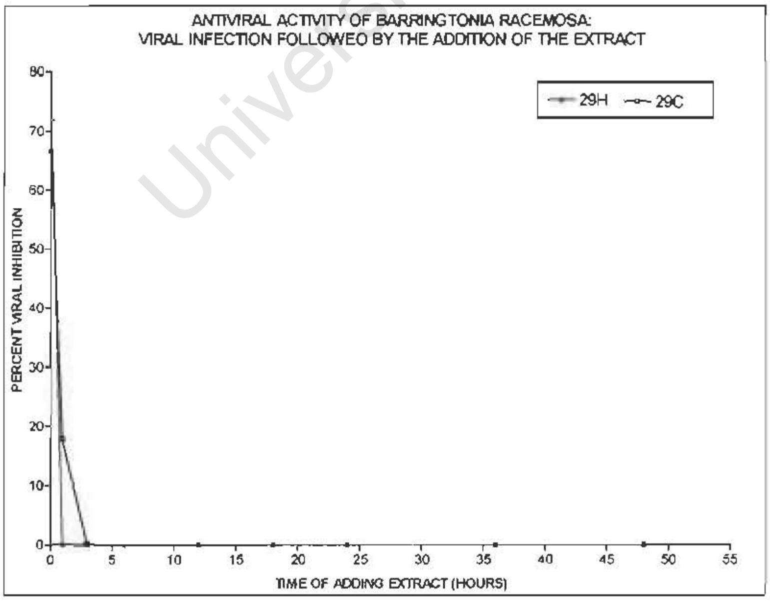
[3] According to Hudson (1994), when the compound is added to cell cultures for a known period before the virus, one is determining the "antiviral state" in cells that induces the production of interferons. Vanden Berghe et al. (1986) refers to the ability of this test mode to non-specifically stimulate host defence mechanisms that act indirectly on virus multiplication.

Graph 11: Infection profile for *Barringtonia racemosa* when adding the virus before the extract.



The virus is being added after incubating the cell system with the extract. Times of addition of the virus were 0, 1, 3, 6, 12, 18, 24, 36 and 48 hours after incubation of the extract. n = 3 of an average of 6 readings.

Graph 12: Infection profile for *Barringtonia racemosa* when adding the extract before the virus.



The extract is being added after incubating the cell system with the virus. Times of addition of the extract were 0, 1, 3, 6, 12, 18, 24, 36 and 48 hours after incubation of the virus. n = 3 of an average of 6 readings.

6.5.3.5. Cytotoxicity Studies

During the plant screening process, the general screening of toxicity caused to the Vero cell system was determined microscopically by examining the change in cell morphology in wells inoculated with the extract only. These cells were compared to cells containing maintenance media alone. Common changes that were observed microscopically in inoculates containing cytotoxic agents included cell lysing resulting in damage to the confluency of the cell layer and subsequent cell death. Table 17, p137 lists the plants that exhibited these general and non specific cytotoxic effects. Plant extracts identified as cytotoxic were re-tested at lower concentrations levels that were decreased by an order of log 10. If no antiviral activity was achieved at a lower concentration, they were excluded from further study.

To ensure that the antiviral activity detected during the screening of *Barringtonia racemosa* was real and direct, and not due to secondary cellular effects brought about by the extract, MTT tests and cell viability determinations were conducted. Results of the cytotoxicity assay methods used are presented in Table 18, p138 and table 19, p140.

Table 17: Cytotoxic plant extracts analysed on a Vero cell system

PLANT NAME	VERNACULAR NAME	EXTRACT
Clivia miniata Regel		MEC/MEH
Crinum bulbispermum	umduze (inembe)	AQC/DCM/MEC
Alepidea amatymbica	ikhatazo	AQC/MEH
Rauvolfia caffra	umhlambamanzi	MEH
Acorus calamus L.	ikalamuzi	MEH
Xysmalobium undulatum	ishongwe	AQC/AQH/MEC/MEH
Bulbine natalensis	ibuchu	DCM
Artemisia afra	umhlonyane	AQC/DCM
Senecio serratuloides	insukumbili	AQH/MEC/MEH
Kigelia africana	umfongothi	DCM/MEH
Warburgia salutaris	isibaha	AQC/MEC/MEH
Eucomis autumnalis	umathunga	AQC/AQH/MEH/MEC/DCM
Tetradenia riparia	iboza	AQC/AQH/MEC/MEH/DCM
Barringtonia racemosa		MEC/MEH
Lippia javanica	umsuzwane	MEH
Siphonochilus aethiopicus *S. natalensis*	isiphephetho (wild ginger)	AQC/AQH/DCM/MEC/MEH

Key: AQC – cold aqueous extract; AQH – hot aqueous extract; DCM – dichloromethane extract; MEH – hot methanol extract, MEC – cold methanol extract.

6.4.3.5.1. Cell Viability Assay

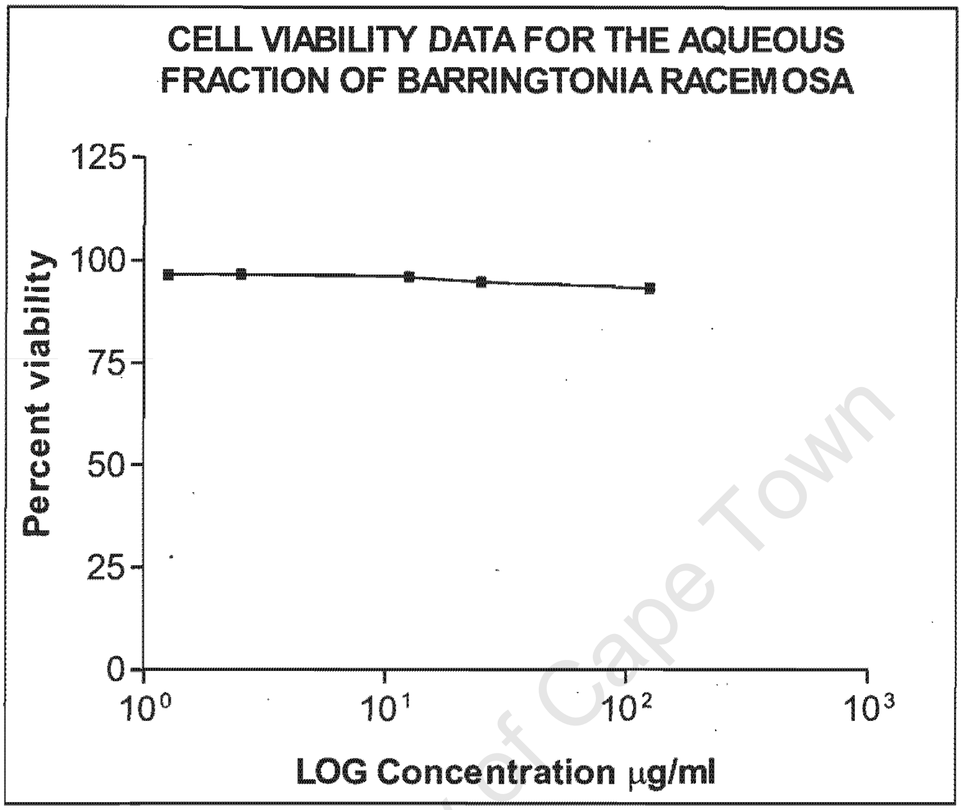
Cell viability assays are useful in determining the degree of cell survival following inoculation of a cell system with a substance of unknown toxicity (Lin, Kuo & Chou 2000). Changes to the confluency of the cell monolayer were observed daily for 5 days following extract inoculation. In addition, possible growth inhibition was investigated by performing cell counts on tryptinised cells. After 5 days of treatment, concentrations of *Barringtonia racemosa* below 125 µg/ml showed no inhibition of Vero cell growth as shown in Table 18 and the corresponding graph 13, p139.

Table 18: Results of cell viability assay

CONC. (µg/ml)	**COLD AQUEOUS EXTRACT**		
	DEAD CELLS	LIVING CELLS	CELL VIABILITY
Blank	11	153	93.3%
1.25	6	110	94.8%
2.5	8	191	96.0%
12.5	5	148	96.7%
25	5	139	96.5%
125	2	157	98.7%

Cell viability was calculated using equation 2, p57. The table is represented graphically in Graph 13, p139.

Graph 13: Cell viability assay



Raw data presented in Table 18, p138.

6.4.3.5.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay

The MTT assay is an instrumental analytical technique that was used to verify cell viability assay results. Optical density measures of cells inoculated with 29AQC and AQH were taken and compared to optical densities of wells containing the positive control and blank. A comparison of the MTT and cell viability assay results indicates that *Barringtonia racemosa* does not possess any cytotoxic activity and can be safely used in *in vitro* systems to a maximum concentration of 125 µg/ml.

Table 19: MTT assay results for *Barringtonia racemosa* cold aqueous extract

COLD AQUEOUS EXTRACT	
Conc μg/ml	Percent survival
0.01	112.9
0.1	96.8
1	112.9
10	129.0
100	96.8

Refer to appendix 5 p222 for raw data.

Both the MTT assay and cell viability determinations clearly show that the cold aqueous extract of *Barringtonia racemosa* is not toxic. Due to the absence of cytotoxicity in the plant tested, the 50% lethal concentration could not be determined. The maximum concentration of extract tested for cytotoxicity is considerably higher than would be normally used. This, therefore, eliminates the need to determine the maximum tolerated concentration.

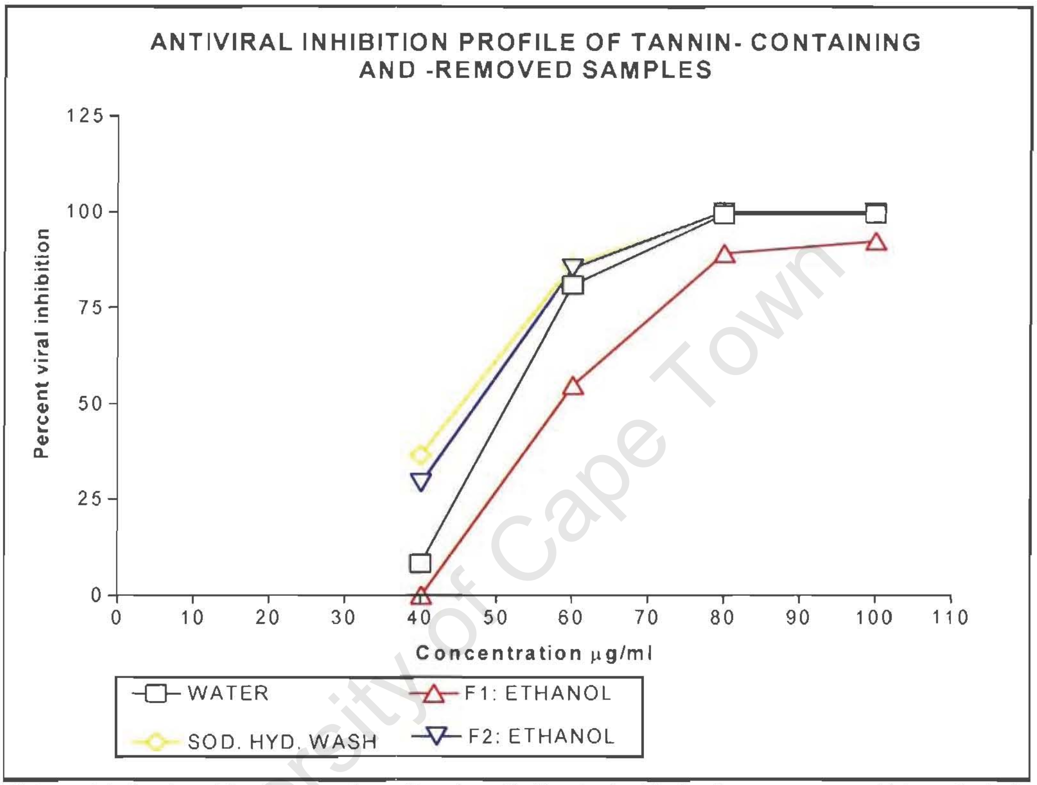
In the development of a new therapeutic entity, it is vital to determine the therapeutic index of the active moiety. The therapeutic index is the ratio between the minimum toxic dose and the therapeutic dose of an extract and it is a measure of the relative safety of the drug. Extract concentration values above the minimum effective limit of the therapeutic index are infective against a 100TCID viral inoculation. As mentioned, the minimum toxic concentration of *Barringtonia racemosa* could not be determined because the extract showed no toxicity, even at exceedingly high concentrations. There are two possible interpretations for such an occurrence. Firstly, it is possible that toxicity occurs at extremely high extract concentrations that were not tested during these investigations or secondly, the therapeutic dose efficacy curve follows a bell shaped dose-response curve whereby the efficacy increases with concentration up to a certain point beyond which it diminishes as the concentration is further increased. In this second setting there exists a therapeutic window of optimal antiviral activity.

6.5.3.6. Tannin And Polyphenolic Removal

Investigations performed to determine major chemical compounds in the extract of *Barringtonia racemosa* confirmed the presence of tannins and polyphenolics in the cold aqueous extract of *Barringtonia racemosa*. Tannins are known to interfere with the identification and isolation of other chemical constituents (Colllins et al. 1998, Claeson et al. 1998, Vanden Berghe, Vlietinck & Van Hoof, Unander, Webster & Blumberg 1995, Thresh 1956). Furthermore, certain tannins have been shown to possess antiviral activity (Verotta 1999, Khan et al. 1992, Khan et al. 1976, Pan & Lungren 1995). Due to these tannin properties, it was essential to determine the antiviral activity of tannin-containing and tannin-free fractions obtained from separation techniques previously described. The method used is described in section 4.2 p. 65. The first fraction collected from the separation procedure was the water fraction in which the extract had been dissolved. The corresponding ED_{50} to this fraction was 50.5 μg/ml. The ethanol fractions were derived from the two consecutive washes of the polyamide column with 80% ethanol. The ED_{50} values obtained from the first and second wash are 57.9 μg/ml and 46.5 μg/ml respectively. The last wash performed on the column used was with sodium hydroxide, which facilitates removal of the tannins bound to the column. The ED_{50} obtained for this final wash was 44.67 μg/ml. The antiviral effect of these fractions is shown in graph 14, p142. The raw data is presented in appendix 12, p241.

All fractions eluted through the polyamide column exhibited some antiviral activity. The above graph confirms that a notable amount of activity was attributed to tannins and polyphenolics present in the sample. Equally, the ED_{50} values of the water and ethanol washes suggest that an equally significant amount of activity is present in the non-tannin containing fractions.

Graph 14: Antiviral profile for tannin removal experiments



Water and both ethanol fractions are free of tannins. Sodium hydroxide fraction possesses a high content of tannin. ED_{50} of water extract = 50.5 µg/ml, ED_{50} of first ethanol extraction (F1) = 57.9 µg/ml, ED_{50} of second ethanol extraction (F2) = 46.5 µg/ml, ED_{50} of sodium hydroxide extract = 44.7 µg/ml.

6.5.3.7. Acid Hydrolysis

A sample of the crude aqueous extract of *Barringtonia racemosa* was put through the same acid hydrolysis process outlined in the section 5.8 p. 102. Acid hydrolysis results in the fragmentation of glycosidic links present in the material. The process involves uncontrolled degradation of the test substance (Linberg, Lonngren & Svensson 1975). When this procedure is coupled with antiviral testing of the hydrolysed sample, valuable information can be obtained about the nature of the starting material being investigated. When the hydrolysed cold aqueous extract of *Barringtonia racemosa* was tested for activity, there was no inhibition of the infectious agent at all concentrations tested. In comparison, it has already been established that the starting material, 29AQC exhibited significant antiviral activity. Therefore, acid hydrolysis resulted in a loss of antiviral activity.

When comparing the chromatograms in figure 13 p155 and figure 17, p159, attained following reverse phase HPLC analysis of 29AQC and the hydrolysed sample, it is evident that there is a change in the composition of the samples. Extract 29 AQC exhibited a broad peak that was detected early during the elution whereas the hydrolysed sample shows numerous smaller peaks eluting over 30 minutes.

6.6. CHROMATOGRAPHY

6.6.1. Thin Layer Chromatography

Thin layer chromatography (TLC) was performed to further investigate the major chemical constituents present in the aqueous extract of *Barringtonia racemosa.* The isolation of chemical groups was dependent on the solubility, polarity and partitioning ability of the extract onto a layer of silica gel adsorbent. Two solvent systems were used to optimise the partitioning of chemical groups. Solvent system A comprised ethyl acetate-methanol-water and aided the separation of polar compounds. Solvent system B comprised toluene-ethyl acetate and facilitated the separation of non-polar compounds. Thin layer chromatographic sheets were prepared to test for polyphenolics, alkaloids, saponins and cardiac glycosides (Wagner & Bladt 1996). The cold aqueous extract of *Barringtonia racemosa* was prepared according to the method outlined in the previous chapter. Duplicate samples were prepared and therefore the sample analysed using TLC was tested in both solvent systems mentioned. Results show that solvent system B failed to partition compounds present in extracts spotted on plates. This indicates that the constituents are highly polar and were therefore unable to partition into the organic phase of the mobile solvent. In contrast, some separation was achieved when using solvent system A. The results obtained are presented in table 20, p144.

Table 20: Summary of TLC results

DETECTION TEST	RESULT
Polyphenolics	
29AQC	[*1]Blue coloured spot visible R*f values* = 0.58, 0.69, 0.66
Alkaloid	No detection
Saponins	No detection
Cardiac glycosides	No detection

[*1] refers to spots detected approximately 3 cm from the base line. n = 3 of an average of 6 readings.

The TLC results indicate that the extract contains polyphenolics. There was no detection of the remaining chemical groups, which were alkaloids, saponins and cardiac glycosides as reflected by their partitioning characteristics. These results further indicate that *Barringtonia racemosa* AQC posseses highly polar compounds. Only qualitative determinations were conducted and therefore the amount of polyphenolics present in each extract was not quantitated.

6.6.2. Liquid Chromatography

Three liquid chromatographic techniques were investigated and the outcomes reveal that a certain degree of fractionation of the compound was achieved and in addition, increased insight to the physico-chemical properties of the agent was obtained.

6.6.2.1. Size Exclusion Chromatography

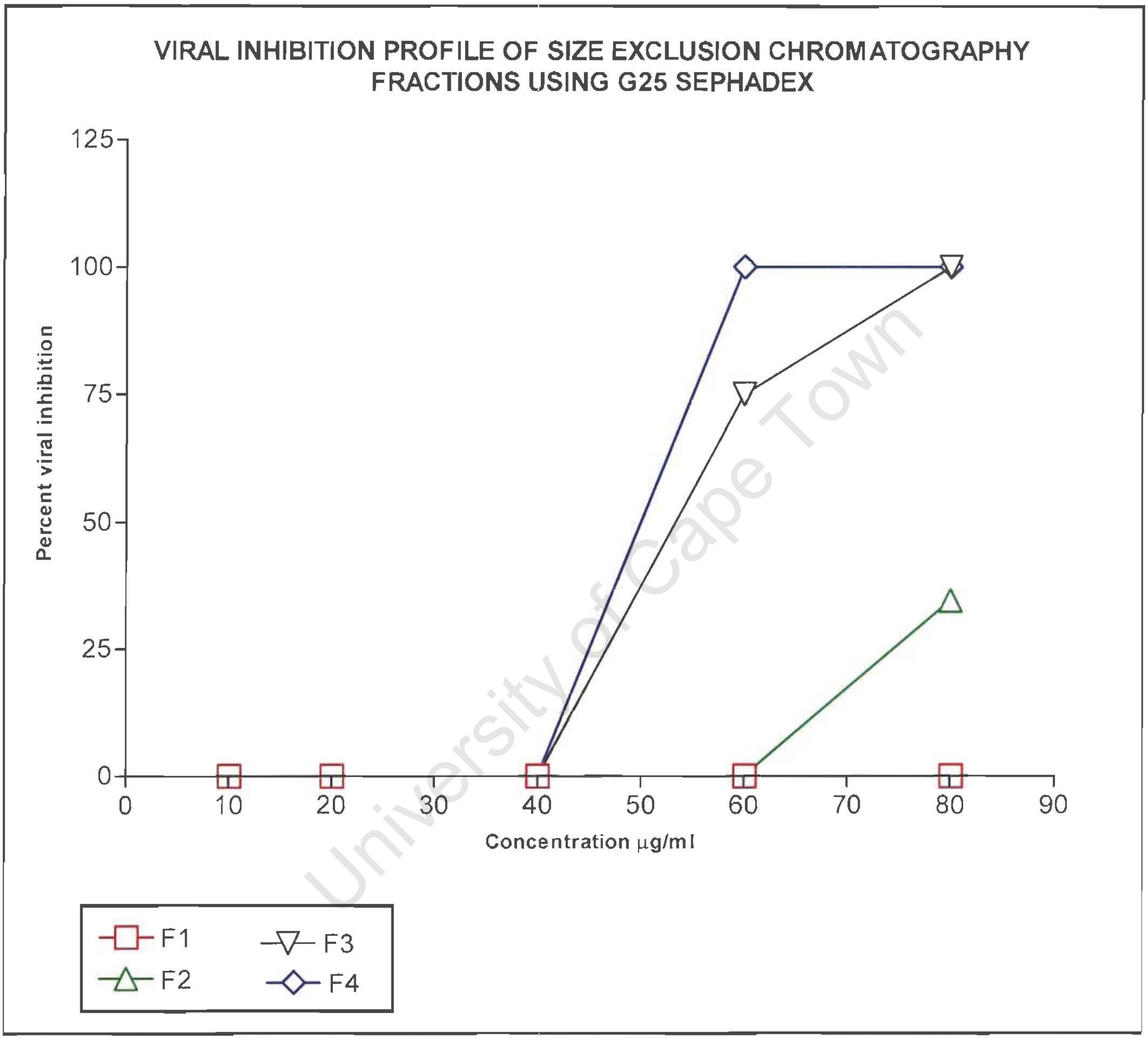
Separations in size exclusion chromatography (SEC) are based on the size of the chemical constituents in the sample in solution rather than their interaction with column packing (Le Maire, Viel & Moller 1989, Lumley, Patel & Cohen 1987). Complete dissolution of the test substance in a given solvent is therefore a requirement of this separation method.

The void volume of a column packed with G25 Sephadex was determined using Blue dextran, a protein standard with a molecular weight of 200 000 000.

The void volume (V_o), which is the amount of mobile phase required for the elution of the blue dextran, was 58 ml. The total volume (V_t), which carried the small molecules that pass inside the polymer beads of the column packing material, was 120 ml for the cold aqueous extract. The larger molecules were eluted in the elution volume (V_e), which was 85 ml for the aqueous extract. The distribution coefficient (K_d) was determined by substituting the values obtained experimentally for V_e, V_o and V_t into equation 10 p88. The calculated distribution coefficient for the cold aqueous extract was 0.435. The cold aqueous crude extract was passed through SEC using water as the eluting solvent. Four fractions were collected when using G25 Sephadex as column packing material. Fractions collected were lyophilised and the antiviral activity is presented in Graphs 15 p145.

Fractions 1 and 2 of graph 15, p146 represent high molecular weight biomolecules that are eluted in the void volume. Fraction 3 represents molecules of intermediate molecular weight and fraction 4 in both graphs represents small molecular weight molecules that elute in the total volume. Graph 15 above indicates that the active antiviral component present in *Barringtonia racemosa* elutes in fractions three and four of the cold aqueous extract. The $ED_{50,}$ for the cold aqueous extract was determined using the Reed and Muench method and was calculated to be $10^{-1.73}$ and $10^{-1.69}$ for fractions three and four, respectively. This translates to effective concentrations of 54.2 µg/ml and 49.0 µg/ml. Fractions 1 and 2 of of the extract showed no antiviral activity.

Graph 15: Viral inhibition profile of 29AQC SEC fractions using a G25 column



Fraction one (F1) represents the large molecular weight components. Fraction 2 (F2) represents large molecular weight fractions and is characterised by the elution of the coloured component of the extract. Fraction 3 (F3) represents substances of intermediate molecular weight as determined by the detection of chloride ions and fraction 4 (F4) represents low molecular weight substances that appear after the detection of chloride ions. The ED_{50} for F3 = 54.2 μg/ml and the ED_{50} for F4 = 49.0 μg/ml.

6.6.2.2. Ion Exchange Chromatography

Ion exchange chromatography (IEC) techniques were used to determine the presence of ionic components in the extract analysed. DEAE Sephacel packing is a weak anionic exchanger that is able to retain positively charged groups. Increasing concentrations of sodium chloride, which are able to lower the retention of ions bound to the column, can displace the charged groups. The anionic column is capable of retaining organic acids, polyphenols and most amino acids (Redgwell, 1980). It is expected that constituents such as sugar moieties will be eluted early in the run. Fractions that had already been isolated using SEC were passed through an IEC column.

A preliminary test that was performed on the IEC fractions collected was the phenol-sulphuric acid tests to identify sugar moieties present in the fractions. Results show that fractions containing sugar moieties were eluted at low concentrations of the displacing salt (Table 21).

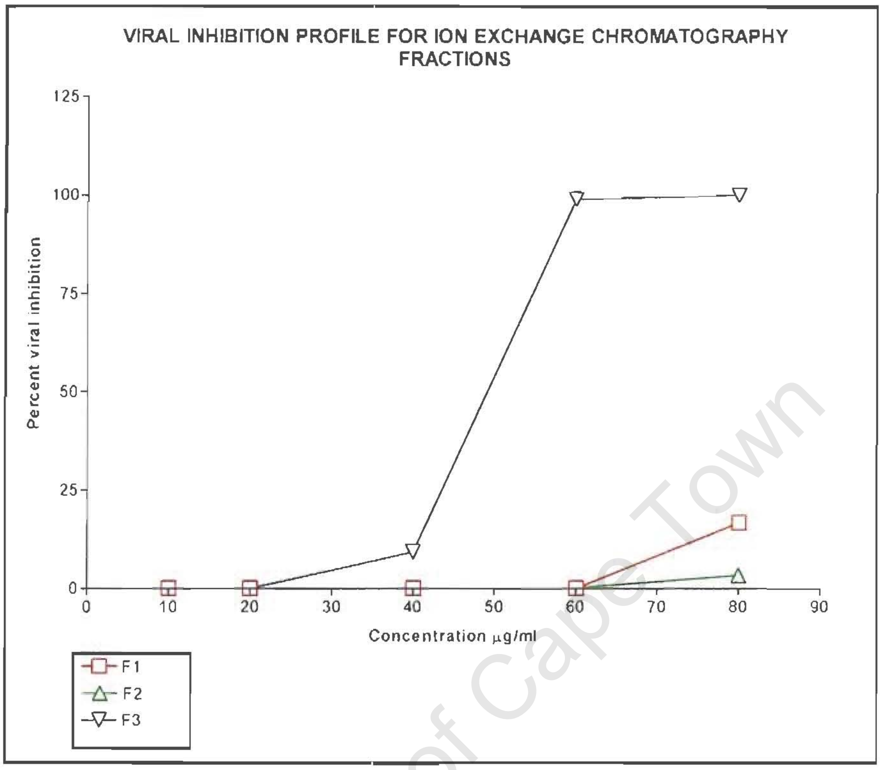
Following the lyophilisation of ion exchange chromatography samples, the fractions were reconstituted in water and put through size exclusion chromatography to remove the displacing salt. Salt-free fractions were then tested for anti-viral activity.

Table 21: Analysis of ion exchange chromatography samples following desalting of samples using size exclusion chromatography techniques

	COLD AQUEOUS EXTRACT	
FRACTION	*[*1]P-S TEST*	*ANTI-VIRAL TEST*
SEC: F1	Negative	Negative
SEC: F2	Negative	Negative
SEC: F3	Positive	Positive

[*1] P-S refers to phenol-sulphuric acid test. [*2] SEC refers to size exclusion chromatography. F1 represents the high molecular weight fraction eluted before the detection of salt ions; F2 represents the elution of intermediate molecular weight compounds during which the salt ion is detected and F3 represents the detection of low molecular weight compounds.

Graph 16: Viral inhibition profile for *IEC sample following desalting on G50 column



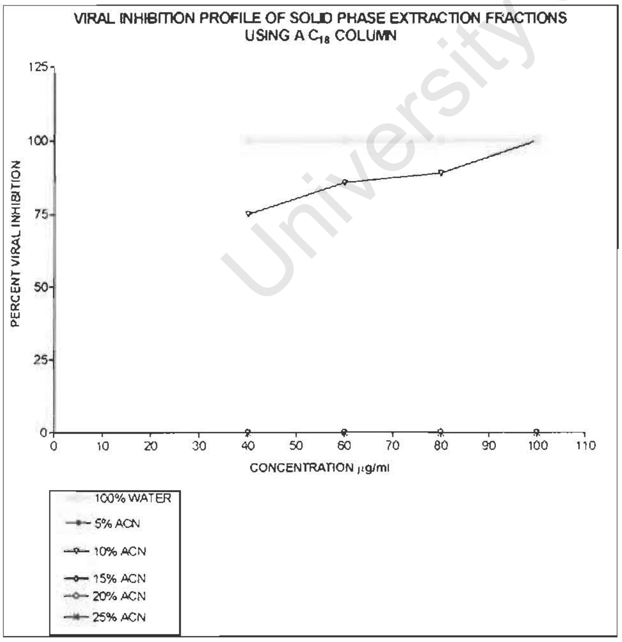
* Ion Exchange Chromatography. F3 – ED_{50} = 48.1 µg/ml. F1 represents high molecular weight compounds derived from desalting the IEC samples using a size exclusion column. F2 represents intermediate molecular weight compounds and F3 represents low molecular weight compounds.

The active fraction derived from IEC eluted in the total volume of the size exclusion chromatography column. This further indicates that the analyte is of small molecular weight. The corresponding ED_{50} value of this fraction was 48.1µg/ml.

6.6.2.3. Solid Phase Extraction

Solid phase extraction (SPE) techniques are particularly useful and convenient for the concentration of sample material prior to chemical analysis and for the removal of contaminants that may mask the identity of the compound of interest. The extraction profiles of test compounds can be determined using different SPE columns. Therefore, it is possible to classify active constituents by their different class characteristics hence, priotirise the sequence of biofractionation procedures (Stefanska 1995). Chemical characterisation results generated thus far clearly suggest that the active compound in the aqueous extract of *Barringtonia racemosa* is highly polar. Two types of SPE columns were therefore used; the first was a normal phase column which is used to isolate polar compounds from a non-polar matrix. In this system the column matrix retains desired molecules whilst interfering compounds are removed by the eluent. The second system used involved the application of a C_{18} reverse phase column which isolates non-polar compounds from a polar matrix. The basis of using a reversed phase column on a highly polar compound is to promote the retention of undesired non-polar contaminants, resulting in the collection of polar constituents in the eluent. In both systems it was possible to influence the elution of compounds by manipulating the acetonitrile-water concentration gradient of the mobile phase. Fractions of the eluting solvent were collected, lyophilised and tested for antiviral activity.

Graph 17: Antiviral profile for C_{18} *SPE fractions



*Solid Phase Extraction. Key: ACN – acetonitrile. 5% ACN indicates 5% acetonitrile and 95% water; 10% ACN indicates 10% acetonitrile and 90% water; 15% ACN indicates 15% acetonitrile and 85% water; 100% water indicates no acetonitrile solvent.

The above graph 17, p149 reveals that a reversed phase column minimally retains the active fraction of crude extract and the material is eluted through the column without any partitioning taking place. As expected, fractions eluted with 20% and 25% acetonitrile showed no antiviral activity.

Ideally, to ensure binding of the active polar agent to a normal phase column, the test material needs to be dissolved in an organic solvent such as acetonitrile and eluted with a polar solvent. Due to solubility problems associated with the cold aqueous extract of *Barringtonia racemosa*, dissolution in water was first required, before adding acetonitrile as a co-solvent. Although this procedure aided dissolution of the plant in an organic solvent, it did not facilitate the binding of active material to the Cyano solid phase extraction cartridge. Consequently, fractions collected after the elution of the test material showed no antiviral activity.

Table 22: A summary of liquid chromatography results

LC Analytical Assay	ED$_{50}$ in µg/ml
Size exclusion chromatography	
[*1]F3 29AQC	54.2
[*2]F4 29AQC	49.0
Ion exchange chromatography	
[*3]F3 following SEC	48.1
Solid phase extraction: C$_{18}$	
5% ACN	ND
10% ACN	ND
15% ACN	ND

Key: AQC – cold aqueous extract; ACN – acetonitrile, ND – not determined due to percent inhibition values of above 50%. [*1] (F3) and [*2] (F4) refer to the intermediate and low molecular weight fraction of 29AQC respectively using a G25 size exclusion column (graph 15, p146). [*3] refers to the low molecular weight fraction obtained from desalting the ion exchange fraction using a G50 size exclusion column (graph 16, p148).

6.7. REVERSE PHASE HPLC

High Performance Liquid Chromatographic techniques were used extensively in the purification and isolation of the active moiety of the aqueous extract of *Barringtonia racemosa*. Prior to performing separation procedures, it is necessary to validate and therefore, confirm the suitability of a chromatographic system developed. HPLC validation methods should include the validation of both chromatographic and pre-chromatographic analyses (Krull & Swartz 1997, Szepesi, Gazdag & Mihalyfi 1989). However, since HPLC techniques were being utilised to obtain qualitative rather than quantitative information about the test sample, it was only necessary to validate the chromatographic separation (Therapeutics Products Programme 1998, Therapeutics Products Programme 1994, Hagan 1994, Das Gupta, Pramar & Parasrampuria 1991, Krull & Swartz 1997, Szepesi, Gazdag & Mihalyfi 1989).

6.7.1. System Validation

During routine system validation of an HPLC method, it is necessary to demonstrate the accuracy, precision, limit of quantitation, sensitivity, linearity and range of the analytical procedure (Gruber et al. 1999). According to validation guidelines approved by the Canadian Health Therapeutics Products Programme, only limit of detection and specificity parameters are necessary for the validation of identity tests of a given compound (Therapeutics Products Programme 1994). Therefore, in adherence to these guidelines, both validation parameters were determined and in addition, precision and sensitivity investigations were carried out.

Two methods were used during system validation; the first determined day-to-day reproducibility and the second, demonstrated intra-day reproducibility. Results obtained during system validation are presented below.

6.7.2. Precision Studies

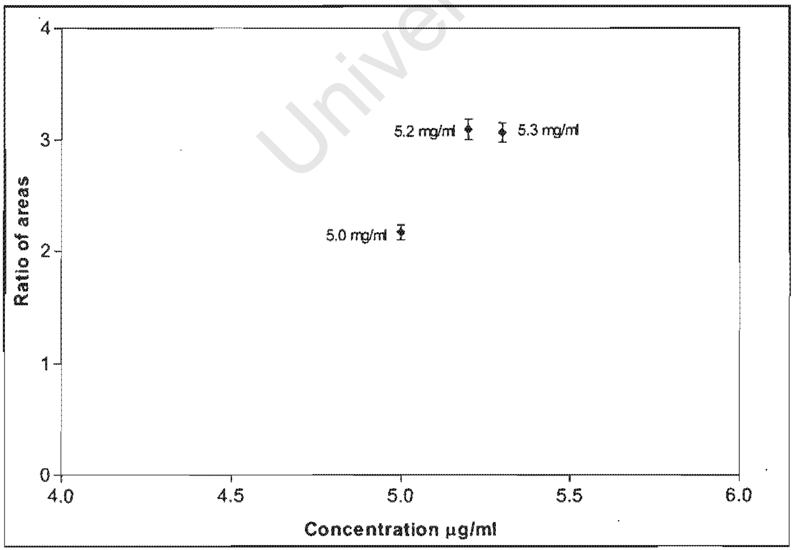
The precision of an analytical procedure determines the degree of closeness of measurements obtained from multiple sampling of a single substance, using the same chromatographic conditions. A total of 10 injections of a 0.5 mg/ml solution of the internal standard were injected into the HPLC system (Table 23 and graph 18). The procedure was repeated over three days of analysis and on each day, the internal standard, mannose, was freshly prepared. The HPLC detector integrated peak data received to give relative concentration data that are based on the amount of extract injected into the system. The peak concentration data obtained were used to calculate the percent relative standard deviation.

Table 23: The percent relative standard deviations for the internal standard

	DAY 1	DAY 2	DAY 3	Inter-day
CONC mg/ml	5.2	5.0	5.3	
% RSD*	9.47 ±0.30	9.566 ±0.21	8.989 ±0.28	2.796 ±0.082

(n = 10 for each intra-day trial). *RSD – relative standard deviation. Refer to equation 11, p97 for calculation method.

Graph 18: Precision assay: HPLC validation data



Graph 19 illustrates the percent relative areas for the HPLC precision determinations taken at each of the three concentrations.

The relative percentage standard deviations values for intra- and inter-day variability are less than 10% thus indicating that HPLC analytical system demonstrates adequate precision (Therapeutics Products Programme 1994).

6.7.3. *Limit of Detection*

Limit of detection tests determine the lowest amount of analyte in a sample that can be detected but not necessarily quantitated. The analysis is useful in determining lower detection limits of the chromatographic system. The lowest concentration of the internal standard prepared that was detected by the chromatographic instrument was 0.01 μg/ml.

6.7.4. *Sensitivity Studies*

The measure for the sensitivity of the systems can be calculated by analysing the percent relative standard deviation values of the precision results. The attainment of percent relative standard deviations of less than 10% during the precision studies indicate that the chromatographic test method used is reliable for the analysis of small concentrations of analyte.

6.7.5. *Specificity Tests*

Specificity tests were conducted using D-glucose, which is structurally similar and closely related to mannose. The purpose of this test was to determine the ability of the chromatographic system to resolve two components that elute close to one another. The chromatographic profile below illustrates reasonable resolution of mannose and D-glucose (figure 16 p158).

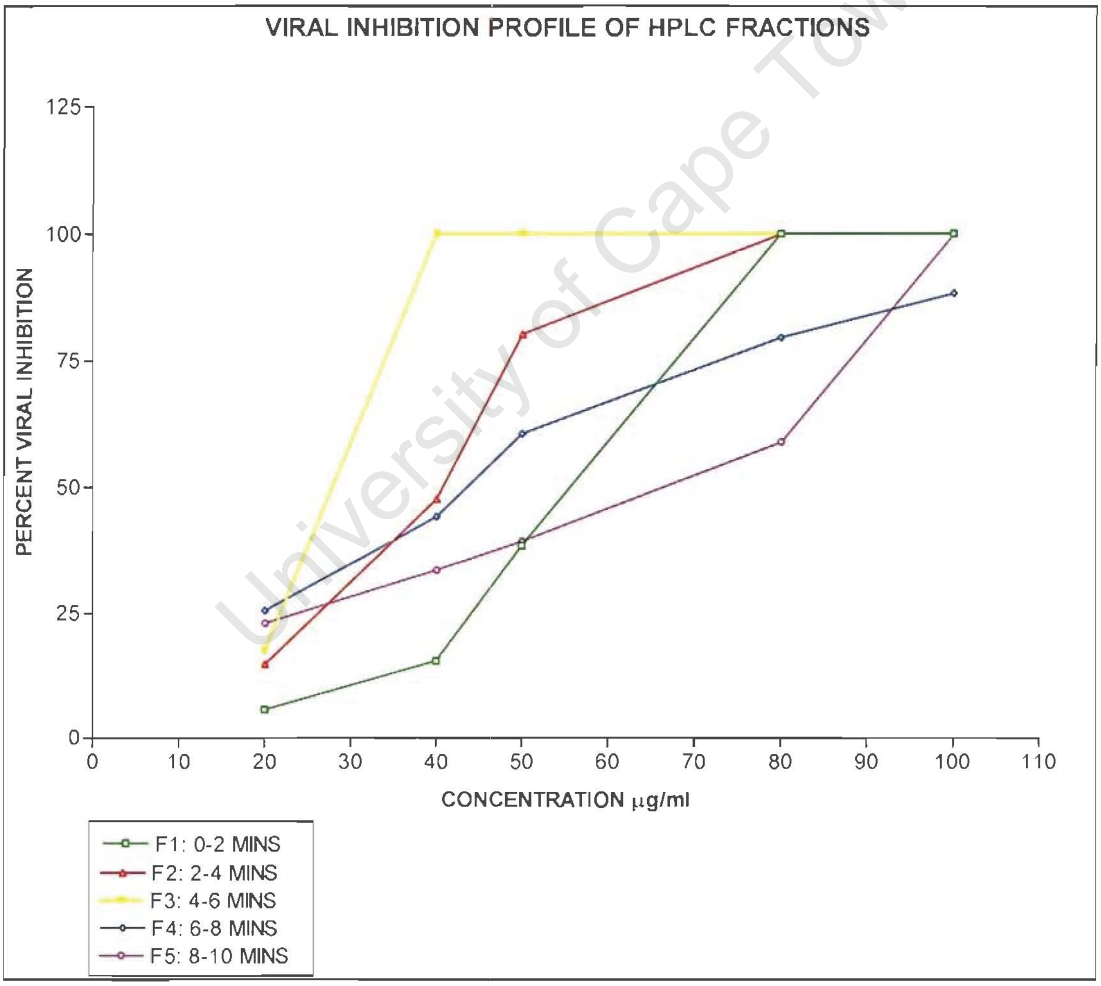
6.7.6. *Summary*

Precision and sensitivity results show a percent standard deviation of less than 10% and a limit of detection of 0.01μg/ml. These results suggest that the HPLC system developed is suitable for the identification, isolation and analysis of *Barringtonia racemosa*

6.7.7. Antiviral Testing

The analytical and preparative applications of reversed phase techniques were used for the separation and purification of the cold aqueous extract of *Barringtonia racemosa*. Samples of the extract were injected into the HPLC system and fractions were collected at 2-minute intervals from the time of injection. A total of five fractions was collected and lyophilised prior to testing for antiviral activity. The bioassay results are presented in Graph 19.

Graph 19: Viral inhibition profile for reverse phase HPLC fractions

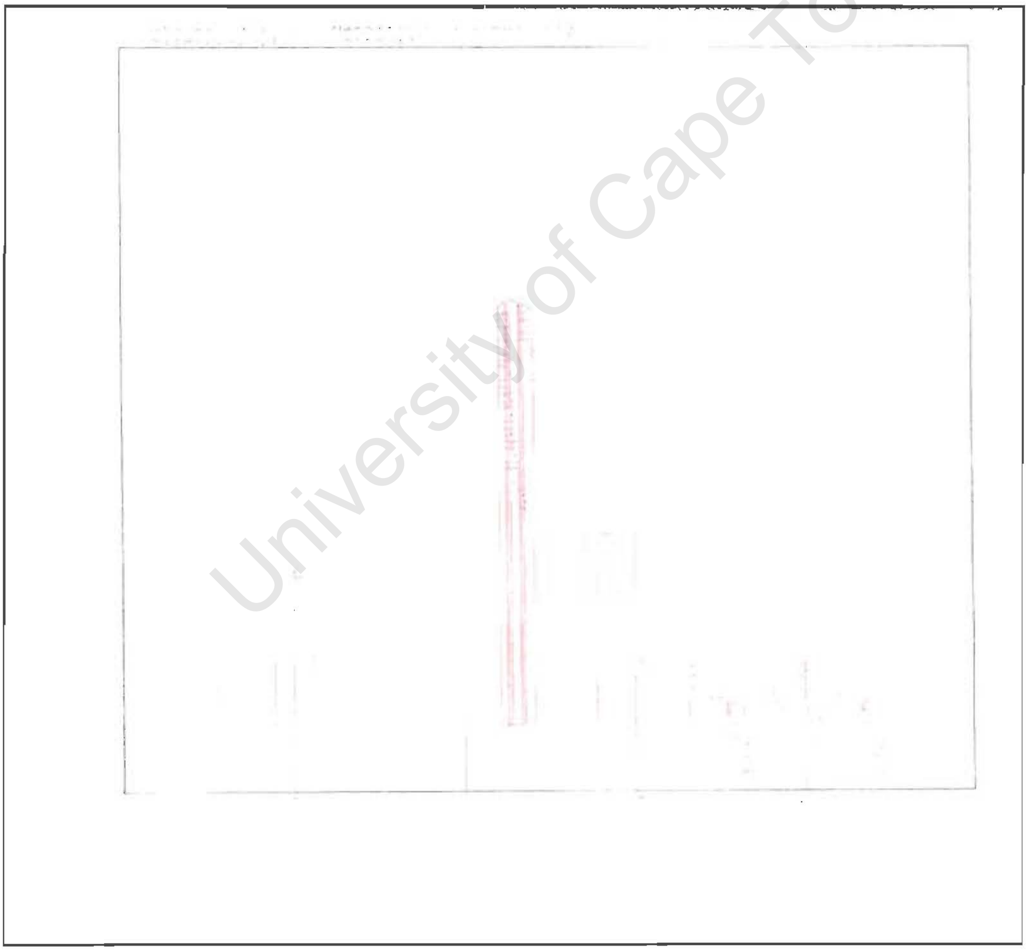


Key: F1 to F5 refer to fraction 1 through to fraction 5 collected at the time intervals indicated in the key above. Readings taken were at Day 5 post-inoculation. n = 1 of an average of 5. readings.

Table 24: ED_{50} values for reverse phase chromatography fractions of 29AQC

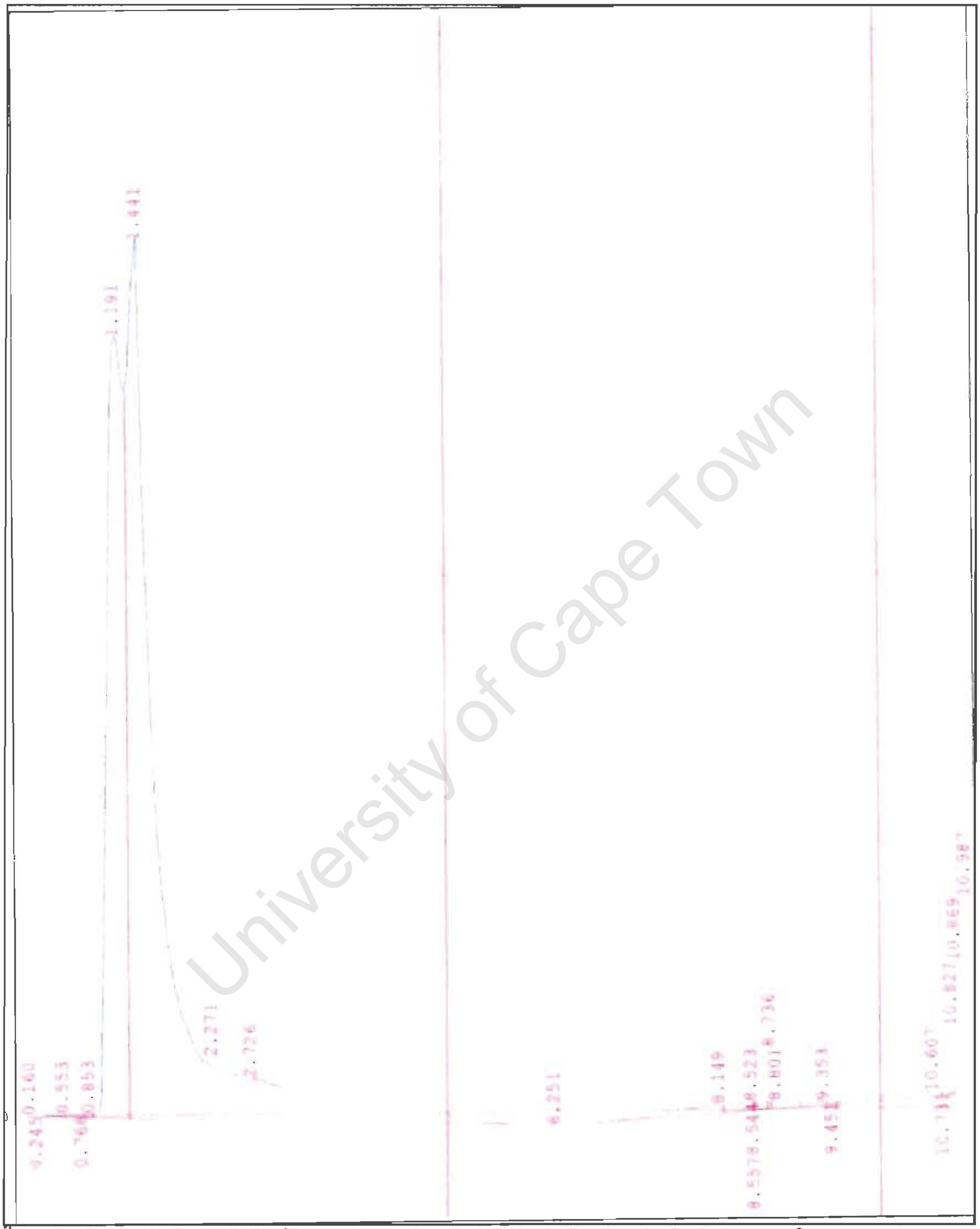
FRACTION	ED_{50} $\mu g/ml$
F1: 0 – 2 minutes □	54.6
F2: 2 – 4 minutes △	40.6
F3: 4 – 6 minutes ▽	26.2
F4: 6 – 8 minutes ◇	43.4
F5: 8 – 10 minutes ○	64.4

Figure 13: Chromatographic profile of 29AQC



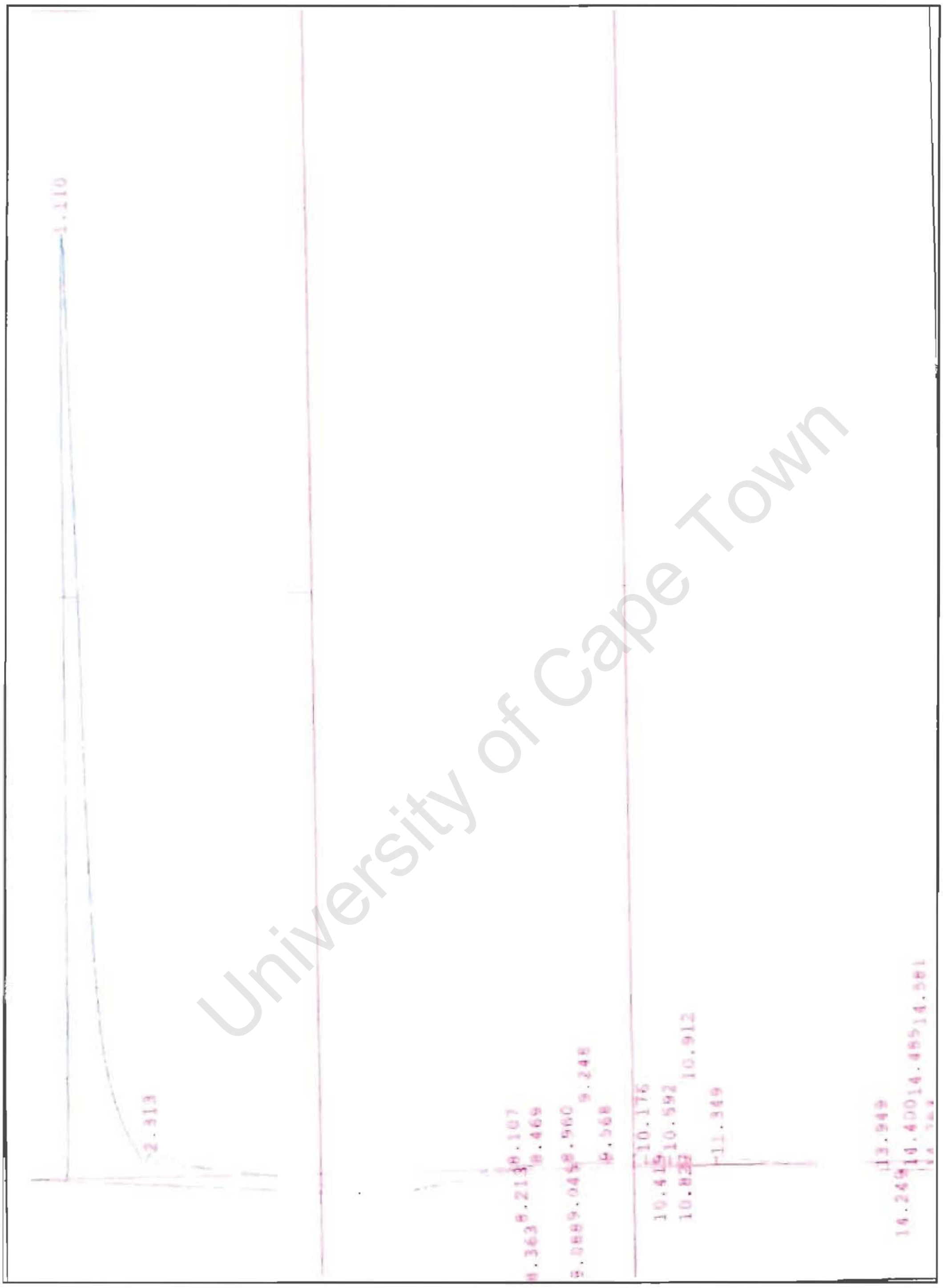
The above profile was obtained using an acetonitrile/ water mobile phase, eluting at a gradient of 0% acetonitrile to 50% acetonitrile over 20 minutes at a flow rate of 1 ml/min using a C_{18} RP-HPLC column. The above chromatogram shows early elution of compounds. The presence of broad peaks indicates the presence of impure compounds.

Figure 14: Chromatographic profile of [*1]fraction 3



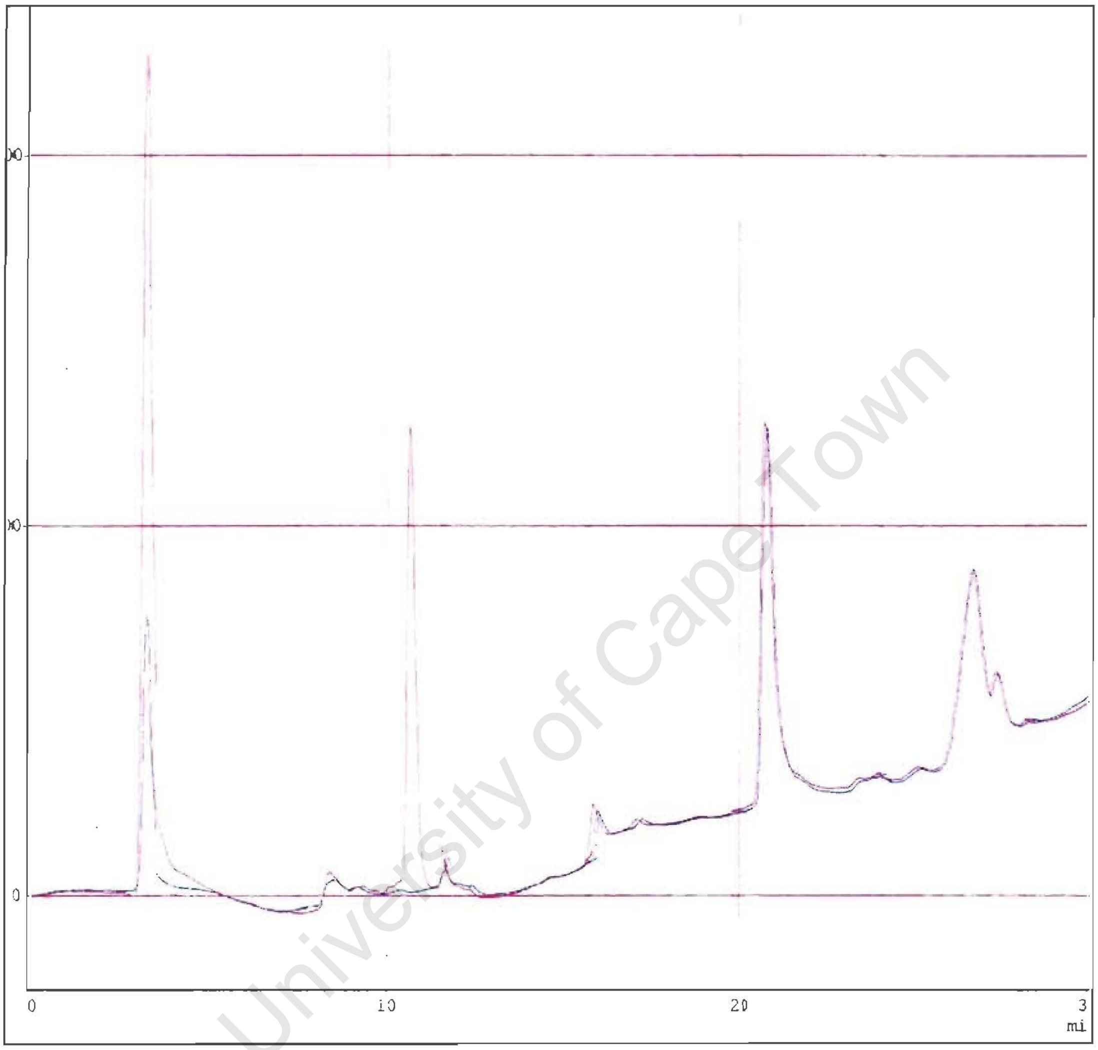
[1] Fraction 3 refers to the analysis of the crude sample collected at retention time 4 – 6 minutes on a C_{18} reverse-phase HPLC semi-preparative column. The above profile was obtained using an acetonitrile/ water mobile phase, eluting at a gradient of 0% acetonitrile to 50% acetonitrile over 20 minutes at a flow rate of 1 ml/min. The chromatogram shows early elution of compounds in Fraction 3. The profile indicates the presence of at least two compounds that elute close to one another.

Figure 15: Chromatographic profile of fraction [*1]4



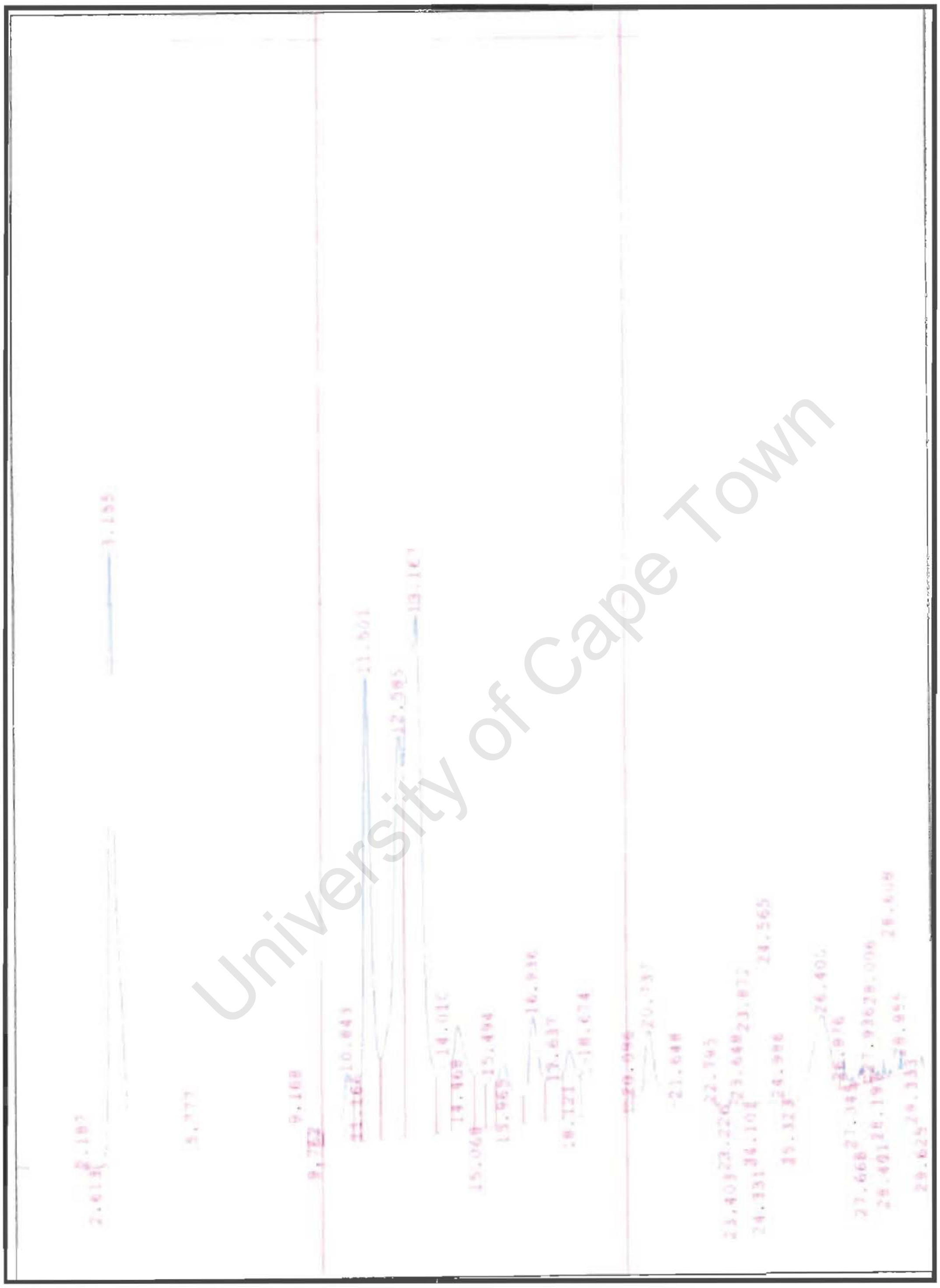
[*1] Fraction 4 refers to the analysis of the crude sample collected at retention time 6 – 8 minutes on a C_{18} reverse-phase HPLC semi-preparative column. The above profile was obtained using an acetonitrile/ water mobile phase, eluting at a gradient of 0% acetonitrile to 50% acetonitrile over 20 minutes at a flow rate of 1 ml/min. The chromatogram shows early elution of compounds in Fraction 4.

Figure 16: Chromatogram showing D-glucose and mannose



The profile in red represents mannose and the profile in blue represents D-glucose. The chromatogram shows the resolution of each saccharide, eluted using a gradient elution of acetonitrile/water on a reverse phase column.

Figure 17: Chromatographic profile of 29AQC following acid hydrolysis



The above figure illustrates the reverse phase HPLC profile of the crude sample of *Barringtonia racemosa* following acid hydrolysis. In comparison to figure 13, p155 the profile shows a greater frequency of smaller peaks eluted over 30 minutes.

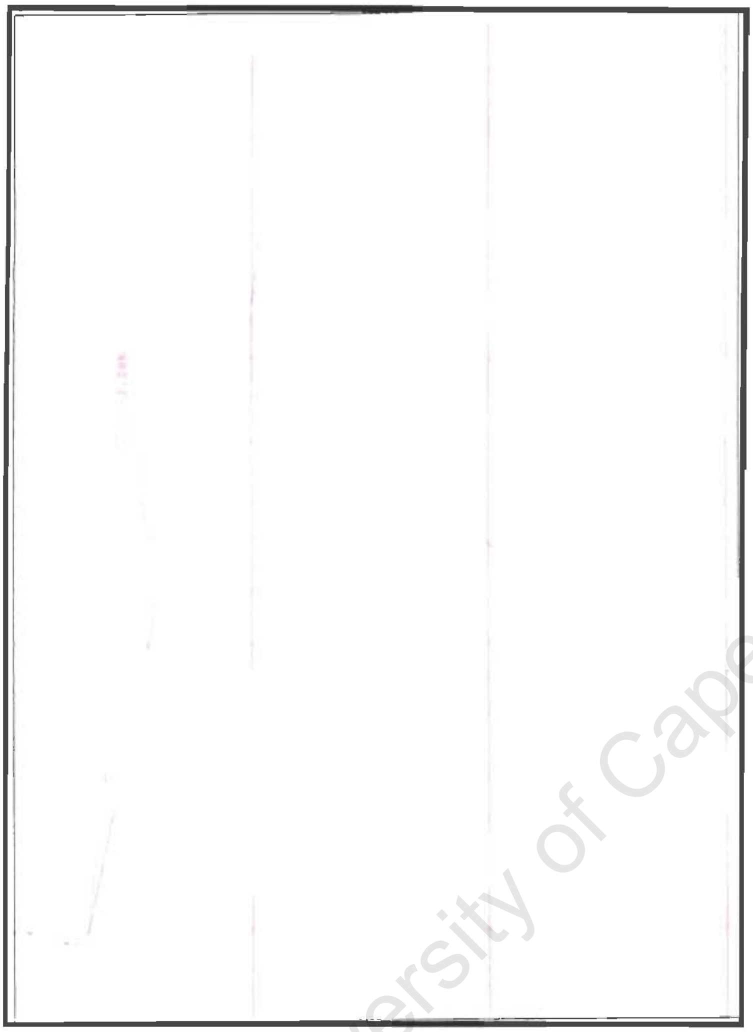
The ED_{50} values in Table 24, p 155 indicate that fraction 3 of the five fractions is the most active. The ED_{50} values of fractions 2 and 4 are very similar, suggesting that there may be residual active material that eluted in either fraction prior and after the main active had been eluted. The chromatograms presented indicate that the analyte elutes very early in the run.

In contrast to spectra obtained in previous chromatograms shown, the peak profile of the acid hydrolysed sample of *Barringtonia racemosa*AQC illustrates the breakdown of the compound into several different compounds.

6.8. ION EXCHANGE HPLC

Ion exchange high performance liquid chromatography (IEHPLC) has been successfully used in the separation of compounds that possess ionic characteristics (Vanecek & Regnier 1980, Heinitz 1988, Yamamoto, Nomura & Sano 1987). The isolation of ionic compounds from the strong anionic exchange column used was dependent on the pH and salt content of the eluent (Heinitz et al. 1988, Yao & Hjerten 1987, Yamamoto, Nomura & Sano 1987). As a result, investigations were made to determine the displacing ability of three buffer salt solutions at a constant pH of 8.0. The displacing salts investigated were 0.5M sodium acetate, 0.5M sodium sulphate and 0.5M sodium chloride. The chromatographic profiles obtained were clearly similar to one another and did not differ significantly from that shown in Figure 18, p161. Ideally, the salt of choice should be an intermediate salt which spreads out the chromatogram resulting in clear resolution of the eluting molecules. However, due to the reported superiority of sodium acetate in IEHPLC analyses, it was selected for use (Kopaciewicz & Regnier 1983, Vanecek & Regnier 1980, Smookler 1971).

Figure 18: Chromatographic profile of 29AQC using IEHPLC



The mobile phase used was phosphate buffer/ sodium acetate running at a gradient of 0% buffer to 100% salt ion over 15 minutes. The flow rate was 1 ml/min. The above chromatogram illustrates the broad peaks obtained during ion exchange HPLC analysis.

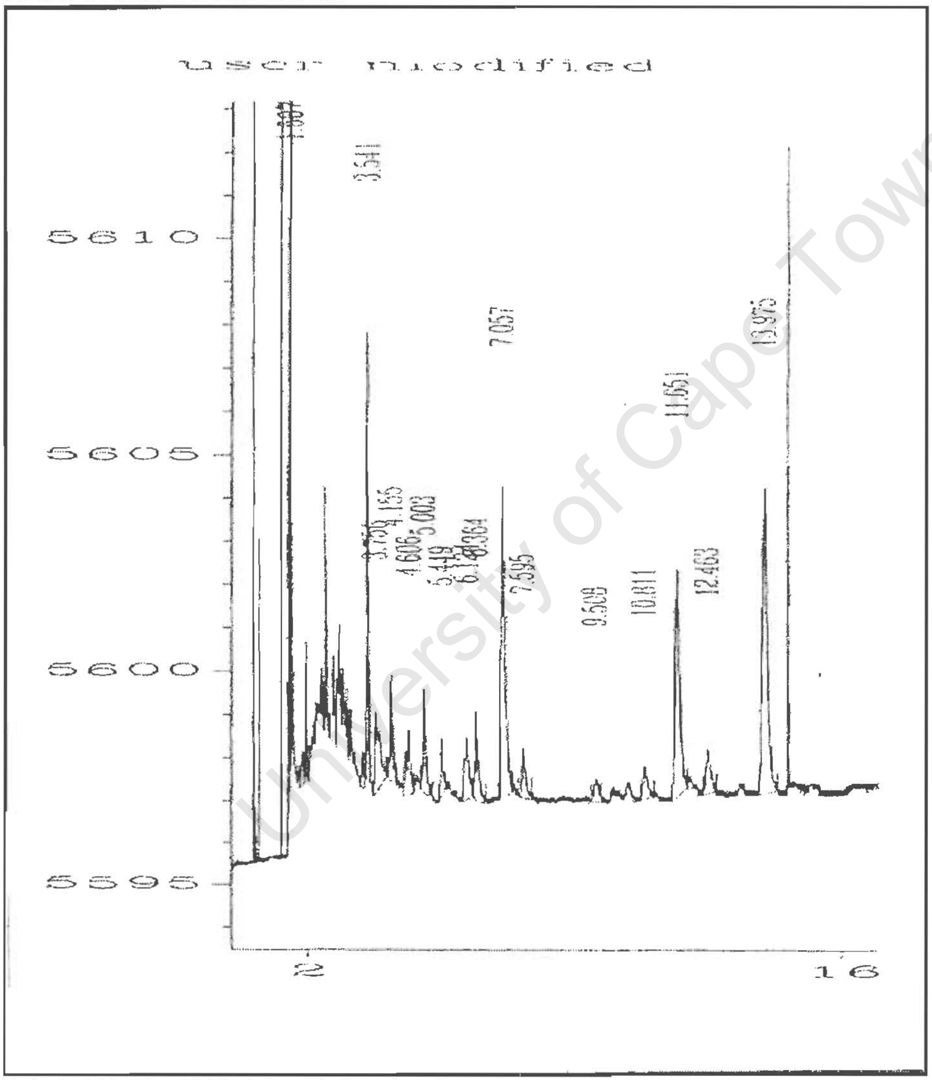
Antiviral analysis of the three fractions collected using a strong anionic ion exchange column showed no inhibition action.

6.9. GAS CHROMATOGRAPHY

Gas chromatography (GC) analysis was performed on the alditol acetate derivative of the cold aqueous crude extract. Table 25 p164 shows the common standards used during analysis and the corresponding retention times obtained. When comparing the GC profile of the standard with the profile of the active, the only peak that corresponds to both profiles is galactose which is eluted at 11.7 minutes.

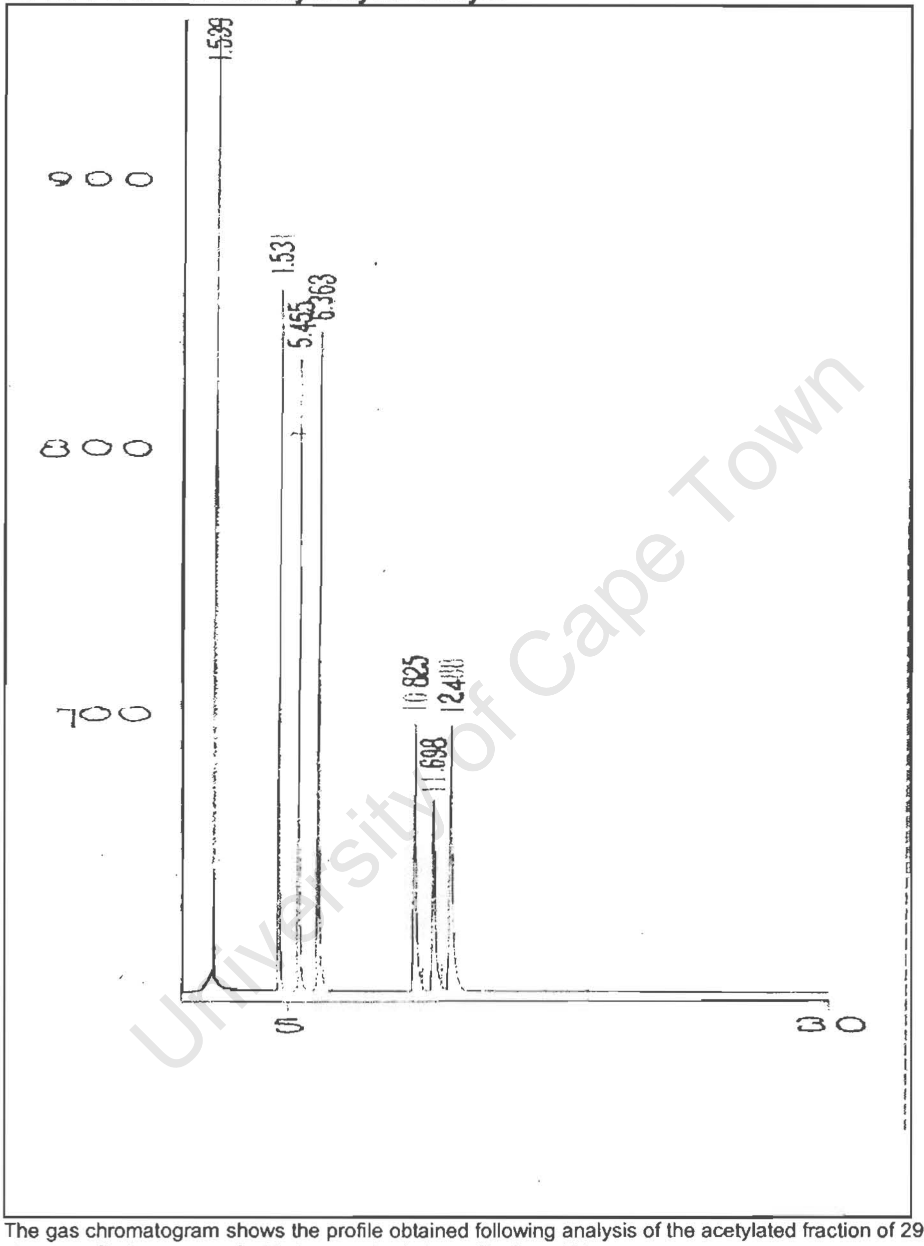
Figure 19: Gas Chromatography Profiles

A: GC Profile for standards.



Refer to table 25, p164 for interpretation of profile.

B: GC Profile for acid hydrolysis analyte



The gas chromatogram shows the profile obtained following analysis of the acetylated fraction of 29AQC. The above profile is compared with the standards in profile A, p. 161.

Table 25: Retention times of monosaccharide internal standards

MONOSACHARIDE STANDARD	RETENTION TIME (minutes)
Rhamnose	4.53
Arabinose	5.46
Xylose	6.36
Mannose	10.83
Galactose	11.70
Glucose	12.49

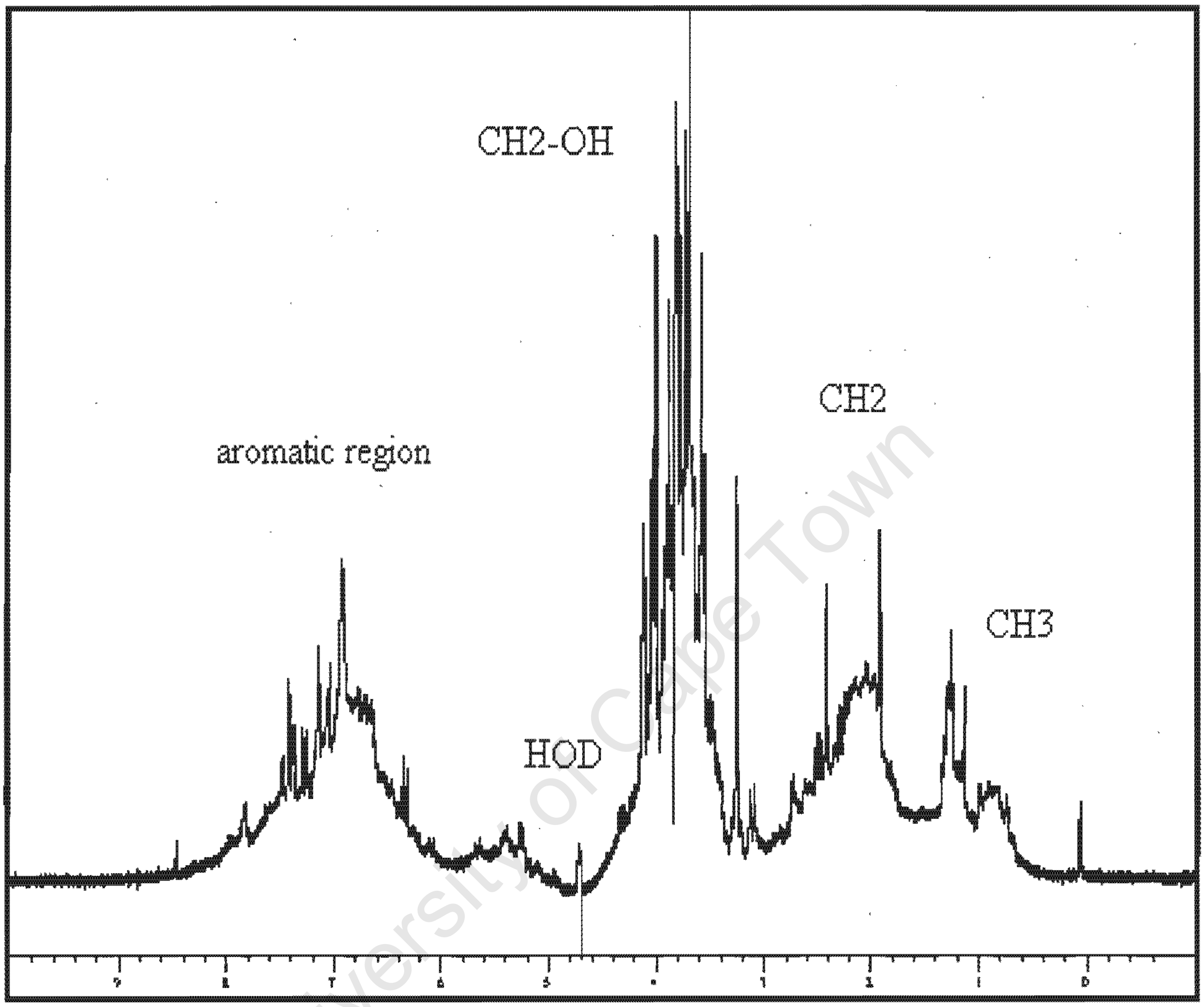
The above table shows the retention times of the internal standards used during GC analysis. The results shown in the table are graphically illustrated in Figures 19a and 19b.pp162-163.

6.10. NUCLEAR MAGNETIC RESONANCE

Proton nuclear magnetic resonance analysis of the plant extract was performed using a 400 MHz spectrophotometer. Presented here are the NMR spectra of the crude cold aqueous extract of *Barringtonia racemosa*, (Figure 20, p165) fraction 3 (Figure 21, p166) and fraction 4 (Figure 23, p168) from reverse phase HPLC and the ion exchange fraction of 29AQC (Figure 24, p169). The NMR profile of the crude extract served as a fingerprint to monitor the purification of subsequent fractions.

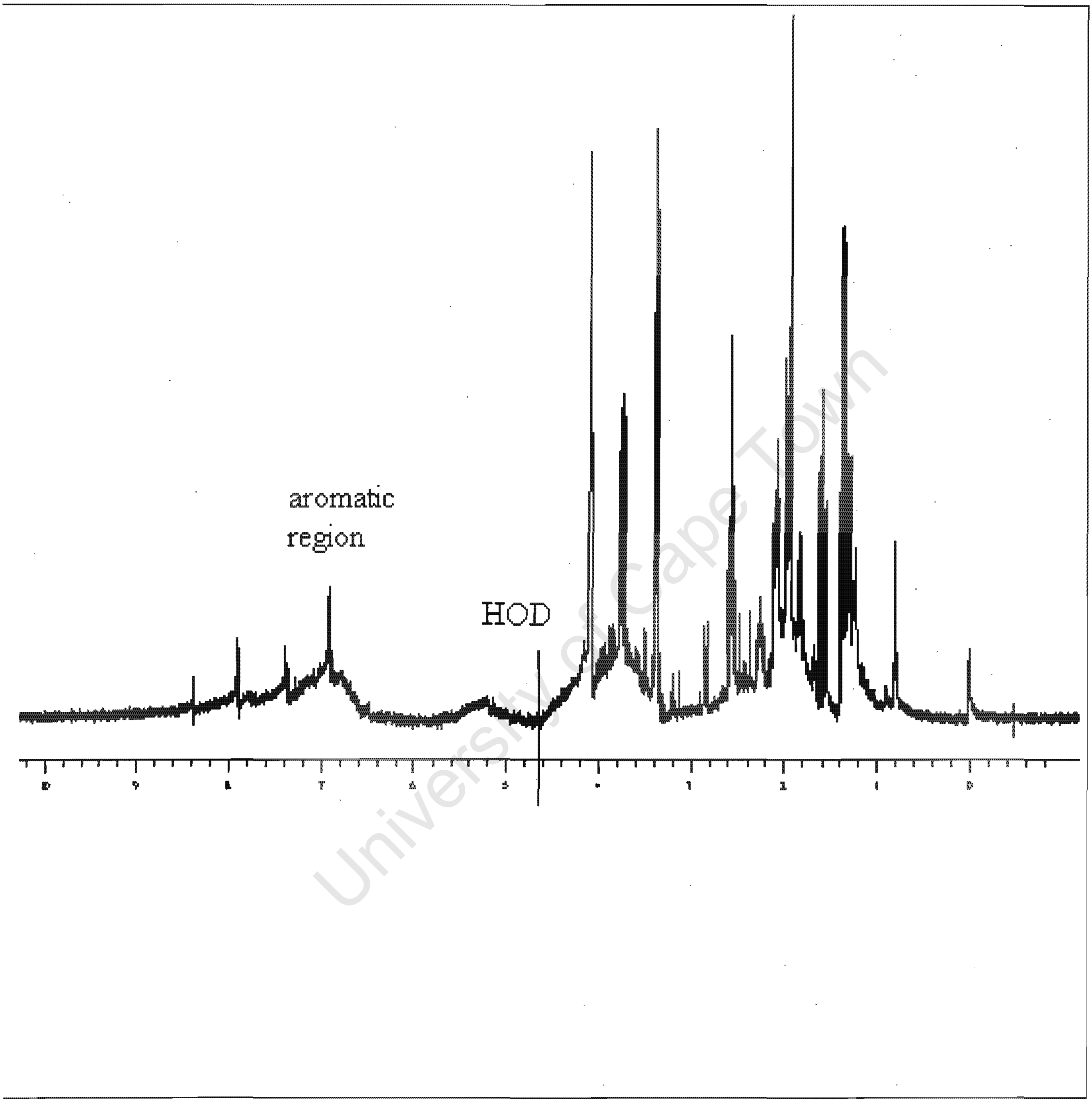
Chemists have introduced a convenient system for presenting the information in an NMR spectrum (Hall 1965). Firstly, each signal in the spectrum was described by stating its multiplicity; secondly, the chemical shift in parts per million downfield from TMS was determined and thirdly, the relative intensity of the signal was given in terms of the number of identical protons that give rise to the signal. It was therefore possible to refer to tables that give chemical shift data characteristic of a variety of proton environments. From this information it is possible to deduce typical carbon and hydrogen groups present in molecules. The carbon and hydrogen groups that could be deduced from the profiles presented are shown on the spectra of each fraction.

Figure 20: 400 MHz H-1 NMR spectrum of 29AQC



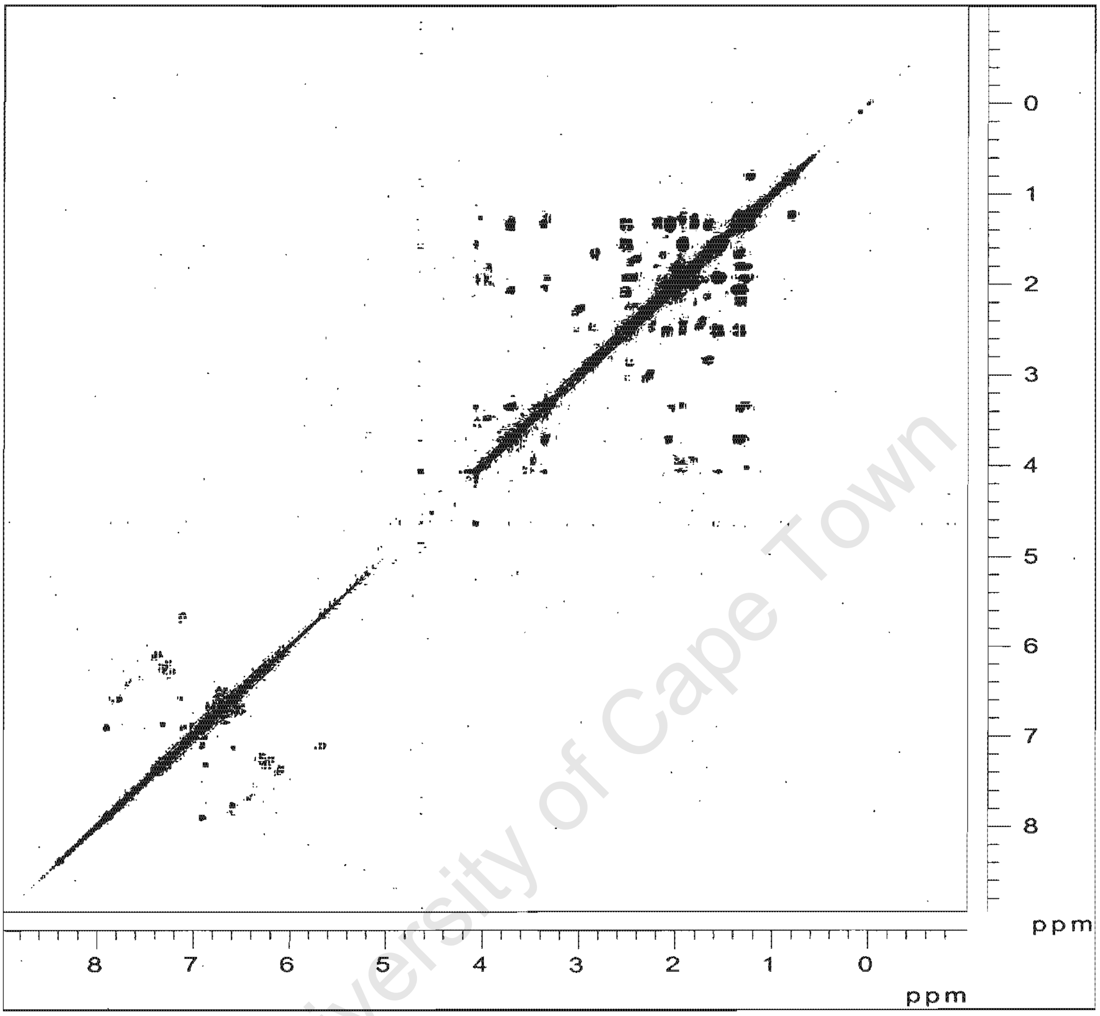
NMR spectrum for the crude cold aqueous extract of *Barringtonia racemosa*.

Figure 21: NMR spectra for fraction 3 of reverse phase chromatography



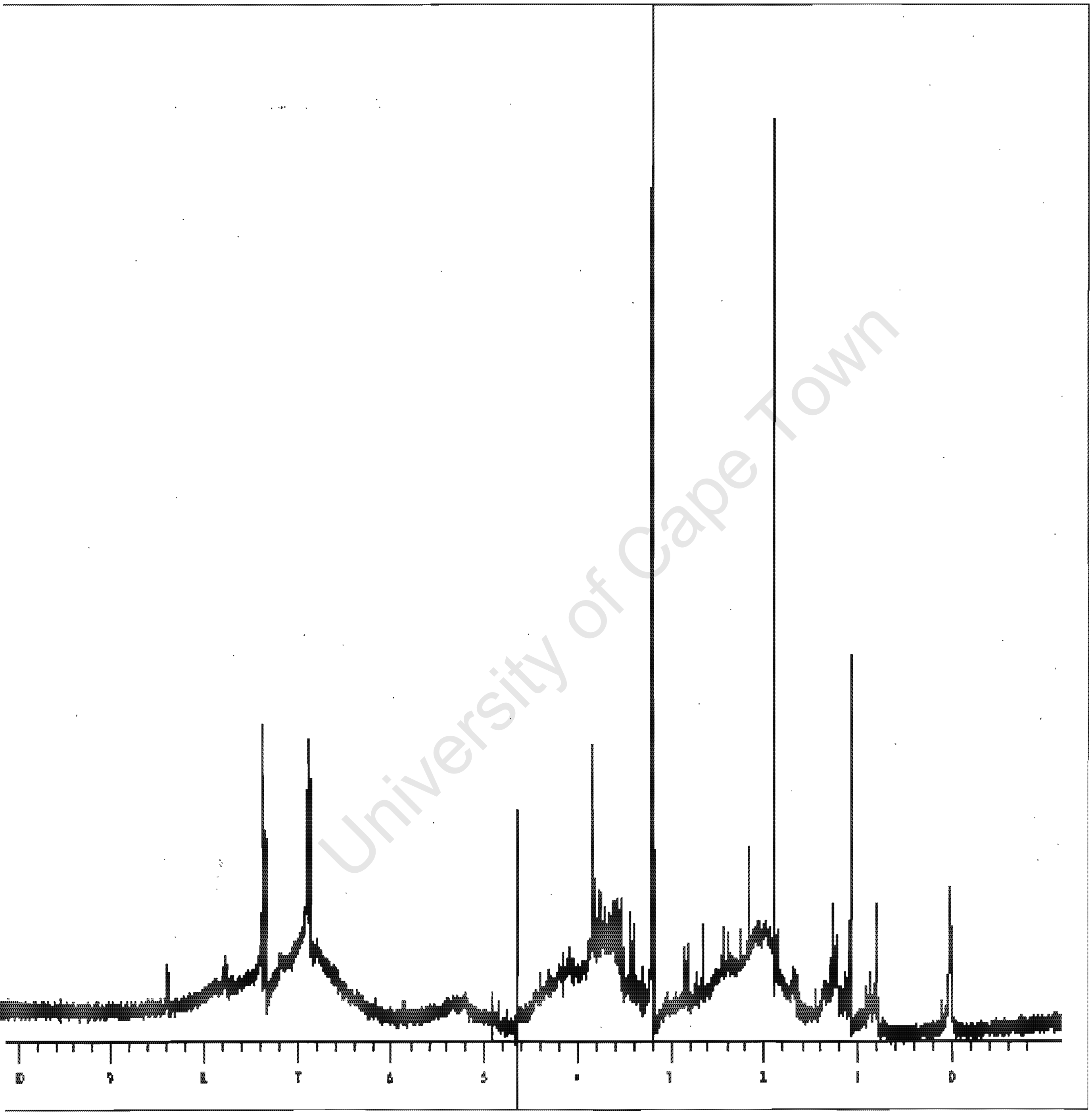
NMR spectrum of fraction 3 obtained at retention time 4 – 6 minutes from the crude aqueous sample of *Barringtonia racemosa* using reverse-phase chromatography.

Figure 22: 400 MHz COSY spectrum of fraction 3



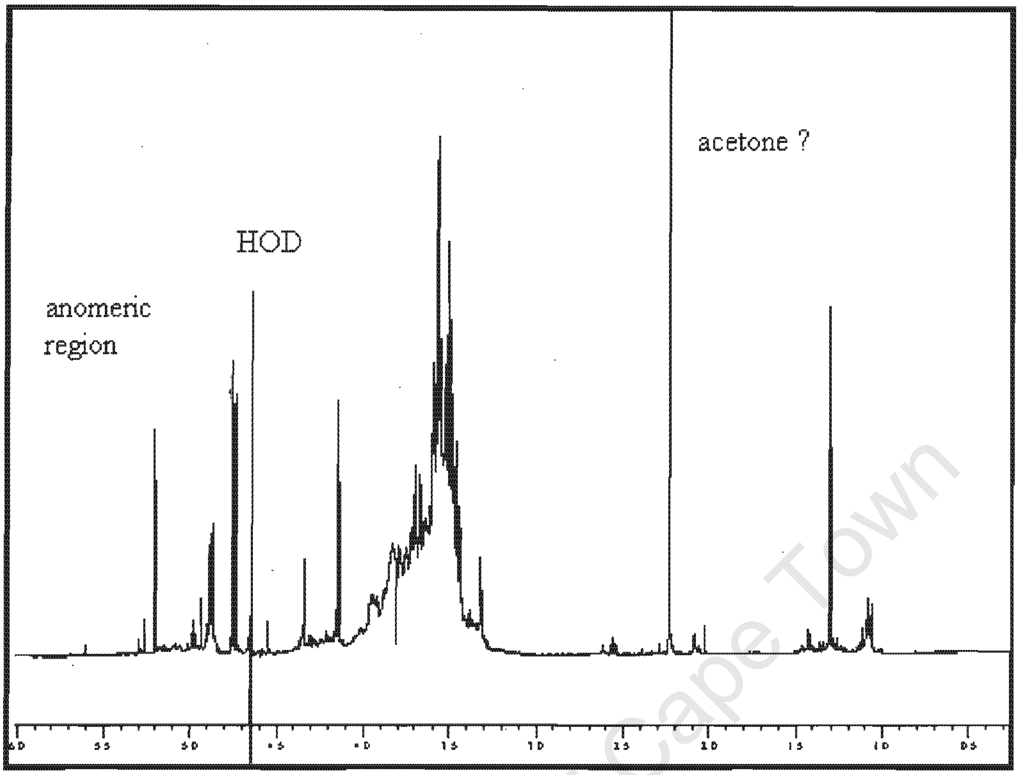
NMR cosy spectrum of fraction 3 obtained at retention time 4 – 6 minutes from the crude aqueous sample of *Barringtonia racemosa* using reverse-phase chromatography.

Figure 23: NMR spectra for fraction 4 of reverse phase chromatography



NMR spectrum of fraction 4 obtained at retention time 6 – 8 minutes from the crude aqueous sample of *Barringtonia racemosa* using reverse-phase chromatography.

Figure 24: 400 MHz H-1 NMR spectrum of 29AQC isolated by IEHPLC



Above profile was the sample obtained following the injection of the crude cold aqueous extract into an ion-exchange HPLC column. H1 at 400 MHz, COSY at 400 MHz, C13 at 300 then 400 MHz, HSQC on 400 MHz.

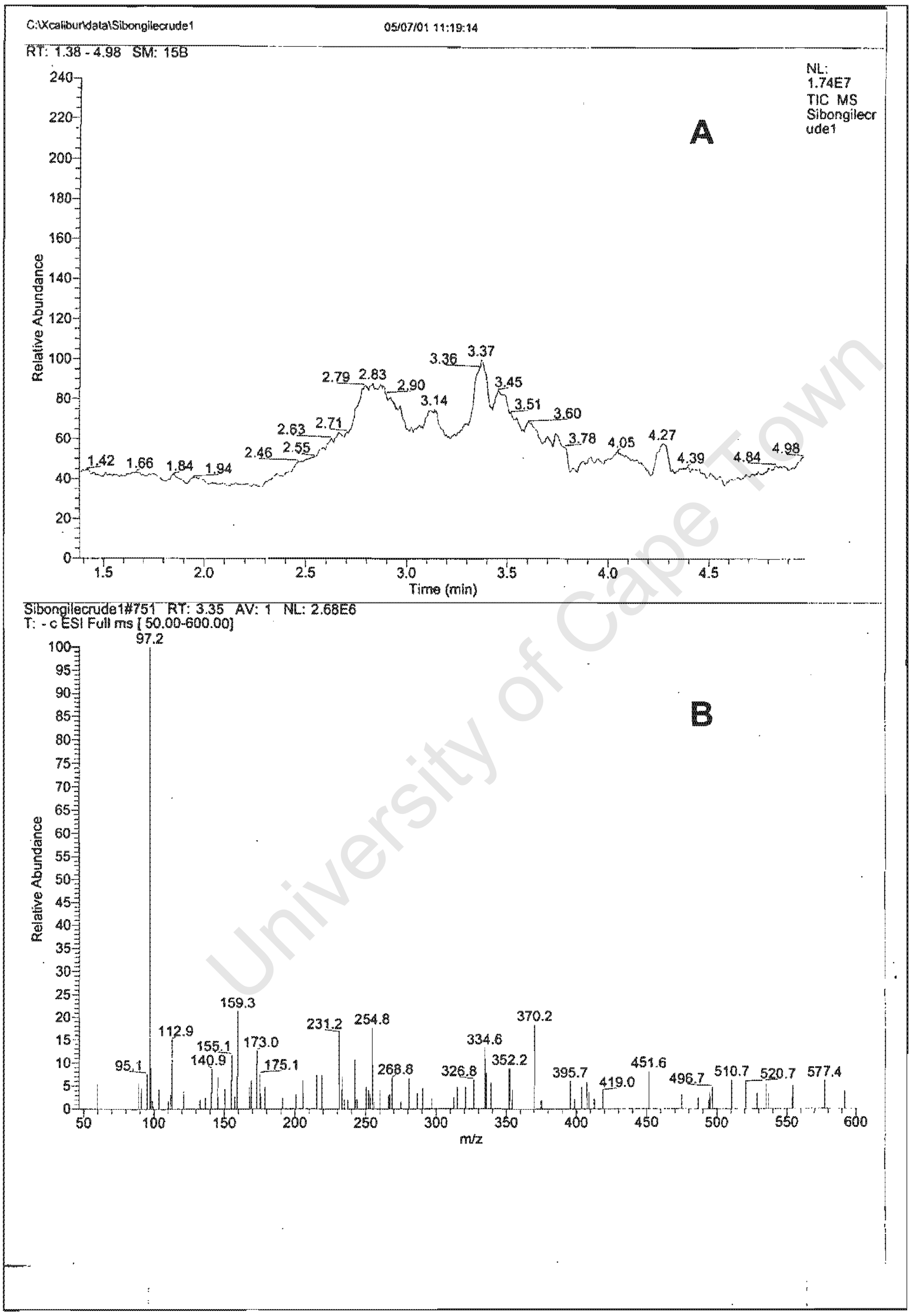
Summary

A close study of the NMR profiles shows that there is minimal change in the spectra of isolated fractions when compared to the crude material. The proton splitting of samples did not yield distinct profiles, making it difficult to determine the number of unique types of carbon atoms in the molecule and the number of hydrogen atoms attached to each carbon. The complexity of the profiles indicates that there is significant interference of the analyte by undesirable impurities. As a result, it was not possible to determine the chemical groups of the active to *Barringtonia racemosa*.

6.11. LIQUID CHROMATOGRAPHY – MASS SPECTROSCOPY

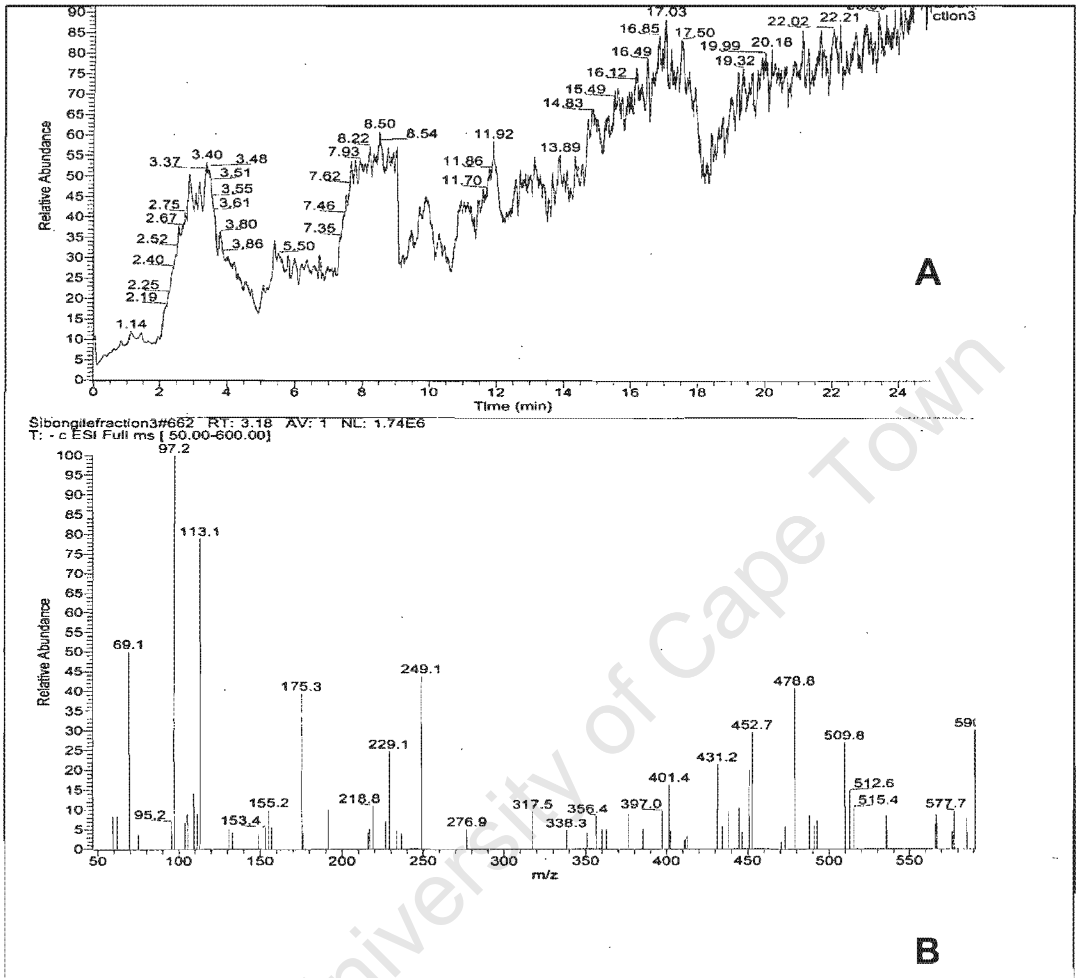
Liquid chromatography (LC) analysis coupled with mass spectroscopy (MS) was used to measure the mass of sample molecules of peaks eluted following the introduction of fractions 29AQC and the active fractions 3 and 4 derived from reverse phase HPLC of *Barringtonia racemosa*. LC/MS is a powerful technique for identifying unknown compounds within a relatively short analysis time. The quantity used during investigations for each fraction analysed was 0.1 µg/ml. Figures 25, 26 and 27, pp171-173 show the total ion current chromatograms obtained following the injection of the crude cold aqueous extract of *Barringtonia racemosa*, and fractions three and four derived from reverse-phase HPLC. The ion current chromatograms suggest that the analyte is eluted in a single peak at a retention time of between 2 to 4 minutes. The peak obtained is broad and not well resolved, thus suggesting the presence of interfering components. The mass charge ratios at different points of the peak were determined and are presented in figures 25, 26 and 27 in the form of an electrospray ionisation spectrum. From these figures, it is evident that the molecular mass range of analyte ions in the samples ranges from 69 to a maximum of 590.

Figure 25: LC/MS profile for the crude cold aqueous extract of *Barringtonia racemosa*



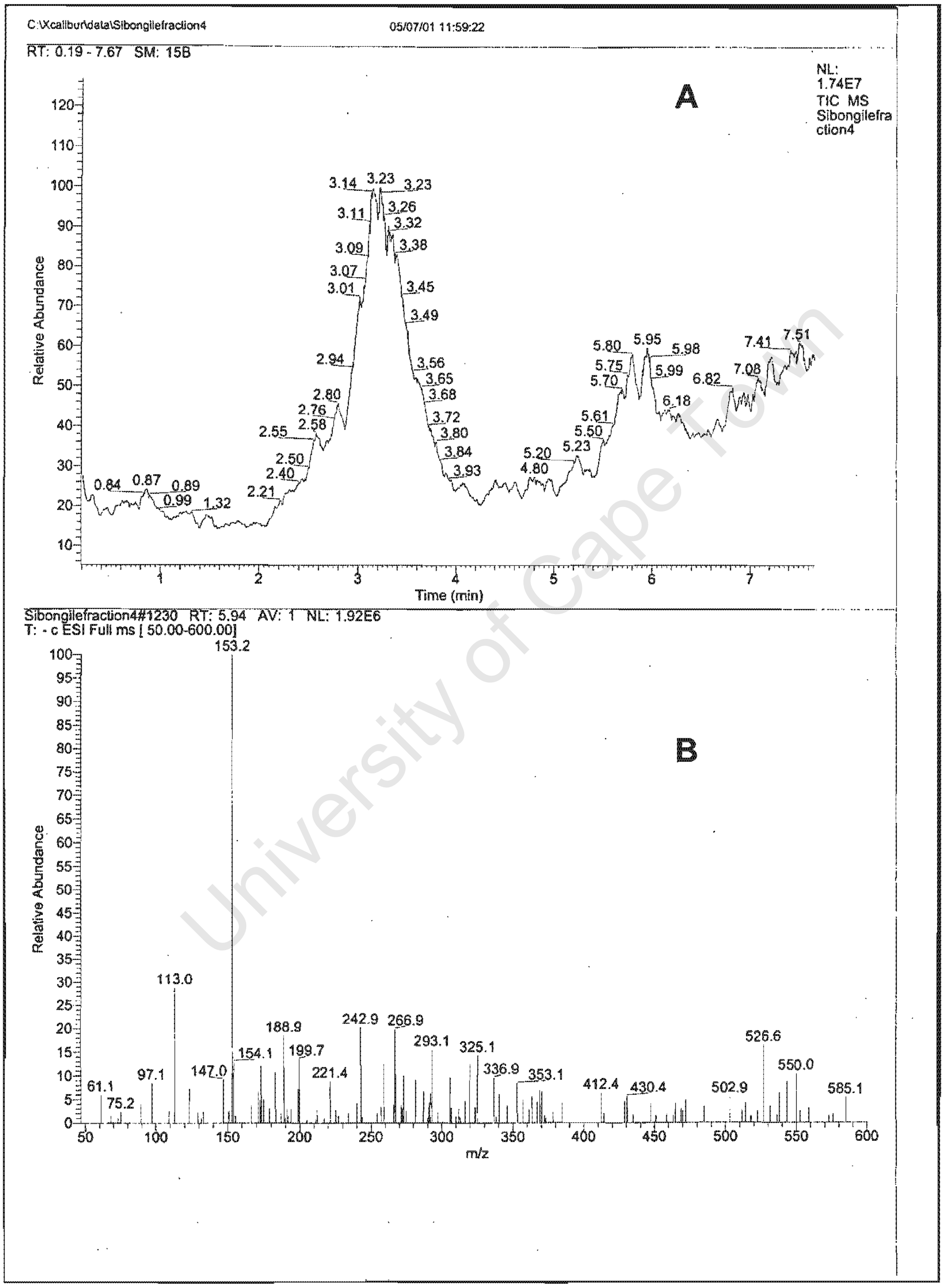
A – chromatographic profile.
B – ion count corresponding to chromatogram. The retention time of around 3.37 minutes is common to 29AQC and the active fractions 3 and 4 derived from reverse phase HPLC.

Figure 3: LC/MS profile for [*1]fraction 3



[*1]Fraction 3 refers to the sample obtained at retention time 4 – 6 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.
A – chromatographic profile. B – ion count corresponding to chromatogram.

Figure 27: LC/MS profile for fraction 4

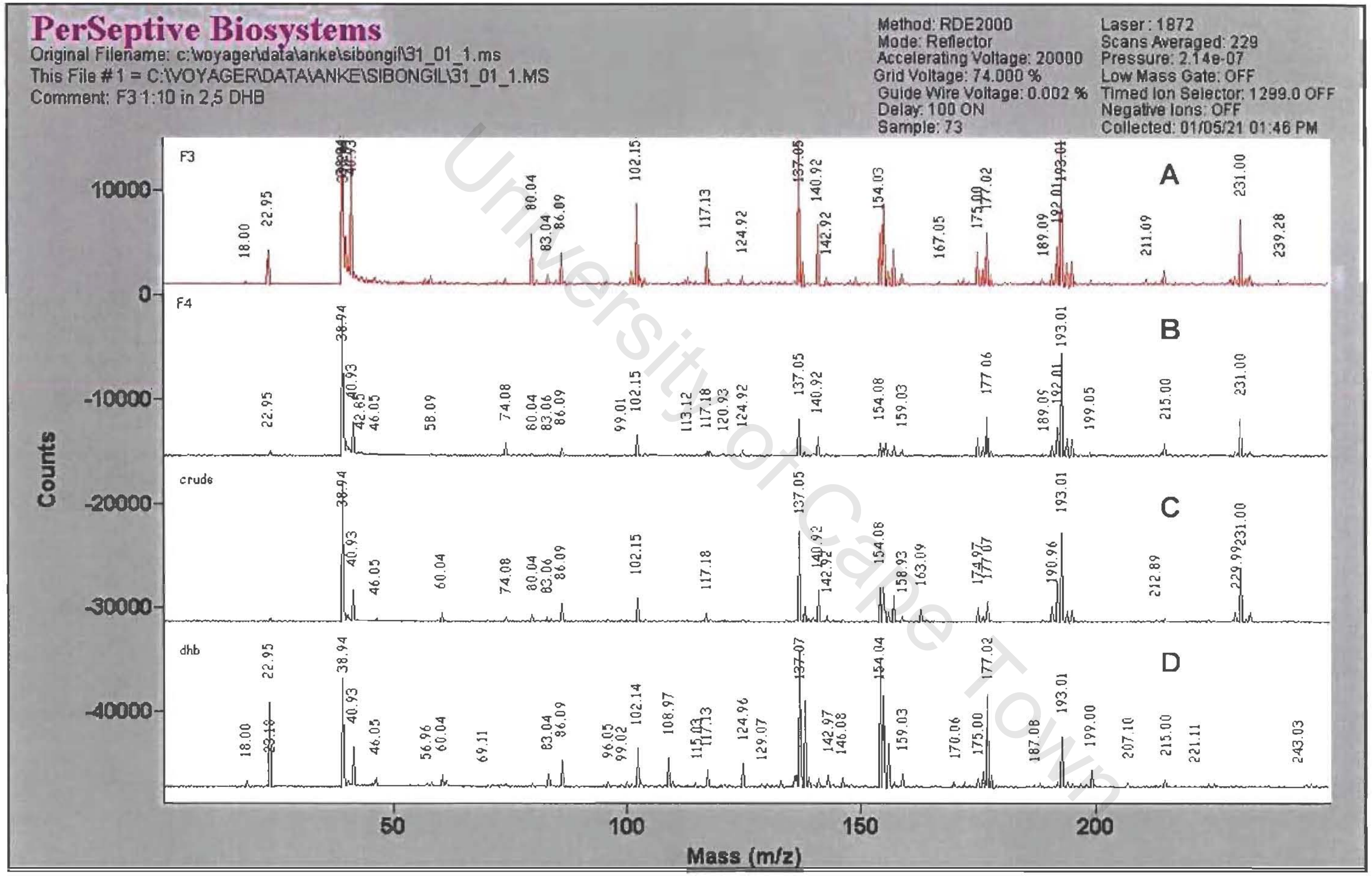


[*1]Fraction 4 refers to the sample obtained at retention time 6 – 8 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system. A – chromatographic profile. B – ion count corresponding to chromatogram.

6.11. MALDI-TOF/MS ANALYSIS

The cold aqueous extract of *Barringtonia racemosa* and fractions 3 and 4 derived from reverse-phase chromatography were analysed using MALDI-TOF/MS. During MALDI-TOF, molecules in the samples were bombarded with pulses of a laser beam that fragmented them into manageable individual molecules whose mass to size ratio was analysed using MS. The mass charge ratios of each sample analysed over a molecular mass range of 0 to 750 are presented in figures 28, 29 and 30 on pp175-177. The three top profiles (A, B, C) of the spectrum represents the mass charge ratio of fraction 3, fraction 4 and the crude sample, respectively and the bottom profile (D) is the mass charge ratio due to ionisation of the solvent. Therefore, peaks that are duplicated in (D) and the above profiles (A – C) are ignored. The upper mass charge ratio limit for all samples was in the region of 670. The spectra shows that the only mass charge ratios that are common in all three test samples and absent in the bottom matrix have molecular mass ratios of 231.0, 310.98 and 429.3.

Figure 28: MALDI-TOF/MS profiles for 29 AQC, fractions 3 and 4.



PerSeptive Biosystems
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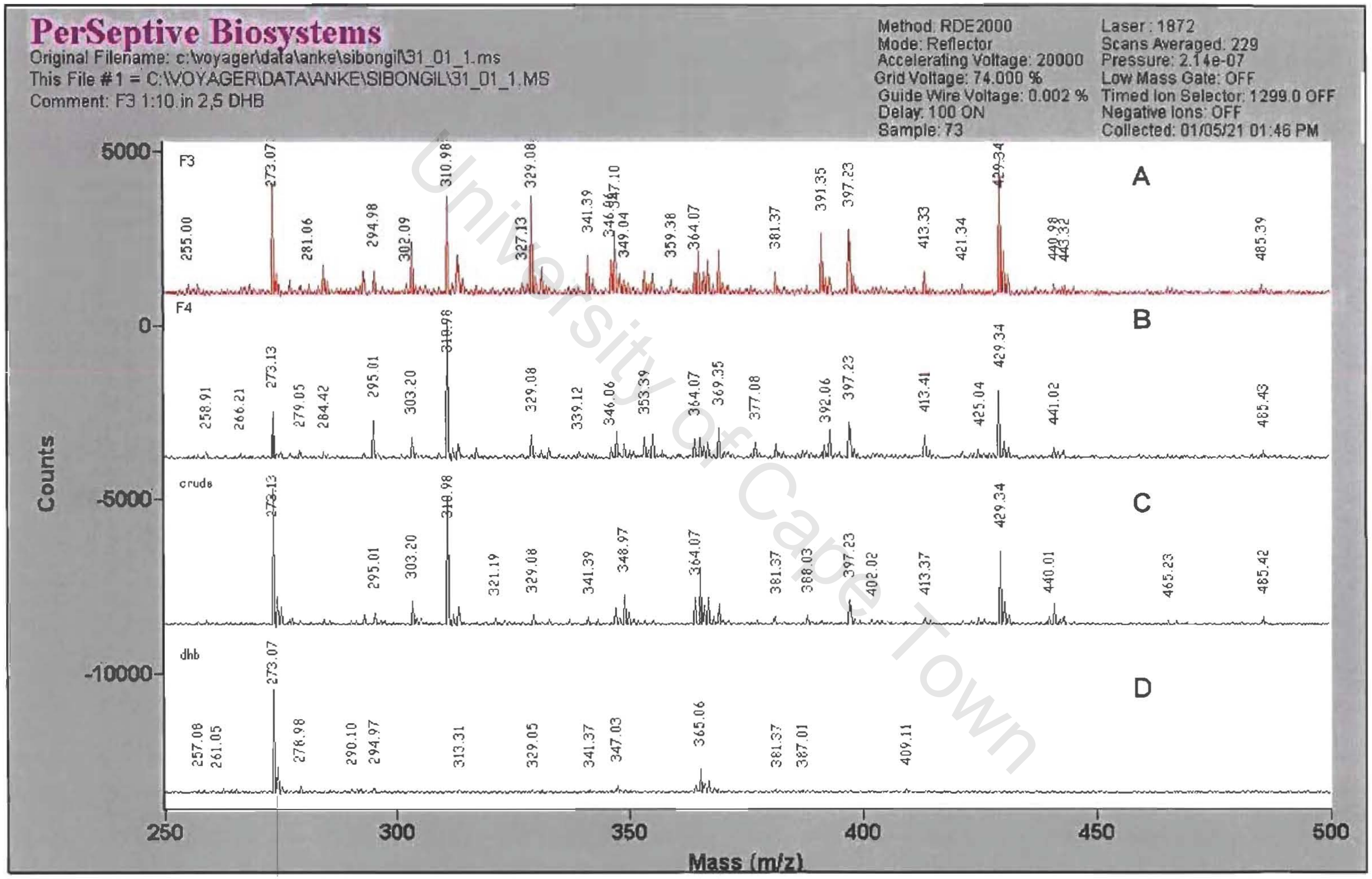
This File # 1 = C:\VOYAGER\DATA\ANKE\SIBONGIL\31_01_1.MS
Comment: F3 1:10 in 2,5 DHB

Method: RDE2000
Mode: Reflector
Accelerating Voltage: 20000
Grid Voltage: 74.000 %
Guide Wire Voltage: 0.002 %
Delay: 100 ON
Sample: 73

Laser: 1872
Scans Averaged: 229
Pressure: 2.14e-07
Low Mass Gate: OFF
Timed Ion Selector: 1299.0 OFF
Negative Ions: OFF
Collected: 01/05/21 01:46 PM

Figure 29: MALDI-TOF/MS profiles for 29 AQC, fractions 3 and 4.

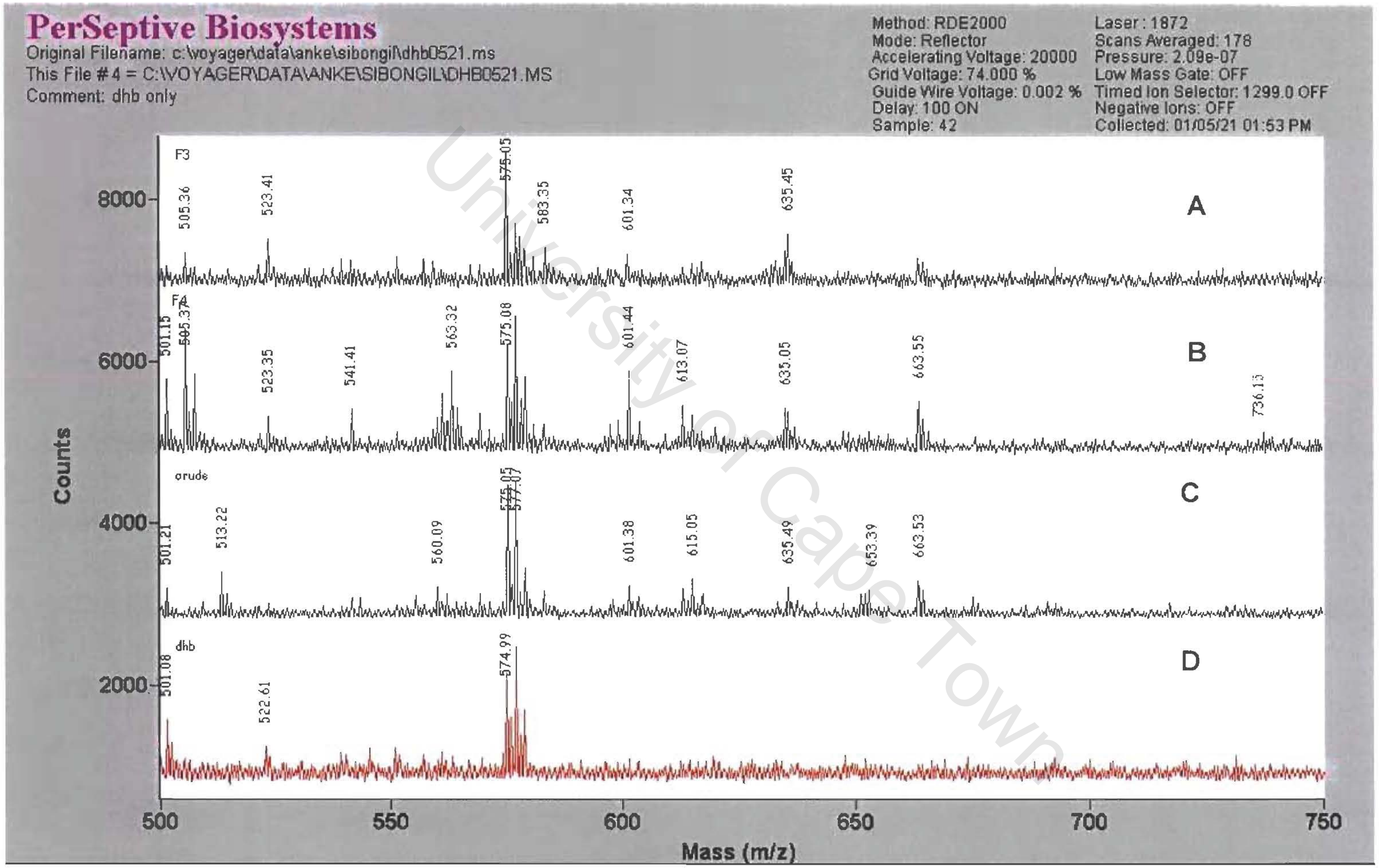


PerSeptive Biosystems
Original Filename: c:\voyager\data\anke\sibongil\31_01_1.ms
This File #1 = C:\VOYAGER\DATA\ANKE\SIBONGIL\31_01_1.MS
Comment: F3 1:10 in 2,5 DHB

Method: RDE2000
Mode: Reflector
Accelerating Voltage: 20000
Grid Voltage: 74.000 %
Guide Wire Voltage: 0.002 %
Delay: 100 ON
Sample: 73

Laser : 1872
Scans Averaged: 229
Pressure: 2.14e-07
Low Mass Gate: OFF
Timed Ion Selector: 1299.0 OFF
Negative Ions: OFF
Collected: 01/05/21 01:46 PM

Figure 30: MALDI-TOF/MS profiles for 29 AQC, fractions 3 and 4.



6.13. AMINO ACID/ PROTEIN ANALYSIS

Free amino acid analysis was performed on aqueous solutions of fractions three and four obtained from reverse-phase HPLC. The samples were first hydrolysed with 6N HCl at 110°C to release free amino acids. The presence of amino acids was determined using a strong cationic exchange column and derivitised amino acids were quantitated by their absorption at 570nm. The quantitative determination of the total amount of each amino acid present in the sample was obtained. The approximate number of nanomoles of each of the different amino acids was calculated relative to the quantity of internal standard added.

Table 26: Amino acid analysis results for [*1]fraction 3 obtained from reverse phase chromatography

COMPONENT NAME	ACTUAL NAME	[*4]RAW AMOUNT F3	[*5]AMINO ACID RATIO
D	Aspartic acid	3.603	1
T	Threonine	0.852	1
S	Serine	1.624	1
E	Glutamine	3.596	1
G	Glycine	3.519	1
A	Alanine	2.010	1
V	Valine	1.355	1
I	Isoleucine	7.330	2
L	Leucine	1.035	1
NL	Int. std.	50.000	
Y	Tyrosine	0.451	1
F	Phenylalanine	0.830	1
K	Lysine	1.943	1
TOTAL AMT		28.148	
TOTAL x 110[*2]		3096.28	
(TOTAL x 110)/1000000[*3]		0.003096	
PERCENT AMINO ACIDS		0.62	

[*1] Fraction 3 refers to the sample obtained at retention time 4 – 6 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

[*2] The total molecular mass is multiplied by a constant that corresponds to the general molecular mass of amino acids.

[*3] The mass available is converted from monograms to milligrams.

[*4] Quantity of material given in monograms.

[*5] Amino acid ratio is relative to the detection of associated amino acid groups.

The above table shows the quantity detected of each amino acid tested in the fractions analysed.

Table 27: Amino acid analysis results for [*1]fraction 4 obtained from reverse phase chromatography

COMPONENT NAME	ACTUAL NAME	RAW AMOUNT F4	AMINO ACID RATIO
D	Aspartic acid	3.322	1
T	Threonine	0.961	1
S	Serine	1.855	1
E	Glutamine	2.840	1
G	Glycine	3.651	1
A	Alanine	2.134	1
V	Valine	1.653	1
I	Isoleucine	0.833	1
L	Leucine	0.944	1
NL	Int. std.	50.000	
Y	Tyrosine	0.815	1
F	Phenylalanine	0.688	1
TOTAL AMT		19.696	
TOTAL x 110[*2]		2166.56	
(TOTAL x 110)/1000000[*3]		0.00217	
PERCENT AMINO ACIDS		0.433	

[*1] Fraction 4 refers to the sample obtained at retention time 6 – 8 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

[*2] The total molecular mass is multiplied by a constant that corresponds to the general molecular mass of amino acids.

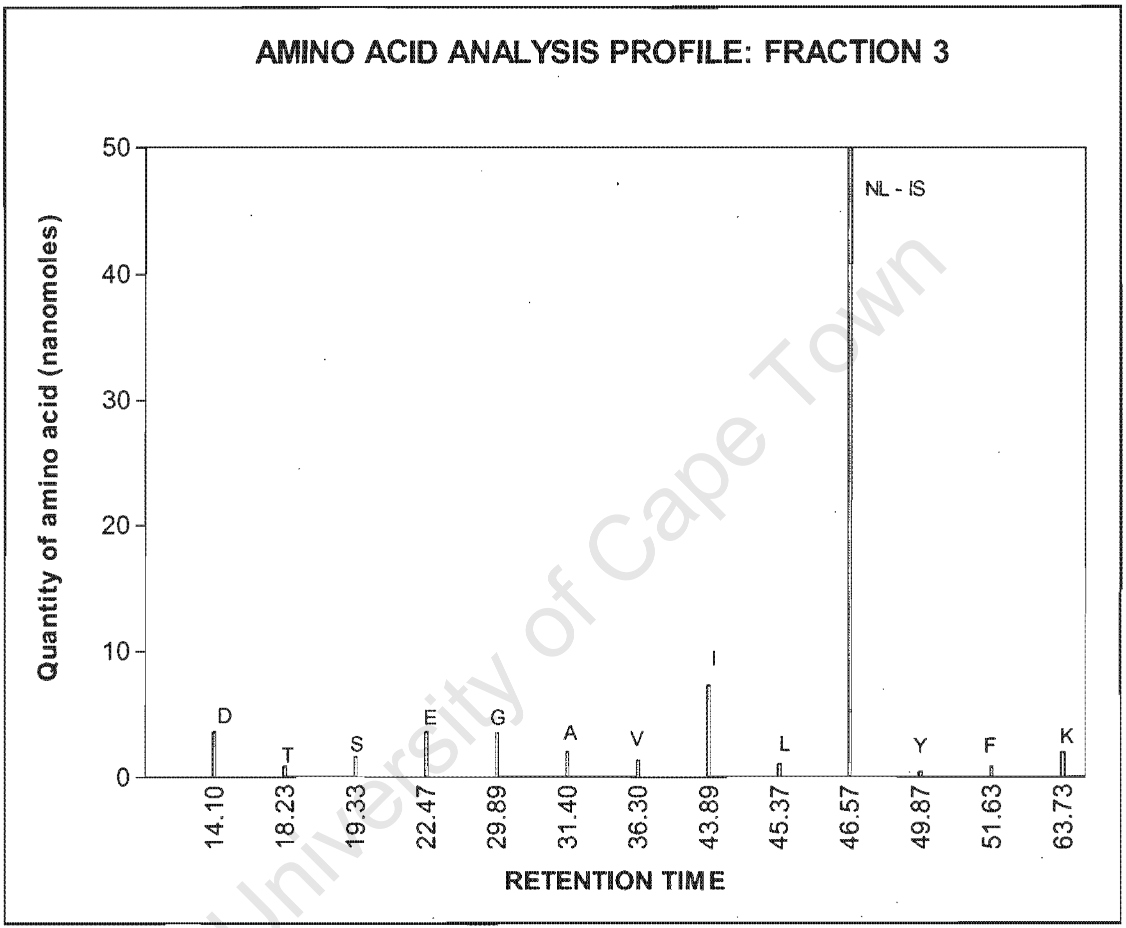
[*3] The mass available is converted from monograms to milligrams.

[*4] Quantity of material given in monograms.

[*5] Amino acid ratio is relative to the detection of associated amino acid groups.

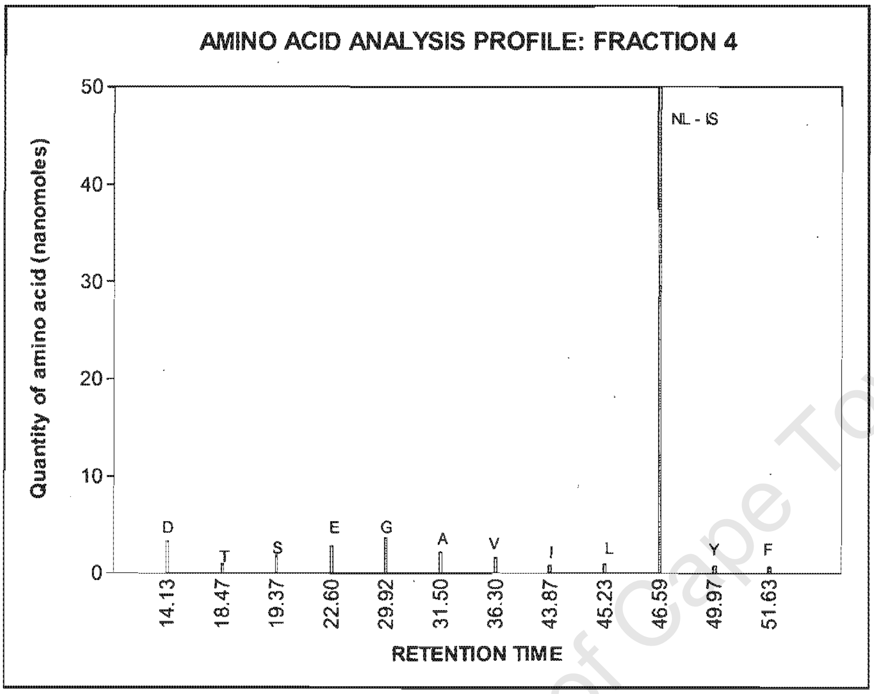
The above table shows the quantity detected of each amino acid tested in the fractions analysed.

Figure 31: Amino acid analysis profile for ^{*1}fraction 3 obtained from reverse phase HPLC



Key: Refer to Table 26, p178 for interpretation of symbols.
[*1]Fraction 3 refers to the sample obtained at retention time 4 – 6 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

Figure 32: Amino acid analysis profile for [*1]fraction 4 obtained from reverse phase HPLC



Key: Refer to Table 27, p179 for interpretation of symbols.
[*1]Fraction 4 refers to the sample obtained at retention time 6 – 8 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

From the results presented in table 26, p178 it was calculated that 0.62% of the weighed material of fraction 3 and 0.43% of the weighed out material of fraction 4 were amino acids (Table 27, p179). This translates to a quantity of 3.1μg and 2.15 μg of amino acid, respectively. The amino acid that exists in greatest abundance in fraction 3 is isoleucine and it is present in at least twice the ratio of other amino acids. The nanomolar compositions of amino acids present in fraction 3 are provided in table 26. In contrast, the amino acids that appear to be of greatest abundance in fraction 4 are aspartic acid and glycine, as shown in table 27. Figures 31 and 32, pgs 180 and 181 illustrate the quantity of the various amino acids present in each fraction tested.

CHAPTER VII

7. DISCUSSION

7.1. Physico-chemical Attributes and Chemical Group Composition

Relatively few studies have been conducted in the area of screening, isolating and identifying lead antiviral agents from natural plant products (Abou-Karam & Shier 1990, Nowotny 1997). This may be a result of several reasons that include: 1. there are limited metabolic differences between the virus and host thus restricting the number of possible target sites; 2. the technical challenges common in antiviral studies may discourage many from working in this area, and 3. most antiviral natural product extracts are derived from aqueous extractions (Cardellina et al. 1993, Xu et al. 1999, Awasthi 1981, Abou-Kaaim & Shier 1990, Eo et al. 1999, Berge et al. 1999, Nowotny et al. 1971, Yukawa et al. 1996). Due to the physico-chemical properties of this group of extracts, their isolation is complex and purification processes are particularly problematic. In the few publications that have reported on the antiviral activity of natural plant extracts, not many have identified the chemical compounds responsible for activity or determined the molecular targets of the agents (Hayashi 1990, Hoshino et al. 1998, Berge et al. 1999, Simons et al. 1989, Kraus 1991, Eo et al. 1992, Eo et al 1999, Serekedjieva & Ivancheva 1999, Nowotny et al. 1997, Yip et al. 1991, Yamada et al. 1991, Pinilla & Luu 1999, Chandresekran, Bemiller & Lee 1978). The work presented here reflects the successes accomplished and some of these challenges experienced in the field of natural plant product antiviral analysis.

During this study the activity of local traditional medicinal plants against herpes simplex virus type 1 was investigated. Of the twenty-two medicinal plants screened, *Barringtonia racemosa* showed the most potent antiviral activity applying a concentration of a 100 $TCID_{50}$ viral titre. Therefore the ensuing isolation and characterisation processes were focused on the cold aqueous extract of this plant. Substances isolated from plant extracts that inhibit viral infection and multiplication have different physico-chemical properties. Hence, the first requisite for biofractionation was to understand the biophysical properties of the extract (Samuelsson, Kyerematen & Farah 1985). The solubility studies of the aqueous extract of *Barringtonia racemosa* showed that the cold aqueous extract is only soluble in water. The crude extract showed minimal solubility in organic solvents. This, therefore, indicates that the compounds present in the extract are more hydrophilic than hydrophobic. Biofractionation results derived from size exclusion chromatography of the cold crude extract of *Barringtonia racemosa* indicate that the active component is of low molecular weight as it was eluted in the total volume of the system. For an agent to possess the type of solubility profile observed with the aqueous plant extract, it is either of low molecular weight and/or it contains a high proportion of polar chemical groups. The SEC results support the theory that 29AQC has a very low molecular weights. The thin layer chromatography results further substantiate the fact that the crude extract contain mainly hydrophilic constituents. Two solvent systems were established during the identification of major chemical groups in the cold crude aqueous extract of *Barringtonia racemosa*. The first solvent system was non-polar and the results show that this system failed to partition samples spotted on TLC plates. In contrast, sample separation was noted during the use of the second solvent system comprising a polar solvent system of ethyl acetate-methanol-water. Application of TLC detection tests revealed the presence of polyphenolic compounds in the cold aqueous extract tested.

Normal phase chromatography was applied to the crude cold aqueous extract of *Barringtonia racemosa* and the results showed that the fractions collected had no activity. This result was unexpected as it was anticipated that the highly polar groups in 29AQC would bind to the normal phase column resulting in the detection of antiviral activity. It is essential to note that during normal phase chromatography the sample is normally dissolved in an organic solvent to promote binding of the polar chemical constituent to the column. To achieve dissolution of the crude extract, it had to be dissolved in water first before adding the organic solvent to achieve co-solvency and therefore promote binding to the hydrophilic column. It is probable that the agent remained dissolved in the water component of the solvent and, therefore, failed to bind to the column. Alternatively, the highly hydrophilic groups may have bound very strongly to the column and, hence, were not displaced by the different concentrations of acetonitrile/water used.

The reverse phase column chromatography results show that the active antiviral agent derived from the cold crude aqueous extract of *Barringtonia racemosa* is eluted during low retention times of less than 10 minutes. This suggests that either the compound did not bind to the column and was therefore eluted through the system without being fractionated or that the bound active constituents were displaced by low concentrations of the organic solvent. Either interpretation of the normal and reverse phase liquid chromatography results further implies that the active constituent is highly polar.

MALDI-TOF/MS analysis was especially useful in determining the masses of fractions present in the actives tested. The crude cold aqueous fraction and fractions 3 and 4 derived from reverse phase chromatography fractionation of the crude material were analysed using MALDI-TOF/MS. Examination of the profiles obtained shows that mass peaks corresponding to all three fractions occur at the molecular masses of 231, 311 and 429. No fragments were observed at masses beyond 700.

The colorimetric tests provided a simple and rapid test to identify chemical constituents present in the cold aqueous crude extract isolated from *Barringtonia racemosa* and subsequent fractions generated. Through the use of the phenol-sulphuric acids test, positive identification was obtained for the presence of saccharides. It may be argued that the brown coloration, which decreased in intensity in succeeding fractionations derived from the crude extract, may have caused masking of the orange colour obtained; however, the attainment of a positive result following the testing of a colourless fraction of the crude extract obtained from ion-exchange HPLC confirmed the presence of saccharide components in the cold aqueous extract of *Barringtonia racemosa*. The gas chromatography tests identified the presence of sugars and reveal that the sugar moiety present is galactose. Sugar moieties have a typical maximum absorption spectrum around 200nm. During HPLC analysis the UV absorption maxima of the chromatograms produced was in the region of 200 to 205 nm.

Although one cannot make concrete conclusions on the identity of constituents based on absorption maxima, this information supports the evidence gathered from the more defining tests (Xu et al. 1988). It is interesting that the solubility profile of sugars is similar to that obtained in the analysis of 29AQC. Sugar moieties are freely soluble in water and show minimal solubility in organic solvents (Biermann & McGinnis 1989, El Rassi 1995).

Saccharides are known to affect the growth of viruses, and this is particularly true of anionic saccharides. Evidence showing *Barringtonia racemosa* could be eluted from DEAE-Sephacel suggests that it is anionic in nature. Anionic saccharides act by successfully competing with the virus for binding sites in host cells resulting in the inhibition of viral propagation (Xu et al. 1988). Studies from other laboratories have reported that polysaccharides inhibit virus attachment to the cell surface and/or penetration into host cells (Gonzalez, Alarcon & Carrasco 1987, Hayashi et al. 1990, Hoshino et al. 1998, Berge et al. 1999, Eo et al. 1999, Jung-Bum 1999, Xu et al. 1999). This mechanism of action has mainly been observed in *in vitro* enveloped viruses, including HSV-1.

The colorimetric tests further revealed that the crude hot and cold aqueous samples contained polyphenolics such as tannins. *Barringtonia racemosa* is a dicotyledonous plant, the bark of which, is known to contain significant quantities of tannins (Hutchings et al., 1996). Information on the antiviral activity of this chemical group has been previously published (Vanden Berghe, Vlietinck & Van Hoof 1986, Serkedjiva & Ivancheva 1999). Polyphenols bind to proteins to form unstable complexes and this class of compounds is therefore able to interact with glycoproteins of the viral envelope of herpes simplex viruses. This process of irreversible denaturation of the virus protein results in a loss in infectivity of the infectious agent (Vanden Berghe, Vlietinck & Van Hoof 1986). It is postulated that polyphenols interfere with the initial stages of viral replication and the release of newly formed virions. Direct interaction with virus capsid protein may be at least one of the inhibitory mechanisms of action.

Lastly, the amino acid analysis performed on fractions 3 and 4 derived from the fractionation of the cold aqueous crude extract using reverse-phase

HPLC indicated trace amounts of isoleucine in fraction 3 and glycine and aspartic acid in fraction 4. The presence of the amino acids in fraction 4 was not as prominent as was noted in fraction 3. The varying binding capacities of amino acids to the reverse-phase HPLC column is likely to have resulted the identification of different amino acids in the fractions 3 and 4.

In summary, the cold aqueous extract of *Barringtonia racemosa* has high polarity and low molecular weight. It contains undetermined quantities of galactose, polyphenolics and isoleucine, glycine and aspartic acid. Due to the low molecular weight of the active constituents, it is unlikely that it possesses more than a trace amounts amino acid group. Major constituents that have been most commonly identified in antiviral plant extracts are proteins, glycoproteins, polysaccharides, polyphenolics and alkaloids (Awasthi 1981,Vanden Berghe, Vlietinck & Van Hoof 1986, Montanha 1995, Renard-Nozaki et al. 1989, Yamamoto et al. 1989).

7.2. Antiviral Bioassays

A concentration dose-dependent relationship was observed in the antiviral activity of 29 AQC. The antiviral validation assays demonstrated the suitability of the system for the analysis of natural plant extracts. Validation analyses were important to ensure the reliability and repeatability of the test system selected. During the screening process, 38% of the extracts showed cell toxicity. This figure is relatively high, considering that the total number of extracts tested was 108. This can be attributed to the high sensitivity of the system that was able to detect low levels of toxicity. It was not surprising to find that aqueous extracts showed the least cytotoxicity compared with dichloromethane and methanol extracts. It is interesting that in investigations in which a large number of plants were tested against a series of different viruses, discoveries of plant extracts against herpes simplex viruses occur less frequently and activity is less marked compared with other viruses such as rhinovirus and poliovirus (Beuscher et al. 1994, Vanden Berghe et al. 1978, Collins et al. 1997). The ED_{50} results obtained for the cold aqueous extract of *Barringtonia racemosa* was 23 μg/ml for trials in which the extract and virus were added together; 28 μg/ml when the extract was added to the system prior to the virus, and 75 μg/ml when the extract was added after the addition of the virus. Previously reported ED_{50} values for natural plant extracts tested at 100 $TCID_{50}$ ranged from 1.7 μg/ml to 1290 μg/ml for extracts considered to have potential lead activity (Abou-Karam & Shier 1990, Aquino 1989, Berge et al. 1999, Eo et al. 1999, Montanha 1995, Xu et al. 1999). It was essential to establish the viral titre used during testing in reports of ED_{50} values of less than 20 μg/ml. When compared with the figures reported, *Barringtonia racemosa* showed good activity, especially as the figures refer to the crude form. The ED_{50} values obtained for acyclovir were between 0.026 and 0.033 μg/ml (Sudo et al. 1994). Comparative ED_{50} values reported in the literature ranged between 0.1 and 0.22 μg/ml. During the time-of-addition studies, there was a marked reduction in activity after one hour following the inoculation of the virus or test substance. These results suggest that *Barringtonia racemosa* needs to be present during the early stages of viral replication to bring about antiviral activity. As reported earlier, tannins and saccharide groups, both of which are present in *Barringtonia racemosa*, act at the initial stages of viral replication. Experiments in which the extract and virus were inoculated simultaneously evaluated the virucidal ability of the compound (Hudson 1994, Yip et al. 1991, Kurokawa et al. 1998, Green, Yeh & Overall 1980). The investigations determine the capacity of 29AQC to inactivate viral replication directly. When 29AQC was inoculated before the virus, it was possible to determine the ability of the test compound to induce an antiviral state in the cells being investigated. To stimulate the existence of an antiviral state involves the production of interferons which are natural cell-coded antiviral proteins. It was not possible to determine the mechanism by which interferon activity was induced. It has been shown, however, that a number of polysaccharides are effective inducers of interferon (Vanden Berghe, Vlietinck & Van Hoof 1986, Gonzalez, Alarcon & Carrasco 1987, De Clercq & Luczak 1976, De Somer et al. 1968, Merigan & Finkelstein 1986). In contrast, results obtained when the virus was inoculated before the extract show that the aqueous extract of *Barringtonia racemosa* showed limited ability to block advanced stages of replication. The hot aqueous crude extract of *Barringtonia racemosa* showed no antiviral activity. The ED_{50} results for fraction 4 and fraction 3 obtained from

29AQC using the same size exclusion chromatography conditions did not vary. The values were 49.0 µg/ml and 54.2 µg/ml for fraction 4 and fraction 3, respectively. When compared with the ED_{50} value of 23 µg/ml obtained for 29AQC, it is evident that size exclusion chromatography did not improve the antiviral activity of this extract; instead it resulted in a significant decrease in activity. A possible explanation for this is that during fractionation the active in fraction 3 was separated from a compound of higher molecular weight and the presence of which is required to potentiate the antiviral activity of the crude extract.

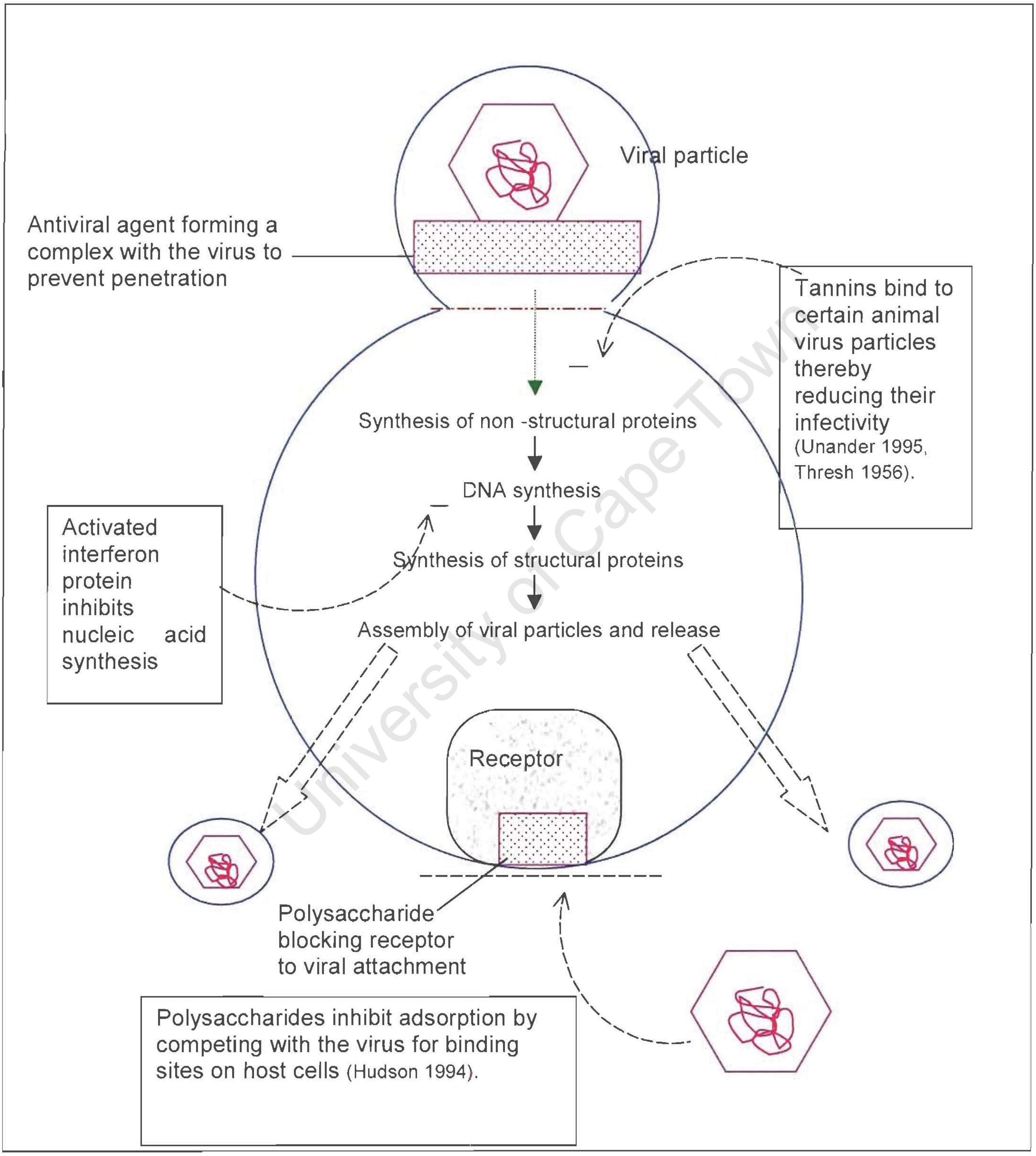
Crude samples of cold aqueous fraction were put through ion exchange column chromatography. To eliminate the salt used to displace the active agent, SEC techniques were used. The results obtained from the fraction derived from ion exchange were in accordance with previous results obtained in SEC. Briefly, the active fraction obtained was of low molecular weight and eluted in the total volume of the system. The ED_{50} value obtained from the de-salted fraction that was originally generated from ion exchange chromatography was 48.1 µg/ml. The value is lower than that of the crude and once more the reason for this may be that during fractionation one experienced the loss of substances that, when present, potentiate the antiviral activity.

Due to a significant number of reports of the antiviral effects of tannins and sugars, both of which have been identified in *Barringtonia racemosa*, it was considered important to test the antiviral activity of tannin containing and non-tannin containing fractions. A separation technique in which tannins were bound to a polyamide column and non-tannin agents were removed from the system using water and 80% ethanol was used during experimentation. The bound tannins were removed from the column using sodium hydroxide. The results show that the non-tannin containing fractions comprising water and the two ethanol elutions had ED_{50} values of 50.5 µg/ml, 57.9 µg/ml and 46.5 µg/ml, respectively. In comparison, the tannin-containing sodium hydroxide fraction had an ED_{50} value of 44.7 µg/ml. These figures indicate that there are two antiviral agents present, one of which is tannin. The ED_{50} values of the tannin and non-tannin fraction reveal that there is no significant difference between the antiviral activities of either fraction. However, when compared to the ED_{50} value of the 29AQC, a loss in activity was noted.

All fractions collected at 2-minute intervals during HPLC analysis were tested for activity. The ED_{50} values were nearly double that of the crude extracts with the exception of fraction 3 which eluted between 4-6 minutes. The ED_{50} value of this fraction was 26.2 μg/ml. The physico-chemical properties of this extract, and in particular its solubility profile, were identical to that of the crude.

It was interesting to note that following acid hydrolysis of *Barringtonia racemosa*, no antiviral activity was detected. The hydrolysed sample contained no tannins and any sugar groups present were reduced to monosacharrides by the hydrolysis process. From these observations, one may deduce that, at least, both the sugar and tannin components are required in combination to retain antiviral activity. Figure 33, p190 illustrates the different mechanisms by which the compounds are able to induce antiviral activity.

Figure 33: Antiviral interactions



As previously reported, aqueous plant extracts present a particular challenge during isolation and characterisation processes. This was especially evident in the NMR results obtained, which revealed the complexities of the material under study. Size exclusion chromatography is considered to be a useful fractionation tool and has been used extensively in saccharide isolation (Lumley, Patel & Cohen 1987, Pinilla & Luu 1999, Andrews 1964, Chen et al. 1999, Claeson et al. 1998, Gonzalez, Alarcon & Carrasco 1987, Jung-Bum et al. 1998, El Rassi 1995). Furthermore, HPLC is considered to be one of the more specific and selective isolation techniques available in natural plant product purification (Kim et al. 1998). The fact that there was no significant difference in the NMR spectra of fractions derived from both these techniques, when compared to the crude material demonstrates the level of difficulty encountered. These limitations ensured that all possible and available isolation techniques were explored during the investigation of *Barringtonia racemosa*.

The cytotoxicity studies of *Barringtonia racemosa* showed that the extract was safe to use at concentrations of at least four times the expected concentration of use. Cytotoxicity examinations are not only important in ensuring that activity is not due to cell death, but also in assessing the safety of the drug for human use.

Ultimately, work of this nature is expected to lead to drug development either through the production of safe, efficacious and affordable botanicals or the introduction of pure, isolated compounds for drug synthesis. From a physiological perspective, a low molecular weight substance is of particular interest because of the relative ease with which these compounds are able to pass through the different barriers of the body and cell membrane, including the skin during topical use. Furthermore, it is easier to manipulate the chemical structure(s) of low molecular weight compounds in order to optimise their absorption and therapeutic efficacy in the body. Whichever drug development route followed, there are pharmacological principles that govern the pharmacodynamics and pharmacokinetics of a drug entity (Jusko 1980). The excellent solubility of this extract in water makes it compatible with physiological systems. Under these circumstances, administration would therefore be simpler and relatively safer. This outcome warrants further analysis of the product for safety in *in vivo* systems. HSV infections frequently occur in moist mucocutaneous regions of the skin. As a result of the aqueous solubility of the extracts investigated, these compounds would be more compatible with this type of skin condition and as a result, this could promote transdermal penetration of the active.

In summary, the results and supporting publications suggest that *Barringtonia racemosa* possesses virucidal activity, since the virus was killed (Vanden Berghe, Vlietinck & Van Hoof 1986, Hudson 1994). Optimisation of the antiviral effect was attained following reverse phase chromatography separation of the crude material. The detailed study of *Barringtonia racemosa* confirmed the presence of more than one biologically active constituent comprising of a polyphenolic, amino acid and sugar moiety. The synergistic interactions between the different chemical components have been shown to be significant in the overall virucidal effect of the agent.

CHAPTER VIII

CONCLUSION

In conclusion, the body of results reported in this thesis shows that *Barringtonia racemosa*AQC possesses activity against herpes simplex virus type 1. By the process of isolation and bioassay guided fractionation antiviral activity has been shown to be in fractions 3 and 4 derived from reverse phase-HPLC.

Using colorimetric, thin layer chromatographic and amino acid analytical techniques it has been possible to establish that constituents present in *Barringtonia racemosa*AQC include polyphenolics, galactose and traces of isoleucine, glycine and aspartic acid. The relative quantities of each constituent could not be established since the nuclear magnetic resonance results were inconclusive. However, evidence presented shows that antiherpes simplex activity is due to a combined effect of different chemical constituents rather than a single entity.

Cell viability and MTT analyses indicated that the active concentrations of 29AQC are non-toxic against Vero cells. The 50% effective dose results showed promising anti-viral activity. The ED_{50} values obtained were 23 μg/ml when the extract and virus were inoculated to the cell system simultaneously; 28 μg/ml when the extract was inoculated to the cell system one hour before the virus; and 26.2 μg/ml for fraction 3 that was derived from reverse phase HPLC. Antiviral activity diminished significantly following an incubation period of more than one hour.

The solubility profile of *Barringtonia racemosa*AQC indicates that the extract is highly polar. Size exclusion chromatography results revealed that the active constituent has low molecular weight and this was further verified by the MALDI-TOF/MS and LC/MS results. Early elution of the active fraction following reverse phase chromatography suggests that the compound of interest is polar and minimally retained by the hydrophobic column.

These findings suggest a framework for ongoing investigation, namely chemical isolation and identification, antiviral mechanism(s) of action and animal test modelling.

CHAPTER XI

IMPROVEMENTS AND FUTURE AREAS FOR RESEARCH

This section of the thesis allows one to reflect on the entire study and critically evaluate recent research effort in order to further future scientific development. The most interesting aspect of this study is that it demanded a multidisciplinary research approach that exposed one to other research specialities including medical anthropology, botany, chemistry, virology, cell physiology and pharmacology (Malone 1993, Kyerematen & Ogunlana 1987). During pursuits within these various disciplines, additional research areas were realised and these are discussed below. The comments serve to help broaden the scope of this research and, in doing so, enhance the significance of drug development in addressing health needs.

1. During the screening of the 108 plant extracts, 100% inactivation at a viral concentration of 100TCID$_{50}$ was used to define antiviral activity of test agents. These conditions were extremely stringent and, as a result, it is likely that several extracts, with partial activity, were not detected. Extracts with partial activity may have contained true antivirals that were present at quantities insufficient to inactivate all infectious virus in the standard viral titre used. A study of antiviral activity at different viral concentrations is more likely to give a higher yield of potential antiviral yields.
2. It is of particular interest to attempt to elucidate the mechanism of antiviral action and determine the targets for the inhibitory action of the extract. This area of research is of particular value because the mechanisms of action of many antiviral agents, including some on the market, have yet to be elucidated. Interpretation of results obtained suggest that the active extract exhibited both virucidal and interferon inducing activity. An understanding of these activities and the interplay between the two can contribute towards improving the specificity and selectivity of future antiviral agents.
3. Attempts to purify and isolate the pure compound will always remain a challenge. Communications with experienced natural plant product chemists have revealed a general avoidance of research into aqueous plant extracts.

Sophisticated equipment and expert advice from scientists experienced in aqueous extract isolation techniques is necessary to accomplish this exploration. This has been achieved in other laboratories and therefore might be accomplished through collaborative partnerships (Eo et al. 1999).

4. The research study focused on herpes simplex virus primarily because of its prominence in HIV and immunocompromised patients and the emergence of resistant strains. Having worked extensively with HSV, it would be of interest to evaluate the activity of the extract against other viruses, including HIV. This investigation may lead to the development of effective agents for other debilitating antiviral conditions.

   A further study of interest would be to test the efficacy of the extract against acyclovir resistant viruses. The importance of such work is self-evident and it would have particular application in immunocompromised patients who commonly present with cases of acyclovir resistance.

5. The work performed here specifically sought to investigate the use of traditional medicinal plants for the treatment of viral skin disorders. In order to closely simulate an *in vivo* skin system further research into the development of an *in vitro* system would be ideal. The benefits of such an investigation would be threefold. First, essential information might be generated on simple keratinocyte propagation techniques; second, this work will make available a test model for the laboratory analysis of agents against viral skin conditions and third, the study would disclose the need to research the active extract further using *in vivo* systems.

6. *In vivo* animal studies are useful in the activity determination of an unknown drug entity in a physiological system. Although one cannot extrapolate results obtained from animal models to human situations, this system provides indicators of possible actions of the test agent in an *in vivo* system. Furthermore, the development of animal test models would provide a readily available *in vivo* assessment technique for future scientific development (Syerjanen et al. 1996, Su et al. 1995, Schnipper et al. 1984, Brice 1988, Namba, Shiraki & Kurokawa 1998). The Balb/c mouse is the most frequently used *in vivo* animal

test model. This model enables researchers to induce anti-HSV-1 activity to duplicate the original condition (Monma, Kawana & Shimizu 1997, Kurokawa et al. 1999, Namba, Shiraki & Kurokawa 1998, Kurokawa et al. 1997). Balb/c mice are albino and have no fur due to the presence of an autosomal recessive nude gene. They have a deficiency in T-cell function. This special feature of bulb/c mice makes them a suitable model to evaluate pathogenic mechanisms.

7. Drug development is incomplete without a suitable delivery vehicle for the active agent. In this study one can consider systemic drug delivery through the use of an oral drug dosage form. In addition a transdermal application might provide a local and more rapid therapeutic effect. Naturally the delivery vehicle would be influenced by the physico-chemical properties of the active agent. Other non-conventional delivery vehicles may include powders and tea preparations. The most suitable delivery vehicle must ensure optimal delivery of the therapeutic agent at the required site of action.

This investigation of the use of traditional medicinal plants in the treatment of viral skin conditions, signified the start of an essential and normally lengthy process of drug development. The project achieved its objectives and, moreover, contributed to a field of study that has immediate application, yet many unanswered questions.

CHAPTER X

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APPENDICES

APPENDIX 1

CONFERENCE PRESENTATIONS

1. Sixth International Congress of Ethnobiology (New Zealand, November 1998): *The Establishment Of New Research Conducts For The Integration Of Indigenous Theory And Practice With Scientific Systems Of Knowledge In The Study Of Ethnopharmacology.*

2. Planning Meeting for the Establishment of an International Interdisciplinary Research Network on Bioactive Natural Products, Common Wealth Science Council (London, 26 – 28 April 1999): *The Status of Biodiversity and Scientific Research on Bioactive Natural Products and Medicinal Plants in South Africa.*

3. Symposium On African Medicinal And Indigenous Food Plants And The Role Of Traditional Medicine In Health Care (Swaziland, October 1999): *A Multidisciplinary Approach to Medicinal Plant Research.*

4. The 3rd African Renaissance Conference: Globalisation, The African Renaissance and Sustainable Development: The Challenge to African Intellectuals (Durban, South Africa, April 2001): *Endogenous Knowledge Systems, Traditional Healers and The Protection of Intellectual Rights.*

5. Building Bridges with Traditional Knowledge, An International Summit Meeting on Issues Involving Indigenous Peoples, Conservation, Sustainable Development and Ethnoscience. (Hawaii, May 28 – June 2, 2001) *A Strategy for the Development of a Traditional Medicines Database: The Integration of Technology and Indigenous Knowledge.*

TITLES FOR INTERNATIONAL JOURNAL PUBLICATIONS:

1. The Screening of South African Plants for Antiviral Activity.
2. The Antiviral Activity of Barringtonia racemosa Against Herpes Simplex Virus Type I.
3. System Validation Procedures for Viral Activity Guided Biofractionation
4. Ethical Considerations in Ethnopharmacology: A South African Perspective.

APPENDIX 2

ETHICAL GUIDELINES USED DURING INTERVIEWS WITH INDIGENOUS PEOPLES ON FIELD TRIPS

1. UCT is resolved to share all intellectual property gains with traditional medicinal practitioners. This refers to intellectual as well as financial gains.
2. Recommended that a trust be established, with equal representation of the university/MRC on the one hand and the traditional medical practitioners on the other to ensure this happens.
3. All UCT/MRC gains will revert to the business of the Traditional Medicines Research Group. There will be no individual gains whatever.
4. It is hoped that the traditional medical practitioners will find a way to agree with this broad principle. It will be set up in a negotiable contract form once traditional medical practitioners have adequately canvassed it.

APPENDIX 3

STAINING METHOD

Slides containing layers of Vero cells of different confluency were fixed with 5 ml of Boiun's fixative. The fixative solution was removed and 5 ml of 96% ethanol was added to the slides for an additional 5 minutes. The 96% ethanol was removed and 70% ethanol was added to the system. The 70% ethanol was left to stand for 5 minutes. The slides were thereafter rinsed under running tap water. Each slide was sequentially dipped for approximately 10 seconds in a series of solvents used to remove traces of the fixative. The solutions used in their sequential order were haematoxylin, Scotts solution, Eosin solution, absolute alcohol solution 1, 96% ethanol, 70% ethanol, absolute alcohol solution 2, absolute alcohol solution 3, xylol solution 1 and to end, xylol solution 2. The slide was mounted using BSX gel and a glass cover slip was placed on top of the slide. The mounted slide was viewed using a microscope. The haematoxylin and eosin were used to develop slides to show the morphology of fixed cells. The formulations used to prepare the solutions are presented below.

FORMULAE FOR STAINING REAGENTS USED DURING CELL STAINING

1. Bouins fixative

Bouins fixative was prepared using the following formula:

1.2% saturated aqueous pioric acid	750 ml
40% Formalin	250 ml
Glacial acetic acid	50 ml

The reagents were added in the order they are presented in the formula and the yellow solution prepared was stored at room temperature.

2. Haematoxylin

Haematoxylin	2.5 g
Absolute alcohol	50 ml
Ammonium sulphate	50 g
Distilled water	500 ml
Mercuric oxide	1.5 g
Glacial acetic acid	20 ml

3. Scotts solution

Sodium carbonate	3.5 g
Magnesium sulphate	20 g
Tap water	1 L

4. Eosin solution

2% eosin solution in water	700 ml
2% phloxine solution in water	300 ml

APPENDIX 4

FORMULAE FOR TLC SPRAYING REAGENTS

1. Vanillin-sulphuric spraying reagent

1% ethanolic vanillin	50 ml
10% ethanolic sulphuric acid	50 ml

2. Dragendorffs spraying reagent

Solution A:	
Bismuth nitrate	0.85 g
Glacial acetic acid	10 ml
Water	40 ml
Solution B:	
Potassium iodide	8 g
Water	30 ml

Stock solution: solution A and solution B are mixed 1:1

Spray reagent:	
Stock solution	1 ml
Glacial acetic acid	2 ml
Water	10 ml

3. Chloramine-trichloroacetic acid spraying reagent

3% aqueous chloramines T solution	10 ml
25% ethanolic trichloroacetic acid	40 ml

APPENDIX 5
MTT ASSAY RESULTS

CONC (μg/ml)	0.01	0.1	1.0	10	100	BLANK	CONTROL
	0.05	0.08	0.05	0.06	0.06	0.02	0.071
	0.07	0.06	0.08	0.07	0.04	0.01	0.077
	0.05	0.01	0.05	0.07	0.06	0	0.068
AVERAGE:	*0.06*	*0.05*	*0.06*	*0.07*	*0.05*	*0.01*	*0.072*
STD. DEV.	*0.01*	*0.036*	*0.017*	*0.006*	*0.01*	*0.01*	*0.005*
% SURVIVAL	112.9	96.8	112.9	129.0	96.8		

The above table gives the raw data of optical densities recorded. n = 3.

APPENDIX 6
TCID$_{50}$ RESULTS FOR n = 12

TCID$_{50}$ I

[*1]Dilution 10^{-n}	0		1		2		3		4		5		6	
A	100	100	100	100	100	100	100	100	100	100	12.5	37.5	0	0
B	100	100	100	100	100	100	100	100	100	100	12.5	12.5	0	0
C	100	100	100	100	100	100	100	100	100	100	37.5	12.5	0	0
D	100	100	100	100	100	100	100	100	100	100	0	0	0	0
E	100	100	100	100	100	100	100	100	100	100	0	0	0	0
F	100	100	100	100	100	100	100	100	100	100	0	37.5	12.5	62.5
G	100	100	100	100	100	100	100	100	100	100	12.5	37.5	0	0
H	100	100	100	100	100	100	100	100	100	100	0	0	0	0
[*2]***AVE***	100	100	100	100	100	100	100	100	100	100	9.38	17.19	1.56	7.81
[*3]***STD. DEV***	0	0	0	0	0	0	0	0	0	0	12.94	17.60	4.42	22.10

TCID$_{50}$ II

Dilution 10^{-n}	0		1		2		3		4		5		6	
A	100	100	100	100	100	100	100	100	100	100	37.5	37.5	0	0
B	100	100	100	100	100	100	100	100	100	100	12.5	37.5	12.5	0
C	100	100	100	100	100	100	100	100	100	100	37.5	0	12.5	0
D	100	100	100	100	100	100	100	100	100	62.5	12.5	37.5	0	37.5
E	100	100	100	100	100	100	100	100	100	100	37.5	37.5	0	12.5
F	100	100	100	100	100	100	100	100	100	100	37.5	12.5	0	37.5
G	100	100	100	100	100	100	100	100	62.5	100	37.5	37.5	0	12.5
H	100	100	100	100	100	100	100	100	100	100	62.5	62.5	12.5	12.5
AVE	100	100	100	100	100	100	100	100	100	100	34.4	32.8	4.69	14.06
STD. DEV	0	0	0	0	0	0	0	0	0	0	16.0	18.8	6.47	15.59

TCID$_{50}$ III

Dilution 10^{-n}	0		1		2		3		4		5		6	
A	100	100	100	100	100	100	100	37.5	37.5	37.5	0	0	100	100
B	100	100	100	100	100	100	100	37.5	37.5	37.5	0	37.5	100	100
C	100	100	100	100	100	100	100	100	62.5	62.5	0	12.5	100	100
D	100	100	100	100	100	100	37.5	37.5	37.5	62.5	0	0	100	100
E	100	100	100	100	100	100	100	62.5	37.5	37.5	0	0	100	100
F	100	100	100	100	100	100	100	37.5	37.5	37.5	0	0	100	100
G	100	100	100	100	100	100	100	12.5	37.5	37.5	0	0	100	100
H	100	100	100	100	100	100	100	37.5	62.5	62.5	0	0	100	100
AVE	100	100	100	100	100	100	92.19	45.31	43.75	46.88	0	6.25	100	100
STD. DEV	0	0	0	0	0	0	22.10	25.82	11.57	12.94	0	13.36	0	0

The above table gives the raw data of infection following the inoculation of 12 wells.
[*1] refers to the dilutions tested of 10^{0} to 10^{-6}. [*2]AVE – average mean value. [*3]SD – standard deviation about the mean value.
The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121. n = 3 of an average of 12 readings taken.

APPENDIX 6 contd.

TCID I

VIRUS TITRE	*SURV.*	*TOTAL (T1)*	*DEAD*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% DEAD*
0	0	0	16	89	89	1	100
-1	0	0	16	73	73	1	100
-2	0	0	16	57	57	1	100
-3	0	0	16	41	41	1	100
-4	0	0	16	25	25	1	100
-5	9	9	7	9	18	0.5	50
-6	14	23	2	2	25	0.08	8

TCID II

VIRUS TITRE	*SURV.*	*TOTAL (T1)*	*DEAD*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% DEAD*
0	0	0	16	74	74	1	100
-1	0	0	16	58	58	1	100
-2	0	0	16	42	42	1	100
-3	0	0	16	26	26	1	100
-4	15	15	1	10	25	0.4	40
-5	15	30	1	9	39	0.23	23.08
-6	8	38	8	8	46	0.17	17.39

TCID III

VIRUS TITRE	*SURV.*	*TOTAL (T1)*	*DEAD*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% DEAD*
0	0	0	16	99	99	1	100
-1	0	0	16	83	83	1	100
-2	0	0	16	67	67	1	100
-3	0	0	16	51	51	1	100
-4	0	0	16	35	35	1	100
-5	0	0	16	19	19	1	100
-6	13	13	3	3	16	0.19	19.0

TCID	1	2	3	**AVE**	**STD**
0	100	100	100	100	0
-1	100	100	100	100	0
-2	100	100	100	100	0
-3	100	100	100	100	0
-4	100	40	100	80	34.64
-5	50	23.07	100	57.69	39.03
-6	8	17.39	18.75	14.71	5.85

n = 3 of an average of 12 readings taken.

APPENDIX 7

ACYCLOVIR (ACV) RESULTS

	CONCENTRATION µg/ml									
DAY 5: ACV I	100	100	10	10	1	1	0.1	0.1	0.01	0.01
A	0	0	0	0	0	0	0	0	100	100
B	0	0	0	0	0	0	0	37.5	100	100
C	0	0	0	0	0	0	0	12.5	100	100
D	0	0	0	0	0	0	0	37.5	100	100
E	0	0	0	0	0	0	0	0	100	100
TOTAL	0		0		0		8.75		100	
DAY 5: ACV II	100	100	10	10	1	1	0.1	0.1	0.01	0.01
A	0	0	0	0	0	0	0	0	100	100
B	0	0	0	0	0	0	0	0	100	100
C	0	0	0	0	0	0	0	0	100	100
D	0	0	0	0	0	0	0	37.5	100	100
E	0	0	0	0	0	0	0	12.5	100	100
TOTAL	0		0		0		5		100	
DAY 5: ACV III	100	100	10	10	1	1	0.1	0.1	0.01	0.01
A	0	0	0	0	0	0	0	0	100	100
B	0	0	0	0	0	0	12.5	12.5	100	100
C	0	0	0	0	0	0	0	0	100	100
D	0	0	0	0	0	0	0	0	100	100
E	0	0	0	0	0	0	0	0	100	100
TOTAL	0		0		0		2.5		100	
DAY 5: ACV IV	100	100	10	10	1	1	0.1	0.1	0.01	0.01
A	0	0	0	0	0	0	100	100	100	100
B	0	0	0	0	0	0	100	100	100	100
C	0	0	0	0	0	0	100	100	100	100
D	0	0	0	0	0	0	0	0	100	0
E	0	0	0	0	0	0	0	0	0	0
TOTAL	0		0		0		60		70	

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121. n = 4 of an average of 5 readings taken

APPENDIX 7 contd.

1. ACV CONC (µg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
100	0	0	5	20	20	1	100
10	0	0	5	15	15	1	100
1	0	0	5	10	10	1	100
0.1	0	0	5	5	5	1	100
0.01	5	5	0	0	5	0	0

2. ACV CONC (µg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
100	0	0	5	20	20	1	100
10	0	0	5	15	15	1	100
1	0	0	5	10	10	1	100
0.1	0	0	5	5	5	1	100
0.01	5	5	0	0	5	0	0

3. ACV CONC (µg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
100	0	0	5	20	20	1	100
10	0	0	5	15	15	1	100
1	0	0	5	10	10	1	100
0.1	3	3	5	5	8	0.63	62.5
0.01	5	8	0	0	8	0	0

4. ACV CONC (µg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
100	0	0	5	20	20	1	100
10	0	0	5	15	15	1	100
1	0	0	5	10	10	1	100
0.1	2	2	5	5	7	0.71	71.43
0.01	5	7	0	0	7	0	0

1 – 4 show the Reed and Muench calculations used to determine the viral percentage inhibition of ACV I to ACV IV.

ACV CONC(µg/ml)	ACV I	ACV II	ACV III	ACV IV	*AVE*	*SD*
100	100	100	100	100	0	0
10	100	100	100	100	0	0
1	100	100	100	100	0	0
0.1	100	62.5	100	71.43	83.48	19.42
0.01	10	0	0	0	2.5	5

n = 4 of an average of 5 readings taken.

APPENDIX 7 contd.

*ACV- PRA	*No. of plaques*					
Conc *(µg/ml)*	*ACV I*	*ACV I*	*ACV III*	*ACV IV*	*Average*	*Std Dev.*
100	0	0	0	0	0	0
10	0	0	0	0	0	0
1	0	0	0	0	0	0
0.1	78	82	61	89	77.5	11.90
0.01	98	73	84	90	86.25	10.53

Key: *ACV refers to acyclovir and PRA refers to plaque reduction assay.
n = 4 of an average of 5 readings.

APPENDIX 8
ANTIVIRAL RESULTS FOR THE COLD AQUEOUS EXTRACT OF BARRINGTONIA RACEMOSA

	CONCENTRATION (µg/ml)									
29AQC I: E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	0	0	0	100	100	100
B	0	0	0	0	0	0	100	100	100	100
C	0	0	12.5	0	62.5	0	100	100	100	100
D	0	0	0	0	0	0	100	100	100	100
E	0	0	0	0	0	0	100	100	100	100
F	0	0	0	0	0	0	0	100	100	100
TOTAL	***0***		***1.04***		***5.21***		***83.33***		***100***	
29AQC II E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	0	0	100	100	100	100
B	0	0	0	0	0	0	62.5	0	100	100
C	0	0	0	0	0	0	100	37.5	100	100
D	0	0	0	0	100	0	100	0	100	100
E	0	0	0	0	0	0	0	0	100	100
F	0	0	0	0	0	0	62.5	12.5	100	100
TOTAL	***0***		***0***		***8.33***		***47.92***		***100***	
29AQC III: E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	100	0	0	100	100	100	100
B	0	0	0	0	12.5	0	100	100	100	100
C	0	0	0	0	0	0	100	100	100	100
D	0	0	0	0	0	12.5	100	100	100	100
E	0	0	0	0	0	0	100	100	100	100
F	0	0	0	0	0	0	100	100	100	100
TOTAL	***0***		***8.33***		***2.08***		***100***		***100***	

Key: E – V refers to bioassays where the extract was added to the cell system 1 hour before the addition of the virus. n = 3 of an average of 6 readings. The concentration range tested was 80 µg/ml to 10 µg/ml.

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 8 contd.

1. 29AQC I CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	36	36	1	100
60	1	1	11	24	25	0.96	96
40	1	2	11	13	15	0.87	86.67
20	10	12	2	2	14	0.14	14.29
10	12	24	0	0	24	0	0

2. 29AQC II CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	39	39	1	100
60	0	0	12	27	27	1	100
40	1	1	11	15	16	0.945	93.75
20	8	9	4	4	13	0.31	30.77
10	23	32	0	0	32	0	0

3. 29AQC III CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	33	33	1	100
60	1	1	11	21	22	0.95	95.45
40	2	3	10	10	13	0.77	76.92
20	12	15	0	0	15	0	0
10	12	27	0	0	27	0	0

29AQC: bioassay	*29 AQC I*	*29 AQC II*	*29 AQC III*	*AVE*	*SD*
80	100	100	100	100	0
60	96	100	95.45	97.15	2.48
40	86.67	93.75	76.92	85.78	8.45
20	14.29	30.77	0	15.02	15.4
10	0	0	0	0	0

1 – 3 show the Reed and Muench calculations used to determine the viral percentage inhibition of 29AQC I to 29AQC III.
n = 3 of an average of 6 readings.

APPENDIX 8 contd.

	CONCENTRATION (µg/ml)									
29AQC I: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	0	0	100	0	100	0
B	0	0	0	0	0	100	0	100	100	100
C	0	0	0	0	0	0	0	100	100	100
D	0	0	0	0	0	0	100	0	100	100
E	0	0	0	0	0	0	100	0	100	100
F	0	0	0	0	0	0	100	100	100	100
TOTAL	***0***		***0***		***8.33***		***58.33***		***91.67***	
29AQC II: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	0	0	100	0	62.5	100
B	0	0	0	0	100	0	100	100	100	100
C	0	0	0	0	0	25	0	0	100	100
D	0	0	0	0	0	0	0	62.5	100	100
E	0	0	0	0	0	0	0	0	100	37.5
F	0	0	0	0	0	0	0	0	0	100
TOTAL	***0***		***0***		***10.42***		***30.21***		***83.33***	
29AQC III: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	0	62.5	100	100	100	100
B	0	0	0	0	0	0	100	100	100	0
C	0	0	0	0	0	0	0	100	100	100
D	0	0	0	0	0	0	100	100	100	100
E	0	0	0	0	0	0	100	100	100	100
F	0	0	0	0	0	0	100	100	100	100
TOTAL	***0***		***0***		***5.13***		***91.67***		***91.67***	

Key: E + V refers to bioassays where the extract and the virus were added to the cell system simultaneously. n = 3 of an average of 6 readings. The concentration range tested was 80 µg/ml to 10 µg/ml.

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 8 contd.

1. 29AQC I E + V CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	41	41	1	100
60	0	0	12	29	29	1	100
40	1	1	11	17	18	0.94	94.44
20	7	8	5	6	14	0.43	42.86
10	11	19	1	1	20	0.05	5

2. 29AQC II CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	43	43	1	100
60	0	0	12	31	31	1	100
40	2	2	10	19	21	0.90	90.48
20	4	6	8	9	15	0.6	60
10	11	17	1	1	18	0.06	5.56

3. 29AQC III CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	12	37	37	1	100
60	0	0	12	25	25	1	100
40	1	1	11	13	14	0.93	92.9
20	11	12	1	2	14	0.14	14.29
10	11	23	1	1	24	0.04	4.17

1 – 3 show the Reed and Muench calculations used to determine the viral percentage inhibition of 29AQC I to 29AQC III.

CONC (μg/ml)	*29 AQC I*	*29 AQC II*	*29 AQC III*	*AVE*	*SD*
80	100	100	100	100	0
60	100	100	100	100	0
40	94.44	90.48	92.86	92.59	2.00
20	42.86	60	14.96	39.05	23.09
10	5	5.56	4.17	4.91	0.70

n = 3 of an average of 6 readings.

APPENDIX 8 contd.

	CONCENTRATION (µg/ml)									
29AC I: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	12.5	37.5	0	37.5	100	100	100	100
B	0	12.5	12.5	37.5	100	100	100	100	100	100
C	0	12.5	12.5	62.5	100	100	100	100	100	100
D	0	37.5	62.5	37.5	100	100	100	100	100	100
E	0	12.5	37.5	62.5	100	100	100	100	100	100
F	0	0	12.5	62.5	100	37.5	100	100	100	100
TOTAL	***6.25***		***37.5***		***81.25***		***100***		***100***	
29AC II: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	12.5	0	0	0	0	100	100	100	100
B	0	0	12.5	37.5	100	100	100	100	100	100
C	0	12.5	37.5	62.5	100	100	100	100	100	100
D	37.5	12.5	37.5	37.5	100	100	100	100	100	100
E	0	12.5	37.5	37.5	100	100	100	100	100	100
F	0	0	12.5	12.5	100	100	100	100	100	100
TOTAL	***7.29***		***27.08***		***83.33***		***100***		***100***	
29AC III: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	12.5	0	0	0	62.5	37.5	100	100	100	100
B	12.5	0	37.5	12.5	100	100	100	100	100	100
C	12.5	0	37.5	62.5	37.5	62.5	100	100	100	100
D	12.5	37.5	37.5	37.5	100	62.5	100	100	100	100
E	0	12.5	37.5	62.5	100	100	100	100	100	100
F	0	12.5	37.5	0	62.5	62.5	100	100	100	100
TOTAL	***9.38***		***30.21***		***73.96***		***100***		***100***	

Key: V – E refers to bioassays where the virus was added to the cell system 1 hour before the addition of the extract. n = 3 of an average of 6 readings. The concentration range tested was 80 µg/ml to 10 µg/ml.

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 8 contd.

1. 29AQC: V-E CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	4	4	8	8	12	0.67	66.67
60	12	16	0	0	16	0	0
40	12	28	0	0	28	0	0
20	12	40	0	0	40	0	0
10	12	52	0	0	52	0	0

2. 29AQC CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	5	5	7	9	14	0.64	64.29
60	10	15	2	2	17	0.12	11.76
40	12	27	0	0	27	0	0
20	12	39	0	0	39	0	0
10	12	51	0	0	51	0	0

3. 29AQC CONC (μg/ml)	*WELLS INF.*	*TOTAL (T1)*	*WELLS UNINF.*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	7	7	5	8	15	0.53	53.33
60	9	16	3	3	19	0.16	15.79
40	12	28	0	0	28	0	0
20	12	40	0	0	40	0	0
10	12	52	0	0	52	0	0

1 – 3 show the Reed and Muench calculations used to determine the viral percentage inhibition 29AQC I to 29AQC III.

29C CONC (μg/ml)	*29AQC I*	*29AQC II*	*29AQC III*	*AVE*	*SD*
80	66.67	64.29	53.33	61.43	7.11
60	0	11.76	15.79	9.18	8.20
40	0	0	0	0	0
20	0	0	0	0	0
10	0	0	0	0	0

n = 3 of an average of 6 readings.

APPENDIX 9

ANTIVIRAL RESULTS FOR THE HOT AQUEOUS EXTRACT OF BARRINGTONIA RACEMOSA

	CONCENTRATION (μg/ml)									
29AQH I: E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	0	0	0	0	0	0
B	100	100	100	100	0	0	0	0	0	0
C	100	100	100	100	0	0	0	0	0	0
D	100	100	100	100	0	0	0	0	0	0
E	100	100	100	100	0	0	0	0	0	0
F	100	100	62.5	100	0	0	0	0	0	0
TOTAL	***100***		***96.88***		***0***		***0***		***0***	
29AQH II: E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	100	100	100	100	100	100	100	62.5
B	0	0	100	100	100	100	100	100	100	100
C	0	0	100	100	100	100	100	100	62.5	100
D	0	0	100	100	100	100	100	100	100	100
E	0	0	100	100	100	100	100	100	100	100
F	0	0	100	100	100	100	100	100	62.5	100
TOTAL	***0***		***100***		***100***		***100***		***90.63***	
29AQH III: E-V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: E – V refers to bioassays where the extract was added to the cell system 1 hour before the addition of the virus. n = 3 of an average of 6 readings. The concentration range tested was 80 μg/ml to 10 μg/ml.

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 9 contd.

29AQH II: E-V: DAY 5	**ANTIVIRAL ACTIVITY**						
1. 29AQH: E-V CONC (µg/ml)	***CELLS DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	100	100	0	3.125	103.1	0.03	3.03
60	96.875	196.875	3.125	3.125	200	0.016	1.56
40	100	296.875	0	0	296.9	0	0
20	100	396.875	0	0	396.9	0	0
10	100	496.875	0	0	496.9	0	0
2. 29AQH: E-V CONC (µg/ml)	***CELLS DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	100	100	0	0	100	0	0
60	100	200	0	9.375	209.4	0.04	4.48
40	100	300	0	9.375	309.4	0.03	3.03
20	100	400	0	9.375	409.4	0.023	2.29
10	90.625	490.625	9.375	9.375	500	0.019	1.88
3. 29AQH: E-V CONC (µg/ml)	***CELLS DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	100	100	0	0	100	0	0
60	100	200	0	0	200	0	0
40	100	300	0	0	300	0	0
20	100	400	0	0	400	0	0
10	100	500	0	0	500	0	0

1 – 3 shows the Reed and Muench calculations used to determine the viral percentage inhibition of 29AQC I to 29AQC III.

29AQH CONC	***29AQH I***	***29AQH II***	***29AQH III***	***AVE***	***SD***
80	3.03	0	0	1.01	1.75
60	1.56	4.48	0	2.01	2.27
40	0	3.03	0	1.01	1.75
20	0	2.29	0	0.76	1.32
10	0	1.88	0	0.63	1.0832

n = 3 of an average of 6 readings.

APPENDIX 9 contd.

	CONCENTRATION (µg/ml)									
29AQH I: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	
29AQH II: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	
29AQH III: E+V: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: E + V refers to bioassays where the extract and virus were added to the cell system simultaneously. n = 3 of an average of 6 readings. The concentration range tested was 80 µg/ml to 10 µg/ml. The above shows no viral inhibition.
The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 9 contd.

	CONCENTRATION (µg/ml)									
29AQH I: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	
29AQH II: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	
29AQH III: V-E: DAY 5	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: V – E refers to bioassays where the extract was added to the cell system 1 hour before the addition of the virus. n = 3 of an average of 6 readings. The concentration range tested was 80 µg/ml to 10 µg/ml. The above shows no viral inhibition.
The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 10

ANTIVIRAL RESULTS FOR TIME-OF-ADDITION ASSAYS: ADDITION OF THE VIRUS AFTER THE ADDITION OF THE EXTRACT

	CONCENTRATION (µg/ml)									
29AQC - 3 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC - 6 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC - 12 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC - 24 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: time of addition assays where the virus is added 3, 6, 12 and 24 hours after the addition of the extract. The concentration range tested was 40 µg/ml to 2 µg/ml. The above shows no viral inhibition.
The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 11
ANTIVIRAL RESULTS FOR TIME-OF-ADDITION ASSAYS: ADDITION OF THE VIRUS BEFORE THE ADDITION OF THE EXTRACT

	CONCENTRATION (µg/ml)									
29AQC - 6 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	37.5	37.5	100	37.5	100	100	100	100	100	100
B	62.5	37.5	37.5	37.5	100	100	100	100	100	100
C	37.5	62.5	62.5	62.5	100	100	100	100	100	100
D	62.5	37.5	37.5	37.5	100	100	100	100	100	100
E	100	37.5	37.5	62.5	100	100	100	100	100	100
F	62.5	62.5	62.5	62.5	100	100	100	100	100	100
TOTAL	***53.125***		***53.125***		***100***		***100***		***100***	

29AQC - 12 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC - 18 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC - 24 HOURS: DAY 5	40	40	20	20	10	10	4	4	2	2
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: time of addition assays where the virus is added 6, 12, 18 and 24 hours after the addition of the extract. The concentration range tested was 40 µg/ml to 2 µg/ml. The above shows no viral inhibition.

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 11 contd.

	CONCENTRATION (µg/ml)									
29AQC - 36 HOURS: DAY 5	**40**	**40**	**20**	**20**	**10**	**10**	**4**	**4**	**2**	**2**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29C - 48 HOURS: DAY 5	**40**	**40**	**20**	**20**	**10**	**10**	**4**	**4**	**2**	**2**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

Key: time of addition assays where the virus is added 36 and 48 hours after the addition of the extract. The concentration range tested was 40 µg/ml to 2 µg/ml. The above shows no viral inhibition.
The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 12
ANTIVIRAL RESULTS TO TANNIN REMOVAL ANALYSES

	CONCENTRATION (μg/ml)							
WATER EXTRACT	**100**	**100**	**80**	**80**	**60**	**60**	**40**	**40**
A	0	0	0	0	0	0	100	100
B	0	0	0	0	0	0	100	100
C	0	0	0	0	0	100	100	100
D	0	12.5	0	0	0	100	100	100
E	0	0	0	0	0	0	0	100
TOTAL	***1.25***		***0***		***20***		***90***	

	CONCENTRATION (μg/ml)			
F1: ETHANOL EXTRACT	**100**	**80**	**60**	**40**
A	100	CD	CD	100
B	0	0	62.5	100
C	0	0	0	100
D	0	0	0	100
E	0	0	62.5	100
TOTAL	***20***	***0***	***31.25***	***100***

F2: ETHANOL EXTRACT	**100**	**80**	**60**	**40**
A	0	0	100	100
B	0	0	0	62.5
C	0	0	0	62.5
D	0	0	0	62.5
E	0	0	0	37.5
TOTAL	***0***	***0***	***20***	***65***

SODIUM HYDROXIDE EXTRACT	**100**	**80**	**60**	**40**
A	CD	0	100	CD
B	CD	0	0	62.5
C	CD	0	0	62.5
D	CD	0	0	62.5
E	CD	0	0	37.5
TOTAL	CD	***0***	***20***	***56.25***

The above table represents raw data collected from antiviral assays using the scoring system as described in Table 13, p121.
KEY: CD represents cell destruction. Ethanol extracts represent two sequential washes of the column with ethanol solution.

APPENDIX 12 contd.

WATER EXTRACT							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	1.25	1.25	98.75	288.75	290	1.00	100
80	0	1.25	100	190	191.25	1.00	100
60	20	21.25	80	90	111.25	0.81	80.9
40	90	111.25	10	10	121.25	0.08	8.25

F1:ETHANOL EXTRACT							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	20	20	80	245.75	265.75	0.92	92.0
80	0	20	100	165.75	185.75	0.89	89.0
60	34.25	54.25	65.75	65.75	120	0.55	54.8
40	100	154.25	0	0	154.25	0	0

F2:ETHANOL EXTRACT							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	315	315	1.00	100
80	0	0	100	215	215	1.00	100
60	20	20	80	115	135	0.85	85.0
40	65	85	35	35	120	0.29	29.2

SODIUM HYDROXIDE EXTRACT							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	323.75	323.75	1.00	100
80	0	0	100	223.75	223.75	1.00	100
60	20	20	80	123.75	143.75	0.86	86.0
40	56.25	76.25	43.75	43.75	120	0.36	36.0

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

APPENDIX 13

ANTIVIRAL TEST RESULTS FOR SIZE EXCLUSION CHROMATOGRAPHY (G25) FRACTIONS OF COLD AND HOT AQUEOUS EXTRACT

1. COLD AQUEOUS EXTRACT

	CONCENTRATION (µg/ml)									
29AQC: F1	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***100***		***100***		***100***		***100***		***100***	

29AQC: F2	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	62.5	100	100	100	100	100	100	100	100	100
B	62.5	62.5	100	100	100	100	100	100	100	100
C	62.5	62.5	100	100	100	100	100	100	100	100
D	62.5	62.5	100	100	100	100	100	100	100	100
E	62.5	62.5	100	100	100	100	100	100	100	100
F	62.5	62.5	100	100	100	100	100	100	100	100
TOTAL	***65.625***		***100***		***100***		***100***		***100***	

29AQC: F3	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	100	100	100	100	100	100	100
B	0	0	0	0	100	100	100	100	100	100
C	0	0	0	0	100	100	100	100	100	100
D	0	0	0	0	100	100	100	100	100	100
E	0	0	0	100	100	100	100	100	100	100
F	0	0	100	0	100	100	100	100	100	100
TOTAL	***0***		***25***		***100***		***100***		***100***	

29AQC: F4	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	100	100	100	100	100	100
B	0	0	0	0	100	100	100	100	100	100
C	0	0	0	0	100	100	100	100	100	100
D	0	0	0	0	100	100	100	100	100	100
E	0	0	0	0	100	100	100	100	100	100
F	0	0	0	0	100	100	100	100	100	100
TOTAL	***0***		***0***		***100***		***100***		***100***	

KEY: F1 – F4 refer to size exclusion chromatography fractions using G25 Sephadex where Fraction one (F1) represents the large molecular weight components. Fraction 2 (F2) represents large molecular weight fractions and is characterised by the elution of the coloured component of the extract. Fraction 3 (F3) represents substances of intermediate molecular weight as determined by the detection of chloride ions and fraction 4 (F4) represents low molecular weight substances that appear after the detection of chloride ions.

The above tables represent raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 13 contd.

29AQC: F1							
Conc µg/ml	*DEAD*	*TOTAL (T1)*	*SURV*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	100	100	0	0	100	0	0
60	100	200	0	0	200	0	0
40	100	300	0	0	300	0	0
20	100	400	0	0	400	0	0
10	100	500	0	0	500	0	0

29AQC: F2							
Conc µg/ml	*DEAD*	*TOTAL (T1)*	*SURV*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	65.6	65.6	34.4	34.4	100	0.34	34.0
60	100	165.6	0	0	165.6	0	0
40	100	265.6	0	0	265.6	0	0
20	100	365.6	0	0	365.6	0	0
10	100	465.6	0	0	465.6	0	0

29AQC: F3							
Conc µg/ml	*DEAD*	*TOTAL (T1)*	*SURV*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	100	175	175	1	100
60	25	25	75	75	100	0.75	75
40	100	125	0	0	125	0	0
20	100	225	0	0	225	0	0
10	100	325	0	0	325	0	0

29AQC: F4							
Conc µg/ml	*DEAD*	*TOTAL (T1)*	*SURV*	*TOTAL (T2)*	*T1+T2*	*T2/T1+T2*	*% INHIB.*
80	0	0	100	200	200	1	100
60	0	0	100	100	100	1	100
40	100	100	0	0	100	0	0
20	100	200	0	0	200	0	0
10	100	300	0	0	300	0	0

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

2. HOT AQUEOUS EXTRACT

	CONCENTRATION (µg/ml)									
29AQH: F2	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	*100*		*100*		*100*		*100*		*100*	

29AQH: F3	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	*100*		*100*		*100*		*100*		*100*	

29AQH: F4	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	100	100	100	100	100	100
B	0	0	0	0	100	100	100	100	100	100
C	0	0	0	0	100	100	100	100	100	100
D	0	0	0	0	100	100	100	100	100	100
E	0	0	0	0	100	100	100	100	100	100
F	0	0	0	0	100	100	100	100	100	100
TOTAL	*0*		*0*		*100*		*100*		*100*	

KEY: F2 – F4 refer to size exclusion chromatography fractions using G25 Sephadex where Fraction 2 (F2) represents large molecular weight fractions and is characterised by the elution of the coloured component of the extract. Fraction 3 (F3) represents substances of intermediate molecular weight as determined by the detection of chloride ions and fraction 4 (F4) represents low molecular weight substances that appear after the detection of chloride ions.
The above tables represent raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 13 contd.

29AQH: F2							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	100	100	0	0	100	0	0
60	100	200	0	0	200	0	0
40	100	300	0	0	300	0	0
20	100	400	0	0	400	0	0
10	100	500	0	0	500	0	0

29AQH: F3							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	100	100	0	0	100	0	0
60	100	200	0	0	200	0	0
40	100	300	0	0	300	0	0
20	100	400	0	0	400	0	0
10	100	500	0	0	500	0	0
29AQH: F4							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	0	0	100	200	200	1	100
60	0	0	100	100	100	1	100
40	100	100	0	0	100	0	0
20	100	200	0	0	200	0	0
10	100	300	0	0	300	0	0

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

APPENDIX 14
ANTIVIRAL ASSAY RESULTS FOR ION EXCHANGE COLUMN CHROMATOGRAPHY SAMPLE

29AQC: F1	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	100	62.5	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	37.5	100	100	100	100	100	100	100	100
D	62.5	100	100	100	100	100	100	100	100	100
E	37.5	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***83.33***		***100***		***100***		***100***		***100***	

29AQC: F2	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	62.5	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100	100	100
C	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100
E	100	100	100	100	100	100	100	100	100	100
F	100	100	100	100	100	100	100	100	100	100
TOTAL	***96.88***		***100***		***100***		***100***		***100***	

29AQC: F3	**80**	**80**	**60**	**60**	**40**	**40**	**20**	**20**	**10**	**10**
A	0	0	0	0	62.5	62.5	100	100	100	100
B	0	0	0	0	100	100	100	100	100	100
C	0	0	0	0	100	100	100	100	100	100
D	0	0	12.5	0	100	100	100	100	100	100
E	0	0	0	0	100	100	100	100	100	100
F	0	0	0	0	62.5	100	100	100	100	100
TOTAL	***0***		***1.04***		***90.63***		***100***		***100***	

KEY: F1 – F3 refer to size exclusion chromatography fractions using G25 Sephadex where Fraction 1 (F1) represents large molecular weight fractions. Fraction 2 (F2) represents substances of intermediate molecular weight as determined by the detection of chloride ions and fraction 3 (F3) represents low molecular weight substances that appear after the detection of chloride ions.

The above tables represent raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 14 contd.

29AQC: F1							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	83.3	83.3	16.7	16.7	100	0.17	17.0
60	100	183.3	0	0	183.3	0	0
40	100	283.3	0	0	283.3	0	0
20	100	383.3	0	0	383.3	0	0
10	100	483.3	0	0	483.3	0	0

29AQC: F2							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	96.9	96.9	3.13	3.125	100	0.031	3.1
60	100	196.9	0	0	196.9	0	0
40	100	296.9	0	0	296.9	0	0
20	100	396.9	0	0	396.9	0	0
10	100	496.9	0	0	496.9	0	0

29AQC: F3							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
80	0	0	100	208.3	208.3	1	100
60	1.04	1.04	99.0	108.3	109.4	0.99	99.0
40	90.6	91.7	9.38	9.38	101.0	0.09	9.0
20	100	191.7	0	0	191.7	0	0
10	100	291.7	0	0	291.7	0	0

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

APPENDIX 15

ANTIVIRAL ASSAYS RESULTS FOR SOLID PHASE EXTRACTION (SPE) SAMPLES USING C_{18} COLUMN

	CONCENTRATION (µg/ml)			
29AQC: 0% ACN	100	80	60	40
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	0	0	0
E	0	0	0	0
F	0	0	0	0
TOTAL	*0*	*0*	*0*	*0*

29AQC: 5% ACN	100	80	60	40
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	0	0	0
E	0	0	0	0
F	0	0	0	0
TOTAL	*0*	*0*	*0*	*0*

29AQC: 10% ACN	100	80	60	40
A	0	100	0	0
B	0	0	0	0
C	0	0	0	0
D	0	100	0	0
E	0	0	0	0
F	0	0	0	0
TOTAL	*0*	*33.33*	*0*	*0*

29AQC: 15% ACN	100	80	60	40
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	0	0	0
E	0	0	0	0
F	0	0	0	0
TOTAL	*0*	*0*	*0*	*0*

Key: ACN – acetonitrile. 5% ACN indicates 5% acetonitrile and 95% water; 10% ACN indicates 10% acetonitrile and 90% water; 15% ACN indicates 15% acetonitrile and 85% water; 100% water indicates no acetonitrile solvent.

The above tables represent raw data collected from antiviral assays using the scoring system as described in Table 13, p121.

APPENDIX 15 contd.

29AQC: 0% ACN							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	400	400	1	100
80	0	0	100	300	300	1	100
60	0	0	100	200	200	1	100
40	0	0	100	100	100	1	100

29AQC: 5% ACN							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	400	400	1	100
80	0	0	100	300	300	1	100
60	0	0	100	200	200	1	100
40	0	0	100	100	100	1	100

29AQC: 10% ACN							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	366.7	366.7	1	100
80	33.3	33.3	66.7	266.7	300	0.889	88.9
60	0	33.3	100	200	233.3	0.860	86.0
40	0	33.3	100	100	133.3	0.750	75.0

29AQC: 15% ACN							
Conc µg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	400	400	1	100
80	0	0	100	300	300	1	100
60	0	0	100	200	200	1	100
40	0	0	100	100	100	1	100

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

APPENDIX 16
HPLC VALIDATION METHODOLOGY

CONC mg/ml	5.2	5.0	5.3	
Relative conc.	2.34	2.00	3.47	
	3.46	2.15	3.10	
	3.22	2.00	3.49	
	3.10	2.10	3.19	
	3.05	2.17	2.84	
	2.99	2.24	2.94	
	3.21	2.01	2.96	
	3.29	2.61	3.01	
	3.13	2.00	3.11	
	3.15	2.44	2.57	
AVE	3.09	2.17	3.07	2.80
[*1]*STD DEV*	0.30	0.21	0.28	0.082
[*2]*% RSD*	9.57	9.57	9.0	2.95

Relative concentration readings obtained from reverse phase HPLC analysis.

[*1] Standard deviation about the mean value.

[*2] Percent relative standard deviation refers to the percentage variation of the standard deviation around the mean.

APPENDIX 17

ANTIVIRAL ASSAY RESULTS FOR FRACTIONS COLLECTED USING REVERSE-PHASE HPLC

	CONCENTRATION (µg/ml)				
29AQC F1: 0-2 MINS	**100**	**80**	**50**	**40**	**20**
A	0	0	100	100	100
B	0	0	37.5	62.5	62.5
C	0	0	62.5	62.5	62.5
D	0	0	100	100	100
E	0	0	100	100	100
TOTAL	***0***	***0***	***80***	***85***	***85***

29AQC F2: 2-4 MINS	**100**	**80**	**50**	**40**	**20**
A	0	0	100	100	100
B	0	0	0	100	100
C	0	0	37.5	37.5	100
D	0	0	37.5	0	62.5
E	0	0	0	12.5	0
TOTAL	***0***	***0***	***35***	***50***	***72.5***

29AQC F3: 4-6 MINS	**100**	**80**	**50**	**40**	**20**
A	100	100	100	100	0
B	100	100	100	100	12.5
C	100	100	100	100	37.5
D	100	100	100	100	0
E	100	100	100	100	37.5
TOTAL	***100***	***100***	***100***	***100***	***17.5***

29AQC F4: 6-8 MINS	**100**	**80**	**50**	**40**	**20**
A	62.5	0	100	100	100
B	37.5	37.5	37.5	37.5	0
C	62.5	37.5	37.5	0	0
D	0	37.5	37.5	37.5	37.5
E	0	37.5	37.5	37.5	37.5
TOTAL	***32.5***	***30***	***50***	***42.5***	***35***

29AQC F5: 8-10 MINS	**100**	**80**	**50**	**40**	**20**
A	0	100	100	100	100
B	0	100	100	62.5	0
C	0	62.5	100	37.5	0
D	0	100	100	37.5	0
E	0	100	100	37.5	0
TOTAL	***0***	***92.5***	***100***	***55***	***20***

The above tables represent raw data collected from antiviral assays using the scoring system as described in Table 13, p121. The bioassay performed is of 29AQC fractionated using reverse phase HPLC according to the time intervals given.

APPENDIX 17 contd.

29AQC F1: 0-2 MINS							
Real conc mcg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	235	235	1	100
80	0	0	100	150	150	1	100
50	80	80	20	50	130	0.38	38.0
40	85	165	15	30	195	0.154	15.4
20	85	250	15	15	265	0.057	5.70

29AQC F2:2-4 MINS							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	315	315	1	100
80	0	0	100	242.5	242.5	1	100
50	35	35	65	142.5	177.5	0.803	80.3
40	50	85	50	77.5	162.5	0.477	47.7
20	72.5	157.5	27.5	27.5	185	0.149	14.9

29AQC F3: 4-6 MINS							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	400	400	1	100
80	0	0	100	382.5	382.5	1	100
50	0	0	100	282.5	282.5	1	100
40	0	0	100	182.5	182.5	1	100
20	17.5	17.5	82.5	82.5	100	0.825	82.5

29AQC F4:6-8 MINS							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	32.5	32.5	67.5	245	277.5	0.883	88.3
80	30	62.5	70	242.5	305	0.795	79.5
50	50	112.5	50	172.5	285	0.605	60.5
40	42.5	155	57.5	122.5	277.5	0.441	44.1
20	35	190	65	65	255	0.255	25.5

29AQC F5: 8-10 MINS							
Conc μg/ml	***DEAD***	***TOTAL (T1)***	***SURV***	***TOTAL (T2)***	***T1+T2***	***T2/T1+T2***	***% INHIB.***
100	0	0	100	152.5	152.5	1	100
80	92.5	92.5	7.5	132.5	225	0.589	58.9
50	100	192.5	0	125	317.5	0.394	39.4
40	55	247.5	45	125	372.5	0.3356	33.6
20	20	267.5	80	80	347.5	0.230	23.0

The above show the Reed and Muench calculations used to determine the viral inhibition of the fractions analysed.

APPENDIX 18
AMINO ACID REPORT FOLLOWING ACID HYDROLYSIS OF FRACTIONS 3 AND 4 FROM REVERSE-PHASE HPLC

1. FRACTION 3 (RP –HPLC)

COMPONENT NAME	ACTUAL NAME	RET TIME (min)	EXPECTED TIME	RAW AMOUNT
D	Aspartic acid	14.1	14.3	3.60
T	Threonine	18.2	18.4	0.85
S	Serine	19.3	19.4	1.62
E	Glutamine	22.5	22.4	3.60
G	Glycine	29.9	29.9	3.52
A	Alanine	31.4	31.4	2.01
V	Valine	36.3	36.4	1.36
I	Isoleucine	43.9	44.0	7.33
L	Leucine	45.4	45.4	1.04
NL	Internal standard	46.6	46.5	50.0
Y	Tyrosine	49.9	50.0	0.45
F	Phenylalanine	51.6	51.7	0.83
K	Lysine	63.7	65.0	1.94
TOTAL AMT				28.15
TOTAL x 110[*1]				3096.28
(TOTAL x 110)/1000000[*2]				0.003
PERCENT AMINO ACIDS				0.62

[*1] Fraction 3 refers to the sample obtained at retention time 4 – 6 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

[*2] The total molecular mass is multiplied by a constant that corresponds to the general molecular mass of amino acids.

[*3] The mass available is converted from nanograms to milligrams.

2. FRACTION 4 (RP-HPLC)

COMPONENT NAME	ACTUAL NAME	RET TIME (min)	EXPECTED TIME	RAW AMOUNT
D	Aspartic acid	14.1	14.3	3.32
T	Threonine	18.5	18.4	0.96
S	Serine	19.4	19.4	1.86
E	Glutamine	22.6	22.4	2.84
G	Glycine	29.9	29.9	3.65
A	Alanine	31.5	31.4	2.13
V	Valine	36.3	36.4	1.65
I	Isoleucine	43.9	44.0	0.83
L	Leucine	45.2	45.4	0.94
NL	Internal standard	46.6	46.5	50.00
Y	Tyrosine	50.00	50.0	0.82
F	Phenylalanine	51.6	51.7	0.69
TOTAL AMT				19.70
TOTAL x 110[*1]				2166.56
(TOTAL x 110)/1000000[*2]				0.002
PERCENT AMINO ACIDS				0.43

[*1] Fraction 4 refers to the sample obtained at retention time 6 – 8 minutes following the injection of the crude cold aqueous extract into a reverse-phase HPLC system.

[*2] The total molecular mass is multiplied by a constant that corresponds to the general molecular mass of amino acids.

[*3] The mass available is converted from nanograms to milligrams.