In vitro characterization of the antiviral activity of Secomet V against vaccinia virus infections

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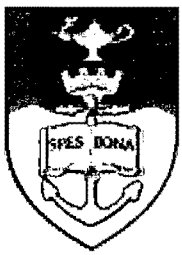

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Foreword:

SECOMET V is a product of the SA based, bio-pharmaceutical company called Secomet Ltd. This fledgling company was founded in 2002, and specializes in the extraction of secondary metabolites from plant cells cultivated in biorectors. Their protocols for the propagation of plant cells in large scale were developed in house and have not been disclosed.

The name SECOMET V is a reference name for a plant extract cultivated from plant stem cells in a bioreactor. The plant has been used as an oral infusion in folklore medicine in South Africa for many generations, and its name has been kept confidential. Furthermore, the company also has a confidentiality agreement with the university itself, so that any data related to this product may only be disclosed after previous approval by Secomet Ltd.

Empirical evidence suggests that this plant might have a broad spectrum of activity. It also appears that the extract may be effective in delaying the onset of AIDS and associated diseases in humans infected with HIV, as well as reducing the amount of circulating virus in the blood.

Currently the company is focusing on this product for the treatment of HIV/AIDS. One of the duties that UCT agreed to undertake was to set up an assay system to test for batch-to-batch consistency of their product, as well as to characterize and identify the active agents.

ACKNOWLEDGEMENTS

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List of figures:

List of abbreviations:

ATP – Adenosine Triphosphate

At – Atmospheres

° C – Degree Celsius

BSA – Bovine Serum Albumin

BSC – Green Monkey Kidney cells

CAM – Chorioallantoic membrane

CC_{50} – Concentration of Extract that Reduces Cell Viability by 50 %

CEV – Cell-associated Enveloped Virus

CMEM – Complete Minimum Essential Medium

CMV – Cytomegalovirus

CPE – Cytopathic Effect

CTP – Cytidine Triphosphate

dH_2O – Distilled Water

dGTP – Deoxyguanosine Triphosphate

DNA – Deoxyribose Nucleic Acid

dTMP – Thymidine Monophosphate

DMSO – Dimethyl Sulfoxide

dTTP – Thymidine Triphosphate

EEV – Extracellular Enveloped Virion

EICAR – 5-alkynyl-1-β-D-ribofuranosylimidazole-4-carboxamide

EC_{50} – Concentration of Extract that reduces the Number of pfu by 50 %

EDTA – Ethylenediaminetetra Acetic Acid

E_6SM – Primary Human Embryonic Skin Muscle Fibroblasts

FCS – Fetal Calf Serum

GTP – Guanosine Triphosphate

HBV – Hepatitis B Virus

HDP - Hexadecyloxypropyl

HIS – Heat Inactivated Serum

HIV – Human Immunodeficiency Virus

HPLC – High Performance Liquid Chromatography

IEV – Intracellular Enveloped Virion

IMP – Inosinate Monophosphate

IMV – Intracellular Mature Virion

ITR – Inverted Terminal Repeats

i.p – Intraperitonial

i.v - Intravenous

mRNA – messenger Ribose Nucleic Acid

MW – Molecular Weight

N/A – not applicable

ODE – Octadecyloxyethy

OMP – Ornithine Monophosphate

PBS – Phosphate Buffered Saline

pfu – Plaque Forming Units

PRK – Primary Rabbit Kidney cells

R_f – Retention Factor

r/t – room temperature

SAH - S-Adenosylhomocysteine Hydrolase

s.c – Subcutaneous

SCID – Severe Combined Immune-Deficiency

(S)-HPMPC - [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine]

$\therefore$ - Therefore

TLC – Thin Layer Chromatography

TFA – Trifluoroacetic Acid

TMV – Tobacco Mosaic Virus

UMP – Uridylate

UTP – Uridine Triphosphate

VV – vaccinia virus

XMP – Xanthylate Monophosphate

Abstract

The anti-poxvirus agent SECOMET V was the reference name for a plant extract produced in a bioreactor from primary plant stem cells, whose antiviral activity has been widely reported in folklore medicine. It exerted its anti-vaccinia virus activity by neutralizing cell-free virus rather than interfering with the downstream events following adsorption. The extract was tested against a virus titer of 10^8 pfu/ml, exhibiting strong vaccinia virus (VV) inhibition (EC_{50} 1150 ± 150 µg/ml, when incubated for 60 s prior to infection) and mild cytotoxicity in BSC-1 host cells (CC_{50} 560 ± 70 µg/ml) when compared to other plant extracts with anti-VV activity. Furthermore, the inactivation of VV was not only dose dependent, but time dependent as well. When incubated with VV at an equal titer, at a concentration equal to that of the EC_{50}, there was complete inhibition of plaque-forming units (PFU) after 6 min of exposure. However, the extract had no impact whatsoever on cell-associated, infectious virus. Moreover, the presence of high concentrations of bovine serum albumin (BSA) completely neutralized the anti-VV properties of the extract. It was also observed that the extract remained active after being subjected to high temperatures and pressure (121 °C, 15 psi for 30 min) and when size-fractionated through a 3 Kda cut-off filter, the active fraction was below 3 Kda in size. These properties suggested that the active agents were not proteins in nature. HPLC and TLC fractionation yielded two distinct active fractions, soluble in both organic and aqueous medium. The antivirals interacted strongly with both polar and hydrophobic matrices, indicating the presence of hydrophilic and hydrophobic moieties. The HPLC conditions developed could be used to further fractionate and purify the antivirals agents in the extract.

Chapter One

1.0 – Literature Review

1.1 - Introduction

The poxvirus family of viruses encompassed a large number of members that infected both vertebrate (chordopoxviruses) and invertebrate (entomopoxviruses) organisms (reviewed in Moss, 2003).

The chordopoxvirus sub-family consisted of eight genera, of which vaccinia virus (VV) was the representative member of the orthopoxvirus genera. The highly pathogenic and now eradicated smallpox virus also belonged to this genus, along with camelpox, cowpox, monkeypox, and four other non-medically significant members (reviewed in Moss, 2003).

VV is to date, the workhorse of poxviruses in the research arena. It remains established that VV inhibitors were effective to a greater or lesser extent against all other members of the poxvirus family. Furthermore, VV was much safer to work with, not requiring high biological containment facilities.

Clinically, the most important of all poxviruses was smallpox (reviewed in Fenner *et al.*, 1988). Variola virus is the etiological agent of smallpox. A highly transmissible disease proved lethal in up to 50 % of cases. It had no natural reservoir and infected humans exclusively.

Due to its severity, a worldwide vaccination campaign was launched, with the ultimate goal of eradicating smallpox. The campaign was successful, and in 1981, smallpox was officially declared eradicated worldwide by the WHO. However, in reality, vaccination in developed countries had been discontinued nearly 15 years prior, and the search for novel more effective and less toxic therapeutic agents soon followed.

A renewed interest in poxvirus prevention and cure was brought about by the terrorist attacks on the USA in September 2001 (reviewed in Rotz *et al.*, 2002). Furthermore, recent advances in molecular biology had made it possible to produce smallpox *in vitro* most likely by using camelpox as a template (Gubser & Smith, 2002), led officials to believe that the world could yet again be vulnerable to smallpox, released deliberately or by criminal intent.

The health hazards posed by poxviruses were further highlighted by the emergence of the relatively rare viral zoonosis, human monkeypox (reviewed in Giulio *et al.*, 2004). This potentially lethal pathogen was introduced in the USA in the spring of 2003 by imported giant Gambian rats sold as exotic pets. Furthermore, nearly 100 cases have been reported in the Democratic Republic of Congo since 1996.

However, in reality, it was the steady rise in the number of human immune-deficiency virus (HIV) infected individuals as well the increase in cancer cases and solid-organ recipients, which has made poxviruses clinically significant pathogens once again. What all these ailments had in common was that they led to a dangerously weakened immune system, increasing the susceptibility to opportunistic pathogens such as *Ecthyma infectiosum* and *Molluscum contagiosum*, two members of the poxvirus family.

The vaccine used during the smallpox eradication campaign consisted of live attenuated vaccinia virus, and as late as 2004, enough was still available to immunize a population as large as that of the USA (Birmingham & Kenyon, 2001). Following vaccination, the virus would go through a few replication cycles, before being cleared by the immune system. This vaccine was highly effective in immunizing individual against not only smallpox, but because of their antigenic relatedness, it conferred protection against all other orthopoxvirus infections as well.

However, the severity of post-vaccination complications (such as encephalitis and disseminated vaccinia) prompted the discontinuation of immunization in the USA as

early as 1972, before smallpox was globally eradicated (reviewed in Lofquist *et al.*, 2003).

Since the late 1980's, we were faced with a very different scenario: the threat of a serious natural orthopoxvirus infection was comparatively almost non-existent; therefore, post-vaccination side effects of such magnitude would have been even less acceptable. Secondly, the emergence of immune-deficient individuals either due to the effects of HIV/AIDS (acquired immune deficiency syndrome) or solid-organ recipients has limited the use of live-virus vaccines in that population bracket. Therefore the only alternative available to this rapidly growing group of individuals, especially in the African continent, would be the use of an effective therapeutic agent.

Agents such as thiosemicarbazones (previously marketed under the brand name Marboran®) have proved its effectiveness in the clinical setting in large-scale treatment of serious poxvirus infections (reviewed in Bauer, 1969). These classes of agents were first reported in the 40's and were conservatively used during the smallpox epidemic in the following decades. However, although effective thiosemicarbazones caused serious vomiting following ingestion, possibly due to the high dosages required for effective therapy (>200 mg/kg). In addition, there was formation of a toxic metabolite.

Moreover, it had to be administered during the prodromal phase of infection, as it proved to be ineffective at later stages. However, these side effects and inconveniences were acceptable as a therapeutic agent in the face of a potentially deadly poxvirus infection.

In today's clinical management of mostly non-lethal poxvirus infections, such side effects may not be tolerated

More recently, a novel drug consisting of the nucleoside analogue cidofovir (Vistide®) was approved for the treatment of the herpes virus cytomegalovirus (CMV) infections in immunocompromised individuals. It has proved to be a potent *in vitro* inhibitor of all known orthopoxviruses, particularly smallpox (reviewed by De Clercq, 2002). It also

proved to be effective in the treatment of certain opportunistic poxvirus infections in patients with HIV/AIDS. The drug only had to be administered once a week, which made it very promising for poxvirus therapy. However, its dose-dependent nephrotoxicity and the need to use the intravenous route for administration sparked a renewed search for less toxic and more effective agents that could be administered orally.

Once again, this search turned to the kingdom of plants. Many therapeutic agents in current clinical use were obtained from this source, from morphine to cancer-fighting agents such as toxol (reviewed in Ming *et al.*, 2003). Furthermore, plants have been the primary source and very often the only source of therapeutic agents since medieval times. A wealth of knowledge regarding plants and their healing properties has been accumulated over millennia. This knowledge extends beyond therapeutic value to include in some instances, the side effects, and precautions regarding their use. In light of this, many medicinal plants were considered safe by virtue of the time that they have been administered in the human population as well as knowledge gained during this period.

Many plant extracts have proved effective in neutralizing a range of viruses from herpes simplex to HIV (reviewed in Jassim & Naji, 2003). However, the effectiveness of these medicinal plants has rarely been proven against poxviruses. Following the eradication of smallpox, the remainders of the poxviruses were not considered clinically significant pathogens for a few decades. Nevertheless, seed extracts from *Musa superba* and *Ensete superbum* as well as the leaves of *Melia azadericta* have been shown to have strong anti-vaccinia and anti-variola activity (Pendse & Lyengar, 1967; Dutta *et al.*, 1968 and Rao *et al.*, 1969).

In this study the anti-vaccinia virus, properties of SECOMET V (the reference name of a plant extract produced in a bioreactor from primary stem cells) were analyzed. The extract prevented *in vitro* infection of BSC-1 cells by the attenuated VV strain VGK5 (Kotwal *et al.*, 1989). The extract acted on cell-free virus particles as opposed to interfering with viral replication in infected cells, like most anti-poxvirus agents reported to date (reviewed in Baker *et al.*, 2003). Moreover, the antiviral activity of the extract

could be neutralized with bovine serum albumin (BSA), a strategy that could be applied to purify the active agents. The data also indicated that the antiviral activity of SECOMET V was due to the presence of at least 2 agents that could be fractionated via HPLC and TLC.

This extract has been used in folklore medicine, either by ingestion or as an ointment, to treat a range of maladies, and is still currently in use. The anecdotal evidence suggests that its oral bioavailability may be sufficiently high for therapeutic purposes, and could probably be used topically.

1.2 - Vaccinia virus: a molecular biology perspective

1.2.1 - Classification

The family *Poxviridae* is subdivided into two sub-families depending on the host range (Francki *et al.*, 1991). VV belongs to the *Chordopoxvirinae* sub-family of viruses, which infects vertebrates. This subfamily comprises 8 genera, of which VV belongs to the *Orthopoxvirus* genus together with 10 other members, named after their respective hosts: buffalopox, camelpox, cowpox, ectromelia (vaccinia sub-species), monkeypox, raccoonpox, skunkpox, Uasin Gishu (African horse pox), variola and volepox. Furthermore, all members of this genus share similar morphology and host range as well as genetic and antigenic relatedness.

In spite of the fact that the natural host of VV has to date not been identified (reviewed in Baxby, 1981), VV was the representative virus in the orthopoxvirus genus of viruses.

1.2.2 - Structure

Poxviruses were the largest of the known mammalian viruses. Their morphology varies according to the preparation method, and four types of virions were readily distinguishable, namely: intracellular mature virions (IMV), intracellular enveloped virions (IEV), cell-associated enveloped virus (CEV), and the extracellular enveloped virions (EEV) (Schmelz *et al.*, 1994).

The IMV is fully infectious and was the most common form of VV used in the research arena. It was a large, brick-shaped structure, 350 × 270 nm, when observed under cryoelectron microscopy (Dubochet *et al.*, 1994). Three different regions were visible, consisting of the external membrane, lateral bodies, and the dumbbell-shaped core (**Figure 1.1**). Furthermore, Easterbrook (1966) showed that the membrane surrounding the core consists of cylinder-shaped subunits, 10 nm in length and 5 nm in width and an inner layer of equal size.

The virions also packaged many enzymes and factors, and up to 16 have been reported to date (reviewed in Moss, 2002). Most were enzymes involved in DNA synthesis and post-transcription modification such as polyadenylation, capping, and methylation. In fact, this system was so complete, that infectious virions were capable of carrying out *in vitro* transcription and processing of mRNAs (Munyon *et al.*, 1967).

Chemically, VV virions consisted mainly of proteins (90 %, including glycosidic residues), lipids (5 %) and DNA (3,2 %) as well as RNA (Roening and Holowczak, 1974) and some trace amounts of the polyamine spermine and spermidine (Lanzer and Holowczak, 1975).

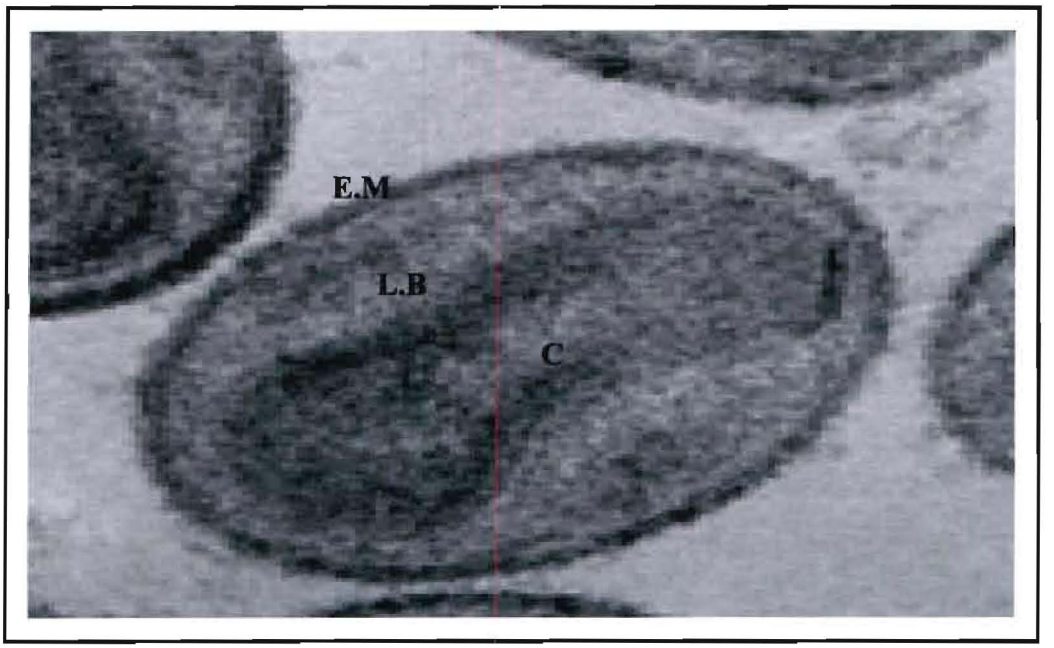

Figure 1.1: Cryo-EM of positively stained, reduced IMV. Three structural features were readily observed: EM – external membrane; L.B – lateral bodies; C – core (www.smbs.buffalo.edu).

1.2.3 - Genome Organization

Poxviruses were classified as type I viruses, in that they had double-stranded DNA genomes, ranging between 130-230 Kbp in size, and around 180 Kbp for VV (Goebel *et al.*, 1990).

The genome was linear and had identical, inverted terminal repeats (ITR) at both ends, containing an A+T rich, incompletely base-paired, flip-flop loops that connected both strands (Baroudy BM *et al.*, 1982) (**Figure 1.2**).

The genome could be fragment into 3 distinct regions, namely: the terminal loop; the variable end-regions, which play a role in pathogen-host interaction; and the conserved central region, which encodes the genes involved in replication (**Figure 1.2**).

One hundred open reading frames (ORFs) have been identified to date encoding both structural and enzymatic proteins.

An interesting feature of the *poxviridae* family regarding the genome organization was that their ORFs were largely non-overlapping. This could have been attributed to the large size of their genome which eliminated the need for extensive overlapping, a common feature in most virus families.

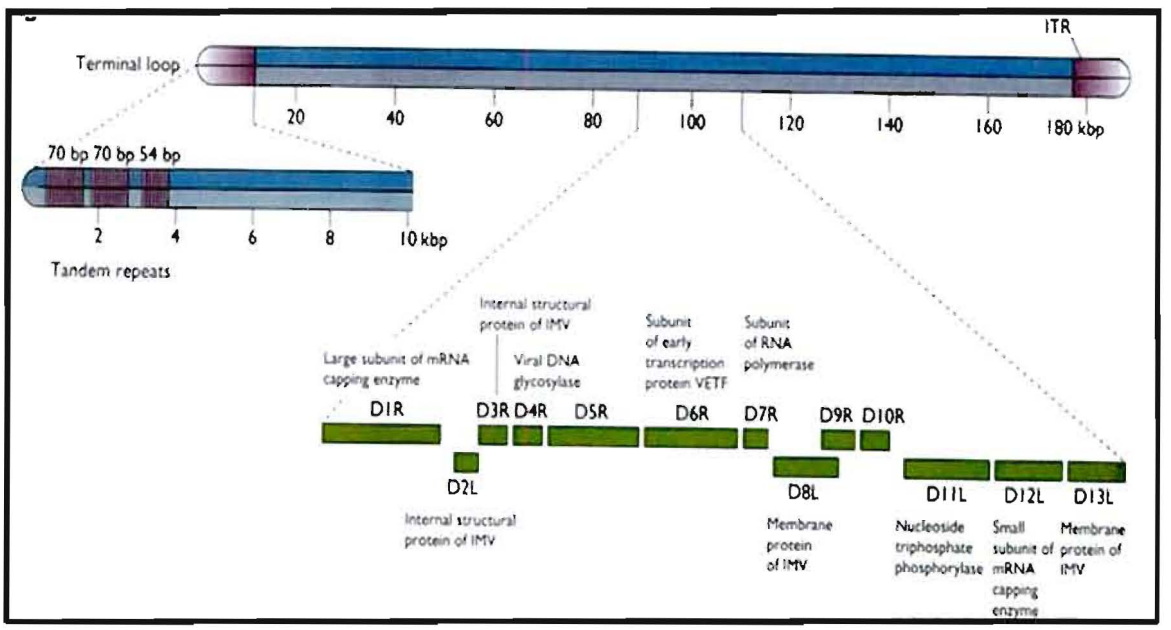

Figure 1.2: Schematic representation of the genomic organization of VV. The terminal loops were 10 Kbp in size and contains inverted terminal repeats. The main structural and functional proteins were encoded in the central region and are highly conserved throughout the genus (www.cornellcollege.edu).

1.2.4 - Gene Expression

Gene expression in VV could be temporarily subdivided into 3 stages (Baldick and Moss, 1993): early stage, beginning 20 min post infection, reaching a maximum after about 90 min; intermediate stage mRNAs could be detected once early gene expression peaks, increasing very rapidly for 30 min, and decreasing just as fast. Late stage gene products were expressed at the time of peak of the intermediate gene products, reaching a maximum after 4 h.

Early-stage gene products included proteins involved in DNA replication (Jones and Moss, 1984 and Lee and Niles, 1988), nucleotide biosynthesis (Hruby and Ball, 1982 and Smith *et al.*, 1989), immuno-modulation (reviewed in Kotwal, 2000), and intermediate gene transcription. About half of the virus genome was translated at this stage, before DNA replication (Oda and Joklik, 1967).

Intermediate and late gene transcripts were expressed after replication and were involved in virion morphogenesis and assembly (reviewed in Broyles, 2003).

1.2.5 - Vaccinia virus life cycle

The life cycle of orthopoxviruses is the most complex of all mammalian viruses known to date. In light of this, it is not surprising that many gaps exist in our knowledge of VV replication. Depicted below is an oversimplified schematic representation of the major steps in the life cycle of VV as well as the sites of action of the various agents reviewed in this chapter (**Figure 1.3**).

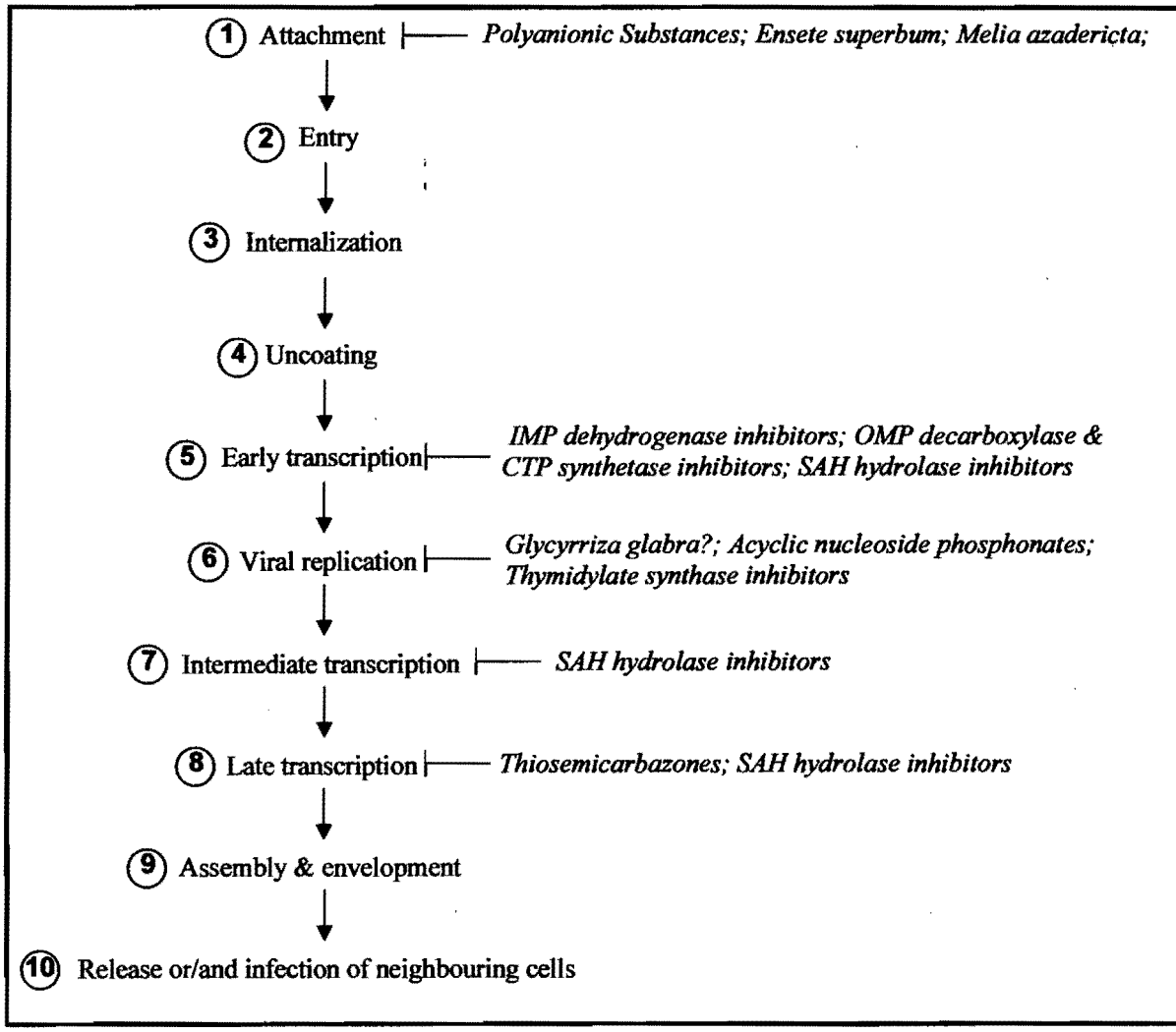

Figure 1.3: Schematic representation of VV life cycle and drug targeting sites. Vaccinia virion attaches to the host cell via surface receptors (1). It then enters the cell (2), is internalised (3) and loses the envelope releasing the core into the cytoplasm (4). Early mRNAs are transcribed and translated. Early mRNs transcribe intermediate transcription factors as well as secretory growth factors and immunomodulatory proteins (5). DNA replication marks the beginning of the intermediate stage (6). Intermediate gene products are transcribed with the aid of the host's nuclear factors and translated into late transcription factors (7). These late transcription factors participate in the expression of structural proteins as well as transcription factors for early gene expression, which will be incorporated into the mature virion (8). Assembly begins in the Golgi apparatus with the formation of scattered membrane intermediates, giving rise to the immature virion (9). Maturation resulted in either the release of virions following cell lysis or infection of neighbouring cells from the host cell, without having to be released into the extracellular milieu (10). Block arrows indicate inhibition by known anti-poxvirus agents at different stages of the life cycle.

1.3 - Known Plants with Anti-Poxvirus Activity

The use of medicinal plants to prevent and/or treat poxvirus infections more specifically smallpox, was widespread across the world. The advent of an effective vaccine against these deadly pathogens greatly reduced the interest in traditional medicinal plants, particularly with the advent of the global eradication of smallpox. However, work was nonetheless undertaken during mostly the last 2 decades of the epidemic to authenticate and characterize the anti-poxvirus properties of medicinal plants in folklore medicine.
In light of this, a review of the very limited studies that where published regarding the subject at hand was undertaken below.

1.3.1 – Ensete superbum (the seeds of Banakadali)

The anti-variola properties of this plant were first brought to the attention of the medical community in 1933 by a Portuguese doctor stationed in the former Portuguese colony of Goa, India (Pendse and Lyengar, 1967). The seeds were subsequently used in the Infectious Disease Hospital in Bombay, greatly decreasing smallpox-associated morbidity and mortality.

However, the results obtained from one the first published studies using rabbits, did not reflect the results obtained by the Infectious Disease Hospital in Bombay nearly two decades earlier (Pendse and Lyengar, 1967). In a non-lethal model, rabbits received orally 100, 500 or 1000 mg/kg of a powdered solution resuspended in water for three days before infection and for four days post-infection. The effectiveness of the extract was measured as a function of the degree of localized skin scarification in control, untreated animals against treated. The temperature of both groups was also monitored, twice daily. However, no difference between control and test rabbits was observed.

More encouraging results were obtained almost in parallel by a different group, in a much more thorough study. In that study undertaken by Dutta *et al.* (1968) using tree increasing

more chemically purified fractions of the starting material of these seeds, they demonstrated that each fraction was decreasingly capable of inhibiting a virus titer of 10^3 pfu/ml *in ovo* at a dose ranging from 0,9 µg to 0,01 µg. The highest particle-forming units (PFU) inhibition was 96,25 % for the most crude fraction and down to 55 % for the most fractionated one. VV and variola showed almost identical susceptibility to the fractions tested. Furthermore, it was observed that the extracts disrupted cell free-virus particles rather then interfering with the downstream events following adsorption (**Figure 1.3**, step 1). For this reason, the extract fractions were incubated with the virus for 1 h prior to infecting the chorioallantoic membrane of embryonated hen's eggs.

The same group provided even more convincing in vivo evidence. Mice where either administered an oral solution containing 28 mg 6 h prior to virus challenge or 14 mg 24 and 180 h post-infection respectively. It was observed that there was a survival rate of 96 % in the pre-challenge treated mice and an almost equally high survival rate in mice treated post-infection (ranging from 85 to 95 %).

Again, as in the results obtained *in ovo*, the least pure of the fractions was the most active, but the difference was not as accentuated. However, once the dosage was doubled (28 mg), there was almost no difference in survival rates across the animals treated with the different fractions, 120 h post-infection.
The work described illustrated that the seeds of Banakadali were indeed active against poxviruses not only *in ovo* but also *in vivo*, while showing no toxic effect at therapeutic dosages.

1.3.2 – Melia azadericta (the leaves of the Margosa tree)

Yet another medicinal plant used in Indian folklore medicine, not only to treat poxvirus-infections but also an array of other ailments.

Rao *et al.* (1969) demonstrated that 0,1 ml of a 10 % (10 mg) solution of the leaves of the Margosa tree effectively reduced the number of PFU by 2 logs, when tested against a virus titer of 10^6 pfu/ml of VV *in vitro*. Furthermore, they also showed that the leaf extract was only active if incubated with the virus before infection. This clearly suggested that the antiviral properties were due to a direct effect on cell-free virus particles and not by interfering with the post-infection events, much like the seeds of Banakadali (**Figure 1.3**, step 1).

Even more compelling evidence originated from the work carried out *in vivo*, where a 3-log drop in virus titters was observed for VV and two logs for variola virus. It was also observed that the virucidal effects of the leaf extract were dose-dependent.

When the work was extended to primates, more specifically the Indian Bonnet monkey (*Macacus radiata*), it was clear that the leaf extract had a positive effect in the normal course of infection. The untreated animals developed localized ulceration and necrosis which lasted for 32 days, whereas the treated animals a much milder ulceration and minor necrosis, lasting for only 12 days.

This was another plant that clearly showed therapeutic potential, but was not exploited in large scale.

1.3.3 – Glycyrriza glabra (the roots)

The active constituent in this case was known to be glycyrrhizic acid, and an array of viruses were tested, but VV was the only member of the poxvirus family. A pure, commercially available preparation was made at a concentration of 8 mM (Pompei *et al.*, 1979). It was observed that the addition of glycyrrhizic acid immediately post infection as well as 3 h later, caused a 3 log drop in the number of PFU from an initial titre of $6,8 \times 10^7$.

Based on these data, the investigators speculated that the virucidal properties of glycyrrhizic acid might be due to the interaction with sensitive virus proteins, during the replication of VV (**Figure 1.3**, step 6). No cytotoxicity was observed at the working dosages.

However, no animal work was undertaken and the study was very limited. Nonetheless, it was shown that a plant metabolite was an effective, non-specific antiviral agent, which could have been further exploited for therapeutic potential.

1.4 - Vaccinia Virus Inhibitors of Known Structure and Mode of Action

The complex life cycle of orthopoxviruses presented many virus-specific drug-targeting sites from entry (**Figure 3**, step 1) to maturation inhibitors (**Figure 3**, step 10).

In this section, VV inhibitors were reviewed and grouped according to structure and pathways inhibited. Furthermore, their *in vitro* and *in vivo* efficacies were described as well as effectiveness and side effects in the clinical setting where applicable.

1.4.1 - Acyclic Nucleoside Phosphonates

Cidofovir [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine] [(S)-HPMPC] belongs to this class of inhibitors. It was commercially available under the brand name Vistide® since the late 1990's, and was approved to treat CMV retinitis in AIDS patients.

The activity of cidofovir against a broad spectrum of DNA viruses (De Clercq *et al.*, 1986) and its anti-vaccinia virus activity was first described over a decade ago (De Clercq *et al.*, 1987).

The mode of action of this antiviral was determined by studies carried out in CMV, and it was observed that it inhibited viral DNA synthesis 1000-fold stronger than cellular DNA synthesis (Neyts *et al.*, 1990). It was an analogue of the nucleotide cytosine and causes chain-termination upon incorporation in a growing DNA strand (**Figure 1.3**, step 6).

Cidofovir proved very effective in neutralizing VV *in vitro* (EC_{50} of 5 μg/ml). Furthermore, its efficacy against orthopoxvirus infections was further strengthen when *in vivo* work was carried out: severe combined immune deficiency (SCID) mice survived ten-fold longer when cidofovir was administered, subcutaneously (s.c.) at dosages ranging from 1 mg/kg/day for 5 days to 20 mg/kg, twice a week, than untreated mice (Neyts *et al.*,1993). Furthermore, when a single large dose (100 mg/kg) was administered 0, 2 and 4 days post-infection via the same route, cidofovir was found to protect mice against a lethal inoculation of cowpox (Bray *et al.*, 2000).

In a parallel study, Smee *et al.* (2000) showed that a single intranasal dose (10, 20 and 40 mg/kg) of cidofovir conferred 90-100% protection against a lethal dose of cowpox virus in mice, when administered 24 h after lethal aerosol or intranasal virus challenge.

The superiority of aerosol-delivered cidofovir against other delivery systems was further strengthened in a study where, mice where inoculated with a lethal dose of aerosolized cowpox and followed by treatment with 0,5-5 mg/kg of aerosolized cidofovir (Bray *et al.*, 2002). The survival rates were much higher, and the virus titers were much lower than in those treated with 25 and 100 mg/kg of cidofovir delivered subcutaneously.
More recently, Chad and colleagues (2003) reported that aerosol delivery of cidofovir in mice resulted in longer retention times of the drug in the lungs, and a marked decreased concentration in the kidneys. Nearly all mice treated with a single dose two days prior and two days post infection survived.

In an attempt to improve cidofovir's oral bioavailability, a novel series of analogs were synthesized by esterification with long-chain alkoxyalkanols. Keith *et* al (2004) showed that hexadecyloxypropyl-cidofovir (HDP-cidofovir) and octadecyloxyethyl-cidofovir

(ODE-cidofovir) were active *in vitro*, against VV with an EC_{50} of 0,52 and 42 μM respectively. The addition of the ester moiety effectively increased the bioavailability of cidofovir by between 88 and 97 % (Ciesla *et al.*, 2003) as opposed to that of 5 % for cidofovir alone (Wachsman *et al.*, 1997).

When the efficacy of these lipid esters was tested in mice, it was observed that their effectiveness was similar to that of parentally administered cidofovir (Quenelle *et al.*, 2004). Furthermore, it was observed that the pharmacokinetics of the lipid esters was almost identical to that of cidofovir alone, in that therapeutic levels in critical organs (lung, liver and kidney) persisted for 72 h after a single oral administration of 10 mg/kg. This equated to a twice weekly oral dose.

In the clinical setting however, only two types of poxvirus have been treated successfully with cidofovir (De Clercq, 2002 review), namely *Ecthyma infectiosum* (orf) by topical application (Geerinck *et al.*, 2001 and MacCabe *et al.*, 2003) and *Molluscum contagiosum* both by local and parental route (Meadows *et al.*, 1997; Davies *et al.*, 1999; Zabawski *et al.*, 1999; Ibara *et al.*, 2000; Toro *et al.*, 2000 and Baxter and Highet, 2004).

However, in spite of being a very effective anti-orthopoxvirus agent, there are two drawbacks: the dose-dependent nephrotoxicity of this antiviral restricted its use in disseminated infections (maximum of 5 mg/kg/day) (Lalezari *et al.*, 1995). This was potentially a very serious setback, since smallpox and monkeypox caused systemic infections rather than localized (as was the case with the other two poxviruses above described).

Moreover, cidofovir-resistant strains of cowpox and vaccinia have been generated *in vitro* (Smee *et al.*, 2002). These resistant strains although less fit, were nearly as lethal as the wild-type strain. It was observed that mice infected with cidofovir-resistant cowpox did not respond to cidofovir treatment and succumbed merely 36 hours later than the control, untreated mice infected with wild-type cowpox.

Cidofovir was the most likely and possibly the sole agent that would have been used in the clinical setting for the treatment of orthopoxvirus infections.

1.4.2 - Thiosemicarbazones

The first antiviral agents discovered belonged to this family of substances. Its anti-vaccinia virus activity was first reported over 50 years ago by Hamre *et al.* (1950) using chick embryos and mice as hosts. They also reported a high EC_{50} of less than one µg/ml in human cervical epithelial cells (HeLa). However, these values are not in agreement with those obtained by Baker *et al.* (2003). They reported an EC_{50} between 45,8 and 52,0 µg/ml for Vero and LLC-MK2 cell lines respectively.

The search for the most active thiosemicarbazones culminated with the discovery of the 1-methyl derivative (Thompson *et al.*, 1953 and Bauer, 1954), which resulted in the first clinical trial 6 years later (reviewed in Bauer, 1960).

The antiviral was marketed under the brand name Marboran®, and several field trials indicated that it was effective enough agent to justify its use in the prodromal treatment, and prophylaxis of smallpox infections (Bauer *et al.*, 1963; Bauer *et al.*, 1969 and Heiner *et al.*, 1971). However, the mode of action of this antiviral was only elucidated 5 years after being approved for clinical trials (Woodson *et al.*, 1965). Through a series of elegant experiments, Woodson *et al.* (1965) demonstrated that thiosemicarbazones exerted its antiviral effect by interfering with the transcription of late mRNA's, thereby inhibiting virion morphogenesis and assembly (**Figure 1.3**, step 8).

As effective as methisazone was against variola, its side effects of extreme vomiting and diarrhea restricted its use in prophylaxis, even as far back as the 1960's (Bauer *et al.*, 1963). The other downside was the large dosages (200 mg/kg first dose, 800 mg/kg spread over 3 days) required for effective therapy (Bauer *et al.*, 1963).

Methisazone was nonetheless widely used, and was the only antiviral, which has proved effective in the clinical setting, for the treatment of poxvirus infections.

1.4.3 – IMP dehydrogenase inhibitors

Fluoroimidazoles, EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide), tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), selenazole (2-β-D-ribofuranosylselenazole-4-carboxamide) and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) all belong to this class of agents (reviewed in De Clercq, 2001). They were nucleoside analogues that acted by blocking the conversion of IMP to xanthylate (XMP), a crucial event in the synthesis of purine mononucleotides (Streeter *et al.*, 1973). This in turn, led to the depletion of GTP and dGTP pools, thereby interfering with the production of early mRNA transcripts (**Figure 1.3**, step 5).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was chemically synthesized in 1972 (Witkowski *et al.*, 1972) and its *in vitro* (Witkowski *et* al., 1973; Huffman JH *et al.*, 1973 and Katz *et al.*, 1976) and *in vivo* (Sidwell *et al.*, 1972) activity against a broad range of viruses including poxviruses was immediately established.

Sidwell *et al.* showed that a 20 % solution of Ribavirin (Virazole) applied topically twice daily for 15 days to the tail, inhibited the development of vaccinia virus lesions in New Zealand albino rabbits. The same group also showed that the concentration of drug required to kill 50 % of the animals was 2000 mg/kg when given orally. EICAR, a derivative of Virazole, exhibited a similar effect in mice inoculated intravenously (in the tail vein), when treated with 25 mg/k/day intraperitoneal for 5 days (De Clercq *et al.*, 1991).

To date, the most effective anti-vaccinia antiviral of the IMP dehydrogenase inhibitors is EICAR, being 100-fold more active than Ribavirin in PRK cells (EC_{50} of 0,2 μg/ml Vs 20 μg/ml) (Kirsi *et al.*, 1983 and De Clercq *et al.*, 1991). Fluoroimidazoles (EC_{50} of 100

μg/ml), tiazofurim (EC_{50} of 45μg/ml) and selenazole (EC_{50} of 3 μg/ml) were significantly less active than EICAR (De Clercq *et al.*, 1976 and Kirsi *et al.*, 1983).

Since this group of antivirals has not been used in the clinical setting, nor have any human trials ever been carried out, its efficacy and side effects were not known.

1.4.4 - OMP decarboxylase/CTP synthetase inhibitors

Both agents worked by blocking the production of cytidine (CTP) by either interfering with the synthesis of uridylate (UMP) from OMP (OMP decarboxylase inhibitors) or blocking the conversion of UTP to CTP (CTP synthetase inhibitors) hence suppressing RNA synthesis (**Figure 1.3**, step 5).

The prototype for the OMP decarboxylase class of inhibitors was Pyrazofurin, which was also the most active (Petrie *et al.*, 1986). It has shown potent activity against VV (EC_{50} of 0,1 μg/ml) in PKR cells, even more so than the IMP dehydrogenase inhibitor Ribavirin. In fact, this agent appeared similar to EICAR in activity, yet its toxicity index in tissue culture was 1000-fold higher than the EC_{50}.

In spite of this, the use of Pyrazofurim in the clinical setting was never pursued as aggressively as other antiviral agents. Furthermore, no literature could be found detailing the efficacy of Pyrazofurin in *in vivo* animal models.

Regarding the CTP synthetase inhibitors, Carbodine (cyclopentyl cytosine [C-Cyd]) as well as cyclopentenyl cytosine (the most active of the two) have demonstrated strong anti-vaccinia virus activity (De Clercq *et al.*, 1990 and De Clercq *et al.*, 1991). With an EC_{50} of 0,04 μg/ml and host cell toxicity of 400 μg/ml in PRK cells, cyclopentenyl cytosine appeared to be one of the most active (20-fold greater inhibition than EICAR) as well as safest anti-vaccinia virus agents reported to date in *in vitro* studies. However, it is

likely that because these inhibitors target host cell enzymes rather than viral, very little interest has been paid to this class of agents.

1.4.5 - Thymidylate synthase inhibitors

This class of antivirals was also known as 5-substituted 2'-deoxyuridines by virtue of the fact that they only differ at the 5' position. About 16 substitutions have been made at the 5'position, all of which proved active against VV (reviewed in De Clercq, 2001).

Inhibition of Thymidylate synthase resulted in inefficient nucleic acid synthesis (**Figure** 1.3, step 6), due to the depletion of dTTP polls brought about by the inhibition of the conversion of dUMP to dTMP (De Clercq *et al.*, 1981).

The two most active forms of Thymidylate synthase inhibitors were achieved when fluorine and bromine were introduced in the 5'position. The IC_{50} in PRK cells was 0,1 μg/ml for both substitutions (De Clercq, 1980). However, it was the iodo substitution (5-iodo-dUrd) that was extensively used in animal models.

In 1967, Boyle *et al.* used the tail-lesion model to test the efficacy of 5-iodo-dUrd. In their work, mice were infected intravenously (i.v) with VV, followed by subcutaneous (s.c) administration of 5-iodo-dUrd at a dose of 100 mg/d for 7 days. The test-substance was successful in curbing the formation of tail lesions.

Further work was carried out by Hyndiuk *et al.* (1976 a) which demonstrated that topical solution of 0,1% of iododeoxyuridine when applied at 2 hour intervals for a total of 8 applications, 36 h post-infection, effectively suppressed keratitis (eye infection). The same group later showed that similar results could be obtained by using a 0,5% solution but applying it only every 4 h (Hyndiuk *et al.*, 1976).

In the same year, the efficacy of the iodo, methyl and thiocyano derivatives were tested in the same tail-lesion model (De Clercq *et al.*, 1976). Successful inhibition of tail lesion formation was obtained when the substances were delivered intraperitoneally (doses ranged from 4, 20 and 100 mg/kg/d) for four days, starting immediately post-infection.

No further *in vivo* work has been carried out using any of the 16 known derivatives of deoxyuridine up to the end of 2004. In light of the above evidence, these compounds merit that further work should be carried out to fully exploit their potential as effective therapeutic agents.

1.4.6 - SAH hydrolase inhibitors

These inhibitors were adenosine analogs that blocked the cleavage of s-adenosylhomocysteine (SAH) into homocysteine and adenosine, its two components (reviewed in Borchardt, 1980). Adenosine was further converted into inosine, adenine, or AMP. When this process was blocked, S-adenosylmethionine dependent methylations were inhibited thus inhibiting the maturation of VV mRNAs (such as 5'-capping; **Figure 1.3**, step 5).

As many as 24 such inhibitors have been described to date (reviewed in De Clercq, 2001), of which those belonging to the class of the carbocyclic adenosine analogues proved to be the most effective, with EC_{50} less than 0.05 μg/ml. Equally strong were Neplanocin A (De Clercq, 1980), 3-Deazaneplanocin A (Tseng *et al.*, 1989), (-)-5'-Noraristomycin (Siddiqi *et al.*, 1994) and (R)-6'-C-Methylneplanocin A (Shuto *et al.*, 1992) in primary human embryonic skin-muscle fibroblasts in stationary culture (E_6SM) as well as PKR. Furthermore, the cytotoxic dose was >20,000 fold higher than the EC_{50}, a very low value.

No further literature was found on work carried out in animal models to demonstrate the true therapeutic effectiveness of this class of agents.

1.4.7 - Polyanionic Substances

It appeared that these agents exerted their antiviral activity by coating cell-free virus particles and blocking the sites required for interaction with cell-surface receptors (Hosoya *et al.*, 1991; **Figure 1.3**, step 1). These substances included sulphated polysaccharides (which are mostly extracted from seaweeds) such as poly-L-lysines and poly-D-lysines, dextran sulphate and pentosan polysulfate to name a few.

These agents were known to have broad-range antiviral activity *in vitro* against enveloped viruses (Baba *et al.*, 1988; Witvrouw *et al.*, 1994 and De Clercq *et* al., 2001). Furthermore, there was a direct correlation between molecular weight (MW) and inhibition. The highest inhibition achieved with poly-L-lysine was 40 μg/ml and higher for poly-D-lysine of identical MW (20 000) in human embryonic skin muscle fibroblasts (ESM), which was the CC_{50}, indicating a weak and toxic antiviral.
The most effective of the polycations was dextran sulphate. The CC_{50} was 10-fold greater than the EC_{50} (20 μg/ml) in the ESM cell line (Hosoya *et al.*, 1991).

A literature search on the effectiveness of these agents in animal models yielded no results.

1.4.8 - Miscellaneous Antivirals

Rifampin, a tuberculostatic agent used in front-line therapy of tuberculosis, has been reported to have anti-VV activity (Subak-Sharpe *et al.*, 1969). However, a high concentration of the drug (100 μg/ml) was required to inhibit viral replication, which immediately ruled out its use in the clinical setting due to toxicity.

Two other antibiotics reported to have similar *in vitro* activity to that of rifampin were congocidine and distamycin A (Becker *et al.*, 1972). Whereas the inhibition of poxvirus replication brought about by rifampin and distamycin A was reversible (EC_{50} of 100 μg/ml) in the case of congocidine, it was irreversible with a lower EC_{50} (± 50 μg/ml).

However, because both antibiotics at such high doses proved to be toxic, they had no therapeutic significance.

Another antibiotic, this time a derivative of the macrolide antibiotic rifamycin, streptoviricin consisted of a mixture of five compounds related to rifamycin. Its anti-vaccinia virus activity was almost negligible at the concentrations used (2 μg/ml) (Quintrell and McAuslan, 1970).

1.5 - Conclusion

The renewed clinical interest in orthopoxviruses was brought about mostly due to the concern that it could be used as a bioweapon. However, emerging poxviruses such as monkeypox should not be ignored as a potential threat to public health.

It was clear that vaccination with the existing vaccines or even novel vaccines was unrealistic and unnecessary. What was really needed were effective therapeutic agents that are well tolerated in immunocompromised patients that are already under severe stress due to the nature of the disease and accompanying therapy.

It was also clear that we do not yet have such therapeutic agents, although cidofovir looks promising. However, the complexity of the poxvirus infectious cycle and the vast array of virus-specific enzymes, presented a large pool of novel and highly specific drug-targeting sites that could be explored in drug design as well as bio prospecting.

However, poxvirus infections were still not significant enough to warrant the attention of the big pharmaceutical and biotechnology enterprises.

Dozens of therapeutic agents have been obtained directly from natural systems, particularly from plants (Ming *et al.*, 2003), including many plants with *in vitro* antiviral

activity; therefore, it is very likely that many novel and clinically effective agents are yet to be discovered.

However, there was rapidly growing need for an effective and inexpensive therapeutic agent with tolerable side effects that could used to minimize the discomfort and severity of opportunistic and introduced poxvirus infections in the African continent.

1.6 - Scope of the project

The goals of this project were to characterize the anti-vaccinia virus properties of SECOMET V by:

1. Establishing the anti-vaccinia virus activity of the extract;
2. Monitoring its cytotoxicity effect on BSC-1 cells and determine a working dose;
3. Determining at which stage in the replication cycle did the antiviral interfered with;
4. Asserting if the antiviral was taken up by the host cells by studying its impact on cell-associated virus particles;
5. Establish parameters for future fractionation and purification of the active agents using HPLC and TLC.

Chapter Two

2.0 - MATERIALS AND METHODS

2.1 – BSC-1 tissue culture (Protocols adapted from the Sigma-Aldrich® hand book *Fundamental techniques in cell culture*).

2.1.1- Maintenance of the cell line

Cells were maintained in three flasks of different surface-areas (25, 75 and 150 cm^2 respectively, Corning Inc, Corning, NY, USA) at 37 °C, 5% CO_2 containing complete minimal essential medium (CMEM; Highveld Biological, Lyndhurst, SA) and enriched with 10% fetal calf serum (FCS, same manufacturer). Antibiotics were added at a concentration of 500 µg/ml penicillin and streptomycin, and 250 ng/ml fungizone (Highveld Biological, Lyndhurst, SA), supplied as a triple mixture concentrated 100-fold. The size of the flask varied depending on the number of cells required. If no cells were needed, then the smaller 25 cm^2 flask was used. Cell confluency in a 150 cm^2 flask was reached at a total number of 2×10^7, whereas in a 25 cm^2 a total of 3×10^6 cells were required to cover the growth area.

To assess the degree of confluency as well as to ensure that there were no contaminants, the cell monolayer was observed under an inverted microscope at ×20 magnification. Only once the monolayer was 100% confluent were the cells sub-cultured. This procedure was carried out by discarding the overlay medium followed by rinsing cell monolayer twice, with equal volumes of pre-warmed (at 37 °C in a water bath) phosphate buffer solution (PBS, Sigma®, ST Louis, MO, USA), pH 7.2 to remove all traces of growth media and anti-trypsin factors that could interfere with the trypsin treatment. Following this, the monolayer was subsequently treated with trypsin/EDTA (1, 2 or 4 ml

depending on the surface area of the flask) for 10 min to detach the cells from the bottom of the flask.

Once the cells were loose, the flask was tapped gently to remove any cells which were still attached. To ensure that all cell-clumps were broken, a 2 ml disposable pipette was used to aspirate the cell-suspension up and down a few times.

If the cells were not used for further work, they were diluted 20-fold and re-seeded either in the same flask (all flasks were re-seeded no more than 3 times) or into a new flask.

2.1.2 - Incubation of BSC-1 cells

BSC-1 cells were incubated in a CO_2 incubator (5 %), which aided in maintaining the physiological pH of the culture medium, preventing it from turning acidic as waste products of cellular metabolism accumulate. The temperature was kept constant at 37 °C and the humidity was also constant at 90 %.

2.1.3 - Cell quantification

Adherent cells were detached via trypsin treatment (section 2.1.1 in this chapter) and were gently aspired a few times to further break up any clumps. This was followed by removing a 20 µl aliquot of suspended cells and incubating it with an equal volume of a 0,2 % solution of trypan blue (Sigma, ST Louis, Mo, USA) for 5 min at r/t, in a microcentrifuge tube.

In the meantime, the coverslip of the haemocytometer (counting chamber, improved Neubauer®, Balu Brand, Germany) was moistened with water and gently swerved over the chamber until Newton's refraction rings were formed. The cell/dye suspension was then added to each chamber at a volume of 8,6 µl/chamber (this was just enough to cover

the surface area of the chamber, without flooding it thereby minimizing overestimating the number of cells). The cell count was then carried out under a light microscope using a ×20 magnification.

The number of viable and non-viable cells was quantified by counting the cells in all 8 quadrants (each cell in a clump was counted). Dead cells stained blue, live cells did not stain. The total number of cells was then calculated by multiplying the correction factor of the chamber (10^4) by the average number of cells counted and by the dilution factor (2) to obtain the total concentration of cells/ml. Percentage cell viability was then calculated by dividing the average number of viable cells by the total number of cells and multiplied by 100.

2.1.4 – Cryopreservation

For this purpose, adherent cells were cultured in a 150 cm^2 tissue culture flask and used at a confluency between 80 and 90 % to ensure to ensure that a healthy population of cells in the exponential growth phase was frozen. Once the cells were detached from the flask using trypsin/EDTA, a cell count was performed. A minimum viability of 90 % was required for cryopreservation.

The remainder of the cells was then centrifuged in a Heraeus® Multifuge 3L-R (Kendro Laboratory Products, Hanau, Germany) at 100 g, 20 °C for 5 min using a swinging-rotor bucket. After the supernatant was discarded, the cells were resuspended gently in cryoprotective media (Biowhittaker, Walkersville, Maryland, USA) containing 15% DMSO, at a density of 2×10^6 cells/ml. The cell suspension was then aliquoted into 2 ml cryovials (Corning Inc, Corning, NY, USA) at a volume of 200 µl per vial. The vials were subsequently stored at -80 °C for 24 h and transferred thereafter to liquid nitrogen for long-term storage.

2.1.5 – Resuscitation of frozen cells

The frozen cells were removed from the liquid nitrogen storage unit and immediately partially thawed in a water bath at 37 °C until a small amount of ice was still present, to minimize DMSO toxicity, which was cytotoxic above 4 °C (if ice was still present it meant that the solution was still below 4 °C).

The thawed cells were then transferred to four volumes of pre-warmed CMEM, 10% FCS, drop wise to dilute out the DMSO. The cells were then recovered by centrifugation as described above (section 2.1.3 in this chapter). Once the supernatant was decanted, the remaining cell pellet was resuspended in 5 ml of growth media and transferred to a 25 cm^2 tissue culture flask and placed in the CO_2 incubator for a period of 24 h.
After 24 h, the overlay medium was discarded and replaced with 10 ml of fresh, pre-warmed media.

2.1.6 – Seeding 6-well and 96-well tissue culture plates (Protocols adapted from Jonczy et al., 2000)

Six-well tissue culture plates (Corning Inc, Corning, NY, USA) were seeded at a density of 10^6 cells/well in a total volume of 2 ml of CMEM, 10% FCS per well, whereas the 96-well tissue culture plates (Corning Inc, Corning, NY, USA) were seeded at a density of 2×10^4 cells/well in a volume of 100 µl/well. Once the tissue-culture plates were seeded, they were transferred to the CO_2 incubator. The tissue culture plates were usedonly once the BSC-1 monolayers were 100% confluent (2 to 3 days after seeding).

2.1.7 – Staining BSC-1 cells in tissue-culture plates

Ten ml of a 0,01 solution of crystal violet (Saarchem/Sigma, Wadeville, Gauteng, SA) was further diluted ten-fold in a solution containing 80 % PBS and 10 % methanol (a

fixating agent) prior to use. This was necessary not only because the crystal violet was suspended in water, which would lyse the cells but also because it was too concentrated, as observed by the formation of a dark violet precipitate on stained cell monolayers.

After the overlay medium was discarded from the culture plates containing BSC-1 cells, the crystal violet solution was added (20 µl/well for 96-well plates and 200 µl/well for 6-well plates) to the monolayer and incubated at room temperature for 1h in order to allow for the cells to take up the dye. Once the time elapsed, the remaining solution was removed and the cells stained violet.

2.2 – Making stock virus and titration (Adapted from Kotwal & Abrahams, 2004)

2.2.1 – Virus propagation

A tissue culture flask with a surface area of 150 cm^2 containing a confluent monolayer of BSC-1 cells was used. Prior to infection, most of the overlay medium was removed (but not discarded), leaving only enough to cover the monolayer to facilitate synchronous adsorption of the viral particles. Virus was then added to the monolayer at a multiplicity of infection (MOI) of 0.01 (1 pfu per 100 cells, for a total of 10^5 pfu).
The virus was allowed to adsorb for 90 min, after which time the remainder of the medium was added back to the flask, and the now infected cells were further incubated for 24 h. The now virus-infected cells where harvested with the aid of a cell scraper (Corning Costar®, Cambridge, MA, USA) and resuspended in 5 ml of PBS at r/t.

The pfu were then released from inside the cells with 3 freeze (at -20 °C)-thaw (at 37 °C) cycles. The resulting cell debris was removed by centrifugation at 1000 g for 10 min at 4 °C in a swinging-rotor bucket.
The virus laden supernatant was collected and subject to two 1 min sonication cycles at 60 % of the maximum, in a Virsonic® 100 sonicator (VirTis, Gardiner, NY, USA) to

release the virus aggregations. The sonication was carried out with the samples on ice, to prevent heat-induced virolysis of the cell-free pfu.

2.2.2 – Virus titration: plaque assays

A 6-well tissue culture plate containing a confluent monolayer of BSC-1 cells was used. A volume of 20 µl of virus suspension was added to the first well. This was followed by 2 100 fold serial dilutions and 3 10-fold dilutions respectively, effectively diluting the virus suspension 10^{-9} fold. The infected cells were incubated for 48 h, after which time a clear cytopathic effect (CPE) was observed under an inverted microscope at a magnification of ×20. After discarding the overlay media, the wells were individually stained with crystal violet solution.

The titer was determined by counting the number of plaques in the lowest dilution that contained more than 10 but less than 100 plaques, and dividing it by the dilution factor (i.e.: 10 plaques in the well corresponding to the 10^{-7} dilution indicates a titer of $10/10^{-7}$= 10^{8} pfu/ml).

2.2.3 – Serial dilution of antiviral-treated virus in 96-well plates

Sterile, flat-bottom tissue culture plates (Corning Costar®, NY, USA) without lids, were used. A volume of 30 µl of MEM, 10% FCS was added to row A, followed by 45 µl to all the remaining rows. After incubating the virus with the test substance, the suspension was transferred to row A, for a total volume of 50 µl (NOTE: the total volume of the virus/test substance mixture was fixed at 20 µl).

Seven 10-fold serial dilutions were carried out, starting from row A, by transferring 5 µl of virus-infected media from row A down to the last row H, from which 5 µl were discarded (NOTE: pipette tips were replaced after each dilution).

Following the serial dilution, the media was then transferred to a 96-well tissue culture plate, containing a confluent monolayer of BSC-1 cells, in the same order as that of the dilution plate.

2.3 – Characterization of the antiviral properties of SECOMET V

2.3.1- Determination of dry weight

Aim: To determine the dry weight of the plant material in order to standardize all further work accordingly.

The plant extract was supplied dissolved in distilled water (dH_2O). The weighing process started by weighing 3 empty microcentrifuge tubes, and labeling them accordingly. The extract was then dispensed into each tube at a volume of 200 µl/tube. The samples were then dried in a vacuum concentrator (Speedvac® SPD111V, ThermoSavant, Holbrook, NY, USA) at 80 °C for 8 h.

After drying, the samples were weighted and the dry weight of each was determined by subtracting the weight of the microcentrifuge/dry material by that of the microcentrifuge alone. Following this, a second identical drying cycle was carried out. If there was no variation in weight, the sample was assumed to be dry, and the dry weight of the material was then determined as the average of the 3 samples.

2.3.2 – Inhibition of viral replication (adapted from Hernández-Corona et al., 2002)

Aim: Investigation of the anti-VV activity of the extract and if such activity was linked to a disruption of the downstream events following infection.

Six-well tissue-culture plates containing BSC-1 cells were infected in triplicate, with a total of 10^2 pfu. The virus-infected plates were incubated for 90 min to allow for viral adsorption. Following the adsorption event, the overlay media was removed and the cell monolayers were washed twice with 1 ml of pre-warmed PBS to remove any unadsorbed viral particles (tips were never used more than once without being replaced). This was followed by the addition of 2 ml of fresh growth media, containing extract at a lethal but non-toxic concentration (100 µg/ml). The virus-infect cells were then incubated for 48 h. Wells were also infected with untreated virus, in triplicate. As a control for VV inactivation, virus at the same titer was incubated with the extract at a concentration of 100 µg/ml for 60 s prior to infection. After 48 h, the cells were stained with crystal violet solution and scored for CPE.

2.3.3 – Inhibition of adsorption (adapted from Hernández-Corona et al., 2002)

Aim: To determine whether the virucidal effects of the extract were due to a direct disruption of cell-free pfu, prior to infection.

Virus at a titer of 10^8 pfu/ml in a volume of 10 µl was incubated with an equal volume of extract (for a final concentration of 11 mg/ml) at room temperature for 60 s. The extract/virus suspension was then used to infect BSC-1 cells, in triplicate, in 6-well plates.
Following the two day incubation period the cells were stained with crystal violet solution and the number of pfu was counted.

2.3.4 –Cytotoxicity assay: CC_{50} (Adapted from Kurokawa et al., 1995).

Aim: To establish a working dose as well as to quantify the cytotoxicity index of the extract for the purpose of comparing to that of other known plant extracts. The CC_{50}

cytotoxicity assay measures the concentration of extract that reduces cell viability (BSC-1) by 50 %, using the trypan-blue vital dye exclusion method.

A 96-well plate containing a confluent monolayer of BSC-1 cells was used. Decreasing volumes of the crude extract were tested, starting at 10 µl (2200 µg/ml) down to 1 µl (220 µg/ml) in 1 µl increments. The lowest dilution used was 0,5 µl (110 µg/ml). Following the addition of the extract, the cells were further incubated for 24 h. The overlay media was then removed, and the cell monolayers were washed 3 times with 100 µl of pre-warmed PBS. Following the PBS wash, the cells were subsequently rinsed once with 20 µl of pre-warmed trypsin, to remove any remaining anti-trypsin factors. The cells were then suspended with the aid of 20 µl of pre-warmed trypsin following 20 min of incubation.

Once the cells were suspended, they were inspected under an inverted microscope to ensure that there were no cell aggregations that would interfere with the counting process. The cells were then stained with an equal volume of trypan blue under the inverted microscope at ×20 magnification (section 2.1.3 in this chapter).
Percentage cell viability was calculated from the following equation:

$$\frac{\text{no. dead cells (stained blue)}}{\text{no. live cells (unstained)}} \times 100$$

The CC_{50} value was then calculated from a curve relating percent cell viability to the concentration of test extract.

2.3.5 –EC_{50} assay (Adapted from Jonczy et al., 2000 and Yoosook et al., 1999).

Aim: This assay provided information regarding the strength of the antiviral activity of the extract as a whole. The EC_{50} consists in the determination of the concentration of test substance that reduces plaque formation by 50%, and is the standardized value used to compare the strength of antiviral agents, usually expressed in µg/ml.

A total of 10 serial two-fold dilutions of the crude extract were tested, carried out in sterile microcentrifuge tubes, using tissue-culture grade PBS as the solvent. The highest dilution was 11 mg/ml and the lowest 110 µg/ml.

The test substance was then incubated with 10 µl of VV at a titer of 10^8 pfu/ml for 60 s, in a final volume of 20 µl in sterile 1,5 ml microcentrifuge tubes. All assays were carried out in triplicate. Controls included were: virus incubated with PBS and untreated virus. The infected plates were then incubated for 48 h and stained. The plaques in the 2nd lowest dilution that still produced plaques were counted. The percentage inhibition was then calculated using the following equation:

$$\frac{\text{No. surviving pfu}}{\text{PBS-treated virus}} \times 100$$

The EC_{50} was then extrapolated from the curve relating the percentage inhibition (y-axis) to the concentration of extract (x-axis).

2.3.6 – Inactivation kinetics

Aim: To study the relationship between the rate of plaque inhibition as a function of time.

One hundred and twenty µl of extract was incubated with an equal volume of VV at a titer of 10^8 pfu/ml for a total time of 10 min, for a final concentration of extract of 1180 mg/ml (the EC_{50}). At 1 min intervals, 20 µl of virus treated extract was taken and used to infect BSC-1 cell monolayers in 96-well plates. Following 48 h of incubation, the monolayers were stained and number of pfu was scored and plotted in a line graph relating percentage pfu inhibition to time in min;

2.3.7 – Investigation of inhibition of cell to cell viral spread (Adapted from Kimiyasu et al., 1992 and Jonczy et al., 2000)

Aim: This experiment indicated whether or not the antiviral agents were taken up by the cell without losing its virucidal activity, by measuring the number of surviving progeny virions. The assay was similar in principle to the one used to determine the EC_{50} (section 2.3.5 in this chapter) except that the maximum concentration did not exceed 500 µg/ml.

A 75 cm^2 tissue culture flask containing a confluent monolayer of BSC-1 cells, was infected with VV at a MOI of 0.01 and incubated for 24 h. The overlay medium was then decanted and the monolayer was rinsed 3 times with 15 ml of pre-warmed PBS to remove any cell-free virions. The cells were then suspended with the aid of 2 ml of trypsin. After 20 min, the now suspended cells were gently aspired a few times, to minimize cell-clumping. The cells were visualized under an inverted microscope to ensure that there were no aggregations of cells. The cells were then recovered by low speed centrifugation at 100 g for 5 min at 20°C in a swinging rotor bucket. After the supernatant was discarded, the cells were resuspended in 1 ml of pre-warmed MEM and used immediately. The crude extract was diluted 9 times in 5 µl increments, starting with a concentration of 220 µg/ml down to 24 µg/ml in a total volume of 10 µl. Following this, 10 µl of the cell-infected virus suspension was incubated with 10 µl of the diluted extract for 1 min prior to infection.

As a control, the virus-infected cells were lysed by two freeze-thawing cycles, and treated with the extract at all dilutions. The EC_{50} value of the control would then be compared to that of the treated.

The virus titer was determined by infecting wells, in triplicate, with 10 µl of the cell-associated virus suspension followed by 5, 10-fold serial dilutions.

2.3.8 – Neutralization of the antiviral activity of SECOMET V with BSA

Aim: To investigate the postulate that the antiviral agents could bind non-specifically to proteins thereby losing their virucidal properties. The non-specific binding would further explain the lack of specificity of SECOMET V.

An 8% BSA (bovine serum albumin) solution was prepared in tissue-culture grade PBS by dissolving 8 gm of BSA in PBS up to 100 ml with the aid of a magnetic stirrer. Equal volumes of the BSA solution was then mixed with the extract (1 ml for a total of 2 ml) and incubated at room temperature for 1 h. Following the incubation, the extract was tested for activity against 10 µl of VV at a titer of 10^8 pfu/ml, in 6-well plates, by incubating 10 µl of the BSA/extract solution with an equal volume of virus for 1 min prior to infection. As a control, BSA alone, extract alone and PBS alone were incubated with VV prior to infection.

2.4 – Characterization of the chemical properties of the active agents in SECOMET V

2.4.1 – Heat inactivation of SECOMET V

Aim: The vast majority of proteins are heat-labile. By submitting the extract to conditions that would inactivate proteins it would give more weight to the argument that the active agents might not be proteins.

A volume of 1 ml of crude extract was transferred to a microcentrifuge tube and placed in an autoclave for 30 min at 15 psi, 121 °C. Once the autoclaving cycle was over, the extract was allowed to cool to r/t. A volume of 10 µl of the cooled, autoclaved extract was then tested for activity against 10 µl of VV at a titer of 10^8 pfu/ml in 6-well plates as described (section 2.3.3 in this chapter).

2.4.2 – Activity-guided size-exclusion fractionation

Aim: This step allowed us to gain some insight into the physical nature of the antiviral agent/s present in the extract with respect to their size. If there was activity in the fraction below 3 Kda in size, this would give more weight to the argument that the antiviral was unlikely to be a protein.

A volume of 15 ml of crude extract was loaded on the outer chamber of a Centriprep® YM-10 (10 Kda cut off, Millipore, USA). The device was subsequently placed in a centrifuge, and the filtration was driven by centrifugation at 3,500 g for 2 h.
The material that collected in the inner chamber was tested for antiviral activity by incubating with stock virus (10^6 pfu) with 10 µl (at a concentration of 10 mg/ml) of the flow through for 1 min prior to infecting a confluent monolayer of BSC-1 cells in 6-well plates. A second identical fractionation step was carried out using the filtrate from the first fractionation, but this time using a Centricon YM-3 (3 Kda cut-off) and tested for activity in the same fashion.

2.4.3 – HPLC fractionation

Aim: To identify the number of antiviral agents in the extract. Furthermore, the fractionation behavior of the active agents would provide more insight into their chemical nature regarding hydrophobicity.

The principle of this separation method lies in the relative hydrophobicity of the desired compound with respect to that of the matrix (phenyl-hexyl) and solvent gradient. The more hydrophobic the sample, the later it will elute, since only when the hydrophobicity of the solvent becomes greater than that of the column resin, will the compounds interact more favorably with the solvent than with the column. Acetonitrile is often the organic solvent of choice due to its low viscosity, miscibility with polar solvents and significant hydrophobicity (see appendix B)

For this purpose, a volume of 20 µl of the active < 3Kda fraction was loaded into an analytical Luna HPLC column, containing phenyl-hexyl as the solid-phase phase matrix, 5 µ pore size and 200 × 40 mm (Vydac, Maryland, USA).

The organic solvent acetonitrile (hydrophobic) was used in conjunction with trifluroacetic acid (TFA) to create a gradient of increasing hydrophobicity, using a Kantron® 325 HPLC apparatus (Kantron Instruments, USA) with the UV detector set at 230 nm. The fractionation was carried out over a 60 min period, during which time 0,8 ml fractions were collected at 1 min intervals (in sterile plastic tubes) for the duration of the run for a total of 62 fractions. The fractions were then transferred to sterile 1,5 ml microcentrifuge tubes and dried under a vacuum for 6 h at 55 °C. The dried samples were then resuspended in 15 µl of PBS and tested against 10^5 pfu/ml in 96-well tissue culture plates containing confluent monolayers of BSC-1 cells.

A volume of 10 µl of each fraction was incubated with 10 µl of VV (10^5 pfu/ml) for 60 s prior to infection. Positive fractions with antiviral activity were those that showed complete pfu inhibition.

2.4.4 – TLC fractionation

Aim: To corroborate the results obtained in HPLC fractionation regarding both the number of active fractions and hydrophobicity of such fractions.

TLC separation exploits similar properties as those in HPLC, except that there is no solvent gradient. The positively charged silica matrix will interact via hydrogen bonding (more specifically dipole-dipole interactions) with any permissive substances present in the extract. More hydrophobic samples will migrate further up the sheet, since they interact less efficiently with the polar matrix and more favorably with the hydrophobic solvent.

The resolution of the compounds up the TLC sheet is defined as the retention factor (Rf). The Rf is measured as the distance traveled by the compound divided by the distance of the solvent front and is reproducible as long as the same conditions are used.

This experiment was carried out using silica sheets (Merck, Darmstadt, Germany) 20 cm long and 6 cm in width. A total of 10 µl of SECOMET V was loaded along a horizontal line, 1 cm from the bottom, traced with the aid of a pencil. The loading of the material onto the sheet was carried out with the aid of glass capillaries, spotting small amounts at a time followed immediately by drying with a hairdryer. The line along which the material was spotted was considered the point zero of the solvent front or retention factor zero ($R_f = 0$) and 18 cm above this line, a second line was drawn which indicated the end of the fractionation front ($R_f = 1$, **Figure 2.1**).

Once all 10 µl were loaded, the sheet was transferred to a glass tank containing 70 % acetone, 30 % H_2O at a depth of less than 1 cm. Once the solvent front reach the R_f point of 1, the sheet was removed from the tank, and was allowed to dry at r/t. Once the sheet was dry, it was divided into 18 pieces, each piece 1 cm in length.

The material was recovered from the sheet with the aid of a wooden spatula, which was used to scrap of the silica of each individual sheet. The scraped material was then transferred to microcentrifuge tubes, followed by the addition of 300 µl of water. To aid in the removal of the material from the silica matrix, once water was added, the material was vigorously mixed with the aid of a vortex for 2 min. The silica-free supernatant was then recovered via centrifugation in a bench-top centrifuge set at 14,000 rpm, for 5 min.

Once the supernatant was transferred to sterile microcentrifuge tubes, the water was removed in a vacuum-drier set at 55 °C for as long as it took to completely dry the suspended material (usually 24 h). The dried material was then resuspended in 100 µl of PBS and assayed for antiviral activity by incubating 10 µl of each fraction with 10 µl of virus (10^5 pfu/ml) for 60 s prior to infecting BSC-1 cells in 96 well tissue culture plates. Positive fractions with antiviral activity were those that showed complete pfu inhibition.

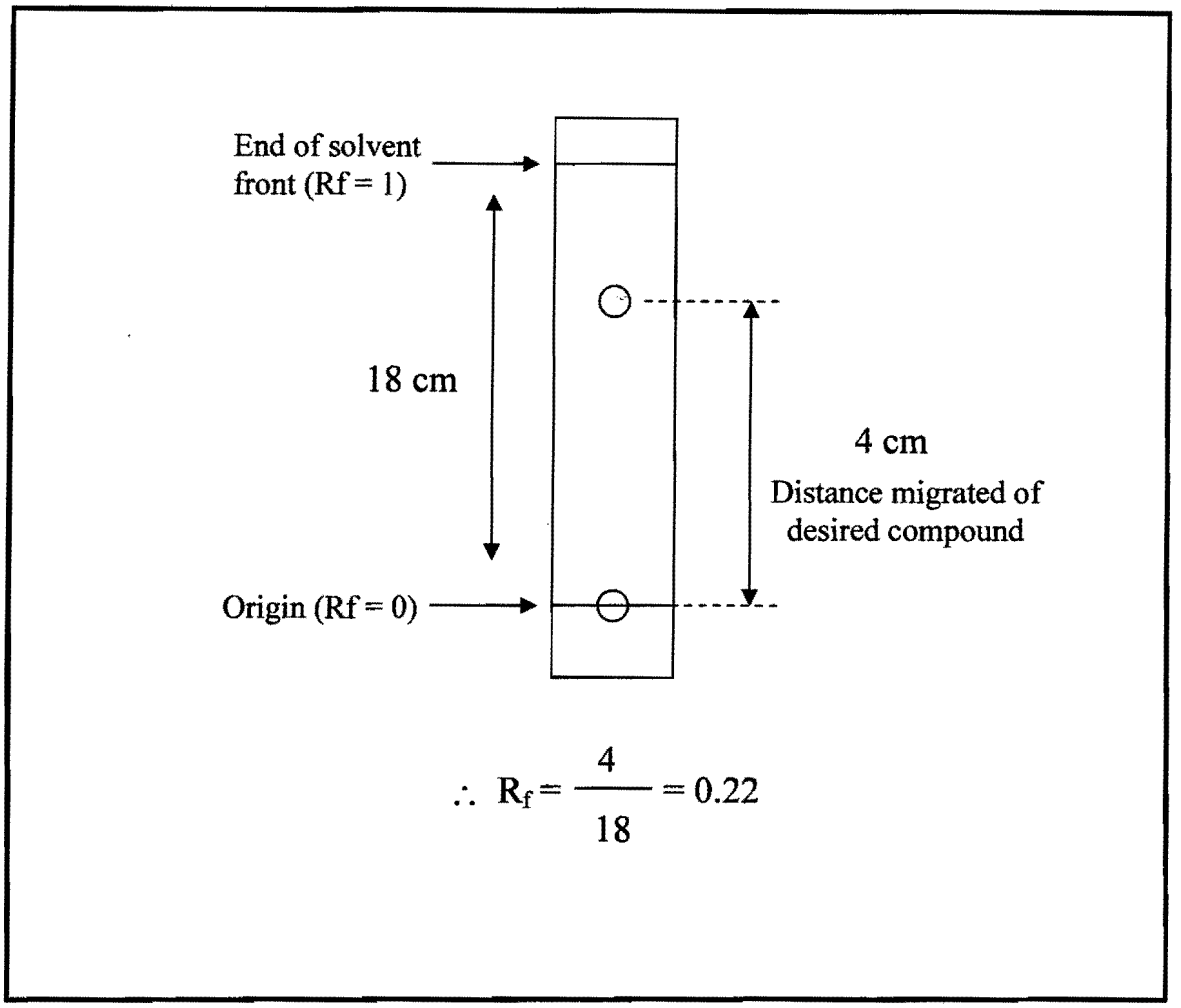

Figure 2.1: Diagrammatic representation of a resolved TLC sheet. The material was spotted at the origin, and once the solvent migrated the pre-determined distance (18 cm, $R_f = 1$), the sheet was probed for the desired compound. In this example, the compound migrated 4 cm from the origin, thereby having an R_f of 0.22.

Chapter Three

3.0 – RESULTS

3.1 – Determination of the dry weight of SECOMET V

Briefly, 6 microcentrifuge tubes were weighed (**Table 3.1**). A volume of 200 µl of the crude extract was dispensed into half of the microcentrifuge tubes. In parallel, an equal volume of the 3 Kda size-fractionated extracted was added to the remaining 3. After recording the weight of the tubes + extract, the samples were placed in a drier for 8 h. Once the time elapsed, the tubes were re-weighted and placed again in the drier for a further 8 h. If there was no change in weight from the first to the second drying cycle, the samples were considered dry.

The actual dry weight of the samples was obtained by subtracting the weight of the samples prior to drying, with that of the dried samples. This then allowed for the calculation the concentration of the samples, expressed in mg/ml. The concentration of the crude material was 22,0 mg/ml whereas that of the < 3 kda fraction was 10 mg/ml (**Table 3.1**).

Table 3.1: Determination of the dry weight of the crude and <3 Kda extract material. The individual weight of each tube was recorded prior to adding the liquid samples. Two identical drying cycles were carried out to ensure complete removal of the carrying medium.

	Weight (mg)					
Fraction	Tube	Tube + dry sample 1st cycle	Tube + dry sample 2nd cycle	Dry	Average	Conc. (mg/ml)
Crude	940,0	945,0	945,0	5,0		
	939,0	943,0	943,0	4,0	4,33	22,0
	942,0	947,0	947,0	5,0		(±2,8)
< 3 Kda	1138,0	1140,0	1140,0	2,0		
	1144,0	1146,0	1146,0	2,0	2,0	10,0
	1134,0	1136,0	1136,0	2,0		

3.2 – Investigation of the anti-VV properties of the extract. Inhibition of adsorption or replication: A diagnostic-type assay

When the extract was received, it was necessary to determine if it inhibited cell-free virions or it interfered with viral replication. For the former, 10 µl (220 µg) of the crude extract was incubated with 10 µl of VV (10^8 pfu/ml) for 60 s prior to infection (assay A). In a parallel experiment, the monolayer was first infected with 10 µl of VV at a titer of 10^4 pfu/ml. An equal volume of extract (20 µl, 440 µg) was then added, 90 min later, once virus had adsorbed (assay B). The absence of pfu in A and presence in B indicated that the extract was exerting its antiviral effect prior to the adsorption event, neutralizing cell-free virions. Based on these findings, all further assays would be carried out by incubating the virus with the extract prior to infection rather than adding extract post infection.

3.3 – Cytotoxicity assay: Determination of CC_{50} and working dose

To shed further light into the properties of the extract, it was necessary to establish a working dose by doing a cytotoxicity study.

For this study, 96-well rather than 6-well plates were used. Starting with an initial concentration of 2200 µg/ml, the extract was diluted in 220 µg/ml increments for a total of 11 dilutions. The cells were exposed to the extract for a period of 24. Following this, the number of viable and non-viable cells were scored using the trypan blue dye exclusion method. The concentration of the extract that reduced cell viability by 50 % was determined from the graph as 560 µg/ml ±70 (**Figure 3.1**). Based on these findings, all further work was carried out at concentrations below the CC_{50} value of 560 µg/ml.

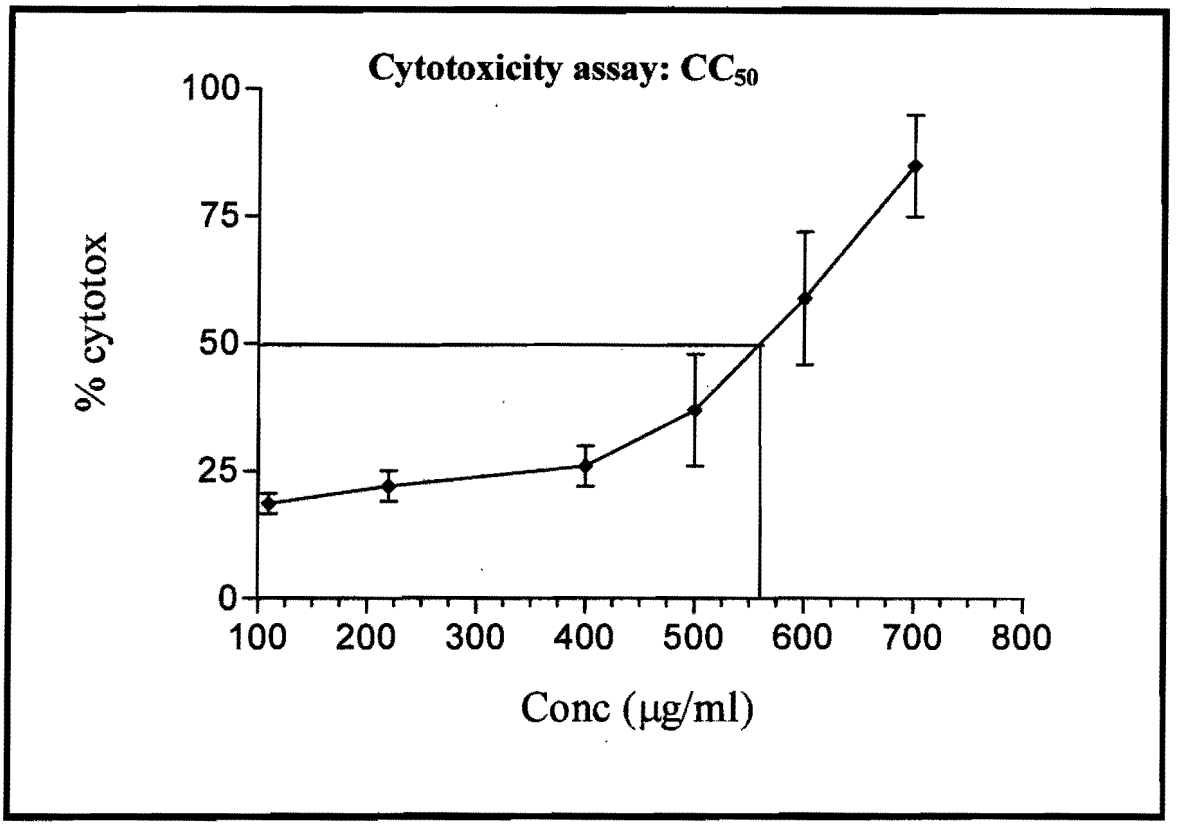

Figure 3.1: Effect of varying concentrations of the extract in cell viability. Confluent monolayers of BSC-1 cells ($\pm 10^5$ cells/well) in 96-well plates were treated with varying concentrations of extract or PBS as a control. After 24 h of incubation the overlay medium was discarded and the monolayer was washed twice with 100 µl of PBS, rinsed once with 20 µl of trypsin, and the cells were brought into suspension with an equal volume of trypsin following a 20 min incubation at 37 °C, 5% CO_2. Cells were then incubated with an equal volume of trypan blue for 5 min and scored under an inverted microscope at a ×20 magnification. The CC_{50} was calculated from the curve as 560 µg/ml ±70. (Results were an average of 3 independent experiments).

3.4 – EC_{50} assay.

Once it was determined that the virus had to be incubated with the extract prior to infection, a more qualitative assay was carried out. For this purpose, 10 µl of a fixed number of pfu (10^8 ml) were incubated with decreasing concentrations of extract for 60 s prior to infection in 96-well plates. Following a 2 day incubation period, the cells were

stained and number of pfu was counted (**Figure 3.2**). The EC_{50} value is the universal method to assess the strength of an antiviral agent.

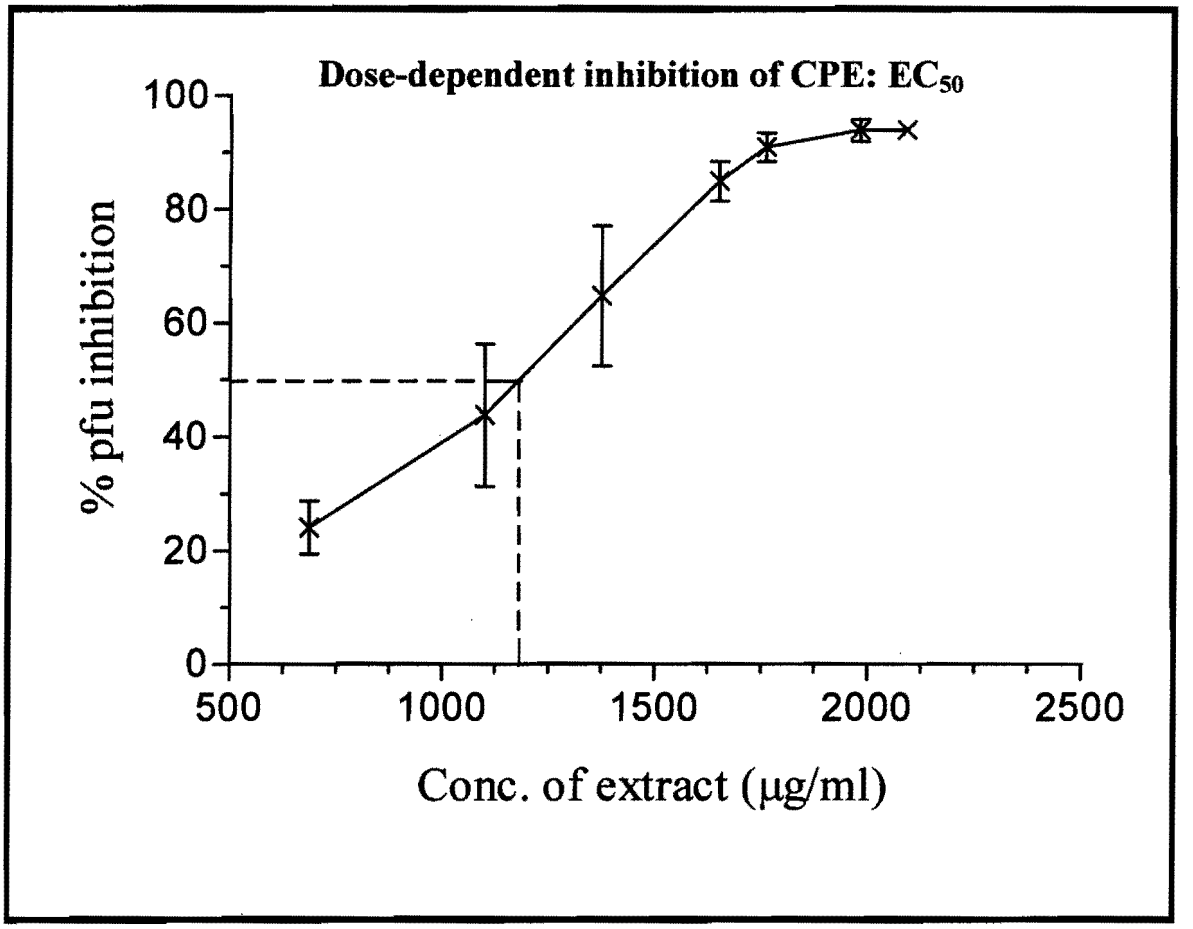

Figure 3.2: Dose-dependent inhibition of CPE. Vaccinia virus (10^8 pfu/ml) at a volume of 10 µl was incubated with decreasing concentrations of the extract, in triplicate, for 60s prior to infection. Following a 48 h incubation period, the cells were stained with crystal violet and the number of pfu was counted. The EC_{50} of the extract was extrapolated from the graph as 1180 µg/ml ± 150. Controls included untreated virus and virus treated with PBS.

3.5 – Inactivation kinetics

The kinetics of inactivation of the extract was assessed by measuring the time-dependent inhibition of pfu (**Figure 3.3**). For this purpose, the final concentration of the extract was

fixed at 1180 µg/ml (the EC_{50}) and the incubation time was manipulated. Five hundred µl of extract was incubated with an equal volume of VV (10^8 pfu/ml) for a final concentration of 1180 µg/ml. A sample of 20 µl was taken every min for 10 min and used to infect confluent monolayers of BSC-1 cells. There was a 50 % reduction in the number of pfu after 60 s of incubation. After 6 min of incubation, no evidence of cytopathic effect was observed.

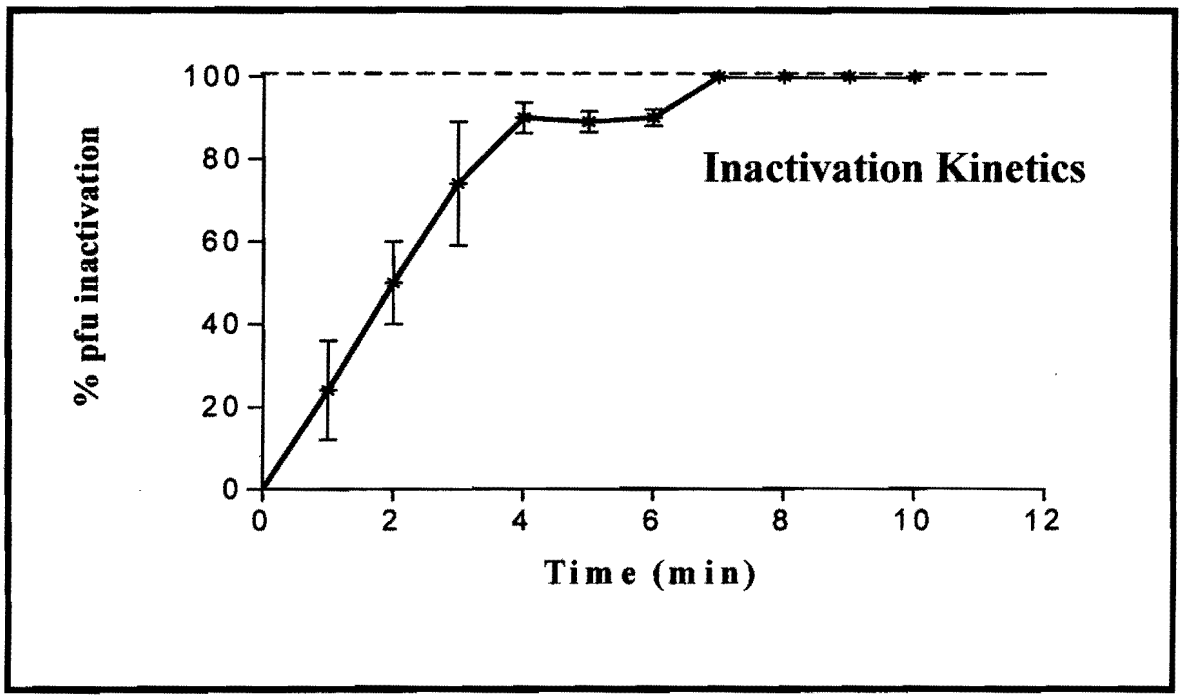

Figure 3.3: Time-dependent inhibition of CPE. A volume of 500 µl of virus (10^8 pfu/ml) was incubated with an equal volume of extract at a final concentration of 1180 µg/ml. At different time points (1-10 min, at 1 min intervals) the monolayer was infected with extract-treated virus, in triplicate. As a control, virus was incubated with an equal volume of PBS prior to infection. Following a 48 h incubation period, the cells were stained with crystal violet for 1 h and the number of surviving pfu was counted. The rate of inactivation was calculated by dividing the number of pfu in test wells by that of PBS treated VV in control wells. Half of the virus was inactivated in the first minute and no evidence of cytopathic effect indicative of virus replication was detected after 6 min of incubation.

3.6 – Inhibition of cell-associated virus

In this study, BSC-1 cells were infected with VV at a MOI of 0,01. Following 24 h of incubation, the cells were harvested. These cells now contained mature, infectious virus particles. To minimise the number of cell-free virus particles, the cells were washed 3 times with PBS. The harvested cells were then used in infectivity assays. By exposing the cells to increasing concentrations of antiviral for 10 min prior to infection, the effectiveness of the extract was assessed in neutralizing cell-associated virus and interfering with viral infection from infected to uninfected cells. It was clear from the results that the extract had no impact on either cell-bound virus or transmission from infected to uninfected cell (**Figure 3.4**).

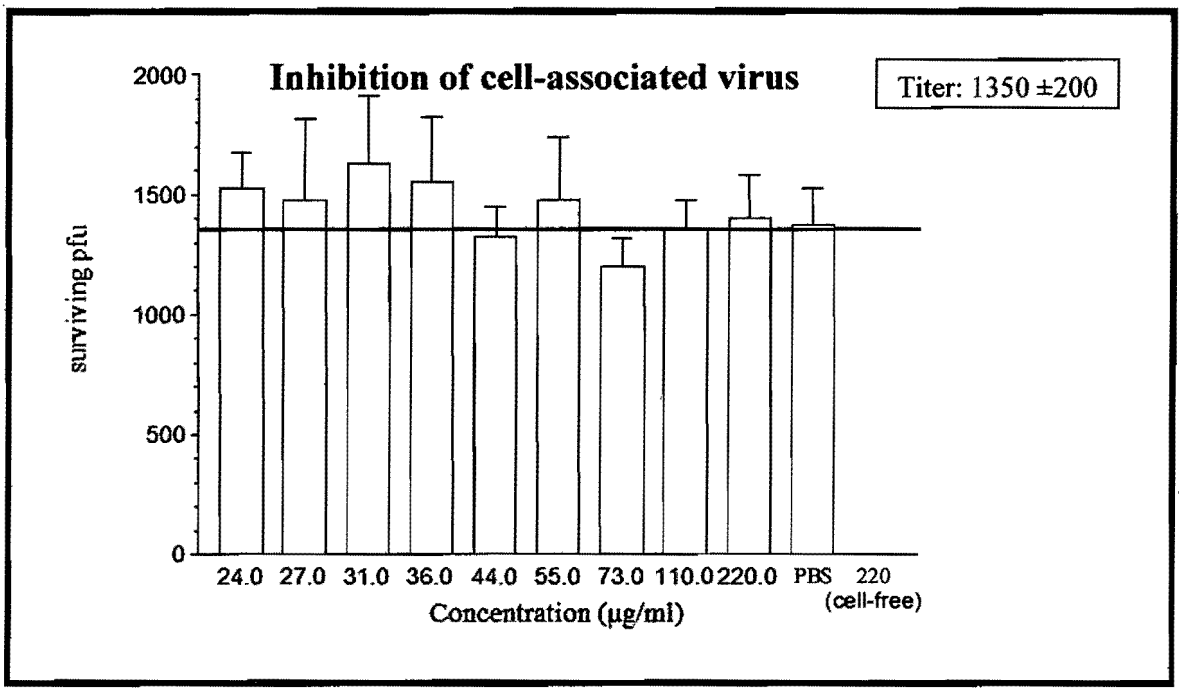

Figure 3.4: Inhibition of cell-associated virus. Cell-associated virus was prepared by infecting a cell monolayer in a 25 cm^2 flask with 3×10^4 pfu (MOI of 0.01) for 24 h. The cells were then harvested by scrapping and resuspended in 1 ml of fresh, pre-warmed MEM. From this stock of cell-associated virus, a volume of 10 µl was used and incubated with an equal volume of difference concentrations of extract for 10 min prior to infecting BSC-1 cells in 96-well plates. As a control, cell-associated virus was disrupted with two cycles of freeze-thawing prior to treatment

with 220 µg/ml. Also, as part of the control, the cells containing virus were treated with an equal volume of PBS prior to infection. The virus titer was determined by infecting cell monolayers with untreated cell-associated virus.
Following 48 of incubation, the wells were individually stained with 30 µl of crystal violet and the number of pfu was counted. The graph illustrates the number of pfu that survived treatment (y axis) with a given concentration of extract (x axis).

3.7 – Neutralization of the antiviral properties of SECOMET V with BSA

For this purpose, an 8 % BSA solution was prepared in PBS and added equal volumes of the solution and the extract. Following a 1 h incubation period at r/t 10 µl of the solution was mixed with 10 µl of VV (10^8 pfu/ml) for 10 min prior to infection. As controls, VV was incubated with either PBS alone or 8 % BSA in PBS. It was observed that neither the presence of BSA nor PBS negatively impacted on virus infectivity indicated by the presence of 10^8 pfu/ml on both assays. As a positive control, the virus was incubated with the extract for 10 min prior to infection. The absence of plaques indicated a virucidal effect exerted by the extract. However, when extract was treated with BSA, it lost its ability to neutralize VV, as indicated by the presence of an equal virus titer (10^8 pfu/ml) in both test and control wells.

3.8 – Heat inactivation of SECOMET V

When SECOMET V was tested for activity after being exposed to a temperature of 121 ° C and a pressure of 15 at for 30 min, it was observed that it had not lost a measurable amount of activity. It completely inhibited plaque formation when tested against 10^8 pfu/ml in 6-well plates. These results were a clear cut indication that exposure of the extract to conditions that would otherwise inactivate the activity of proteins, did not affect the antiviral activity of SECOMET V to a measurable degree.

3.9 – Activity-guided size-exclusion fractionation

This assay was carried out to gain some insight into the nature of the antivirals. A volume of 10 µl of the size-fractionated extract was incubated with an equal volume of stock virus (10^8 pfu/ml) for 60 s. After the incubation, BSC-1 cells in 6-well plates were infected with the virus/extract stock. As controls, virus was incubated with dH_2O instead of extract or an equal volume of crude, unfractionated extract, prior to infection. The absence of plaques in the < 10Kda and < 3 Kda fractions indicated that the antivirals were smaller than 3 Kda in size.

3.10 – HPLC fractionation

Analytical fractionation of SECOMET V using a hydrophobic phenyl-hexyl solid-phase resin and acetonitrile-TFA in a gradient of increasing hydrophobicity as the liquid-phase solvent, yielded two active fractions that eluted after 42 and 56 min respectively. The time-gap of elution between the two active samples indicated that they were different compounds; the one eluted first being more hydrophilic than the second. Furthermore, the active compounds did not absorb light at a wavelength of 230 nm, as observed by the absence of distinct peaks at the time of elution of the antiviral agents (**Figure 3.5**). All other samples showed no measurable activity against VV, as observed by the presence of an equal number of plaques in all of the remaining wells when compared to control, untreated wells.

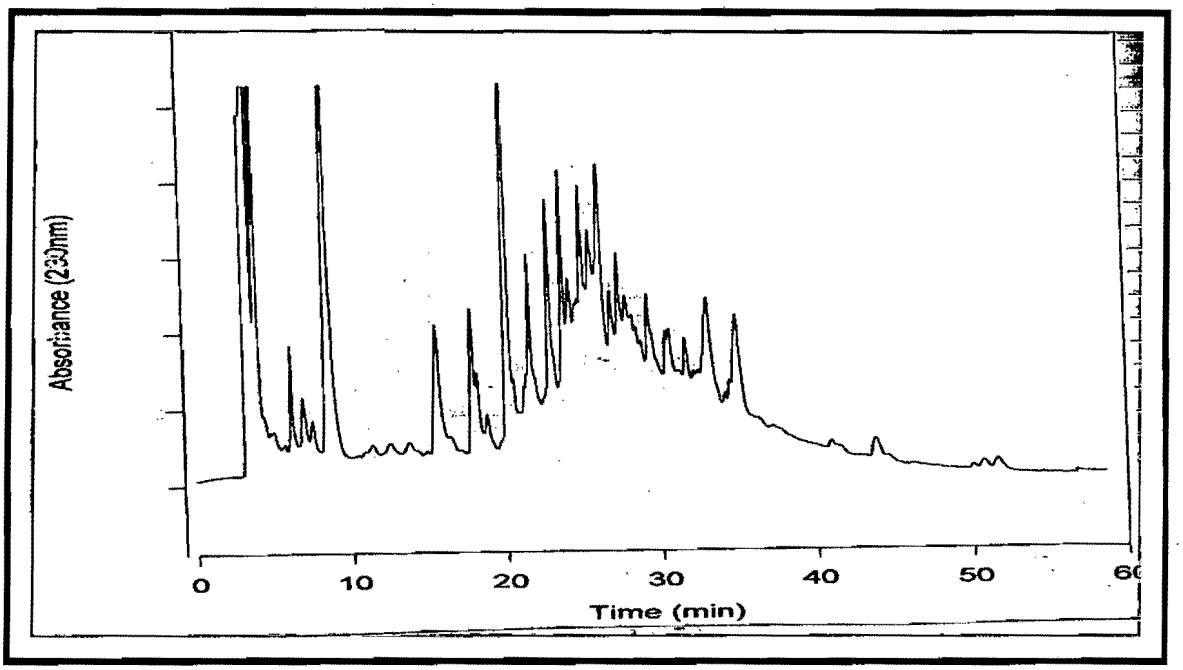

Figure 3.5: HPLC fractionation of SECOMET V. Using an analytical phenyl-hexyl column as the solid-phase matrix and an acetonitrile-TFA gradient of increasing hydrophobicity, SECOMET V was fractionated in a time-dependent fashion over 60 min. Fractions were collected in 1 min intervals (0,8 ml/fraction) for a total of 62 fractions. The fractions were dried under vacuum at 55 °C for 24h. The precipitated material was then resuspended in PBS and tested for activity as described (Chapter 2, section 2.3.3) against 10^5 pfu/ml. Two active fractions were obtained at 42 and 54 min respectively.

3.11 – TLC fractionation

The resolution pattern of SECOMET V on silica sheets using 70 % acetone as the solvent, yielded 2 active fractions out of a total of 18 tested. The first active fraction was recovered less than 1 cm from the start of the solvent front (R_f value between 0,0-0,05), whereas the second antiviral resolved a further 5 cm up the silica sheet (R_f between 0,33-0,38). This clearly indicated the presence of at least 2 antiviral agents, which could be fractionated based on hydrophobicity. The fraction that migrated the furthest (R_f between 0,33-0,38) was the least polar of the two.

Chapter Four

4.0 – DISCUSSION

4.1 – Determination of the dry weight of the extract

The plant extract (SECOMET V) was produced in a bioreactor from primary plant stem cells. It was supplied in liquid form, and the dry weight was not known. It was necessary to determine the dry weight in order to generate quantitative data that could be independently verified.

The reason why two identical drying cycles were used was to ensure that there was complete removal of any trace solvent. If there had been any further drop in weight following the second drying cycle compared to the first, the sample would be dried as often as needed until there was no detectable variation in weight. This clearly did not happen as illustrated in table 3.1.

Once it was established that the dry weight was 22 mg/ml, the remainder of the batch (approximately 30 ml) was carefully labelled and stored to avoid confusion with other batches received later. All work was then carried out using this batch exclusively, since the degree of activity varied from batch to batch (data not shown).

4.2 – Investigation of the anti-VV properties of the extract. Inhibition of adsorption or replication: A diagnostic-type assay

In order to further characterize the plant extract, it was crucial to have some insight into its mode of action. There were two possible hypotheses: it could either interfere with viral replication or it could neutralize cell-free virions prior to infection. The extract could also have exerted its activity at cellular level, either by blocking cell-surface receptors

necessary for virus entry or it could induce an antiviral state within the cell, thereby interfering with virus replication.

Firstly, the attention was focused on direct interference with the virus. The results showed that the extract disrupted cell-free virus rather than interfering with the replication machinery. Had the extract had an impact on the replication cycle of VV, a drop in the number of pfu would have been observed.

4.3 – Cytotoxicity assay: Determination of CC_{50} and working dose

This was a quantitative study on the impact of the extract on cell viability. It is known that any antiviral agent, regardless of how it exerts its effect, at a given concentration has a negative effect on the host cells. By determining the concentration at which a given antiviral agent becomes toxic to the BSC-1 host cells, an *in vitro* working dose can be established.

The cytotoxicity index or CC_{50} refers to the concentration of the test substance required to kill 50 % of a given cell population. Once this value was determined at 560 µg/ml ±70, all further work was carried out at concentrations below this value. This was because if more extract was used than what the cells could tolerate, no useful data could be extracted simply because the cells would not be viable. Furthermore, it is important to point out that the CC_{50} value is inversely proportional to the degree of toxicity incurred by the antiviral agent tested. That is, the higher the CC_{50} the lower the toxicity effect of a given substance, because the cells can tolerate higher concentrations of the agent before being adversely affected.

The calculated CC_{50} value for the extract was not a direct reflection of the true CC_{50} value of the active antiviral agents. In view of the fact that the extract was a heterogeneous mixture of several dozen, probably hundreds of different compounds, the CC_{50} value of the individual antivirals could be much lower than 560 µg/ml. On the other

hand it could also be higher, since there may be substances other than the antivirals that are quite noxious to mammalian cells, even more so than the antiviral agents.

It is widely known that tannins are very ubiquitous in the plant kingdom and also have broad-range activity against viruses, prokaryotic and eukaryotic pathogens; however, they are also somewhat toxic to mammalian cells (reviewed in Cowan MM, 1999). Based on this knowledge, it was reasonable to speculate that some of the toxicity exhibited by the extract may be due to the effect of tannins and other cytotoxic substances. On the other hand one cannot neglect the likelihood that other cytotoxic agents could have been less toxic than the antiviral/s. In this case, the CC_{50} of the purified antiviral/s would have been lower.

The cytotoxicity value of the SECOMET V extract was within the range reported for other extracts showing antiviral activity against other viruses. One of the least toxic plant extracts which exhibited antiviral activity against human herpes simplex virus 2 (HSV-2) was *Clinacanthus nutan*, which presented a CC_{50} value of 6,67 mg/ml (Yoosook *et al.*, 1999). On the other hand, one of the most toxic plant extracts appeared to be that reported for *Rhus javanica* which had a CC_{50} of 130 µg/ml and was active against herpes simplex virus type 1 (Kurokawa *et al.*, 1995).

4.4 – EC_{50} assay

The significance of the information provided by this study was to determine the strength of the extract as well as to carry out *de facto* comparisons with other plant extracts.
As previously stated, the EC_{50} was a measurement of the concentration of extract that inhibited plaque formation by 50%. Furthermore, like the CC_{50}, the EC_{50} was inversely proportional to the rate of inhibition.

As previously stressed, the discrepancy between the EC_{50} (1180 µg/ml) and the CC_{50} (560 µg/ml,) was due to the way in which the extract exerts its antiviral effect. By

incubating the antiviral with the extract in a total volume of 20 µl prior to infection, the actual concentration to which the virus was exposed was 5 times greater than what the cells were exposed to. This was because, once the extract/virus suspension was added to the cells, the final volume was 100 µl, effectively diluting the extract below the CC_{50} value.

The strength of the antiviral was quite high. A comparison between the extract (EC_{50} = 1180 µg/ml) and a known anti-VV agent such as cidofovir EC_{50} = 5 µg/ml) may indicate otherwise. However, two points must be considered when comparing these two antivirals: firstly, cidofovir was tested in its pure form as a homogenous solution, whereas the plant extract was a heterogeneous mixture of several dozen, probably hundreds of other unknown substances, greatly adding to the overall dry-weight of the solution; secondly, most antiviral agents are tested against a comparatively lower virus titer. Such titer usually ranges between 10^2 and 10^4 pfu, as opposed to 10^6 used in this study. This was a very important difference, as a higher virus titer leads to a higher EC_{50} value, since more antiviral would be required to neutralize more pfu. Had a lower titer been used, the EC_{50} would have been equally lower, as clearly described by Baker *et al.* (2003).

When comparing these results to those reported by Rao *et al.* (1969), the potency of SECOMET V becomes even more apparent. In their study, they used 10^6 pfu and incubated it with 100 µl of a 10 % solution of the Margosa tree leaves for 1 h prior to infection. They succeeded in reducing virus titer by 2 logs. On the other hand, a 2,2 % solution of SECOMET V reduced virus titer by more than 6 logs , indicating that it was at least 10^4 orders of magnitude stronger than that of the Margosa tree leaf extract reported by Rao's group.

Semple *et al.* (1998) carried out a comprehensive screening of Australian medicinal plant for activity against a range of unrelated viruses. They used approximately 20 pfu, and reported EC_{50s} ranging from 6 µg/ml to 250 µg/ml depending on the virus and plant. This was nearly 10^5 fold lower than what was used to characterize SECOMET V.

However, the process of comparing the strength of anti-VV agents across independent studies was not very accurate, since there was no standard way set to determine the EC_{50}.

In light of the arguments presented, it can be safely stated that the plant extract SACOMET V was a very potent inhibitor of VV and further work should be pursued to identify the active agents so that more studies that are more comprehensive can be undertaken.

4.5 – Inactivation kinetics

When the rate of VV inactivation was measured in a time-dependent fashion, it was observed that the 60 s incubation period that was used to establish the EC_{50} value did not reflect the true extent of VV inactivation upon exposure to the extract. In reality, had VV been allowed to incubate for longer than 60 s, the EC_{50} value would have been significantly lower, since 1180 µg/ml completely inactivated 10^6 pfu after 6 min of exposure prior to infection (**Figure 3.4**). Furthermore, had a longer incubation period been coupled with a lower virus titer (in the order of 10^3 pfu) the EC_{50} value would have been significantly lower.

The results obtained from the inactivation kinetics study provide more weight to support the statement that the extract SACOMET V was indeed a very strong inhibitor of VV infection.

4.6 – Inhibition of cell-associated virus

As the results of this study clearly illustrate, the plant extract had absolutely no effect whatsoever in intracellular mature virions (**Figure 3.5**). In order for the extract to have had any effect on intracellular virus, the antivirals had to be taken up by the cell. This either did not happen or the cell could have taken it up (possibly by pinocytosis) and

converted it into inactive metabolites. But because the structure of such antivirals were not known, it was not possible to draw any conclusions regarding what happened to the antiviral/s if they were taken up.
This was the downside of the study, since one of the mechanisms of VV infection was via infection of neighboring cells, without leaving the intracellular milieu (**Figure 1.3**, step 10).

These results give even more weight to the theory that the extract exerts its virucidal effect by interfering with cell-free virions prior to adsorption.

4.7 – Neutralization of the antiviral properties of SECOMET V with BSA

The extract was found to inhibit tobacco mosaic virus (TMV), a plant pathogen that infects a wide range of plants (personal communication, Stephen Leivers). The host in this case, belongs to the plant kingdom as opposed to that of VV, whose host belongs to the animal kingdom. This clearly indicated that the antiviral was not specific to VV, but rather it had a broad spectrum of activity.

With these in mind, the rationale in designing the experiment was that the only common denominator between these two completely unrelated viruses was the fact that they were made up of proteins. Therefore it was plausible to postulate that the antivirals could bind non-specifically to proteins.

The observation that the presence of BSA completely inactivated the antiviral activity of the extract corroborated the initial theory.

It was observed that when BSA was added to the extract, a yellow precipitate formed almost immediately. This was probably attributed to the precipitation of BSA on contact with the antiviral.

Based on this, it was postulated that the precipitation may be due to the fact that the antivirals have both hydrophilic and hydrophobic groups, with the polar groups allowing for their solubility in an aqueous environment.
However, it the presence of proteins, which have a very hydrophobic core, the antivirals induced their denaturation by coupling with their hydrophobic cores, removing both substances from solution, explaining the formation of an insoluble, inert precipitate.

4.8 – Heat inactivation of SECOMET V

It was clear that the antiviral agents were resistant to both high temperatures under pressure, conditions that would otherwise denature any proteins. However, non-protein antiviral agents are quite stable under such conditions (reviewed in Jassim and Naji, 2003). It is equally important to stress the fact that SECOMET V was never refrigerated or protected. Even under these harsh conditions, no loss in activity with time could be detected, since the EC_{50} assays were performed over the course of one year.

4.9 – Activity-guided size-exclusion fractionation

Again, these assays were qualitative assays, in that the goal was to determine whether or not the active antiviral compound in the extract was smaller than 3 Kda. Any substance below this value was unlikely to be a protein, since it would have to be less than 27 amino acids in length, a size below that of the minimum folding threshold (Stryer L, 1996). Furthermore, of all antiviral and anti-microbial agents reported to date, only two were proteins, well in excess of 50 amino acids in length (reviewed in Cowan MM, 1999). Substances other than proteins with antiviral activity are usually below 3 Kda in size.

4.10 – HPLC fractionation

The presence of two active fractions eluted 12 min apart, provided information regarding the number and relative hydrophobicity of the active agents. There clearly were at least two distinct anti-VV agents, the most polar being eluted first and the least polar 12 min later. The phenyl-hexyl column matrix and the TFA-acetonitrile gradient used proved to be very effective in separating the antiviral agents in the extract. Nonetheless, it was somewhat surprising to obtain such good separation with a hydrophobic resin, seeing that the extract is suspended in water. Furthermore, the late elution of both agents suggests an even higher degree of hydrophobicity.
Clearly, the antivirals must have a hydrophilic and a hydrophobic group, since they were soluble in water, but interacted strongly with the column matrix.

The relative strength of each antiviral could not be established because the amounts recovered from the analytical column were too small for more comprehensive studies. A preparative column that would bind more material would have been required in order to measure the EC_{50} of each active fraction. However, it could be simply argued that this barrier could have been overcome had several identical fractionation cycles been carried out and the active fractions pooled. In theory this could have been done, but after careful consideration, it was decided that this was not feasible.

No further information could be extracted from this study, seeing that no peaks were present at 230 nm at the time of the elution of the active fractions (**Figure 3.5**). One other drawback was that the detector used only had a single diode, which did not allow for the use of more than one wavelength. Furthermore, the fractions recovered were so small that there was not enough of it to carry out a scan in a separate spectrophotometer.

However, the HPLC protocols developed for this study could be used as a spring board for further fractionation and ultimately, isolation of the active agents in SECOMET V.

4.11 – TLC fractionation

Thin layer chromatography also yielded 2 active fractions, perfectly corroborating the results obtained in the HPLC fractionation. However, in this case the stationary phase was very polar (silica) as opposed to the hydrophobic column (phenyl-hexyl) used in HPLC.

The solvent of choice was random, but with emphasis on a predominantly hydrophobic solvent, since in HPLC the antivirals eluted under hydrophobic conditions (see chapter 3, section 3.11) and also because it decreases the resolution time. Furthermore, it is important to point out that TLC was attempted with other solvents as well, but the one that yielded the best results (70 % acetone) was the one reported in this study.

The fact that one of the antivirals barely migrated up the sheet (chapter 3, section 3.12), indicated the presence of a very hydrophilic group. This could possibly have accounted for the strong dipole-dipole interaction. Even the second antiviral that migrated 1/3 of the way was likely to have significant polar groups. The further they migrated, the least polar they were.

TLC is often used as a quick method to determine the best resolution conditions prior to HPLC fractionation. However, in both cases, very good separation of the antivirals was obtained with either method.

4.12 – Concluding remarks

The characterization of the anti-orthopoxvirus properties of SECOMET V culminated with the knowledge that it was a very strong and possibly non-specific inhibitor of cell-free virus. Furthermore the antiviral effects can be attributed to the presence of at least two autoclaving-resistant agents, which were not proteins in nature and could be readily discerned by their chromatographic behavior.

The seemingly lack of specificity opens up the possibility that SECOMET V could be active against other, more clinically relevant pathogens such as: human immunodeficiency virus (HIV), human herpesvirus (HSV-1 & 2), hepatitis B and C virus (HBV & HCV) and rotavirus (leading cause of diarrhea-associated deaths in African children) to name a few. Other pathogens, including bacteria and eukaryotes such as fungus and parasites should also be included, particularly *Plasmodium spp* (causative agent of malaria). Furthermore, the fact that many plant extracts are active against unrelated viruses gives further weight in the argument that SECOMET V could be active against other viruses, and therefore should be tested against such pathogens.

However what should take priority is the isolation and identification of the active agents, to ensure that such agents are novel; to avoid working on something that has already been characterized. On the other hand, according to the supplier SECOMET SA, the antiviral properties of the extract SECOMET V have never been documented.

It was established that this could be achieved with different conditions. HPLC would be the method of choice to separate and isolate the antivirals, since it gives better resolutions and allows for much bigger sample volumes.
The column of choice would be silica, simply because it is many times cheaper than phenyl-hexyl. Acetone is also cheaper than acetonitrile, and lower material and production cost would reflect in a cheaper, more affordable end product.

In the meantime, it would be very informative to carry out some *in vivo* mouse work. One could compare how mice responded to oral treatment with the extract in lethal and non-lethal mice models, using VV. Accentuated differences between control and test mice would certainly fuel more in depth studies. It would also provide much needed information regarding dose and toxicity, vital data if the extract is to be used in small-scale human trials.

Knowing that SECOMET V has been used in folklore medicine to treat humans for many generations, already provides some evidence suggesting that it may be safe for human

consumption. However, one of the main goals of the company is to use this extract in HIV/AIDS patients, thus providing a source of cheap and possibly effective anti-retroviral agents. Some work is currently being undertaken in this regard, and the results so far were extremely encouraging.

HIV/AIDS is a chronic illness requiring patients to take antiretrovirals for life (reviewed in Richman, 2001). This fact will be no different for the plant extract. However, continuous, non-stop consumption of the extract could lead to serious side-effects, as happens with all antiviral agents in clinical use to date (reviewed in De Clercq, 2004). The observation that the antivirals from the extract seem to bind to and denature proteins, further strengthens this argument. If such results are a true reflection of what happens *in vivo*, then continuous non-stop usage of the extract could prove deleterious. Furthermore, traditionally the extract was only used for the duration of time required to treat a specific illness. Therefore, it may be safe for therapy lasting a limited time, but no empirical information could be obtained regarding safety for long-term therapy.

According to the supplier, the production cost was relatively low, and much more could be done to significantly lower these costs even further. The cost factor is very important in the African continent, often restricting the use of life-saving drugs to an elite few.
If this product is to benefit those in desperate need, than it must be cheap enough so that the impoverish nations our continent can afford them.

Empirical evidence suggests that SECOMET V can be taken orally. Furthermore, the finding that neither high temperatures nor light have a detrimental effect on the activity of the extract, further lowers production and delivery costs, since it eliminates the need for refrigeration at any step along the manufacturing process.

However, in light of all the information obtained with these series of studies, it is imperative that more work should be carried out both *in vitro* and in animal models before setting up small-scale human trials.

Appendix A

Media and reagents used:

BSC-1 tissue culture medium (MEM, 10% FCS and antibiotics)

- 500 ml MEM;
- 50 ml FCS;
- 5,5 ml antibiotics (100 X solution).

Crystal violet staining solution

- 80 ml PBS;
- 10 ml crystal violet solution (0,01 % in water);
- 10 ml methanol

70% acetone for TLC

- 70 ml acetone;
- 30 ml water.

8% BSA solution

- 800 mg BSA;
- Tissue-culture grade PBS up to 10 ml

Appendix B

Relative hydrophobicity of organic solvents:

HPLC/TLC reference table of the relative hydrophobicity of organic solvents

Increasing Hydrophobicity (migrate faster up the TLC sheet)

- Alkanes
- Alkenes
- Ethers
- Halogenated hydrocarbons
- Aromatic hydrocarbons
- Aldehydes and ketones (acetone)
- Esters
- Alcohols
- Amines
- Carboxylic acids

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