ANTI-VIRAL ACTIVITY OF QUILLAJA SAPONARIA MOLINA EXTRACTS AGAINST HIV-1 AND HIV-2

by

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ABSTRACT

ANTI-VIRAL ACTIVITY OF QUILLAJA SAPONARIA MOLINA

EXTRACTS AGAINST HIV-1 AND HIV-2

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It has been well established that extracts from the bark of the South American tree *Quillaja saponaria* Molina have the capacity to stimulate strong immune responses against exogenous antigen when used as an adjuvant. However, the anti-viral capacity of Q. saponins has not been well established. We document the anti-viral capacity of *Quillaja saponaria* extracts against HIV-1 and HIV-2. The extract appeared to interact with the gp120 subunit of HIV-1 and HIV-2 to produce a low anti-viral effect by blocking viral attachment. The extract was also observed to coat cells in a time dependent manner and block attachment to CD4 receptors. Furthermore, no lasting

protection was conferred when cells were pre-treated, and the extract containing media removed, the cells subsequently could again be infected with HIV-1 and HIV-2.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Background on HIV

Since the identification of Acquired Immunodeficiency Syndrome (AIDS) in the early 1980's, the epidemic has resulted in more than 20 million deaths worldwide. According to the World Health Organization, an estimated 40 million people are currently living with Human Immunodeficiency Virus (HIV). In the more than 20 years since the identification of HIV, considerable progress has been made in understanding the HIV replication scheme and designing mortality reducing treatment strategies. Nonetheless, the pathogenesis and determinants of endogenous control of viral replication are poorly understood. It is clear that an understanding of these components will be critical in designing new prevention strategies to control or suppress viral replication. New therapeutic agents must also be considered to provide potential resources for prevention of infection as well.

HIV is a member of the lentivirus family which includes retroviruses that exhibit variegated genomes (1). Mature HIV virions have a spherical morphology of 100-120 nm in diameter and are enveloped by a lipid bilayer that protects a truncated, cone-shaped core (2). The lipid bilayer is of host cellular origin and contains two surface glycoproteins, gp41 and gp120, which mediate binding to cellular receptors of CD4+

cells (Fig. 1). A matrix protein (MA, p17) lines the inner surface of the viral membrane and is vital for the integrity of the virion. The capsid protein (CA, p24) comprises a conical core that houses the two identical 9.2 kb single-stranded RNA molecules, viral protease (PR), reverse transcriptase (RT), integrase (IN), and the packaged viral accessory proteins, Nef, Vif, and Vpr (2). Virus particles also contain additional accessory proteins, Rev, Tat, and Vpu that are not packaged and appear to function in the host cell (3). A staggering feature of HIV infection is the extreme within-host genetic variation of the virus. The viral population within one infected person is non-homogenous due to the ongoing appearance of new variants each having its own infection rate. The competition for reproduction in the same cell line leads to a selection and direct evolution of higher cellular infection rates (1). This evolutionary process caused by the highly developed immune evasion strategies, has lead to the challenging search for improved treatment methods and the potential for vaccine development.

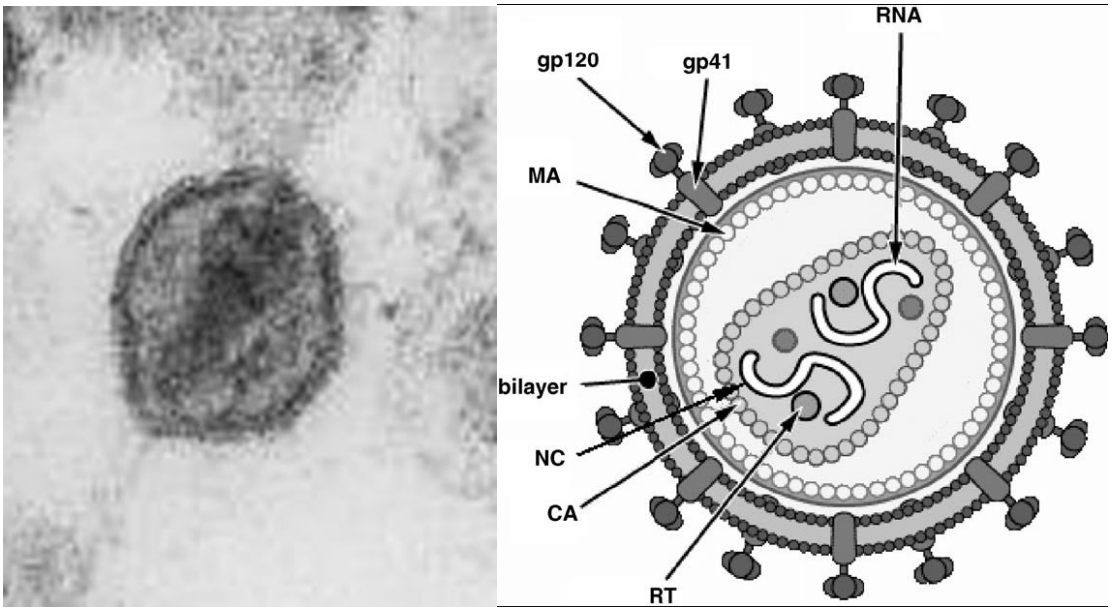

Fig.1. Electron micrograph (13) and diagram of mature HIV virion (2).

HIV needs to establish itself within an uninfected host via an infectious unit that consists of cell-free virus or virus infected cells. The host cells must be infected at the point of entry and disseminate throughout the body and propagate infection in cells at other tissue sites. Three mechanisms for spread of HIV have been proposed. The first mechanism involves the binding of cell-free virus to a host cell through the appropriate receptor interactions, which induces fusion of viral and host cell membranes (18). Secondly, an HIV infected cell can spread infection by cell-to-cell transmission without releasing virions into the extracelllar environment (18). Thirdly, dendritic cells of the immune system could capture virus, without becoming infected, and present the virus to a permissive cell though a synapse (18). No one mechanism has been shown to be dominate, therefore a greater understanding of the modes of infection and pathogenesis must be a priority in developing methods to interfere with attachment and replication.

1.1.1 HIV Replication

As a retrovirus, HIV must complete an ordered sequence of events to establish a successful infection (Fig. 2). The virus binds specifically via interactions between the viral glycoprotein (gp120) and the amino-terminal immunoglobulin domain of the CD4+ cells of the immune system. Prevention of this initial binding is the focus of our research. Following initial binding and fusion with the cellular membrane, the viral core is released into the cytoplasm and uncoated by several cellular factors as well as the viral proteins MA, Nef, and Vif, The viral RNA genome then undergoes reverse transcription to produce a double-stranded DNA molecule that localizes to the nucleus as a pre-integration complex. The pre-integration complex facilitates the integration of the HIV DNA into the host chromosome via the use of integrase (4). After the proviral DNA has been integrated, the initial rounds of transcription occur through RNA polymerase by the binding of cellular factors to the viral long terminal repeats (LTR) (2). Each LTR contains sequences that signal a transcriptional unit. Both segments can serve to initiate transcription, but the U3 segment has the promoter elements and a cap addition site, while the U5 segment contains a poly-A-site (Fig 3). The spliced and unspliced mRNAs are transported out of the nucleus for translation. The spliced HIV RNA transcripts encode the regulatory proteins Tat, Rev, and Nef, whereas the unspliced transcripts are used in Gag and Gag-Pol synthesis. Small amounts of the Gag-Pol precursor proteins then associate with the Gag polyprotein at the cellular membrane to form immature particles which encapsidate two copies of the viral RNA genome (3). In addition, the cellular tRNAlys3 primer and other cellular compounds associate to the

immature core (2). The polyproteins are cleaved to produce MA, CA, and NC structural proteins which combine to form infectious viral particle (Table 1). After assembly of the infectious viral particle, budding occurs and the virion is released from the cell.

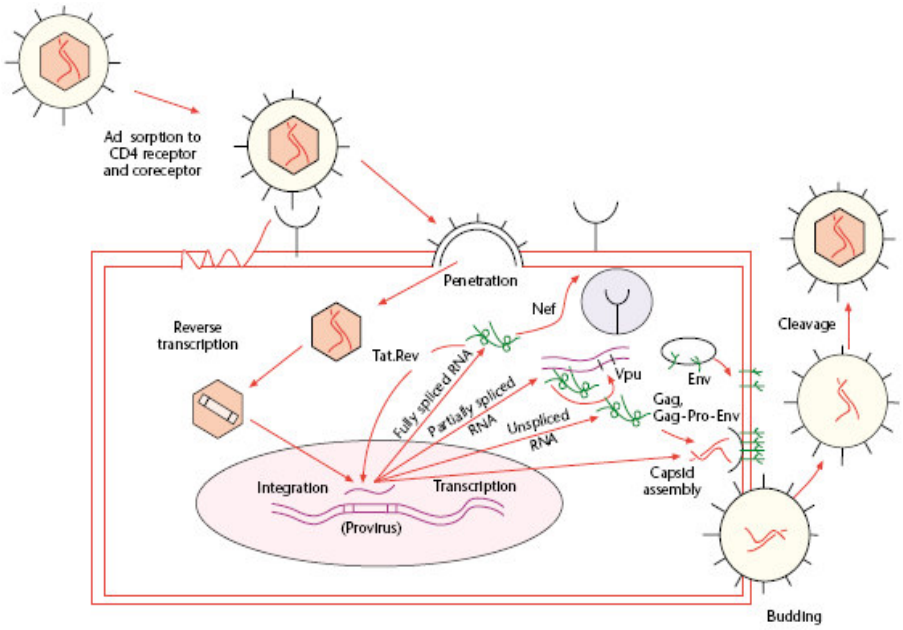

Fig. 2. Replication scheme of human immunodeficiency virus implicating the main steps involved: penetration, DNA synthesis, integration, transcription, processing of RNA, protein synthesis, and assembly and release (14).

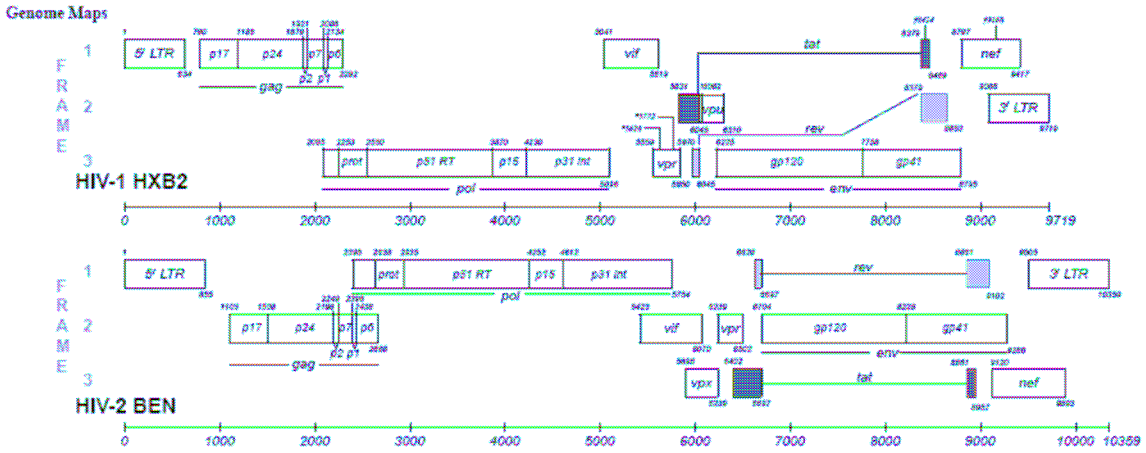

Fig. 3. Genome organization of HIV-1 and HIV-2 (24).

Table 1. HIV proteins and their corresponding size and function. *Indicates characteristics found in retroviruses. ° Indicates characteristics found in lentiviruses that are involved in transcription regulation.

Protein	**Size (kd)**	**Function**
Gag*	p25 (p24) p17	Capsid (CA) Matrix (MA)
Polymerase (Pol)*	p66, p51	Reverse transcriptase (RT); RNAse H
Protease (PR)*	Plo	Post-translational processing of viral proteins
Integrase (IN)*	p32	cDNA integration
Envelope (Env)*	gp120 gp41	Envelope surface protein Envelope transmembrane protein
Tat°	p14	Transactivation
Rev°	p19	Regulation of viral mRNA expression
Nef°	p27	Pleiotropic, virus suppression
Vif°	p23	Increases infectivity of virus and cell-to-cell transmission; assists in proviral DNA synthesis and possible in virion assembly
Vpr°	p15	Virus replication, transactivation
Vpu°	p16	Virus release
Vpx°	p15	Assists infectivity
Tev°	p26	Tat and Rev activities

1.1.2 Specifics of HIV and Cellular Interaction

The HIV envelope glycoprotein (Env) is synthesized in the rough endoplasmic reticulum in the host cell as an amino acid precursor, glycoprotein 160 (gp160). The gp160 molecules are glycosylated in the Golgi system and cleaved to generate the mature envelope glycoproteins, gp120 and gp41 (15). The mature Env contains three gp120 subunits that are noncovalently bonded to gp41 transmembrane subunits and are transported to the host cell membrane where the cytoplasmic tail of gp41 is able to bind to HIV nucleocapsids (16). The budding of virions from the cellular membrane contains the envelope proteins that form mature virus particles.

HIV entry requires the interaction of the gp120 subunit with the primary host receptor CD4 and a co-receptor. The two major HIV co-receptors are chemokine receptors, specifically CXCR4 (X4) and CCR5 (R5). Binding of gp120 causes a conformational change (Fig. 4), resulting in the exposure of a highly conserved surface on Env that is capable of interacting with X4 or R5 (17). The interaction of Env with X4 or R5 largely contributes to the differences in HIV tropism among CD4 positive cells. HIV strains that use CCR5 preferentially infect CD4+ memory T cells and macrophages. However, it has been noted in approximately 50% of infected individuals that the onset of symptomatic disease (AIDS) is preceded by the emergence of HIV strains able to bind CXCR4, increasing the tropism to CD4+ memory and CD4+ naïve T cells (16). In addition, viruses that use R5 are largely responsible for HIV transmission and individuals lacking a functional R5 receptor due to a 32-base pair deletion, are highly resistant to infection. The X4 strains seem to emerge later in

infection and their appearance correlates with a rapid decline in CD4+ cells and a faster progression to AIDS (18).

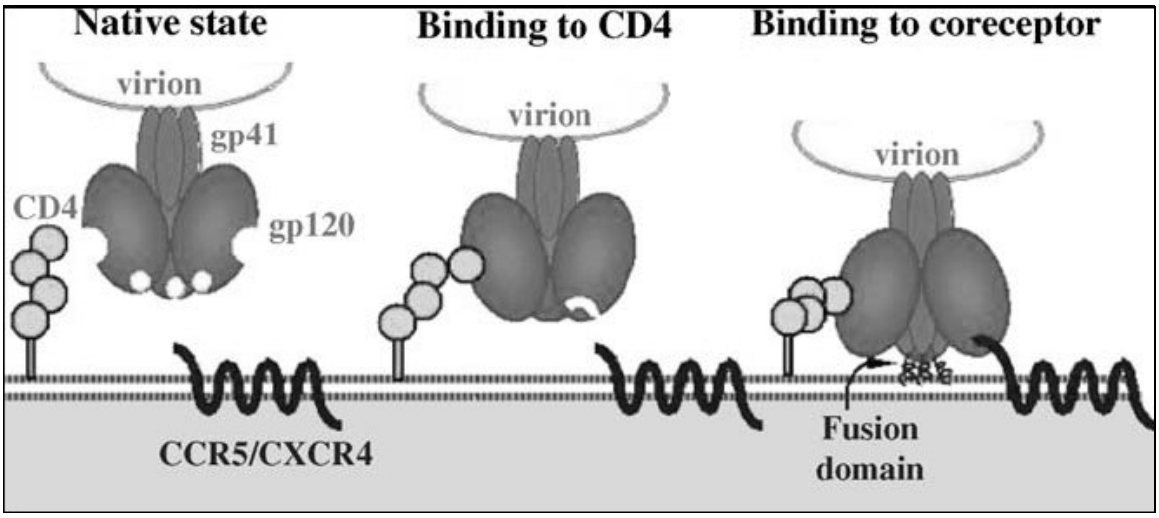

Fig. 4. Proposed model for HIV attachment and entry (2). The gp120 receptor interacts with the CD4 receptor on the cellular surface. This triggers a conformational change in gp120 and exposes the conserved region, allowing co-receptor binding.

Understanding the entry mechanism for HIV has provided alternatives for the identification of new targets for drug therapy. The need for new classes of antiretroviral therapy is becoming more apparent due to the long-term toxicity and the spreading of HIV variants that are resistant to current treatment methods. Entry-inhibitors have recently emerged as intriguing alternatives, and this research provides a potential candidate for this type of therapy.

1.1.3 HIV Pathogenesis

Clinically, HIV infection can be divided into three phases (Fig 5). During primary infection the virus replicates mainly in the Th1 cells of the immune system which represent the pool of target cells. Approximately 50% of cases of primary infection remain asymptomatic, making it difficult to provide initial treatment without a conclusive blood test. At this time, virus titers are extremely high and accompanied by a significant reduction of Th1 CD4 cells. Before a latent period is reached, an HIV specific immune response is mounted by the activation of viral specific cytotoxic CD8+ T lymphocytes (CTL) which temporarily decreases the viral load. If therapy could be provided at this particular stage of infection, which is of particular interest to this research, it would be possible to sustain an immune response and therefore decrease mortality rates. The second phase of infection, clinical latency, is characterized by a long asymptomatic period. During clinical latency, the number of Th1 CD4 cells decrease continually until the patient's immune system is incapable of fighting opportunistic infections. The rate of T cell decrease differs from patient to patient and is dependent upon the level of permanent viral replication (1). The final stage of infection is classified as AIDS and is designated by an outnumbering of Th1 CD4 cells by Th2 CD4 cells, being an antibody response. At this point, opportunistic infections occur due to the depletion of a cell mediated immune response.

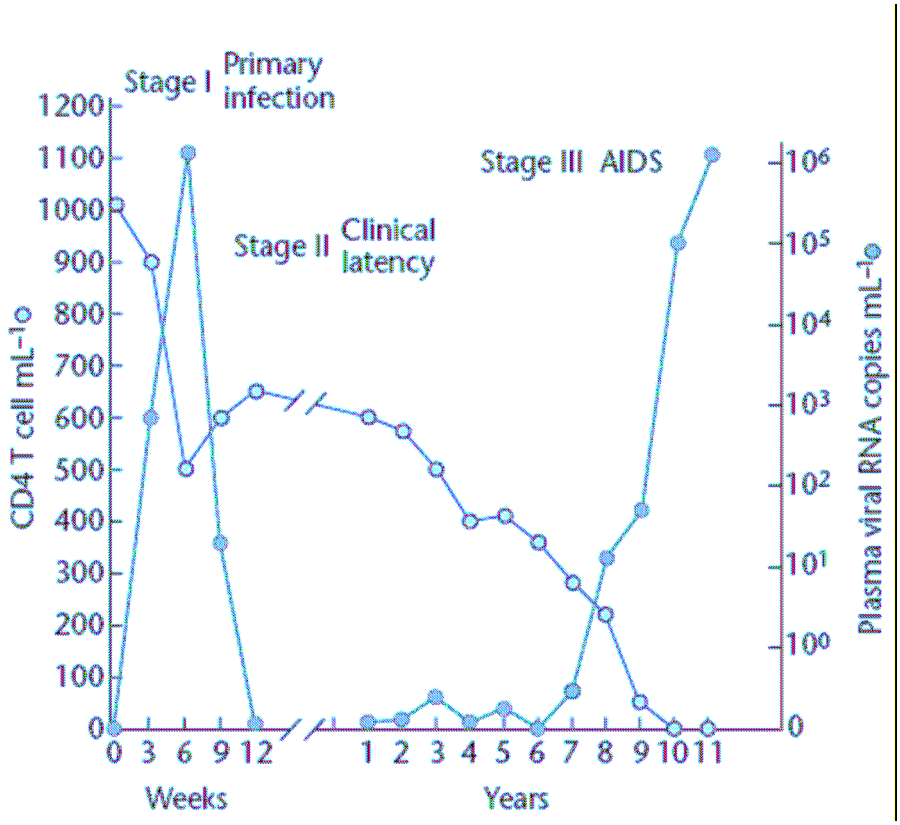

Fig 5. The change in CD4 counts and viral load over time during infection and disease progression in HIV. The areas illustrated above show progression from primary infection to clinical latentcy to AIDS (14).

The mechanisms involved in the cessation of early phase infection to the onset of a latent phase are poorly understood. Several data support the role of HIV-specific CTL and humoral responses in reducing the level of replication from the peak in primary infection to the set-level in the latent period. It has long been presumed that a CTL response is more effective in controlling viral replication than a humoral (Th2) response. CTL mediated responses play a significant role in conserving HIV replication and disease progression, but can be limited by the emergence of escape mutants (19). There are various methods for CTL mediated immune response. CTL recognition of

virus infected antigen-presenting cells occurs through binding to the T cell receptor (TCR). The CTL TCR binds to viral peptides that are associated with MHC class I molecules at the surface of infected cells and triggers the release of proteases that lyse the infected cells (2). A second method is the direct interaction of the Fas ligand (FasL) on the CTL surface with the Fas molecule on the target cell, leading to lysis (2). CTL can also directly bind to HIV infected cells and release chemokines which are the natural co-receptors for HIV and inhibit infection (2).

The contribution of an antibody response seems to play a minor role in controlling HIV infection and is dependent on the amount of neutralizing antibodies rather than the amount of total specific antibodies (2). HIV is able to escape a humoral response as efficiently as it does a CTL response through several routes. First, Env is highly variable in certain regions that form the viral surface. Second, gp120, being covered by a carbohydrate shield, is able to shift epitopes by point mutations (19). Third, conserved regions of Env are only partially exposed (19).

1.1.4 Origins of HIV

Early AIDS research recovered many HIV strains that indicated the virus was highly heterogenous in a variety of features including latency, cytopathicity, and genetic structure (6). Shortly after the discovery of HIV, it was concluded that two subtypes existed, HIV-1 and HIV-2. Cloning and sequence analysis showed that the HIV-2 subtype differed by more than 55% from HIV-1 strains with the greatest difference residing in the envelope glycoproteins (6). It is believed that a cross-species transmission of Simian Immunodeficiency Virus (SIV) from an unidentified primate

species resulted in HIV. Primates that are naturally infected with SIV appear not to develop AIDS, in contrast to HIV infection of humans which is characterized by a loss of CD4+ cells. According to various phylogenetic analyses, SIV isolated from chimpanzees (SIV-1, SIVcpz) was shown to give rise to HIV-1, and SIV isolated from sooty mangabees (SIV-2, SIVsmm) was shown to give rise to HIV-2 (7). The analysis provided further evidence that HIV-1 strains fall into three categories (M, N, O) that are more closely related to SIVcpz, which indicates three different introductions of SIVcpz into the human population (33). HIV-1 groups N and O are mainly restricted to western equatorial Africa, whereas group M has spread globally. HIV-2 strains that have been studied yield 8 categories. Groups A and B contribute to the majority of HIV-2 infections, and groups C through H have only been identified in single individuals (34). Recently, and international research team has centered in on the geographic origin of the AIDS virus and determined by sequence analysis that chimpanzees living in Camaroon are the natural reservoirs of HIV-1 (39). The clinical differences between the two lie in the general characterization of HIV-1 having shorter clinical latency periods, higher viral loads, and a higher rate of transmissibility than HIV-2. Both HIV-1 and HIV-2 will be the focus of this research.

1.2 Background on *Quillaja saponaria*

Productive therapies for HIV infection are in demand to curb the epidemic. New prevention strategies have turned to the use of plant extracts as potential inhibitors of viral replication. We are interested in the use of a natural, aqueous extract of triterpenoid saponins from *Quillaja saponaria* Molina, Chilean Soap Bark Tree, as a

candidate for inhibition of HIV attachment and replication. Q. saponins constitute a group of immune stimulating complexes that have been widely used for centuries. In general, Q. saponins have been shown to exhibit certain viralcidal properties including: 1) destruction of viral envelopes, 2) interaction with host cell membrane leading to loss of viral binding sites, 3) induces cell signaling cascade, 4) induces a Th1 immune response (CTL) (10). A Th1 immune response is required for the production of CD8+ CTL which would be of particular importance to sustain immunity against HIV (9). These saponins have been shown to demonstrate strong adjuvant activity that has been exploited in animal and human vaccines (20, 21, 22). Though these saponins do pose as potential contributors against viral replication by blocking viral attachment, there are certain side effects from the use of saponins that must be remedied. Q. saponins are highly surface active, cytolytic, and hemolytic (8). Such damage is reduced by using purified fractions, or decreasing the concentration to a minimum, a goal of this research. We examined the *in vitro* effectiveness of Q. saponins as potential candidates to prevent HIV infection. The saponin extract used in this study is only cytotoxic at high concentrations and is currently approved for use in food and beverages and organic foods by the FDA.

The Q. saponin extract used here, is a complex mixture of triterpenoids built around a common quillaic acid. (Fig 6). All these identified structures have in common the terpene quillaic acid with either a di- or one of two tri-saccharides attached to C-3 and several oligosaccharides at C-28 (23). The differences within the members of this family lie in the level of oxidation around the acid skeleton, the type, location and number of sugars, and the number, type, and location of acyl moieties. It is believed that

the fatty acid acyl moiety is the determinant of toxicity and immune stimulating responses and is critical for adjuvant activity. It can be presumed that the quillaic acid moiety serves as a scaffolding element, which can then order the oligosaccharides in the proper order for interaction with cellular targets. Therefore, the saponin extract would be able to bind to the CD4 receptor and prevent viral attachment, or bind to the gp120 envelope protein and similarly prevent attachment to cells.

Recent research has investigated the use of *Quillaja saponaria* as antigen-presenting systems in the form of ISCOMS. The *Quillaja saponaria* Molina extract allows for hydrophobic interactions consisting of cholesterol, phospholipid, and antigen, and can function as an adjuvant in vaccines (35, 36). The most active component of this extract, designated as QS-21, is a water-soluble triterpene that can be mixed with a soluble antigen to create a soluble vaccine (37, 38). In recent clinical trials, QS-21 was shown to elicit a strong humoral and cellular response to low doses of HIV-1 gp120 (38).

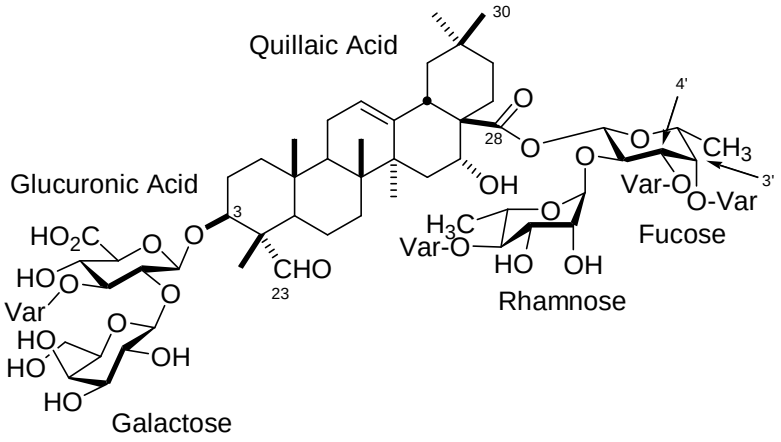

Fig. 6. General structure of the *Quillaja* saponins.

We attempt to define the conditions under which saponin blocks HIV infection by examining a series of questions, being imposed through three treatment regimes. We hypothesize that the *Quillaja saponaria* extracts have anti-viral activity that blocks infection extracellularly by coating cells to prevent viral attachment and reduces spread by blocking infection from cells that do manage to become infected, while the extracts cannot confer long-lasting resistance when cells are pre-treated followed by removal of extract and subsequent infection.

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintenance and Infection of Cells

2.1.1 Maintenance of Cells

CEMx174 cells were maintained in cell culture flasks and grown in suspension. CEMx174 cells (NIH) are a fusion product of the human B cell line 721.174 and the human T cell line CEM (25). RPMI 1640 was used to maintain the cells and supplemented with 10% fetal bovine serum (FBS) and antibiotic containing penicillin [100 U/ml] and streptomycin [100 µg/ml] (P/S). Cells were maintained between $1x10^5$ and $1x10^6$ cells per milliliter. The cell-containing media was added to 50mL conical centrifuge tubes and spun at 2,000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 25 mL of media. Cells were then aliquoted into flasks depending on the dilution ratio needed and incubated at 37°C and 5% CO_2.

2.1.2 Infection of Cells

Twenty-five cm^2 flasks were seeded with 1×10^6 cells in RPMI 1640 containing 10% FBS and 1% P/S and incubated overnight at 37°C and 5% CO_2. HIV-1 and HIV-2 (NIH) were used to infect separate flasks at volume of 100 µl. The flasks were allowed

to incubate until approximately 80% of the cells were lysed. The cells were sonicated and the lysate was frozen at -80°C until further use.

2.2 Cytotoxicity Assay

Cells were plated at a concentration of $1x10^6$ cells per well in a 24-well plate and were incubated for approximately 24 hours to allow the cells to divide to yield $3.4x10^6$ cells per well. *Quillaja* extract solutions (Ultra Dry 100-Q, Desert King International, San Diego, CA) were prepared by dissolving dried material in MEM to yield a 5% solution, which was then filter sterilized. From this stock the saponin was diluted in MEM containing 10% FBS to produce desired concentrations. Ten-fold serial dilutions of *Quillaja* extract were prepared, beginning at an initial concentration of 0.05 and diluted in RPMI 1640 supplemented with 10% FBS and 1% P/S. The appropriate concentration was added to each well at a volume of 250 µl and allowed to incubate for 96 hours at 37°C and 5% CO_2. Cytotoxicity was measured microscopically by counting 500 cells/well with 4 wells at each concentration, using trypan blue staining procedures. Assays were performed in duplicate.

2.3 Virus Titer Assay

Cells were plated at a concentration of $1x10^6$ cells per well in a 24-well plate and were incubated for approximately 24 hours to allow the cells to divide to yield $3.4x10^6$ cells per well. Two-fold serial dilutions of HIV-1 and HIV-2 were prepared using RPMI 1640 supplemented with 10% FBS and 1% P/S. The appropriate concentration was added to each well at a volume of 100 µl and allowed to incubate for 120 hours at 37°C and 5% CO_2. Cell viability was determined by counting 500

cells/well with 2 wells at each concentration. Neutral red staining procedures were used at a concentration of 2% of the total volume contained in each well. Assays were performed in duplicate.

2.4 Differential Treatments of Cells and HIV-1 and HIV-2

2.4.1 Treatment of HIV-1 and HIV-2 with Quillija Extract and Infection of Cells

To measure the effect of pre-treatment of the virus before infection of cells, 150 µl of HIV-1 and HIV-2 were added to 24-well plates. Two-fold dilutions were made of each virus, beginning at the concentration of virus determined above. The virus was treated with 150 µl of extract concentration of *Quillaja* previously determined. After 1, 2, 4, and 6 hours, $3.4x10^6$ cells were added to each well and incubated for 120 hours at 37°C and 5% CO_2. Cell viability was determined by counting 500 cells/well with 2 wells at each concentration. Neutral red staining procedures were used at a concentration of 2% of the total volume contained in each well. Assays were performed in duplicate.

2.4.2 Treatment of Cells with Quillaja Extract and Infection with HIV-1 and HIV-2

To measure the attachment of HIV-1 and HIV-2 to the host cell, $1x10^6$ cells were seeded in a 24-well plate and incubated overnight to yield $3.4x10^6$ cells per well. The cells were treated with 150 µl of the extract concentration of *Quillaja* determined above, with the extract diluted in RPMI 1640 supplemented with 10% FBS and 1% P/S. After 1, 2, 4, and 6 hours of treatment 150 µl of HIV-1 and HIV-2 were added to the

cells at two-fold dilutions beginning at the appropriate titer determined above. The plates were incubated for 120 hours at 37°C and 5% CO_2. Cell viability was determined by counting 500 cells/well with 2 wells at each concentration. Neutral red staining procedures were used at a concentration of 2% of the total volume contained in each well. Assays were performed in duplicate.

2.4.3 Treatment of Cells with Quillaja Extract, Removal of Extract, and Infection with HIV-1 and HIV-2

To measure the attachment of HIV-1 and HIV-2 to the host cell, $1x10^6$ cells were seeded in a 24-well plate and incubated overnight to yield $3.4x10^6$ cells per well. The cells were treated with 150 µl of the extract concentration of *Quillaja* determined above, with the extract diluted in RPMI 1640 supplemented with 10% FBS and 1% P/S. After 6 hours of treatment the plates were spun in a centrifuge at 500 rpm for 5 minutes with no brake. The saponin containing media was removed, only allowing for 10-15% loss of viable cells. HIV-1 and HIV-2 were added to the cells at a volume of 150 µl per well at 2, 6, 12, and 24 hours after removal of the extract and the plates were incubated for 120 hours at 37°C and 5% CO_2. Longer treatment times were used to illustrate when cellular protection reached close to 0% protection. Cell viability was determined by counting 500 cells/well with 2 wells at each concentration. Neutral red staining procedures were used at a concentration of 2% of the total volume contained in each well. Assays were performed in duplicate.

CHAPTER 3

RESULTS

3.1 Cytotoxicity Data

Figure 7 shows the results of the cytotoxicty assay performed with the CEMx174 cell line after 96 hours of saponin extract treatment. The concentration of saponin extract that was most minimally cytotoxic was determined to be 0.00001%. As the concentration increases, there is a corresponding decrease in viable cells (Table 2). This concentration left the greatest number of viable cells after the incubation period and was the lowest concentration tested from 10-fold serial dilutions.

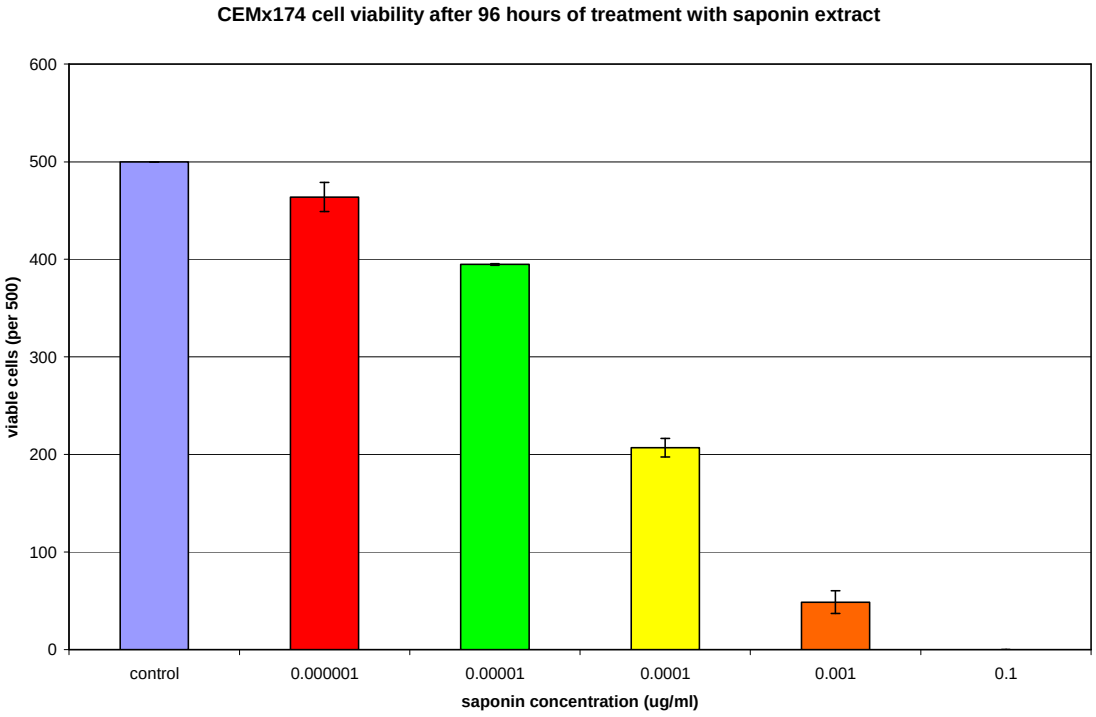

Figure 7. The maximally viable concentration of saponin extract after 96 hours of treatment

Table 2. Viability of CEMx174 cells after 96 hours of saponin treatment.

Viability	Concentration of saponin extract (ug/ml)					
	0	0.000001	0.00001	0.0001	0.001	0.01
Mean	500	452.5	389	211	50.25	0
SD	0	14.8847	0.0816497	9.486833	11.67262	0

3.2 Concentration of HIV-1 and HIV-2

The desired concentration of HIV-1 and HIV-2 to be used were determined by incubation of infected cells for 96 hours. A 50 % reduction in cell viability was needed to yield the appropriate virus concentration to be used in the three treatment regiments. It was determined that the viral concentration of 0.16 was required to cause approximately half the number of cells to die after 96 hours of infection in both HIV-1 and HIV-2 (Fig. 8). HIV-1 appears to have a slightly lower threshold for infection than HIV-2, which is consistent with what is known about the two strains. Therefore, it can be concluded that 16% of the cell lysate was needed to induce cell death in 50% of observed cells.

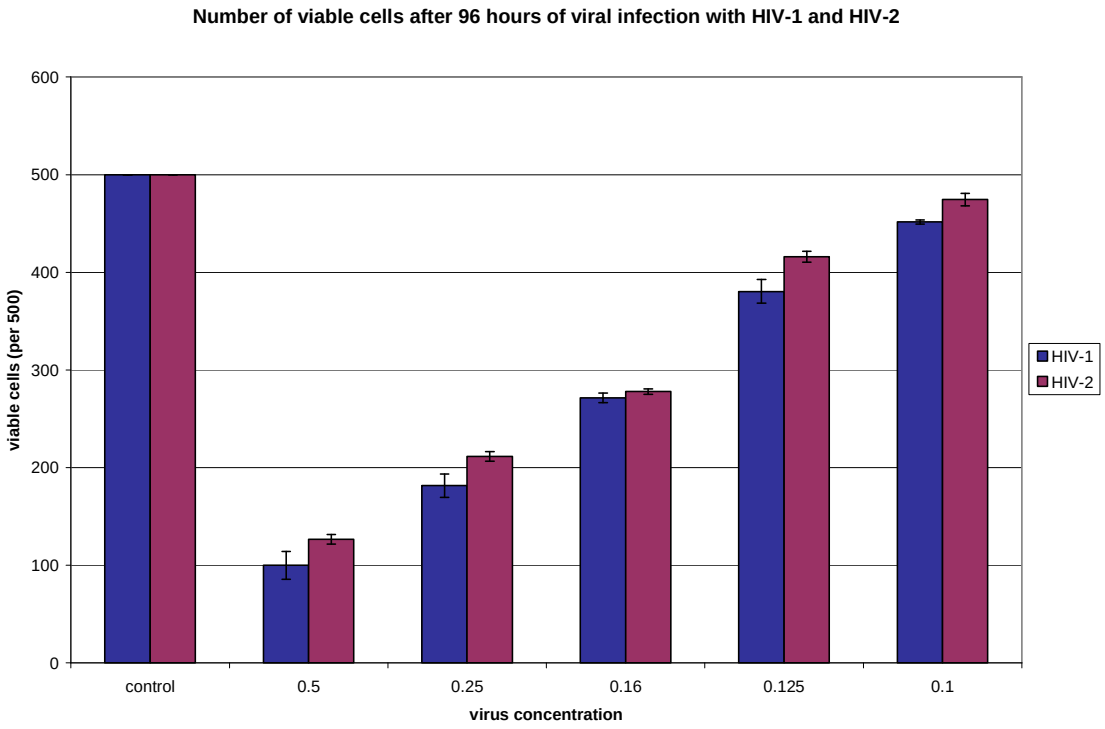

Figure 8. The effect of HIV-1 and HIV-2 infection on CEMx174 cells after 96 hours.

Table 3. Viability of CEMx174 cells after 96 hours of infection with HIV-1 and HIV-2.

Viability	Concentration of saponin extract (ug/ml)					
	0	0.000001	0.00001	0.0001	0.001	0.01
Mean	500	452.5	389	211	50.25	0
SD	0	14.8847	0.0816497	9.486833	11.67262	0

3.3 Pre-treatment of Virus with Extract Data

In order to determine the effect of saponin on the viral envelope, HIV-1 and HIV-2 were pre-treated with the extract at a concentration of $1.6x10^{-6}$ µg/ml before

infection of cells. For HIV-1 there was a consistent increase in the number of protected cells at each concentration as the incubation time increased (Fig. 9). The maximum amount of protection appeared to be at a virus concentration of 0.04 with a steady increase in the percentage of protected cells from 1-6 hours (Table 3). There also was a considerable increase as the time doubles from 2 to 4 hours with the values balancing off at 6 hours.

Treatment of HIV-2 produced similar results. The total number of protected cells appeared to be higher than that of HIV-1, but can be attributed to different infection rates. The percentage of protected cells increases with a corresponding increase in incubation time (Fig.10). The virus concentration of 0.04 allowed for the maximum amount of protected cells through each incubation point (Table 4). As with HIV-1, there is an ample increase in the percentage of protection as the time double from 2 to 4 hours.

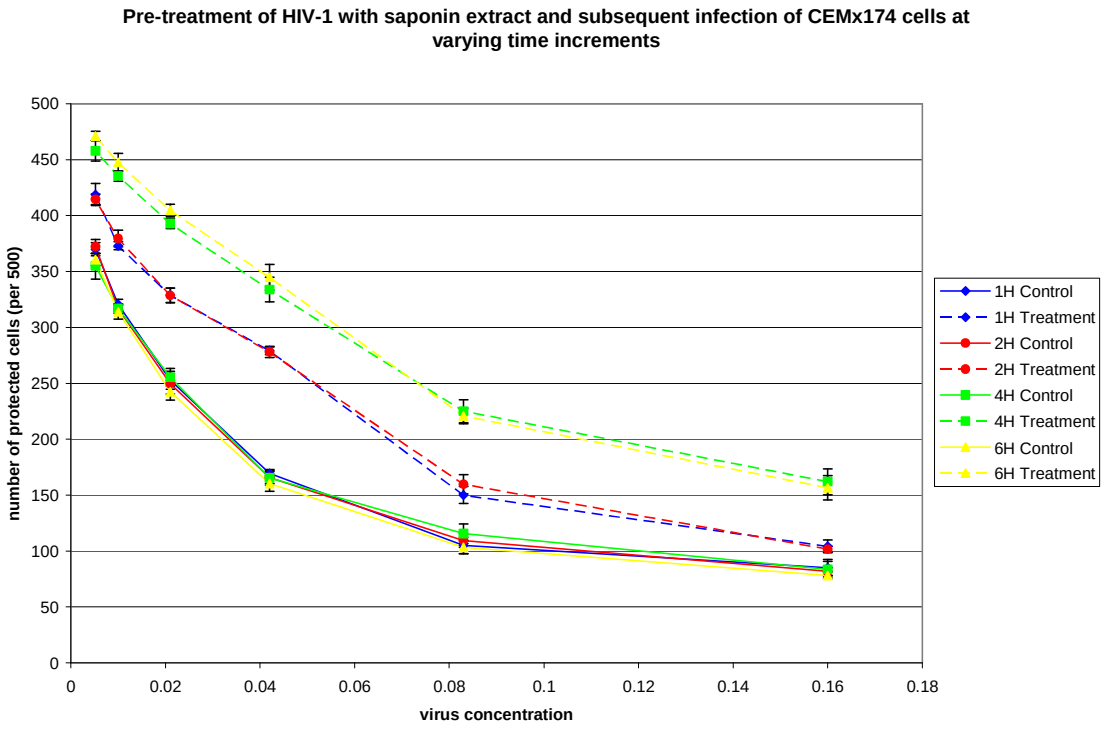

Figure 9. HIV-1 treatment with saponin extract followed by infection of CEMx174 cells at sequential incubation points.

Table 4. Standard deviations of HIV-1 pre-treatment with saponin extract.

HIV-1	Standard Deviation hours of saponin extract treatment of virus 0		1		2		4		6	
	con	exp	con	exp	con	exp	con	Exp	con	exp
$1.6x10^{-1}$	0	0	7.14	5.74	3.59	2.63	7.14	11.6	3.10	10.8
$8.3x10^{-2}$	0	0	7.11	7.53	6.14	8.66	8.74	10.2	5.68	6.99
$4.2x10^{-2}$	0	0	3.32	3.56	5.44	4.97	6.66	11.1	7.0	11.4
$2.1x10^{-2}$	0	0	8.06	6.40	9.15	6.56	7.85	4.57	7.55	5.45
$1.0x10^{-2}$	0	0	4.66	3.65	2.87	7.42	4.19	4.65	6.83	7.94
$5.2x10^{-3}$	0	0	5.91	9.98	6.29	4.73	11.6	9.29	5.57	4.08

Table 5. Calculated percent of protected cells after pre-treatment of HIV-1 and infection of CEMx174 cells at varying incubation points.

HIV-1	% protected cells hours of saponin extract treatment of virus				
	0	1	2	4	6
$1.6x10^{-1}$	0	3.8	4.0	15.7	15.7
$8.3x10^{-2}$	0	9.0	10.1	21.9	23.5
$4.2x10^{-2}$	0	21.9	22.5	33.7	36.9
$2.1x10^{-2}$	0	15.2	15.8	27.5	32.4
$1.0x10^{-2}$	0	10.5	12.6	23.7	26.7
$5.2x10^{-3}$	0	9.8	8.5	20.6	22.1

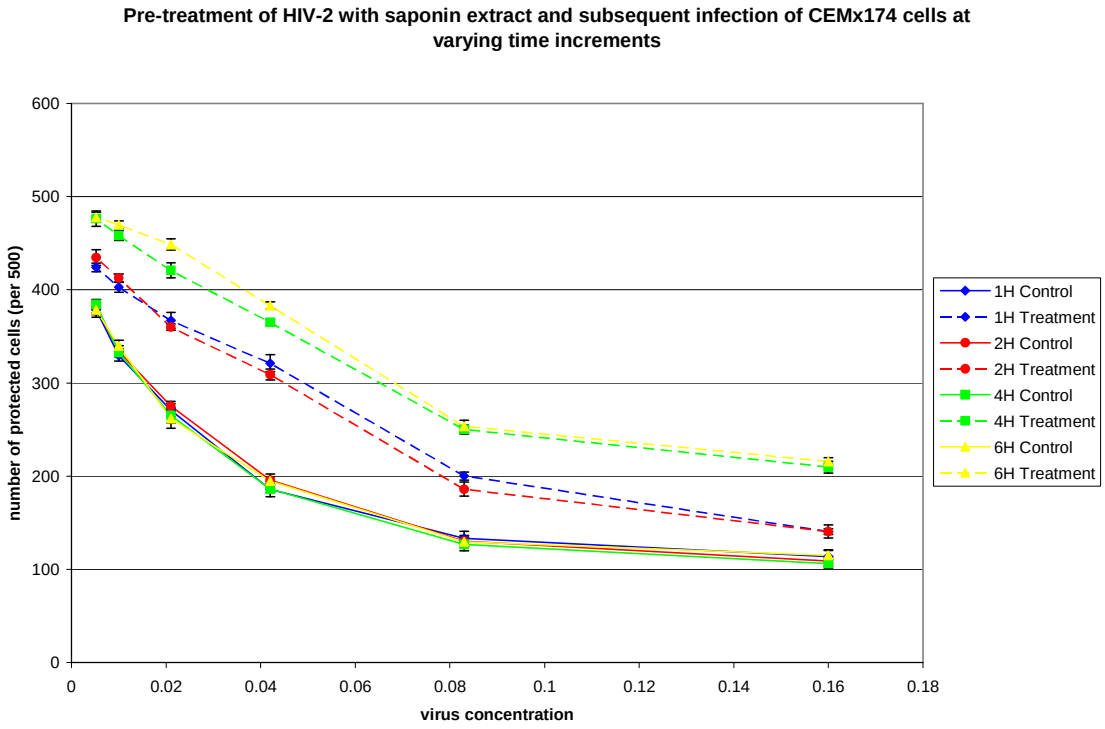

Figure 10. HIV-2 treatment with saponin extract followed by infection of CEMx174 cells at sequential incubation points.

Table 6. Standard deviations of HIV-2 pre-treatment with saponin extract.

HIV-2	Standard Deviation hours of saponin extract treatment of virus									
	0		1		2		4		6	
	con	exp	con	exp	con	exp	con	exp	con	exp
$1.6x10^{-1}$	0	0	7.18	7.0	4.86	3.11	4.79	6.34	4.86	4.03
$8.3x10^{-2}$	0	0	7.80	4.27	6.18	7.53	6.90	4.69	5.12	6.66
$4.2x10^{-2}$	0	0	8.25	9.03	3.59	5.89	4.03	3.37	7.59	3.92
$2.1x10^{-2}$	0	0	6.99	8.76	5.29	3.37	8.30	7.97	10.1	5.97
$1.0x10^{-2}$	0	0	5.68	5.62	6.0	4.43	4.03	5.38	6.18	4.11
$5.2x10^{-3}$	0	0	3.10	4.55	8.06	8.43	5.12	8.10	7.85	5.32

Table 7. Calculated percent of protected cells after pre-treatment of HIV-2 and infection of CEMx174 cells at varying incubation points.

HIV-2	% protected cells hours of saponin extract treatment of virus				
	0	1	2	4	6
$1.6x10^{-1}$	0	5.4	6.4	20.7	20.1
$8.3x10^{-2}$	0	13.4	11.2	24.7	24.8
$4.2x10^{-2}$	0	27.1	22.7	35.8	37.7
$2.1x10^{-2}$	0	19.3	17.0	31.1	37.3
$1.0x10^{-2}$	0	14.7	15.7	25.1	26.0
$5.2x10^{-3}$	0	9.2	10.7	18.4	19.9

3.4 Pre-treatment of CEMx174 Cells with Extract Data

Cells were pre-treated with saponin extract at a concentration of $1.6x10^{-6}$ µg/ml to demonstrate the effect of extract on the cellular membrane with subsequent viral infection. Two-fold serial dilutions of HIV-1 and HIV-2 beginning, at 0.16, were used to infect CEMx174 cells after 1, 2, 4, and 6 hours of incubation with saponin extract. Infection with HIV-1 showed a consistent increase in the number of cells protected as the incubation time increased (Fig. 11). Maximum protection appeared to be with 6 hours of pre-treatment at a viral concentration of 0.08, producing 60.8% of cellular protection (Table 5). Additionally, over 50% of cells were protected at a viral concentration of 0.042 with 6 hours of pre-treatment.

Infection with HIV-2 produced comparable results. There was an equivalent increase in the percentage of cells protected as the incubation time increased (Fig 12). The maximum percentage of protected cells appeared to be at a viral concentration of 0.083 with 6 hours of pre-treatment with saponin extract (Table 6). Viral concentration of 0.042 with 6 hours of incubation al showed over 50% cellular protection as with HIV-1.

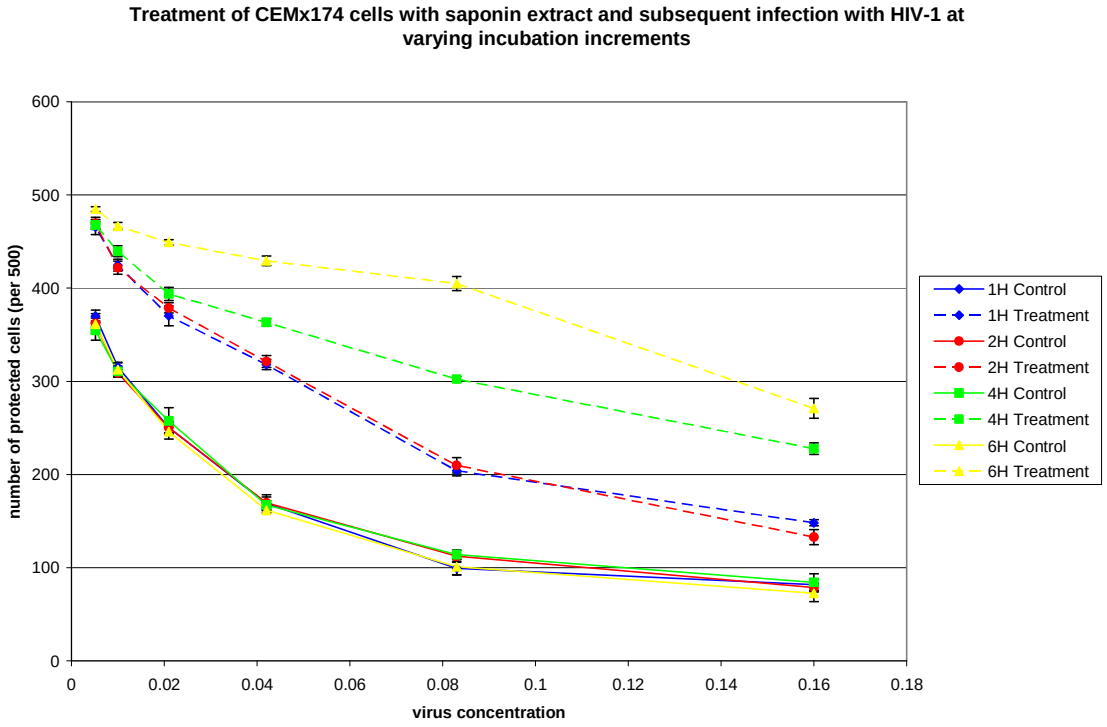

Figure 11. Cell viability of CEMx174 cells that have been pre-treated with saponin extract and infected with HIV-1 at varying time points.

Table 8. Standard deviations of CEMx174 pre-treatment with saponin extract and infection with HIV-1.

HIV-1	Standard Deviation hours of saponin extract treatment of cells 0		1		2		4		6	
	con	exp	con	exp	con	exp	con	exp	con	exp
$1.6x10^{-1}$	0	0	6.24	3.10	4.51	8.08	9.29	6.18	8.81	10.6
$8.3x10^{-2}$	0	0	7.42	5.29	6.40	8.30	5.10	3.20	8.29	7.62
$4.2x10^{-2}$	0	0	6.98	5.48	8.62	6.40	4.57	4.03	2.75	5.25
$2.1x10^{-2}$	0	0	5.57	10.6	6.50	5.56	14.0	7.18	7.87	2.94
$1.0x10^{-2}$	0	0	5.31	6.45	4.03	7.14	5.74	5.80	7.23	3.70
$5.2x10^{-3}$	0	0	6.55	8.06	10.6	6.61	10.4	3.87	6.29	2.5

Table 9. Calculated percent of protected CEMx174 cells after pre-treatment of cells and infection with HIV-1 at differing time increments.

HIV-1	% protected cells hours of saponin extract treatment of cells				
	0	1	2	4	6
$1.6x10^{-1}$	0	13.3	10.9	28.7	39.7
$8.3x10^{-2}$	0	20.9	19.5	37.7	60.8
$4.2x10^{-2}$	0	29.8	30.3	39.2	53.5
$2.1x10^{-2}$	0	24.0	25.7	27.3	40.6
$1.0x10^{-2}$	0	21.9	22.6	25.8	30.8
$5.2x10^{-3}$	0	19.9	21.5	22.6	24.7

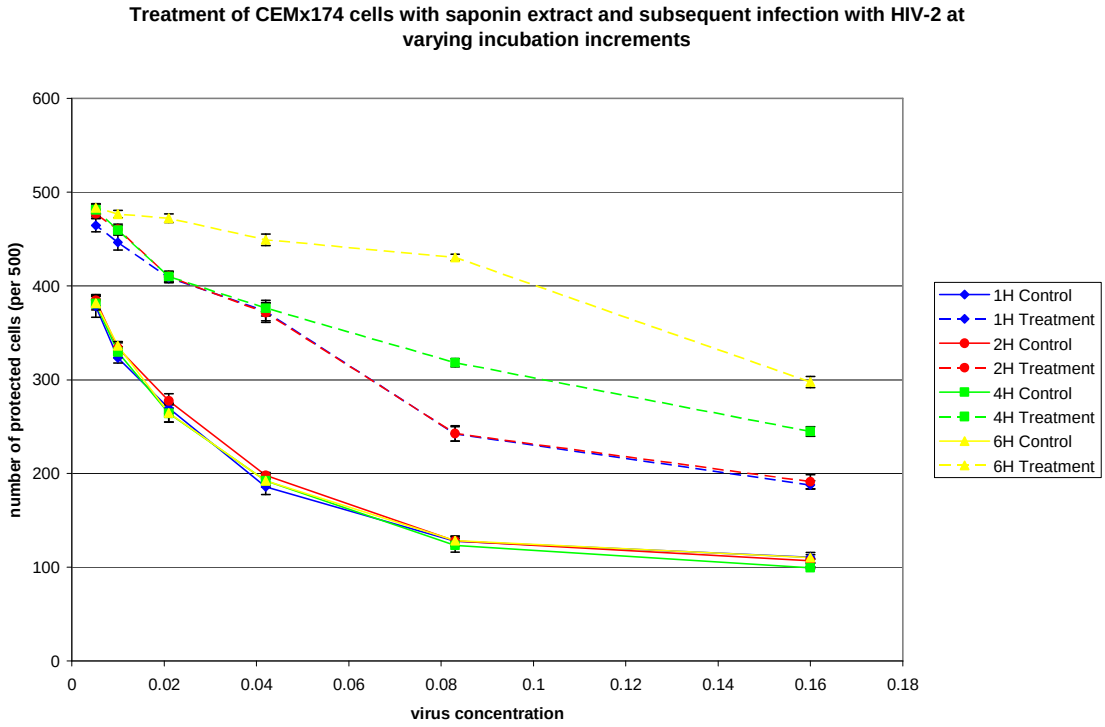

Figure 12. Cell viability of CEMx174 cells that have been pre-treated with saponin extract and infected with HIV-2 at varying time points.

Table 10. Standard deviations of CEMx174 pre-treatment with saponin extract and infection with HIV-2.

HIV-2	Standard Deviation hours of saponin extract treatment of cells									
	0		1		2		4		6	
	con	exp	con	exp	con	exp	con	exp	con	exp
$1.6x10^{-1}$	0	0	2.5	4.35	2.31	7.41	1.29	5.12	5.56	6.03
$8.3x10^{-2}$	0	0	5.5	7.63	4.24	8.26	7.18	4.35	4.80	3.42
$4.2x10^{-2}$	0	0	8.02	9.81	2.94	10.1	6.58	8.42	4.79	6.02
$2.1x10^{-2}$	0	0	5.5	5.48	7.59	5.38	9.27	6.06	9.78	4.43
$1.0x10^{-2}$	0	0	5.57	8.02	5.44	5.68	5.80	4.80	4.57	4.03
$5.2x10^{-3}$	0	0	11.4	7.14	6.02	4.40	4.43	6.65	7.14	3.70

Table 11. Calculated percent of protected CEMx174 cells after pre-treatment of cells and infection with HIV-2 at differing time increments.

HIV-2	% protected cells hours of saponin extract treatment of cells				
	0	1	2	4	6
$1.6x10^{-1}$	0	15.4	16.9	29.1	37.5
$8.3x10^{-2}$	0	22.9	23.0	39.0	60.4
$4.2x10^{-2}$	0	37.4	34.7	36.9	51.4
$2.1x10^{-2}$	0	27.9	26.6	29.2	41.5
$1.0x10^{-2}$	0	24.6	25.4	26.0	28.1
$5.2x10^{-3}$	0	17.3	18.5	20.0	20.4

3.5 Pre-treatment and Removal of Extract Data

The lasting effects of cellular protection from saponin extract were examined by infection of CEMx174 cells at 2, 6, 12, and 24 hours after removal of extract containing media. The removal of saponin extract produced opposite results from previous treatments in which the highest percentage of protected cells occurred closest to extract removal (Fig. 13). As more time passed, more cells were vulnerable to infection with almost no protection at 24 hours after removal of saponin extract (Table 7). The most amount of protection from HIV-1 infection was 2 hours after removal at a viral concentration of 0.042. There was also a dramatic decrease in the percentage of protected cells when the removal time was doubled from 6 to 12 hours and 12 to 24 hours.

HIV-2 infection after removal of saponin extract produced comparable results. The highest percentage of protected cells were seen to be closest to the least amount of time between removal and infection and the lowest percentage of protected cells furthest from removal time and infection (Fig. 14). The greatest amount of protection came from infection 2 hours after removal at a viral concentration of 0.042 (Table 8). When the infection time was doubled from 6 to 12 hours and 12 to 24 hours, there was a considerable decrease in the percentage of cellular protection.

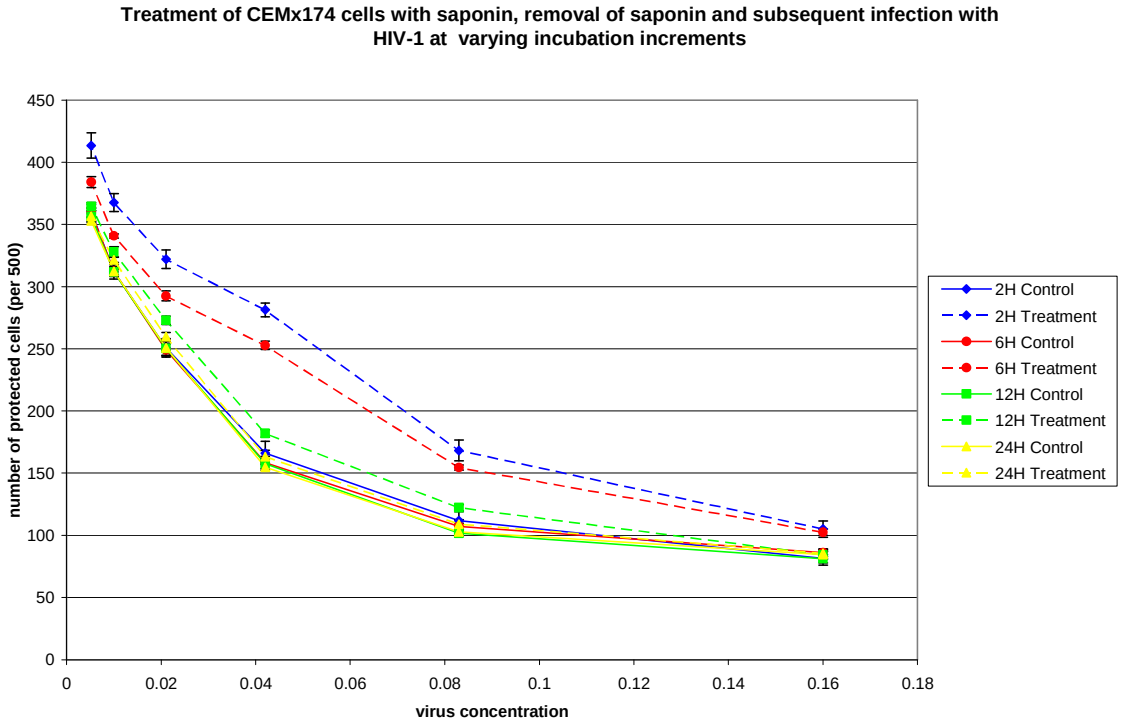

Figure 13. Cell viability of CEMx174 cells that have been pre-treated with saponin extract with the extract removed and infected with HIV-1 at varying time points.

Table 12. Standard deviations of CEMx174 pre-treatment with saponin extract, removal of extract, and infection with HIV-1.

HIV-1	Standard Deviation hours after saponin extract removal from cells and infection with virus 0		2		6		12		24	
	con	exp	con	exp	con	exp	con	exp	con	exp
$1.6x10^{-1}$	0	0	5.45	6.58	2.99	2.65	3.30	2.87	3.70	2.89
$8.3x10^{-2}$	0	0	7.37	8.30	2.63	2.08	2.5	2.5	2.5	3.74
$4.2x10^{-2}$	0	0	9.43	5.38	5.0	3.30	3.87	2.94	2.5	5.12
$2.1x10^{-2}$	0	0	7.41	7.53	4.83	4.04	5.44	3.37	6.34	3.40
$1.0x10^{-2}$	0	0	5.77	7.14	3.19	1.70	2.22	4.08	4.11	2.08
$5.2x10^{-3}$	0	0	7.41	10.1	3.87	4.40	4.79	1.73	2.94	4.20

Table 13. Calculated percent of protected CEMx174 cells after pre-treatment of cells and removal of extract containing media and infection with HIV-1 at differing time increments.

HIV-1	% protected cells hours after removal of saponin extract from cells and infection with virus				
	0	2	6	12	24
$1.6x10^{-1}$	0	4.7	3.3	0.6	0.2
$8.3x10^{-2}$	0	11.3	9.5	4.1	1.3
$4.2x10^{-2}$	0	23.0	18.9	4.9	1.7
$2.1x10^{-2}$	0	14.3	8.7	4.6	1.8
$1.0x10^{-2}$	0	11.1	5.7	3.3	1.9
$5.2x10^{-3}$	0	10.7	4.9	1.7	0.7

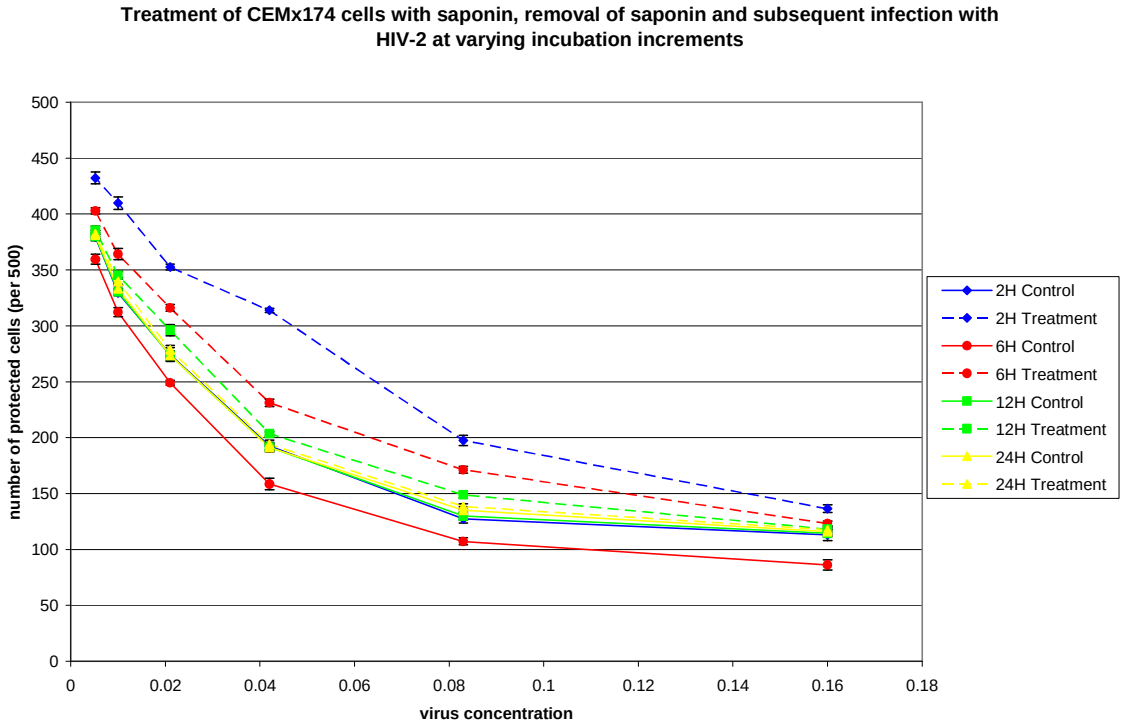

Figure 14. Cell viability of CEMx174 cells that have been pre-treated with saponin extract with the extract removed and infected with HIV-2 at varying time points.

Table 14. Standard deviations of CEMx174 pre-treatment with saponin extract, removal of extract, and infection with HIV-2.

HIV-2	Standard Deviation hours after saponin extract removal from cells and infection with virus 0		2		6		12		24	
	con	exp	con	exp	con	exp	con	exp	con	exp
$1.6x10^{-1}$	0	0	4.97	3.42	4.65	2.22	3.11	1.71	3.16	2.99
$8.3x10^{-2}$	0	0	3.79	4.51	3.10	2.99	3.56	3.16	2.99	2.38
$4.2x10^{-2}$	0	0	5.26	1.89	5.12	3.30	2.65	1.91	3.30	1.83
$2.1x10^{-2}$	0	0	6.24	2.65	2.16	2.94	4.04	4.97	1.73	3.87
$1.0x10^{-2}$	0	0	2.38	5.67	3.86	4.97	3.30	3.5	4.32	3.51
$5.2x10^{-3}$	0	0	3.57	5.19	4.5	2.87	1.89	3.70	4.24	2.99

Table 15. Calculated percent of protected CEMx174 cells after pre-treatment of cells and removal of extract containing media and infection with HIV-2 at differing time increments.

HIV-2	% protected cells hours after removal of saponin extract from cells and infection with virus				
	0	2	6	12	24
$1.6x10^{-1}$	0	4.7	1.7	0.8	0.4
$8.3x10^{-2}$	0	14.0	8.3	3.8	0.7
$4.2x10^{-2}$	0	24.3	9.0	2.4	0.6
$2.1x10^{-2}$	0	15.7	7.6	4.5	1.0
$1.0x10^{-2}$	0	16.1	6.6	3.0	1.3
$5.2x10^{-3}$	0	10.6	4.2	1.2	0.3

CHAPTER 4

DISCUSSION AND CONCLUSION

Aqueous extracts of triterpenoid saponins from *Quillaja saponaria* Molina have been shown to demonstrate a strong cytotoxic effect on the CEMx174 cell line. It was determined that the maximum viable concentration of saponin extract was 0.00001%, causing a minimal amount of cytotoxicity after 96 hours. The low value could be the result of various interactions between the structure of saponin and the cellular membrane. Q. saponins derive their high toxicity from the acyl fatty acid moiety present on the molecular structure (10, 25). These close interactions, occurring through the coating of the cells, places phospholipid portions of the membrane in vulnerable contact with the acyl fatty acid moiety. A higher concentration would therefore be more cytotoxic and cause destruction of the cellular membrane.

Q. saponins were able to partially prevent viral attachment when HIV-1 and HIV-2 were pre-treated with the most minimally cytotoxic concentration of saponin extract. The maximum amount of virus inactivation resulted in 37% of cells protected from infection at a viral concentration on 0.042, allowing for the maximum amount of saponin extract to bind to the gp120 subunit on the viral envelope and prevent attachment to the CD4 receptor on the cellular membrane. Both HIV-1 and HIV-2 were seen to be inactivated immediately at 1 hour of treatment, with the trend continuing at longer treatment times with more neutralization of the viral envelope. Curiously, lower

concentrations of the pre-treated virus were not inactivated as much as higher concentrations possibly due to excess amounts of saponin that did not attach to viral envelopes, destroying cellular membranes. It should also be noted that protection almost doubles between infection times 2 and 4 hours. Likely, some sort of cooperative binding occurs at this point between the side groups of the saponin and the gp120 subunit of HIV-1 and HIV-2, causing the large increase in the percentage of protected cells (26). Even at higher concentrations of virus a significant portion of HIV-1 and HIV-2 are inactivated, leading to the conclusion that this treatment could be used as a new therapy.

When cells were pre-treated with the most minimally cytotoxic concentration of saponin extract, there was a considerable increase in the percentage of protected cells. Similar to the previous treatment, a greater percentage of cells are protected when the pre-treatment time is extended. When cells are pre-treated, the maximum amount of protection was determined to be at a viral concentration of 0.083 with 6 hours of pre-treatment. This concentration, though the second highest, allows for the most amount of binding between side group on the structure of saponin to the CD4 receptor on the cellular membrane. The highest concentration of HIV-1 and HIV-2 was able in infect large portions of cells at lower pre-treatment times due to the ratio between larger amounts of virus particles and the least amount of CD4 receptor neutralization. After 6 hours of pre-treatment, there is also a greater percentage of protected cells than at the lowest concentration. Counter-intuitively, protection decreased steadily after 0.083 percent and can be attributed to virucidal properties of the extract. By looking at the results, a viral concentration of 0.042 conferred the most protection consistently until 6

hours of pre-treatment. Although protection is still over 50%, infection occurs slightly more at the 0.042 concentration which can be due to some extent of cell death due to saponin or some amount of extract un-binding or falling off the CD4 receptor. Protection almost doubles at 6 hours with concentrations at 0.083%, 0.042%, and 0.021% possibly due to a type of cooperative binding mechanism (26). This treatment regiment could potentially be used as a new therapy in the future, as opposed to pre-treatment of HIV-1 and HIV-2, as greater than 50% protection is conferred at two viral concentrations with pre-treatment of cells with 0.00001% *Quillaja saponaria* Molina extract.

It was then examined if saponin extract allowed for continued protection after the extract was removed and cells were infected with HIV-1 and HIV-2. It was predicted that protection would decrease when the extract was removed from the media and the cells subsequently infected. Cells were pre-treated with the maximum viable concentration of the extract for 6 hours with the extract containing media removed thereafter. Cells were infected at longer removal times than previous treatments in order to see if protection ever reached 0%. As expected, the greatest amount of protection came at 2 hours, the least amount of time after removal. The viral concentration of 0.042% was seen to have the highest percentage of protection, almost double than that of any other concentration. Protection decreased dramatically when infection after removal time was doubled from 6 to 12 hours and 12 to 24 hours, indicating that functional CD4 receptors are exposed to infection with HIV-1 and HIV-2. Therefore, it can be concluded that *Quillaja saponaria* Molina extracts do not confer resistance after

cells are pre-treated followed by extract removal. The extract must be present to continuously prevent infection from HIV-1 and HIV-2.

There is one possible mechanism to explain the interaction between the saponin extract and the gp120 subunit on the envelope of HIV-1 and HIV-2. The envelope is highly glycosylated with 50% of carbohydrates representing its molecular weight. Carbohydrates play a critical role in determining the structural domains involved in receptor binding (27, 28). It is possible that the side groups located on the structure of Q. saponins, specifically the fatty acids, can prevent glycoprotein induced cell fusion. Previous studies have demonstrated that binding of gp120 was greatly reduced after the removal of the gp120 carbohydrates (27). If the saponin extract is able to bind to the carbohydrate moieties, the extract functions as a glucosidase inhibitor and causes reduced infectivity of HIV-1 and HIV-2. It is also indicated that major variable loops (V1/V2) in the structure of gp120 is the location of the specific neutralization. V1/V2 are well exposed and binding of Q. saponins could prevent the conformational changes in gp120 needed for CD4 binding (29). Further studies would need to be performed to confirm this.

Another possible mechanism to explain the high percentage of cells protected from HIV-1 and HIV-2 infection could be due to electrostatic interactions. The CD4 molecule consists of over 400 amino acid residues (31). It is plausible that the fatty acid side chains on the structure of saponin react with the amino acids and cause the extract to coat the cells and prevent viral attachment. Saponification would therefore occur at lower concentrations and prevent some degree of cytotoxicity, as determined earlier.

The crystal structure of CD4 reveals four domains (D1, D2, D3, D4) that are comprised of amino acids and function in membrane flexibility (31, 32). The D2-D3 junction is highly flexible and is required for proper binding to gp120. If this region were to be neutralized by the binding of saponin extracts, infection of HIV-1 and HIV-2 would be greatly reduced. Further studies would need to be performed to confirm this. Since the CD4 receptor is the major receptor for HIV infection, studies would need to confirm the degree to which Q. saponin extracts block infection and if there is any effect on the chemokine co-receptors.

Although the potential for vaccine development using the *Quillaja saponaria* Molina extract as an adjuvant would require long-term research, there are other possibilities for short-term development. The extract could be added to current spermicidal foams that are available for use to create a product that can be both spermicidal and virucidal. The current foams available on the market, N-9 (nonoxynol) spermicides, have substantial advantages including ease of use, no direct side effects, do not require a prescription, and can be controlled by women (40). With the most minimally cytotoxic concentration of saponin extract being determined to be $1.6x10^{-6}$, it would be possible to increase the concentration to $1.6x10^{-5}$ since the treatment is topical. Since 100-1000 virus particles are typically transferred, these concentrations could cause the most amount of destruction to sperm and HIV virions, as well as being the least harmful to the host. Of course, the extract added to the foam would not confer 100% protection against HIV infection, but could provide enough of a barrier to block low viral concentrations.

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BIOGRAPHICAL INFORMATION

Born and raised in Oak Cliff, I have been a lifelong resident of Dallas, Texas. After graduating from Tyler Street Christian Academy in 1998, I received my undergraduate degree in Biology from the University of Dallas in 2003. While originally intent of attending medical school, I gradually realized that I can better serve society by devoting my time and efforts to research. After graduation, I accepted a job as a research technician at the University of Texas Southwestern Medical Center. While at UTSMC, I was first introduced to HIV research, assisting in the investigation of the lypodistrophic side-effects of anti-viral therapies currently on the market. Engrossed by my HIV research in the lab, I was inspired to return to school and do my part in continuing fight to end the epidemic.

HIV research has now become a life-long commitment of mine. By increasing social awareness of this problem, it is possible to overcome ignorance of the disease and secure the funding necessary to assist the 40 million people currently infected and to lower infection rates in the future. Current treatment methods are becoming more and more toxic to the host and new alternatives are needed to provide better long-term therapy. My goal is to assist in this cause by working side by side with my fellow researchers to find better treatment regiments as well as with HIV infected individuals to reduce social prejudice.