ANTI-VIRAL CAPACITY OF QUILLAJA SAPONARIA MOLINA EXTRACT AGAINST VACCINIA AND HERPES SIMPLEX VIRUS-1

by

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July 7, 2006

ABSTRACT

ANTI-VIRAL CAPACITY OF QUILLAJA SAPONARIA MOLINA

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It is well known that Quillaja saponins have the ability to elicit a strong immune response when used as an adjuvant in an ISCOM matrix. However, to date, the antiviral capability of Quillaja saponins has not been documented. Using extracts from the Chilean Soap Bark Tree (Quillaja Saponaria Molina) we document the anti-viral capacities of Quillaja saponins against HSV-1 and Vaccinia. The saponin was ineffective against Vaccinia Virus, however neutralized HSV-1 in a time and temperature dependent manner. In addition, Quillaja Saponins were determined capable of preventing cell-to-cell spread when introduced in solution to cell monolayers.

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CHAPTER 1

INTRODUCTION

1.1 Background on Quillaja Saponins

Quillaja saponins obtained from the Chilean Soap Bark tree, *Quillaja saponaria* Molina, are known to be physiologically active triterpenes. They are commonly used in animal vaccines and are under current clinical trial for human vaccines. The saponin extract used in this study is complex. In general, the quillaic acid skeleton serves as a frame to present the oligosaccharide chains usually found at positions C3 and C28. It is believed the quillaic acid does not serve as the physiologically active part of the saponin, instead it is the oligosaccharide chains that are presented to cellular or viral targets. Differences among the multiple types of saponins are based on the oxidation around the quillaic skeleton, which in turn is based on the types, location and number of sugars and acyl moieties (1, 9, 18).

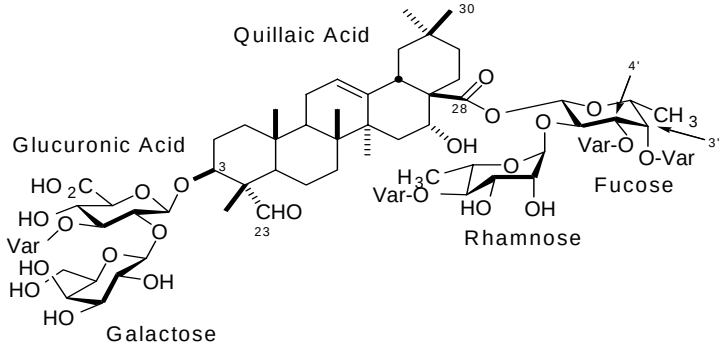


Figure 1. General structural features of the Quillaja saponins

It has been shown that saponins have an immunoenhancing and immunmodulating capacity when incorporated as adjuvants in vaccines, yet have been considered to be far too toxic for human use in crude form. Recent purified fractions, such as QS-21, have shown to elicit both humoral and cytolytic T-cell responses to peptide and protein antigens used in subunit vaccines (2). One major problem encountered when using subunit or non-replicating vaccines is their weak immunogenicity, thus an adjuvant must be used. Currently, the only adjuvants approved for human use are calcium and aluminum salts (4). Unfortunately, these adjuvants are not easily used for many subunit vaccines since they lack the ability to stimulate, enhance and modulate the immune response (5, 6). Specifically, their capacity to stimulate the production of interlukin-2 and interferon-gamma is reduced. These two cytokines along with others, are responsible for activation of T-helper cells (5, 6). It has been shown that the incorporation of Quillaja saponins into ISCOM matrices is sufficient to evoke strong Th-1 and CTL responses. These are required to protect against intracellular pathogens as well as malignant cells, and modulate that response through activation of IL-4, IL-5, IL-6, IL-12 (3, 4, 5, 6, 7).

Unfortunately, degradation of saponins is easily accomplished at high temperatures in a short period of time. Recent evidence indicates that the fatty acid acyl moiety of Quillaja saponins are responsible for their high toxicity and immune stimulating capacities. The ester bond can be easily hydrolyzed above a pH of 5.5 resulting in a deacylated saponin. Upon deacylation, the saponin loses the capacity to stimulate a strong immune response (7). Marciani et al. tested deacylated saponins'

immunoenhancing and immunomodulating capacities using HSV-1 gD as the antigen. They showed that upon immunization of mice with different levels of partially degraded saponin, the ability to produce antibodies to exogenous antigen dropped, and the reduction corresponded to the level of degradation of the saponin. In addition, the deacylated forms allow the shift between a Th-1 like response to a Th-2 like response (7). This change may not affect a vaccine whose effect is through the production of neutralizing antibodies, but in the case of intracellular pathogens, where complement or antibody-dependent cell cytolysis is the major route of clearance, degradation of the saponin would limit the vaccines usefulness (7, 8).

1.2 Background on Vaccinia Virus

Vaccinia virus was used in a global effort to eradicate another member of the orthopoxvirus genus, variola. Vaccinia is one of the largest animal viruses and can be visualized by light microscopy. The virion has a characteristic oval-shaped morphology with a noninfectious linear 191 kb double-stranded DNA genome (11, 12). The envelope structure is of high importance to this experiment and will be discussed in detail below. There are three known infectious forms of Vaccinia; the EEV (Extracellular Enveloped Virion) and the IMV (Intracellular Mature Virion). The IMV and EEV differ phenotypically as well as pathologically. The IMV is a doubly wrapped infectious form of Vaccinia, while the EEV is a triply wrapped infectious virion derived from the quadri-wrapped IEV (Intracellular Enveloped Virion). The IMV does not participate in the second wrapping process utilized by the EEV. The difference between

these infectious forms has led to the discovery of different roles for the EEV and IMV (34, 36). Blasco et al. demonstrated that the EEV is involved in long range spread while the CEV (Cell-associated enveloped virion) is mainly responsible for cell-to-cell spread, however, the IMV lacks the ability to form viral plaques by itself (12, 34, 36). These virions attach to cells differently but a cellular receptor has yet to be identified for either (10).

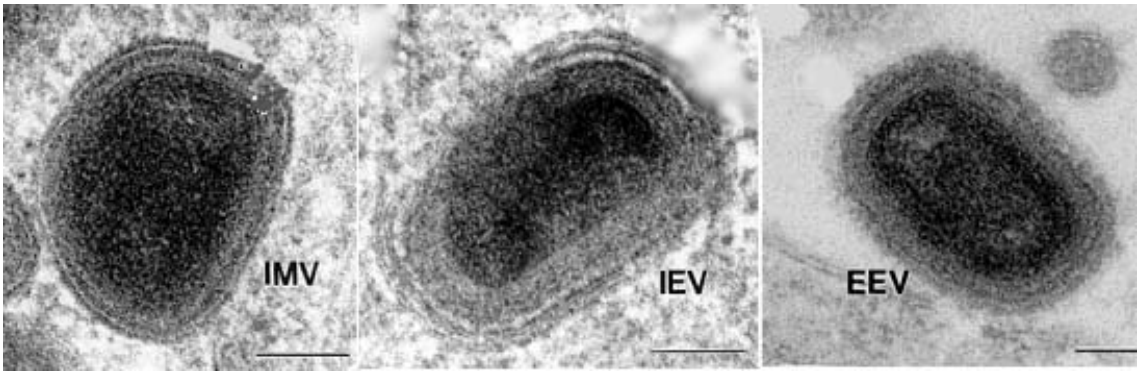


Figure 2. Electron Micrographs of the IMV (left), IEV (middle), and EEV (right). Figures according to Risco et al (39).

Several different membrane proteins have been implicated in attachment of Vaccinia to the host cell, specifically A27L, D8L, and H3L (13,14,15). Since Vaccinia has a wide host range, these viral attachment receptors must bind to the cell surface using ubiquitous cellular receptors. This theory was developed in recent publications, implicating that Vaccinia binds to cell surface heparin sulfate and chondroitin sulfate (13, 16, 17). These two glycosoaminoglycans (GAGs) are common on the surface of most mammalian cells, and would fit the necessary limitation against Vaccinia. Chung et al. demonstrated that Vaccinia A27L protein binds to heparin sulfate on the surface of

host cells along with H3L, while D8L has a chondroitin sulfate biding motif (13, 14, 15). Vasquez et al. revealed that the N-terminus of the A27L envelope protein of Vaccinia virus is responsible for binding heparin sulfate while the binding motifs of D8L and H3L remain unknown (13).

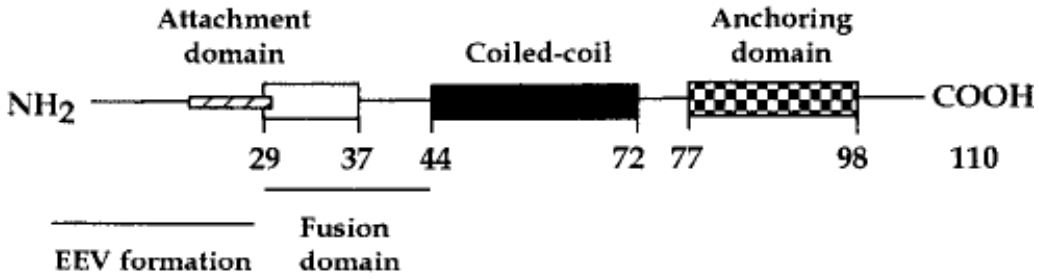


Figure 3. Structure of the A27L protein of Vaccinia Virus illustrating the attachment domain to heparin sulfate. Figure According to Vasquez and Esteban

The second step of viral infection, fusion, is complicated by the presence of two different infectious forms of the Vaccinia virus. The entry of the EEV is mediated by pH-dependent endocytosis, while the fusion of the IMV may be accomplished in a low-pH-independent manner (14, 40). However, the fusion process for both infectious forms has been attributed to the action of the 14kDa protein encoded by the A27L gene, giving dual specificities to this protein (14).

Upon membrane fusion of the virion to the cell, the next step in the replication life cycle of Vaccinia virus is uncoating. Studies on this process have defined two different stages of uncoating. The first stage involves the loss of virion proteins and lipids while the genome is still within the core. This is followed by the release of the

core particle into the cytoplasm of the host cell (11). At this point, the genome of the virus is accessible to DNases; this indicates the presence of nucleic acids in the cytoplasm of the host cell (10, 11).

After the uncoating process is achieved, or arguably during the uncoating process, early gene expression begins. The virion's transcription machinery immediately releases a class of polyadenylated transcripts. Two of the known transcripts are 82 and 77 kDa peptides that likely bind in the form of a dimer. This polypeptide has been shown to bind and initiate transcription from early promoters within the virus genome. These early gene products include DNA polymerase, thymidine reductase, thymidylate kinase and ribonucleotide reductase. The latter three gene products are of importance to the virus since they are responsible for generating precursors for DNA synthesis (11).

Replication of the viral genome takes place in the cytoplasm of the host cell. This attribute is specific to poxviruses, Vaccinia included. The onset of replication begins within 2 and 5 hours post-infection and can yield up to 10,000 copies. There does not seem to be an origin of replication in the Vaccinia genome, unlike many other viruses. Recombination of the viral genome occurs frequently in Vaccinia infections. Typically, the termini of the genome seem to be the most variable due to the lack of a tight packaging mechanism, which explains the length of the genome. Following replication of the genome, the onset of late gene expression begins with the production of several structural transcripts involved in virion assembly post-translation. These

transcripts have a much shorter half-life than that of the early-gene transcripts, and seem to halt early-gene transcription (11).

Due to the complexity of Vaccinia virus, the assembly of virion progeny remains unclear. However, some important data is not beyond the scope of this publication and should be examined. Unlike other enveloped viruses, Vaccinia does not seem to acquire its envelope from the host cell membrane. Rather, the virion is assembled in the host cell's cytoplasm or viroplasm, to form an immature particle. The immature particle is formed in viral factories called viral crescents found in the intermediate compartments located between the ER and the Golgi. The spherical immature particle matures into the brick shaped IMV (34). Afterward, the doubly wrapped IMV may undergo a second wrapping from the trans-golgi yielding the IEV or may be released as the IMV (34, 36). As the IEV egresses out of the cell it loses its outermost membrane yielding the three layered EEV, or it may stay attached to the cell producing the CEV (38).

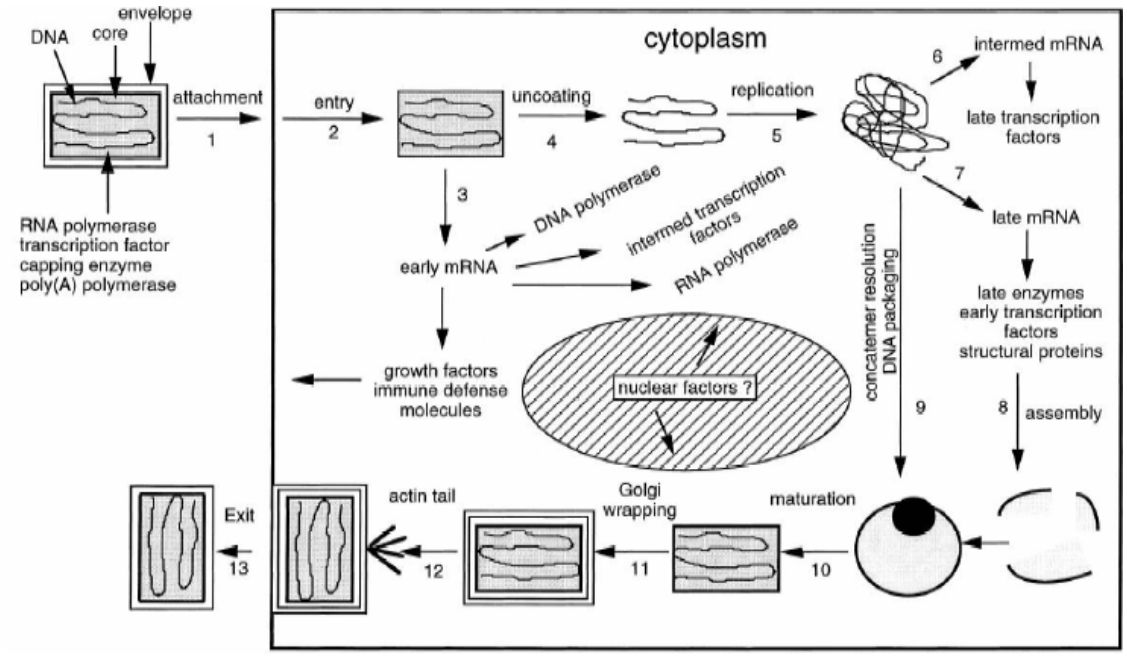


Figure 4. Basic Replication Mechanism of Vaccinia Virus according to B.F. Fields (31).

1.3 Background on Herpes Simplex Virus 1

Currently, it is estimated that 60-95% of the world's population is infected with HSV-1 (20). Infection with this virus can have multiple clinical manifestations including corneal blindness, encephalitis, gastrointestinal disorders and roughly 25% of genital herpes infections (22). HSV-1 is a singly enveloped double stranded DNA virus that replicates in the nucleus of the host cell (19, 35). The genome minimally encodes 84 polypeptides, of which the envelope glycoproteins are of importance to this study (19). It is hypothesized that the interaction between viral glycoproteins and cell surface glycosaminoglycans provides the initial event leading to attachment. Multiple viral glycoproteins are involved in attachment and membrane fusion. The initial attachment event is mediated by gB and gC. These membrane proteins bind to heparin and

chondroitin sulfate moieties of GAGs on the surface of the cell and are responsible for the initial attachment event (23, 24, 26). The heparin and chondroitin sulfate binding domains on gC overlap from residues 129-160, suggesting multiple roles for these glycoproteins. For example, if the HSV virion were to contact a cell lacking either heparin or chondroitin sulfate moieties, the virus still maintains infectivity (23, 26). This event is followed by attachment of gD to one of the three entry receptors. These entry receptors include HVEM (herpes virus entry mediator) a member of the TNF family of receptors, nectin-1 and nectin-2, members of the immunoglobulin super family of receptors, and lastly specific sites in heparin sulfate modified by 3-O-sulfotransferases (23, 28). The binding of gD is not enough to trigger membrane fusion. gB as well as a gH/gL heterodimer bind to entry receptors facilitating entry of the core particle into the cell (23). Attachment differs slightly in polarized epithelial cells lines. Although attachment in nonpolarized cell lines is mediated mainly by gB, it is known that gC- and gG- mutant viruses lack the capacity to attach to apical cell membranes of polarized epithelial cells. However, their ability to attach to the basal membranes remains unhindered. Nonpolarized cell lines used in this study express the necessary attachment receptors for both gC+ and gC-, as well as gG+ and gG- attachment (25).

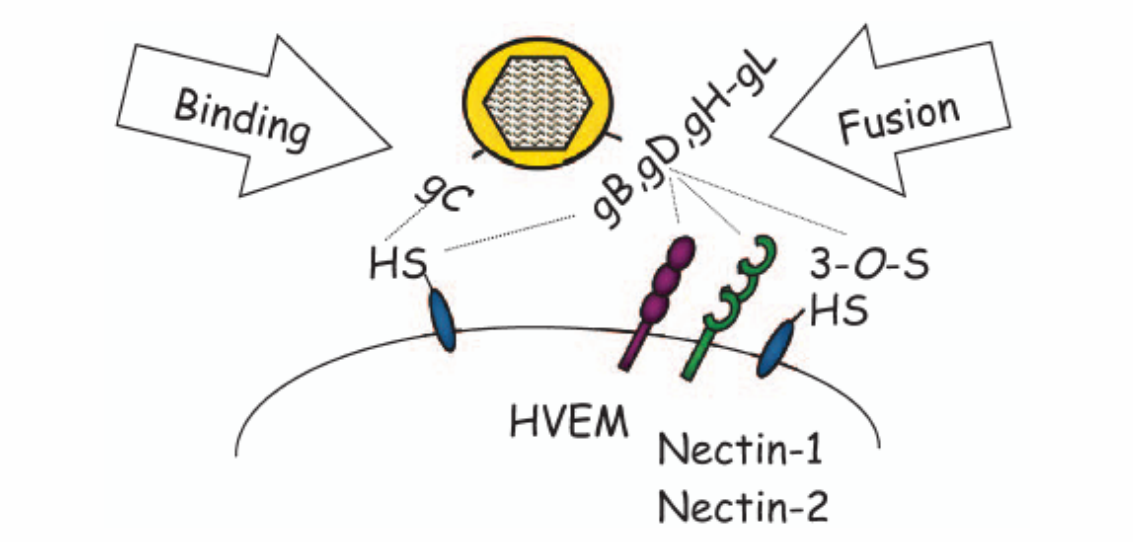


Figure 5. Illustration of Attachment to Cell Surface by Herpes Simplex Virus Type 1 according to Spear (23).

Table 1. Surface glycoproteins of HSV-1 according to Rajcani (29).

Glycoprotein	Gene	Function
gB	UL27	Interaction with GAGs during early attachment/adsorption, participates in membrane fusion
gC	UL44	Cooperates with other glycoproteins during adsorption, interacts with GAGs and complement protein C3b. Specific antigen domain.
gD	US6	Interacts with cell surface receptors (HVEM, nectin1,2, and sites modified by 3-O-sulfotransferases. Participates is cell to cell spread.
gE	US8	Mediates cell to cell transmission and neuronal spread.
gG	US4	Unknown
gH	UL22	Forms complex with gL. Initiates membrane fusion and stabilizes late adsorption.
gI	US7	Complexes with gE
gK	UL53	Involved in virion transport in cytoplasm and in environment of mature capsids.
gL	UL1	Forms complex with gH.

Following viral fusion to the host cell plasma membrane the nucleocapsid is released into the cell cytoplasm. This “naked” nucleocapsid travels along a network of microtubules to the host cell nucleus where the viral DNA is released into the nucleus by means of a pore. Once in the nucleus, transcription of the early genes follows (30). Host cell RNA polymerase II is responsible for viral transcription and is initially stimulated by viral VP16 (31). Once the immediate-early genes have been transcribed, they are followed by the early and late genes sequentially. Viral translation occurs exclusively in the cytoplasm of the host cell. Upon translation of the early gene products, they are relocalized in the nucleus to aid in replication of the viral genome.

Following viral replication, viral capsid particles move to the nucleus and begin polymerization of the nucleocapsid. The viral DNA is then packaged inside the nucleocapsid and buds through the inner nuclear membrane. Following budding through the nuclear envelope, the exit of the virion is a controversial topic. There are two theories that are concerned with this process. The first is the reenvelopment pathway. The enveloped particle fuses with the outer membrane of the nucleus losing its envelope and becoming a "naked" nucleocapsid. Once in the cytoplasm the virion then moves into the trans-golgi network and is released via a secretory vesicle. It then undergoes modifications through a fusion event between the viral vesicle and the Golgi network (31, 37). In the second hypothesis, called the luminal pathway, the enveloped virus particle moves into the endoplasmic reticulum or into the trans-golgi network, or the golgi vesicles themselves. It is at this location that final maturation of viral glycoproteins takes place. Eventually the mature virus particle is released, either through secretory vesicles or through cell to cell junctions (31).

1.4 Previous Antiviral Studies Using Organic Extracts

Antiviral studies involving organic compounds are not a novel concept. Several different studies have taken place in recent history giving a perspective that needs to be discussed. Anti-Poxvirus and Anti-HSV activity of organic compounds have been studied before with mainly African plants. Sindambiwe et al. demonstrated a powerful Anti-HSV effect of a Rwandan medicinal plant that was given to the indigenous population by local medicine men (32). Also, Kotwal et al. effectively neutralized

Vaccinia virus with a small load of Secomet-V, an acidic plant extract from the species Trifollium (33). Other saponin extracts have demonstrated an ability to prevent viral infection. Anagllis arvensis, Primulaceae demonstarted anti-viral activity against herpes simplex virus type 1 and poliovirus type 2 by preventing the attachment of the virion to the host cell plasma membrane (42). In addition, Tokuda et al. showed that triterpene glycosides are able to prevent attachment of Epstein-Barr virus in vitro (43). In order to properly examine the ability of Quillaja Saponins to prevent infection or neutralize common human viral infections we chose Vaccinia virus and HSV-1. These two viruses differ in their mode of initial infection of host cells and their common replication mechanisms. It is intended to use this compound as a broad spectrum anti-viral agent, therefore the saponins' ability to prevent infection and/or neutralization capacity must be ascertained. Quillaja Saponaria Molina as we are using in this publication is only toxic at high concentrations (>1 mg/ml). This compound is currently approved for human consumption under by the FDA (under CFR 172.510, FEMA number 2973 NON-GMO) and is approved for use in organic foods (under N.O.P. 205.605).

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth of Vaccinia Virus

A 150 cm flask of 143 cells containing 5 ml of Minimum Essential Medium Eagle was infected at an MOI of 1. The flask was rocked at 15 minute intervals to prevent the cells from drying out. After 1 hour, the flask was supplemented with 20 ml Minimum Essential Medium Eagle 5% Fetal Bovine Serum and 1% Penicillin/Streptomycin and incubated at 37°C, 5% CO_2. Once 80% or more of the cells lysed, the flask was briefly sonicated at an amplitude of 60 on an Ultrasonic Processor and stored at -20 °C until further use.

2.2 Growth of HSV-1

A 150 cm flask of Vero cells containing 5 ml of Minimum Essential Medium Eagle was infected at an MOI of 1. The flask was rocked at 15 minute intervals to prevent the cells from drying out. After 1 hour, the flask was supplemented with 20 ml Minimum Essential Medium Eagle 5% Fetal Bovine Serum and 1% Penicillin/Streptomycin and incubated at 37°C, 5% CO_2. Once 80% or more of the cells lysed, the flask was briefly sonicated at an amplitude of 60 on an Ultrasonic Processor and stored at -20 °C until further use.

2.3 Cytotoxicity Assay

To determine the maximally viable concentration of saponin, Vero and 143 cells were seeded in 24 well plates at 60% confluency. After 24 hours of incubation the media was aspirated and the cell monolayers were overlaid with Minimum Essential Medium with Eagle's Salts, 5% FBS, 1% Penicillin/Streptomycin and saponin. After 96 hours, the media was aspirated and the cell monolayers were stained with Trypan Blue for 5 minutes to visualize viable and dead cells.

2.4 Plaque Assay

A plaque assay was performed to ensure that enough virus was present to run the experiment. Two 6-well plates were seeded with 143/Vero cells at 60% confluency, and allowed to incubate overnight at 37 °C and 5% CO_2. Minimum Essential Medium with Eagle's Salts was used to set up 10-fold dilutions of lysate in 1 ml microcentrifuge tubes. 300 microliters of each dilution (-3 to -7) was added to the correct well and plates were incubated for 1 hour at 37 °C and 5% CO_2 while rocking the plates at 15 minute intervals. After 1 hour, the plates were covered with Minimum Essential Medium with Eagle's Salts supplemented with 5% FBS and 1% P/S. After 48 hours, the overlay was removed and the cell monolayers were stained with 0.1% crystal violet for 5 minutes to visualize plaques. The titer of the virus was determined using the following equation: # of plaques/ [(volume of dilution added) X (dilution concentration)].

2.5 Vaccinia Plaque Reduction Assay

6-well plates were seeded with $1X10^6$ cells of 143 cells and allowed to incubate at 37 °C and 5% CO_2 for 24 hours. Afterward, the cells were pretreated for 6 hours with the maximally viable concentration of saponin. The media was then aspirated and the cell monolayers were infected according to standard plaque assay protocols.

2.6 Herpes Simplex Virus Plaque Reduction Assay

6-well plates were seeded with $1X10^6$ cells of Vero cells and allowed to incubate at 37 °C 5% CO2 for 24 hours. Afterward, the cells were pretreated for 6 hours with the maximally viable concentration of saponin. The media was then aspirated and the cell monolayers were infected according to standard plaque assay protocols.

2.7 Virucidal Assay

$1X10^6$ PFU's of virus were treated with maximally viable concentrations of saponin for 1, 2, 4, and 6 hours respectively at 37 °C and 4°C. After the respective treatments, the virus was used to infect cell monolayers in 12-well plates according to standard plaque assay protocols. From initial data gained from this assay, the saponin concentrations were then reduced by 10, 100, 1000 fold to discover the minimum concentration that exhibited virucidal capacity.

2.8 Saponins Effect on Viral Spread

Plaque areas were measured by scanning 12-well plates with an EPSON 1640 SU photo scanner. The images were analyzed with Image J from NIH. Eliptical selections were used to determine the average area of the plaque. The data was then analyzed using a linear mixed model ANOVA to determine significance between the mean areas due to treatment and temperature.

2.9 Isolation of Saponin

The saponin extract used was purchased from Desert King International, San Diego, CA. The material Ultra Dry 100-Q is the spray dried purified extract of the Chilean Soap Bark Tree (*Quillaja Saponaria Molina*) consisting of > 93% Quillaja soids and moisture content < 7%. This material was then solvated in methanol to yield a 5% stock solution which was further diluted to yield the CCID 10.

CHAPTER 3

RESULTS

3.1 Cytotoxicity Data

Cells were seeded in 24 well plates at 60% confluency. After 24 hours of incubation, the saponin was serially 1:2 diluted starting at 0.01% down to 0.000625%. The CCID 10 (cellular cytotoxicity index <10% cell mortality) concentration of saponin was determined from this assay and was used to determine the concentration for the plaque reduction and virucidal assays. The CCID 10 concentration of saponin was determined to be 0.00125% for both the Vero and 143 cell lines.

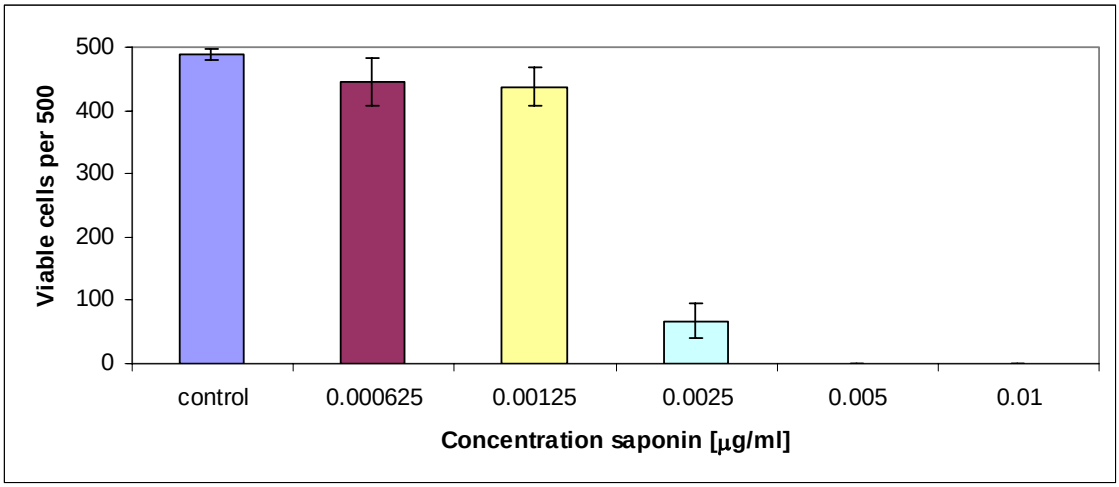


Figure 6. Effect of saponin on viability of Vero cells. Cells were treated for 96 hours and cell viability determined as described in materials and methods.

Table 2. Effect of saponin on viability of Vero cells. Cells were treated for 96 hours and cell viability determined as described in materials and methods.

Viability	Concentration of saponin extract [ug/ml]					
	0	0.000625	0.00125	0.0025	0.005	0.01
Mean	488.55	444.8182	436.73	67.45	0	0
SD	7.72	38.71	30.92	28.11	0	0

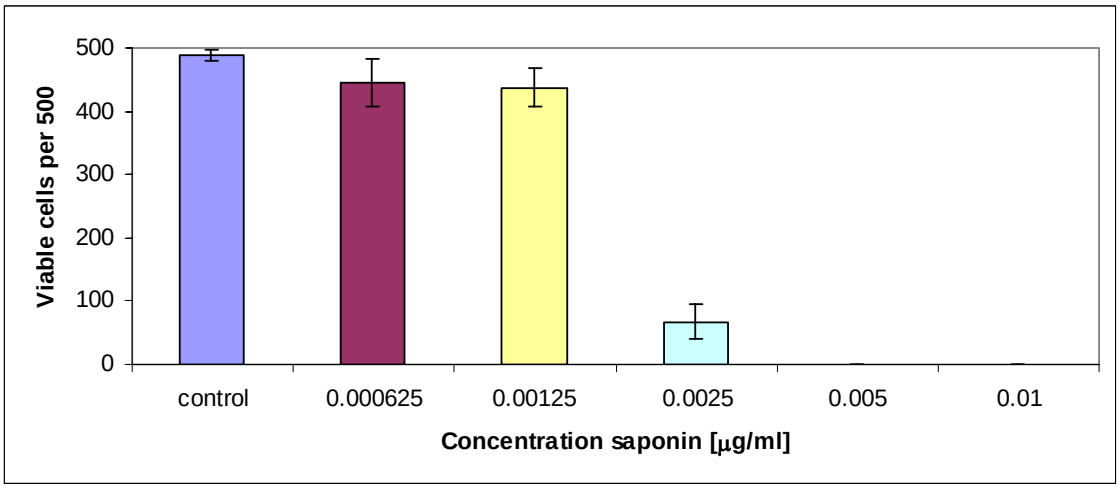


Figure 7 Effect of saponin on viability of 143 cells. Cells were treated for 96 hours and cell viability determined as described in materials and methods.

Table 3. Effect of saponin on viability of 143 cells. Cells were treated for 96 hours and cell viability determined as described in materials and methods.

Viability	Concentration of saponin extract [ug/ml]					
	0	0.000625	0.00125	0.0025	0.005	0.01
Mean	499.64	482.27	488.72	22.91	0	0
SD	0.92	25.63	13.23	6.26	0	0

3.2 Plaque Reduction Assay Data

Cells were seeded in 6 well plates at 60% confluency. After 24 hours of incubation, the medium was aspirated from the well, and the cell monolayers were treated with the CCID 10 concentration of saponin for 6 hours. Following treatment,

the cell monoloyers were infected according to standard plaque assay protocols. There was not a significant reduction in plaque formation in the Vero or 143 cell lines. The experimental and control values were very similar.

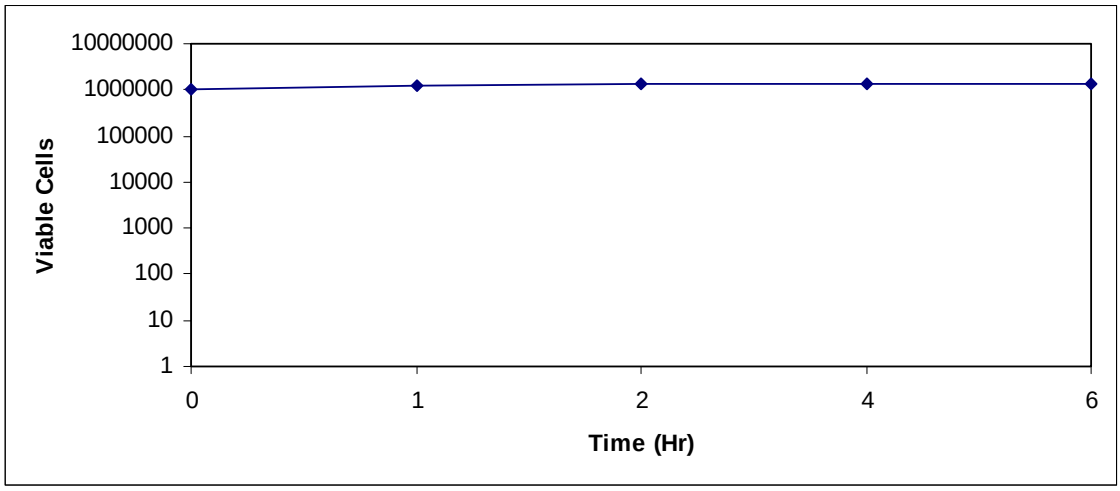


Figure 8. Pretreatment of Vero cells with saponin [0.00125 μg/ml] at multiple times prior to infection with HSV-1

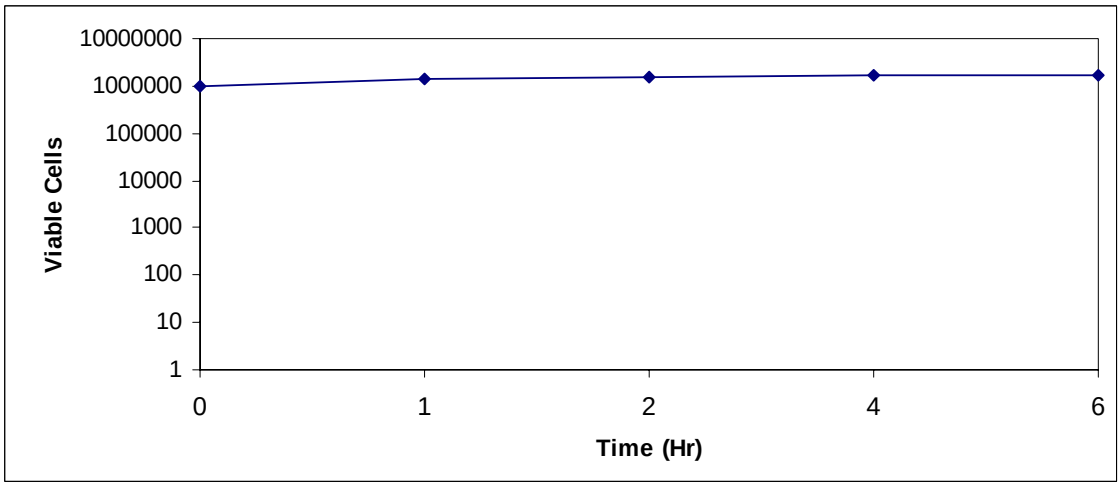


Figure 9. Pretreatment of 143 cells with saponin [0.00125 μg/ml] at multiple times prior to infection with HSV-1

3.3 Virucidal Assay Data

$1X10^6$ PFU's of virus were treated with CCID 10 concentration of saponin for 1, 2, 4, and 6 hours respectively at 37 °C and 4 °C. After the incubation period the viral dilutions were used to infect cell monolayers in 12-well plates. Vaccinia did not display any reduction of infectivity following treatment of saponin at any temperature for any pre-determined period of time. However, HSV-1 responded to saponin treatment in a time and temperature dependent manner. As shown in the figures below, treatment of HSV-1 at 4 °C for up to 6 hours resulted in a 1.5 log reduction of plaque formation as compared to the control. In addition, at 37 °C the HSV-1 was effectively neutralized after 4 hours of treatment.

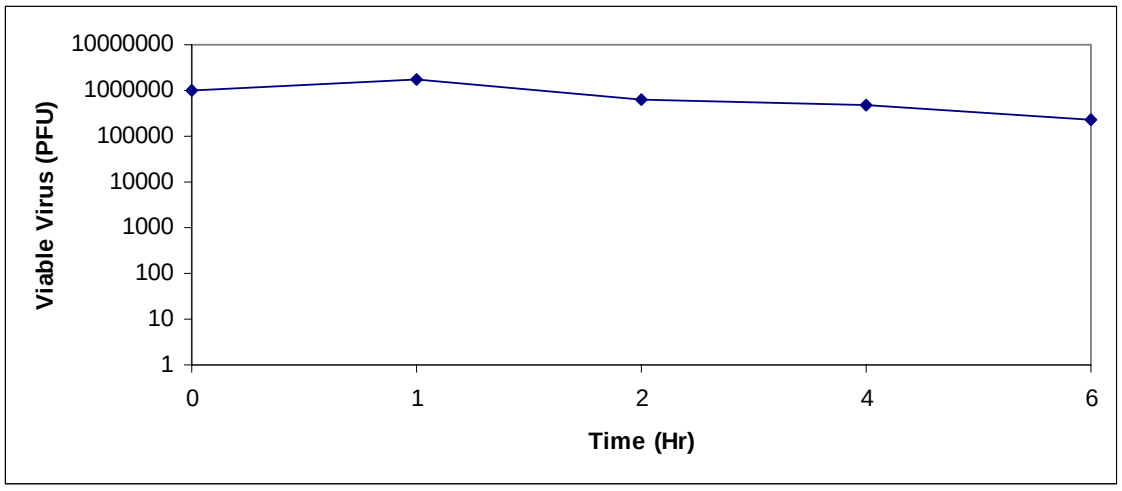


Figure 10. Control treatment of HSV-1 with MEM at 37 °C. HSV-1 [$1x10^6$ PFU] were incubated in MEM for up to 6 hours. The viable virus remaining determined by plaque assay as described above.

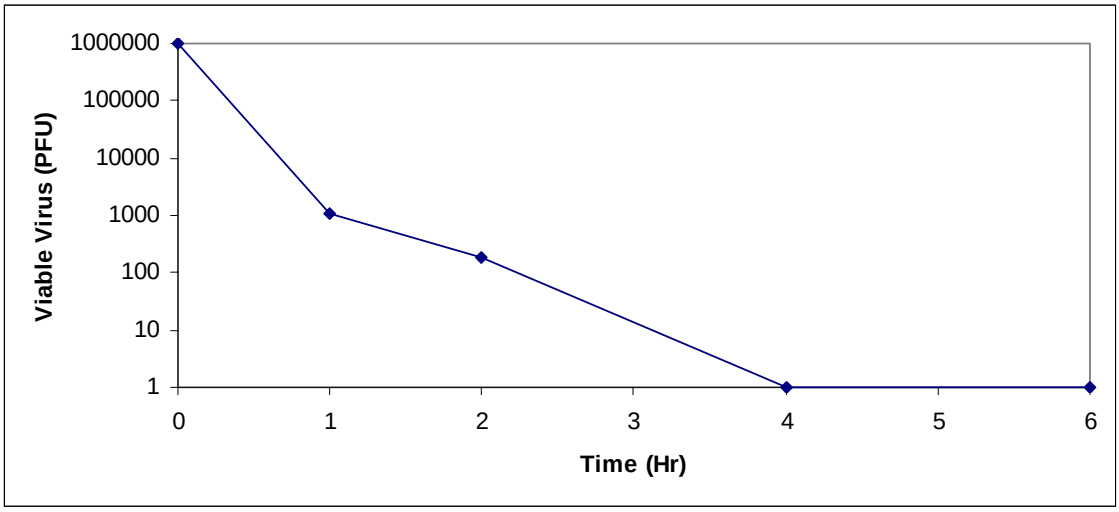


Figure 11. Treatment of HSV-1 with saponin at 37°C. HSV-1 [1x10^6 PFU] were treated for up to 6 hours with saponin [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

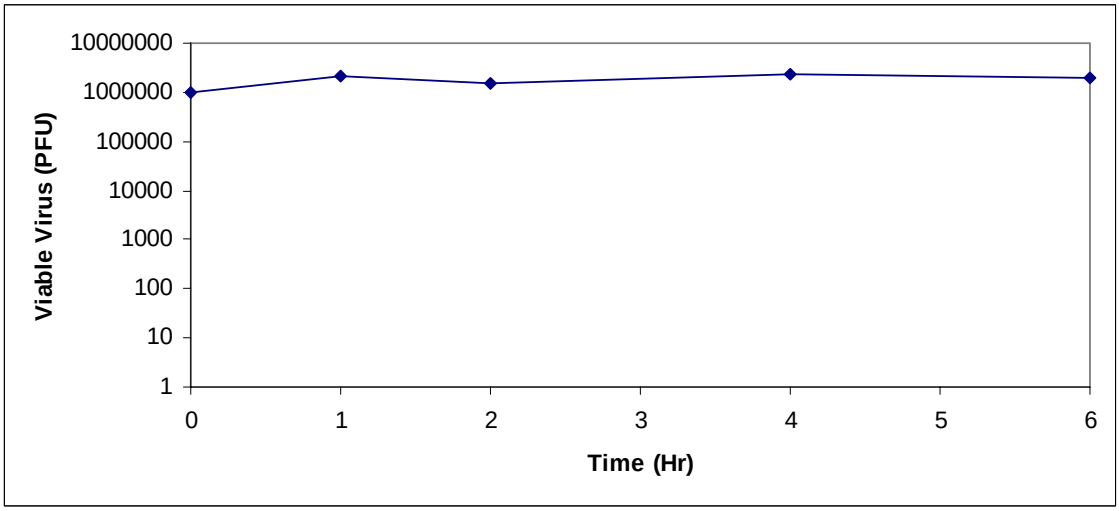


Figure 12. Control treatment of HSV-1 with MEM at 4 °C. HSV-1 [1x10^6 PFU] were incubated in MEM for up to 6 hours. The viable virus remaining determined by plaque assay as described above.

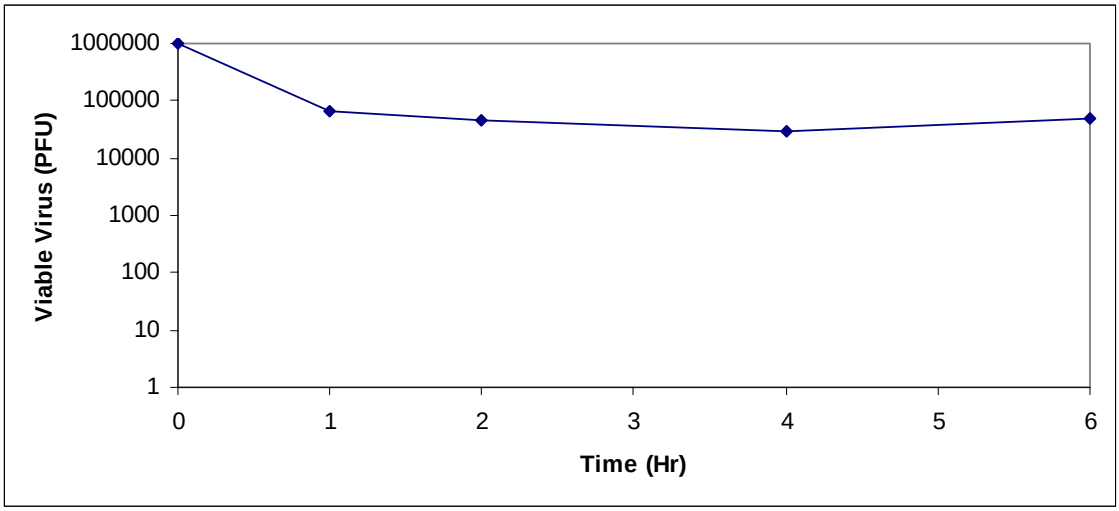


Figure 13. Treatment of HSV-1 with saponin at 4 °C. HSV-1 [1x10^6 PFU] were treated for up to 6 hours with saponin [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

Table 4. Summary of HSV-1 Titers for the Virucidal Assay

Concentration of Saponin μg/ml	T °C	Time after Saponin Treatment			
		1 hr	2 hr	4 hr	6 hr
0.0125 μg/ml	4	6.46E+04	4.62E+04	3.04E+04	4.74E+04
0.0125 μg/ml	37	1.11E+03	1.78E+02	1.00E+00	1.00E+00
0 μg/ml	4	2.10E+06	1.55E+06	2.28E+06	1.91E+06
0 μg/ml	37	1.76E+06	6.11E+05	5.01E+05	2.31E+05

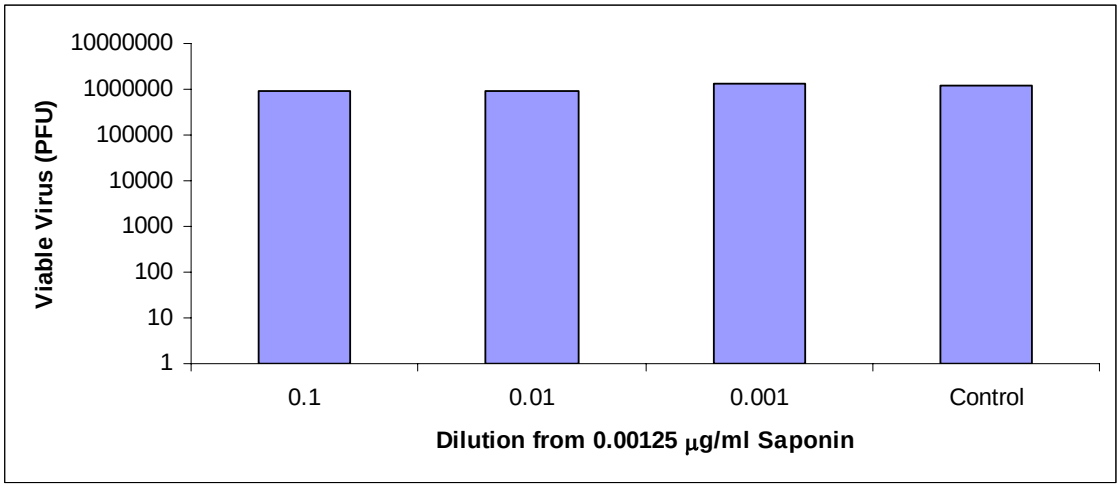


Figure 14. Treatment of HSV-1 with saponin at 4 °C. HSV-1 [1x10^6 PFU] were treated for up to 6 hours with 1:10 dilutions saponin from the CCID 10 [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

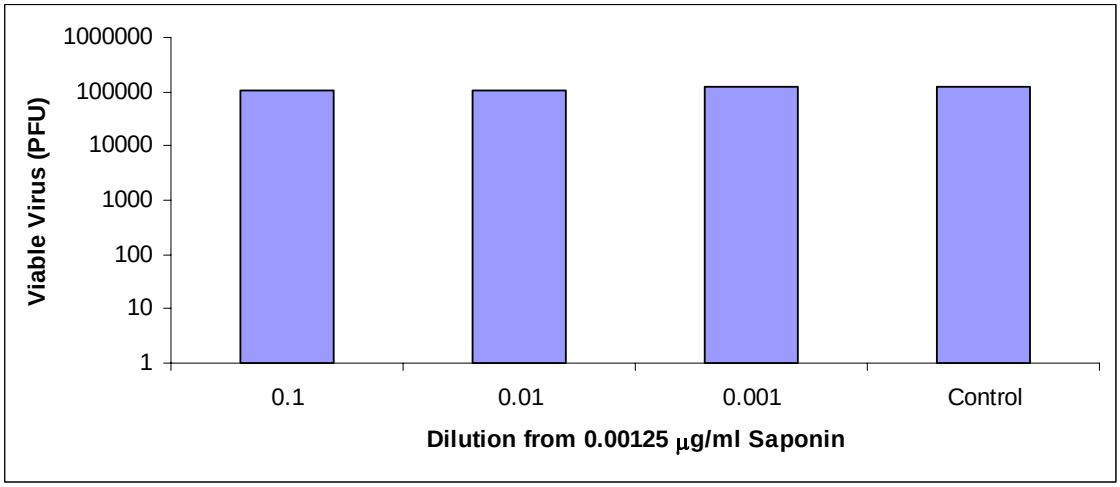


Figure 15. Treatment of HSV-1 with saponin at 37 °C. HSV-1 [1x10^6 PFU] were treated for up to 6 hours with 1:10 dilutions saponin from the CCID 10 [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

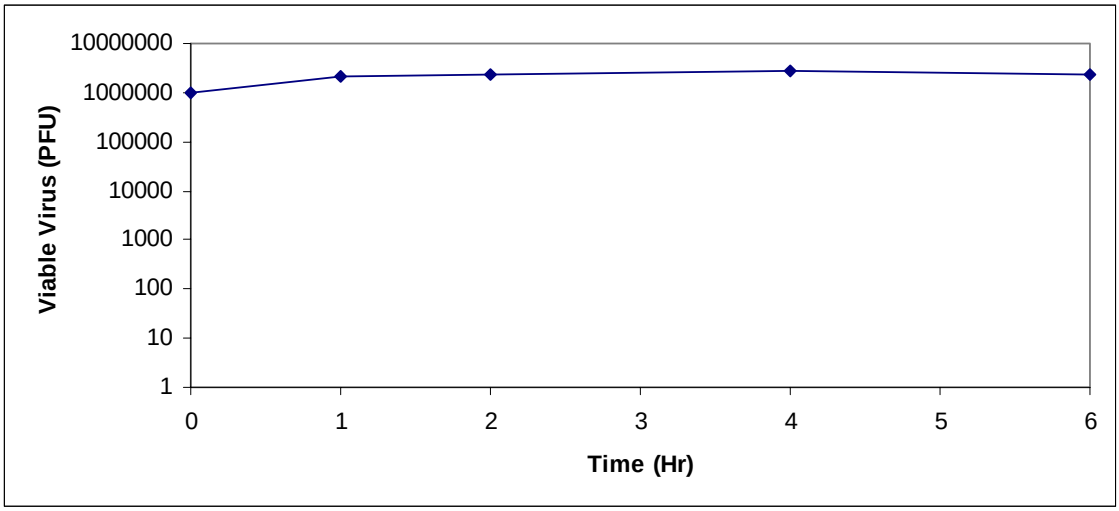


Figure 16. Control treatment of Vaccinia virus with MEM at 4°C. Vaccinia virus [1x10^6 PFU] were incubated in MEM for up to 6 hours. The viable virus remaining determined by plaque assay as described above.

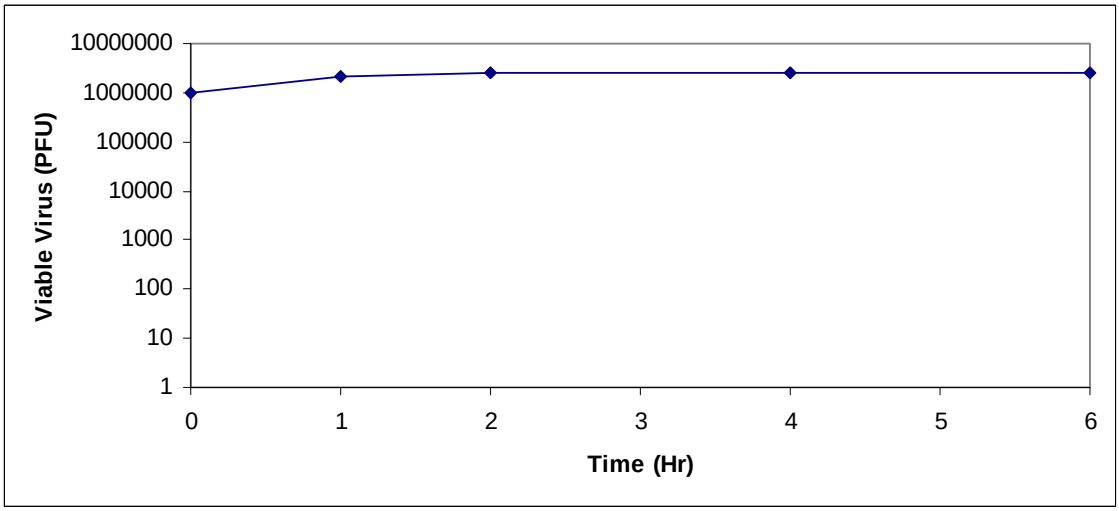


Figure 17. Treatment of Vaccinia virus with saponin at 4 °C. Vaccinia virus [1x10^6 PFU] were treated for up to 6 hours with saponin [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

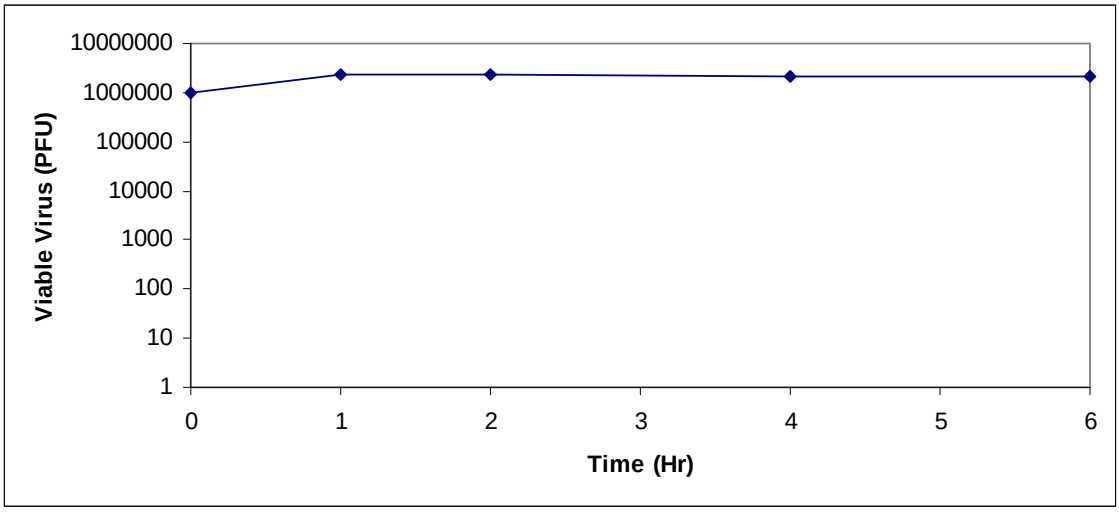


Figure 18. Control treatment of Vaccinia virus with MEM at 37 °C. Vaccinia virus [1x10^6 PFU] were incubated in MEM for up to 6 hours. The viable virus remaining determined by plaque assay as described above.

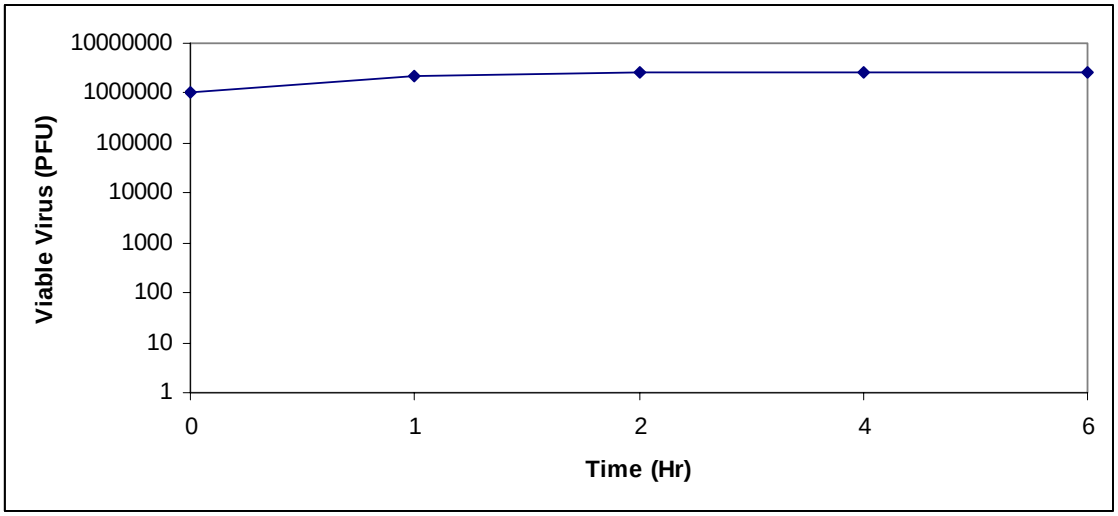


Figure 19. Treatment of Vaccinia virus with saponin at 37 °C. Vaccinia virus [1x10^6 PFU] were treated for up to 6 hours with saponin [0.00125 μg/ml]. The saponin was removed as the viable virus remaining was determined by plaque assay as described above.

Table 5. Summary of Titers of Vaccinia virus for the Virucidal assay.

Concentration of Saponin μg/ml	T °C	Time after Saponin Treatment			
		1 hr	2 hr	4 hr	6 hr
0.0125 μg/ml	4	2.15E+06	2.65E+06	2.62E+06	2.71E+06
0.0125 μg/ml	37	2.14E+06	2.51E+06	2.55E+06	2.49E+06
0 μg/ml	4	2.28E+06	2.35E+06	2.13E+06	2.07E+06
0 μg/ml	37	2.06E+06	2.34E+06	2.71E+06	2.31E+06

3.4 Saponins Effect on Viral Spread

It became apparent that the saponin used in this experiment has a virucidal capacity against HSV-1. To determine the effect of the saponin against viral spread the plaque areas were measured in the control and treatments using Image J from NIH. Fifty data points were randomly selected and measured to determine mean plaque area for each treatment. Analysis of the means was completed using a linear mixed model ANOVA. The mean areas show a reduction in plaque size compared to the control, indicating a potential reduction in cell-to-cell spread when saponin is in solution.

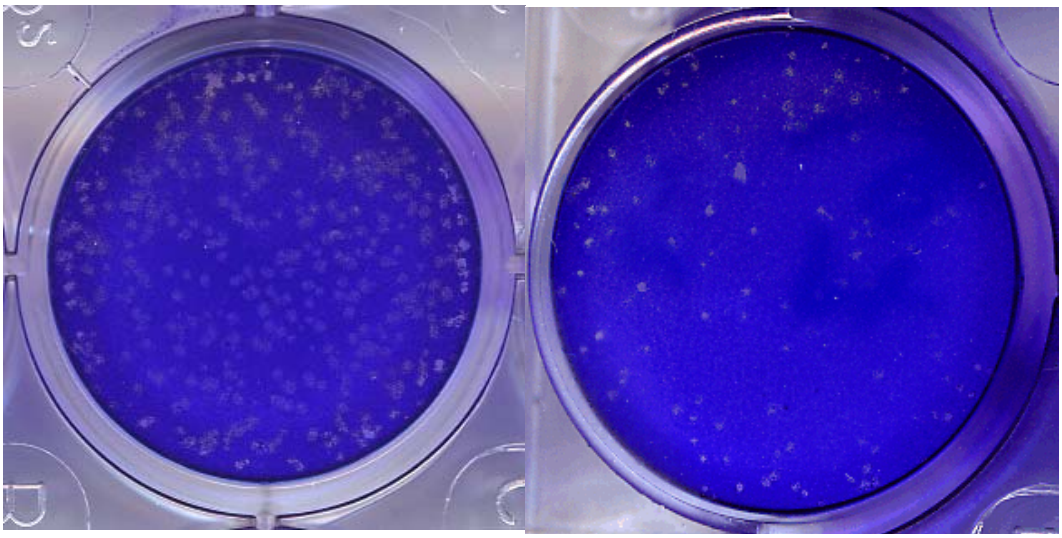


Figure 20. Plate Images of HSV-1 plaques treated with saponin [0.00125 μg/ml] at 4 °C. Control treatment of HSV-1 for 6 hours (left). Saponin treatment of HSV-1 (right).

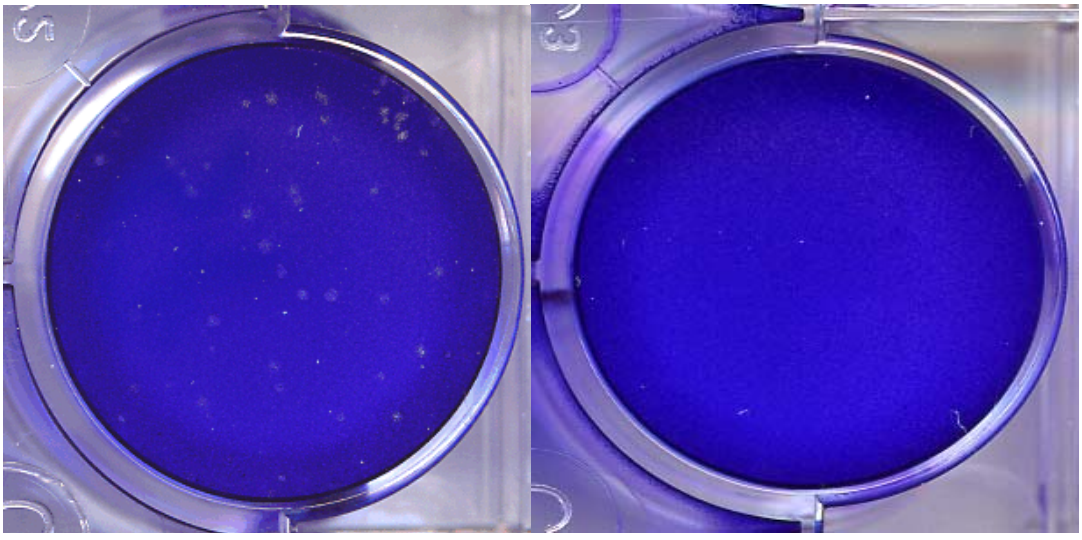
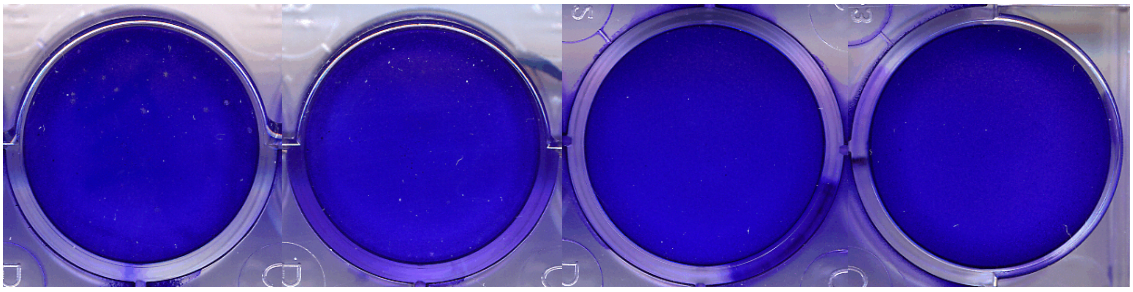
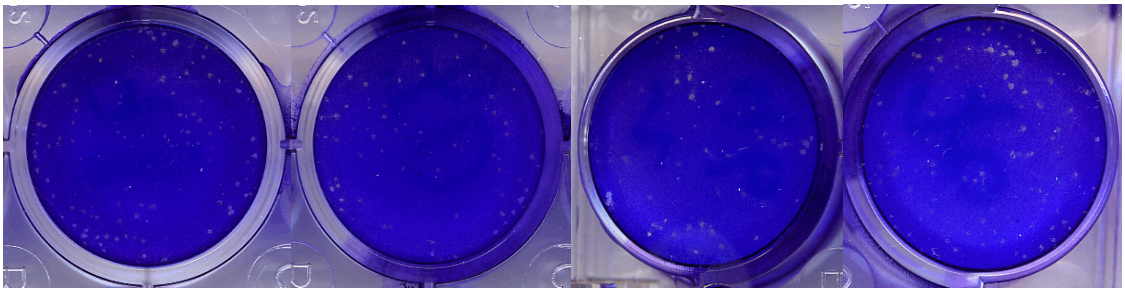


Figure 21. Plate Images of HSV-1 treated with saponin [0.00125 μg/ml] at 37 °C. Control treatment of HSV-1 for 6 hours (left). Saponin treatment of HSV-1 (right).



A B C D

Figure 22. Plate Images of HSV-1 plaques treated with saponin [0.00125 μg/ml] at 37 °C (A) Saponin treatment for 1 hour, (B) Saponin treatment for 2 hours, (C) Saponin treatment for 4 hours, (D) Saponin treatment for 6 hours.



A B C D

Figure 23. Plate Images of HSV-1 plaques treated with saponin [0.00125 μg/ml] at 4 °C (A) Saponin treatment for 1 hour, (B) Saponin treatment for 2 hours, (C) Saponin treatment for 4 hours, (D) Saponin treatment for 6 hours.

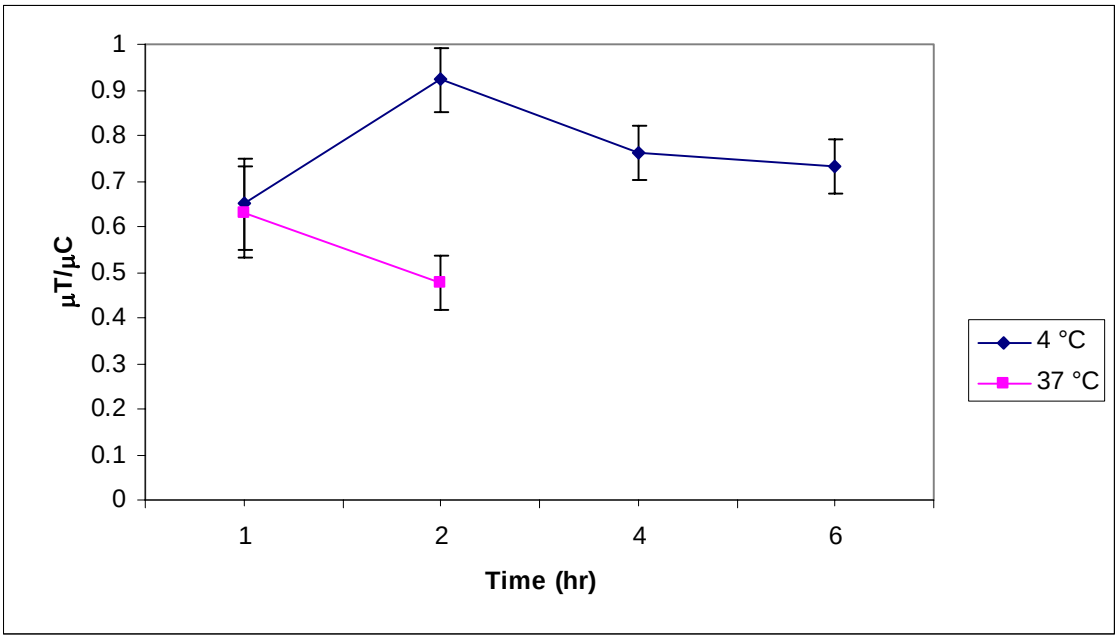


Figure 24. Reduction in plaque size of HSV-1 treated with saponin [0.00125 µg/ml] during plaque assay. µT/µC indicates the mean plaque areas for saponin [0.00125 µg/ml] treatment divided by the mean plaque areas of the control.

Table 6. Reduction in plaque size of HSV-1 treated with saponin [0.00125 µg/ml] during plaque assay. µT/µC indicates the mean plaque areas for saponin [0.00125 µg/ml] treatment divided by the mean plaque areas of the control.

Temperature	Time in Hr	μ_T/μ_C	P	SE
4 ^{o}C	1	0.651	<0.0001	0.1008
	2	0.923	0.2653	0.07168
	4	0.763	<0.0001	0.06494
	6	0.733	<0.0001	0.06346
37 ^{o}C	1	0.631	<0.0001	0.09558
	2	0.477	<0.0001	0.06128

CHAPTER 4

DISCUSSION AND CONCLUSION

Aqueous saponin extract from the Chilean Soap Bark Tree, Quillaja *saponaria* Molina, demonstrated a cytotoxic effect when introduced to Vero and 143 cell lines. Both cell lines demonstrated a similar level of response to the saponin concentration. From this analysis it was determined that the CCID 10 concentration of saponin was 0.00125 μg/ml from a 5 μg/ml methanol stock solution. This relatively low value may be explained by a simple chemical reaction between the saponin and the cell membrane. The toxicity of saponins is due to the acyl moiety present on the carbon skeleton (1, 7). Likely, the acyl moiety on the carbon skeleton is interacting with the hydrophilic portion of the phosopholipids present on the cell membrane through saponification, resulting in the destruction of the cell membrane.

Quillaja saponins were not able to prevent the attachment of the HSV-1 or Vaccinia to the cell membrane. Cell monolayers treated for up to 6 hours with the CCID 10 concentration of saponin yielded similar results as the control following infection, indicating that Quillaja saponins lack the ability to prevent attachment of HSV-1 and Vaccinia to the cell membrane in culture. These results are logical due to the mechanisms that HSV-1 and Vaccinia use to attach to the cell membrane. Vaccinia and HSV-1's initial attachment receptors are heparin and chondroitin sulfate chains

present on the surface of the cell (12, 13, 14, 16, 24, 26, 27). In order for Quillaja saponins to prevent attachment of the virus to the cell membrane, the saponin would have to coat the membrane or block the initial attachment receptors. Since heparin and chondroitin sulfate chains are comprised of sulfated sugar residues possessing an overall negative charge, this interaction is unlikely to occur due to the structure of the saponin (see Fig. 1). Therefore, the necessary limitation that is attributed to the saponin would be to bind to another molecule of similar charge. Alternatively, the saponin would need to bind to one of the other attachment receptors for HSV-1 and Vaccinia. Since the attachment receptor(s) for Vaccinia virus remain(s) unknown, this possibility can not be examined. However, HSV-1's attachment proteins are well known (23, 24, 28). In order to block these attachment events, the saponin would have to bind or coat one or all attachment receptors, for example, the saponin would have to bind to HveA and prevent the interaction between gD (23, 24, 28). Even if the saponin used in this experiment were to block one of the attachment receptors, it seems unlikely that it would block all three possibilities.

However, when HSV-1 was pretreated with the CCID 10 concentration of saponin, plaque formation was significantly reduced in a time dependent manner at 37 ^{o}C and 4 ^{o}C. Under the same conditions Vaccinia did not respond to treatment. Underlying this discrepancy may be the difference in envelope structure and/or composition. There is ambiguity as to where HSV-1 and vaccinia virus derive their envelope, however the thickness of the envelope seems to be agreed upon. HSV-1 originally buds through a nuclear pore. Upon dissemination the virus loses this

membrane and gains one derived either from the trans-golgi network, or from a vacuole (31, 37). However, Vaccinia's envelope may be two, three or four layers thick, depending on the type of virion (34, 36, 38, 40). Since the membrane of HSV-1 is a single bilayer of phospholipids, the reaction between the saponin and the membrane would be more lethal. Instead, the multiple layers of phospholipids that Vaccinia acquires through maturation and release may result in greater stability of the virion during treatment. Secondly, the composition of the viral envelope may provide insight into the differences in the experimental results. The presence of different phospholipids may provide a more resistant structure, preventing the saponification of the envelope (37, 41).

It has been proposed that Quillaja saponins may interact with the viral attachment receptor via electrostatic interactions. The attachment domains for both HSV-1's gC and Vaccinia's A27L are both comprised of basic amino acid residues and bind the heparin sulfate chains on the cell surface (14, 27). If this reaction were to proceed, Vaccnia's replication should have been diminished somewhat. Also, for this instance to be plausible the saponin would need multiple specificities to be able to block the gC/HS;CS interaction as well as the gD/HveA; Nectin-1,2 or other interactions in order to prevent all attachment as is present in the data. This seems unlikely since Vaccinia and HSV-1 both use heparin sulfate and chondroitin sulfate as initial attachment receptors (13. 23, 24, 27). The more plausible interaction is between the saponin's acyl group and the lipids in the viral envelope. When the saponin

concentration was reduced by factors of 10^1 to 10^3 the virucidal capacity was greatly reduced if not eliminated, strengthening the conclusion of saponification.

Upon determining that Quillaja saponins have the ability to neutralize HSV-1 in solution we wanted to determine if saponin in solution would prevent cell-to-cell spread. To determine this possibility, plaque areas were analyzed using Image J from NIH. After running a mixed model factorial ANOVA, we determined that all but one treatment had a significant ($p < 0.0001$) reduction is plaque size. From the statistical data, it is likely that saponin in solution does reduce spread in HSV-1. The possibility was not examined for Vaccinia since the saponin did not have any effect on the virus prior to infection. Therefore, it was unnecessary to determine this possibility.

It seems likely that the effect of Quillaja saponins on HSV-1 and Vaccinia are dependent on the envelope structure. Due possibly to the differences in envelope composition and layers, HSV-1 was neutralized in a time dependent manner as described above. However, Vaccinia evaded destruction when treated at the CCID 10 concentration of saponin. With this information it may be possible to use this compound in spermicidal foams and/or topical treatments for HSV-1. However this compound's use against pox virus infections are unlikely.

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BIOGRAPHICAL INFORMATION

Matthew Sean Spinks graduated with a Bachelor's of Science in Biology in May of 2004. During that time he focused on matriculating to medical school. However, due to the untimely death of his sister, he was unable to prepare for the rigors of medicine. Instead he chose to pursue other interests in the medical and microbiological fields. In January of 2005 he began performing experiments in the Virology lab under the supervision of Dr. Michael Roner. After graduation from the University of Texas at Arlington with a Master's in Science he will be matriculating to medical school at the University of Texas Medical Branch in Galveston where he will pursue a career in medicine and microbiology.