Antiviral activity obtained from aqueous extracts of the Chilean soapbark tree (Quillaja saponaria Molina)

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INTRODUCTION

Aqueous extracts of the Chilean soapbark tree (Quillaja saponaria Molina) contain several physiologically active triterpenoid saponins that display strong adjuvant activity when used in either human or animal vaccines. In this paper, we describe studies that demonstrate a novel antiviral activity of Quillaja extracts against six viruses: vaccinia virus, herpes simplex virus type 1, varicella zoster virus, human immunodeficiency viruses 1 and 2 (HIV-1, HIV-2) and reovirus. We demonstrate that microgram amounts of extract, while exhibiting no cell cytotoxicity or direct virucidal activity, prevent each of the six viruses tested from infecting their host cells. In addition, the presence of residual amounts of extract continue to block virus infection and render cells resistant to infection for at least 16 h after the removal of the extract from the cell culture medium. We demonstrate that a Quillaja extract possesses strong antiviral activity at concentrations more than 100-fold lower than concentrations that exhibit cell cytotoxicity. Extract concentrations as high as 100 μg ml⁻¹ are not cytotoxic, but concentrations as low as 0.1 μg ml⁻¹ are able to block HIV-1 and HIV-2 virus attachment and infection.

Saponins offer some novel mechanisms of antiviral action, including interactions with viral envelopes leading to their destruction, interactions with host-cell membranes leading to a loss of virus binding sites and coating of cells to prevent virus binding (Amoros et al., 1987; Apers et al., 2001; Gosses et al., 2002; Tokuda et al., 1988; Verma & Raychaudhuri, 1970). In this paper, we examine the ability of a Quillaja extract to block virus infection and reduce virus spread.

METHODS

Virus and cell lines. The viruses used in this study were reovirus serotype 3 (ST3) strain Dearing (Ramos-Alvarez & Sabin, 1958), vaccinia virus strain WR (Mackett et al., 1982), herpes simplex virus type 1 (HSV-1) strain GHSV-UL46 (ATCC VR-1544); varicella-zoster virus (VZV) strain Ellen (ATCC VR-1367), HIV-1 (NIH-AIDS Research and Reference Reagent Program, HIV-1 89.6) and HIV-2 (NIH-AIDS Research and Reference Reagent Program, HIV-2 2CDC310248). Mouse L929 fibroblasts (ATCC CCL-1) were used to support the growth of reovirus, human 143 cells (Patel & Pickup, 1987) to support the growth of vaccinia virus, Vero cells (ATCC CCL-81) to support the growth of HSV-1, BS-C-1 cells (ATCC CCL-26) to support the growth of VZV and CEMx174 and LuSIV cells (NIH-AIDS Research and Reference Reagent Program) to support the growth of HIV-1 and HIV-2. All cell lines were propagated in monolayer cultures using minimal essential medium (MEM) with Earle’s salts, supplemented with 10 % fetal bovine serum (FBS). Cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures using 0.05 % trypsin with 0.02 % EDTA.

Preparation of Quillaja extract. The Quillaja extract used was obtained from Desert King International (San Diego, CA, USA). The...
material, UltraDry 100-Q, is the spray-dried, purified, aqueous extract of the Chilean soapbark tree (*Quillaja saponaria* Molina), consisting of >93% *Quillaja* solids and with a moisture content of less than 7%. The extract was prepared by dissolving the dried material into MEM at a concentration of 1.07 g 100-Q per 100 ml and filter-sterilized to yield a 10 mg ml⁻¹ stock. From this working stock, material was transferred to MEM with 10% FBS to yield the indicated final concentrations and the medium was added to the cell cultures.

The extract is a complex mixture of triterpenoids (saponins) (van Setten et al., 1995, 1998; van Setten & van de Werken, 1996). It has been established that these terpenes are built around a common quillaic acid which is decorated with oligosaccharides at C3 and in most cases C28. The differences in the members of this family arise from the level of oxidation around the quillaic acid skeleton (typically at C23 and C30), the type, location and number of sugars and the number, location and types of acyl moieties (most often on the C28 fucose, at C3′ and C4′). Presumably, the quillaic acid moiety simply serves as a scaffolding element, which then presents the oligosaccharides in the appropriate orientation and spatial distribution for interaction with the cellular target(s). The general structure of the *Quillaja* saponin is illustrated in Fig. 1.

**Determination of Quillaja extract cytotoxicity.** *Quillaja* extract cytotoxicity was determined by plating the indicated cells at a concentration of 5 × 10⁴ cells per well (6-well plate) in MEM with 10% FBS and incubating the plates at 37 °C in 5% CO₂ for approximately 24 h or until the cells divided to yield 1 × 10⁵ cells per well. At this time, the medium was removed and replaced with MEM with 10% FBS and the indicated concentration of *Quillaja* extract and the cells were incubated for 96 h in the presence of the extract. Cytotoxicity was measured microscopically after 96 h by counting the cells in three individual wells of a 6-well plate, 500 cells per well, and using the trypan blue dye exclusion procedure (Björkerud & Bondjers, 1972). Assays were performed in triplicate to generate nine measurements per time point. The results are presented as the concentration of the candidate drug that results in the death of 50% of the host cells. This value is commonly referred to as the median cellular cytotoxicity concentration and is identified as the CCIC₅₀.

**Direct virucidal effect of Quillaja extract.** The ability of the *Quillaja* extract to inactivate each of the six viruses directly was examined by a standard plaque-reduction assay or, for HIV-1 and HIV-2, an infectious centre assay. This technical difference was examined by a standard plaque-reduction assay or, for HIV-1 and HIV-2, an infectious centre assay. The extracts were treated with 10 mg ml⁻¹ of each virus directly was

Virus attachment to cells. The ability of these viruses to attach to their host cells was determined by adding 1 × 10⁶ p.f.u. or i.u. to 1 × 10⁶ of the appropriate host cells in 6-well plates in MEM with 10% FBS. After 5, 10, 15, 20, 30, 45 or 60 min, the virus-containing medium was removed and the cells were washed three times with 1 ml fresh MEM with 10% FBS. The three washes were combined and the ‘free’ virus, i.e. virus that did not remain attached to the host cells, was determined by plaque assay or infectious unit assay. The assays were repeated using three individual wells of a 6-well plate. Assays were performed in triplicate to generate nine measurements per time point. We selected this experimental design because it allowed us to follow the role of both unattached virus and virus attached to cells in the presence of *Quillaja* extract. Virus that was unable to attach was assayed for infectivity to examine the possibility that the *Quillaja* extract inactivated the free virus rather than preventing it from attaching to the cell monolayers as we had hypothesized. For virus that did attach, we examined the cells to see whether the attached virus was still able to initiate an active infection or was destroyed and/or released but damaged and was subsequently not detected in the assays for infectious virus that we carried out on the supernatant and cell washes. This assay mimics conditions that would exist when viruses are ingested, inhaled, injected or otherwise introduced into the human body, conditions under which saponins could be used to protect various body surfaces.

To validate this method, we also used the standard technique of adding radioisotope-labelled virus (³⁵S-methionine/cysteine) to cells and measuring cell-associated radioactivity. Briefly, radioisotope-labelled virus was prepared by infecting the indicated host cell line at an m.o.i. of 20 in the presence of ³⁵S-methionine/cysteine (20 µCi ml⁻¹) for 48 h and the viruses were extracted from cell debris as described by Balazs & Caldarella (1981). The ability of each virus to attach to their host cells was determined by adding 1 × 10⁶ c.p.m. of each virus to 1 × 10⁶ of the appropriate host cells in 6-well plates in MEM with 10% FBS. After 5, 10, 15, 20, 30, 45 or 60 min, the virus-containing medium was removed and the cells were washed three times with 1 ml fresh MEM with 10% FBS. The cells were then harvested and the radioactivity (bound virus) was determined by liquid scintillation counting of triplicate samples. The amount of virus bound is expressed as a percentage of the total input 1 × 10⁶ c.p.m.

Infectious centre assay to measure cells infected following treatment with Quillaja extract. The ability of each virus to infect their host cells following *Quillaja* extract treatment was determined by incubating 1 × 10⁶ of the appropriate host cells in 6-well plates in MEM with 10% FBS supplemented with the indicated concentration of *Quillaja* extract. Following incubation for 1 h at 37 °C in 5% CO₂, the *Quillaja* extract-containing medium was removed, the cells were washed three times with MEM without extract and the cells were then infected by adding 1 × 10⁶ p.f.u. or i.u. of the indicated virus to duplicate wells of the appropriate host cells in 6-well plates in MEM with 10% FBS. After 5, 10, 15, 20, 30, 45 or 60 min, the virus-containing medium was removed and the cells were washed with 1 ml fresh MEM with 10% FBS. After washing three times to remove unbound virus, the cells were harvested into 1 ml MEM without serum and then pipetted forcefully to generate a single cell suspension and serial 10-fold dilutions were prepared. The diluted cell suspensions were plated onto cell monolayers as

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**Fig. 1.** General structural features of the *Quillaja* saponins. Var, Variable group.
described for the standard viral plaque assays or for HIV-1 and HIV-2 i.u. assays. Cells infected in the presence of the Quillaja extract that are able to support virus replication and produce infectious progeny virus will release this new virus and it will infect cells of the cell monolayers and generate plaques or, in the case of HIV-1 and HIV-2, spread to adjacent cells. Assays were performed in triplicate to generate six measurements per time point. These assays examined the ability of these viruses to establish a productive infection in Quillaja extract-treated cells and for the infection to spread to adjacent cells to produce a visible ‘plaque’ or, in the case of HIV-1 and HIV-2, increased numbers of infected cells.

Infectious centre assay to measure the lasting effect of Quillaja extract treatment on cells. The lasting effects of Quillaja extract treatment were examined using a standard infectious centre assay as described above. Cells (1 x 10^6) were treated with Quillaja extract as described above for 1 h. The Quillaja extract-containing medium was removed, the cells were washed three times and fresh MEM with 10% FBS was added. Immediately (t = 0) or after 1, 2, 4, 8, 12, 16 or 24 h, the cells were infected by adding 1 x 10^6 p.f.u. of the indicated virus to duplicate wells of the appropriate host cells in 6-well plates in MEM with 10% FBS. After an additional 60 min, the virus-containing medium was removed and the cells were washed with 1 ml fresh MEM with 10% FBS. The remaining procedure was identical to that described above for the infectious centre assay.

Reovirus and vaccinia virus plaque assays. L929 or 143 cells were grown to confluence in 6-well plates in MEM with 10% FBS. The medium was removed and the cells were infected with either reovirus or vaccinia virus at serial 10-fold dilutions ranging from 1 x 10^6 to 10 p.f.u. per well in 250 μl MEM without serum. After 60 min, the medium was removed and replaced with 2 ml MEM with 5% FBS for the 143 cells or 2 ml MEM with 5% FBS and 1% noble agar for the L929 cells. After incubation at 37°C in 5% CO2 for 48 h, the 143 cells infected with vaccinia virus were stained with crystal violet (1%) in methanol (50%) and the plaques were counted (Mackett et al., 1982). After a total of 96 h, the L929 cells infected with reovirus were stained with neutral red (0.1%) and the plaques were counted (Roner & Joklik, 2001). Assays were performed in triplicate.

HSV-1 and VZV plaque assays. Viruses were diluted in MEM with 10% FBS and assayed on monolayers of Vero cells for HSV and BS-C-1 cells for VZV. After adsorption for 2 h at 37°C, 4 ml low-melting-temperature agarose (0.75%) in MEM with 2% FBS was added. After 4 days for HSV and 6 days for VZV, the agarose was removed and cells were rinsed with PBS, fixed for 7 min with a solution of methanol/acetic acid (3:1), stained with crystal violet (1%) in methanol (50%) and rinsed with water (Cole & Grose, 2003). Assays were performed in triplicate.

HIV-1 and HIV-2 infectious units assays. Viruses were diluted in RPMI without serum and assayed in CEMx174 cells (Stefano et al., 1993) and/or LuSIV cells (Roos et al., 2000). After adsorption for 2 h at 37°C, cells were placed in RPMI with 5% FBS for 5 days. Infected cells were detected by staining with neutral red and counting 500 cells for the CEMx174 cells or assayed for luciferase activity using LuSIV cells, a cell line that is highly sensitive to infection by primary and laboratory strains of HIV/simian immunodeficiency virus (SIV), resulting in Tat-mediated expression of luciferase, which correlates with viral infectivity. Assays were performed in triplicate.

RESULTS

Highest concentration of Quillaja extract tolerated by cell cultures

Growth of L929, 143, Vero, BS-C-1 and CEMx174 cells was unaffected by concentrations of 0.1 mg Quillaja extract ml\(^{-1}\) or less in MEM with 10% FBS (Table 1). The CCIC\(_{50}\) values for these cells were 10-fold higher, at 1.0–0.9 mg ml\(^{-1}\). Cells maintained in this Quillaja extract-containing medium continued to divide during the 96 h treatment and, when the Quillaja extract was removed and replaced with Quillaja extract-free medium, the cells suffered no long-term effects and were maintained in the laboratory for at least 3 months. Based on these results, we tested for antiviral activity at Quillaja extract concentrations of less than 1.0 mg ml\(^{-1}\).

Direct inactivation of viruses by Quillaja extract

Also summarized in Table 1 are the results of tests to examine the ability of Quillaja extract to inactivate each of these viruses directly at concentrations from 0 to 1 mg ml\(^{-1}\). The results are presented as the ED\(_{50}\), the dose of a drug that is effective at inactivating 50% of the treated virus within 1 h at 37°C. After incubation for 1 h at 37°C Quillaja extract concentrations of 0.01 mg ml\(^{-1}\) or less, we measured no reduction in virus infectivity for the five enveloped viruses, vaccinia virus, HSV-1, VZV, HIV-1 and HIV-2, while the non-enveloped reovirus was unaffected by concentrations as high as 1 mg ml\(^{-1}\). Increasing the Quillaja extract concentration 10-fold to 0.1 mg ml\(^{-1}\) resulted in a 50% reduction in virus infectivity (ED\(_{50}\)) for all the viruses except reovirus. Reovirus is non-enveloped and highly resistant to disruption by the Quillaja extract. The Quillaja extract does demonstrate direct antiviral activity, but at concentrations only 10-fold lower than those cytotoxic for the cell lines themselves.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CCIC(_{50})</th>
<th>Virus</th>
<th>ED(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929</td>
<td>1.0 ± 0.1</td>
<td>Reovirus</td>
<td>&gt; 1.0 ± 0.1</td>
</tr>
<tr>
<td>143</td>
<td>0.9 ± 0.1</td>
<td>Vaccinia virus</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Vero</td>
<td>0.9 ± 0.1</td>
<td>HSV-1</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>BS-C-1</td>
<td>0.9 ± 0.1</td>
<td>VZV</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>CEMx174</td>
<td>1.0 ± 0.1</td>
<td>HIV-1</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>CEMx174</td>
<td>1.0 ± 0.1</td>
<td>HIV-2</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>
(c) VZV

(i) Virus attached to cells (pfu)

(ii) Virus attached to cells (%)

(iii) Cell-associated virus (cpm)

(iv) Cell-associated virus (%)

(d) HSV-1

(i) Virus attached to cells (pfu)

(ii) Virus attached to cells (%)

(iii) Cell-associated virus (cpm)

(iv) Cell-associated virus (%)

Saponins as antivirals
Can Quillaja extract be used to treat cells and to reduce infection efficiency?

To determine whether Quillaja extract-treated cells were now resistant to virus attachment, we treated cells with Quillaja extract for 1 h, replaced the medium with Quillaja extract-free medium and then measured the amount of virus that could bind to treated or untreated cells within an additional 60 min. We tested Quillaja extract concentrations ranging from the highest that demonstrated no cell cytotoxicity (0.1 mg ml$^{-1}$) across a 4-log reduction to 0.00001 mg ml$^{-1}$. The ability of each virus to attach to their host cells following treatment of the cells was determined as described above. We measured virus binding using two methods, the first an assay for infectious virus, the second an assay to measure binding of radiolabelled virus to the treated cells. The infectious assay measures the removal (binding to cells) of virus from the medium during the 5–60 min test period. In each panel of Fig. 2, graphs (i) and (ii) show the results of the infectious virus concentrations ranging from the highest that demonstrated no cell cytotoxicity (0.1 mg ml$^{-1}$) across a 4-log reduction to 0.00001 mg ml$^{-1}$. The ability of each virus to attach to their host cells following treatment of the cells was determined as described above. We measured virus binding using two methods, the first an assay for infectious virus, the second an assay to measure binding of radiolabelled virus to the treated cells. The infectious assay measures the removal (binding to cells) of virus from the medium within 1 h. See Methods for details. The concentration of Quillaja extract (mg ml$^{-1}$) is shown as: ■, 0; △, 0.1; ○, 0.01; □, 0.001; ▲, 0.0001; ●, 0.00001.

As shown in Fig. 2, within 45 min of virus addition to untreated cells, more than 95% of each of the six viruses tested attached to their host cells (i.e. were removed from the test medium) in the absence of Quillaja extract. Following treatment with non-cytotoxic amounts of Quillaja extract (0.0001 to 0.1 mg ml$^{-1}$), less than 0.25% of each of the six viruses was able to attach to their host cells after 60 min and remained free and infectious in the cell culture medium. The Quillaja extract-treated cells demonstrated no cytotoxicity yet remained highly resistant to virus binding during both of the virus binding assays. More than 99.75% of the original $1 \times 10^6$ p.f.u. of added infectious virus remained free and infectious in the supernatant and less than 1% of the radiolabelled virus was able to bind to these cells. It should be noted that the infectious assay measures ‘loss’ of virus from the test medium during the incubation period. This is because the test medium is removed, the cells are washed to remove unbound virus and the combined rinse medium is tested for infectious virus. If any residual Quillaja extract is acting on the viruses directly and rendering them non-infectious, they would not be detected in the plaque assay of the medium performed at the end of the treatment period. This is not what we found; virus added to cells treated with Quillaja extract does not absorb to cells but remains free in the supernatant and infectious during the 60 min test period. In separate experiments, we have demonstrated that the concentrations of Quillaja extract that we are using have no direct effect on virus infectivity. The results using the radiolabelled virus assay in combination with the infectious virus assay support our hypothesis that the Quillaja extract prevents virus binding to/association with treated cells.

Quillaja extract-treated cells do not demonstrate active virus infection

We were also interested in the fate of the few cells that appeared to be binding virus following treatment with Quillaja extract. To pursue this, we examined Quillaja extract-treated and infected cells using an infectious centre assay. This technique, as described in Methods, involves recovering treated and potentially infected cells and plating them onto new cell monolayers or adding them to uninfected cells (HIV-1 and HIV-2) to assay for infectious virus produced and released by these treated cells. This assay allows us to examine the fate of the adsorbed virus during the 60 min absorption period following treatment of the cells with Quillaja extract. If the viruses were able to attach, infect the cells and initiate an infection, the progeny virus would infect cells of the new monolayer and generate a plaque. As shown in Fig. 3, allowing virus to attach to untreated cells for 60 min followed by incubation for an additional 6 h results in each of the six virus–host cell systems demonstrating active virus infections in 16–55% of the cells. Following infection of cells pretreated with the smallest active amounts of Quillaja extract (0.001 or 0.0001 mg ml$^{-1}$), less than 0.005% of each of the six virus–host cell systems displayed active viral infections. Quillaja extract treatment appears to alter the cells and to suppress virus replication even in the rare event that the viruses were able to attach to and infect the cells. Quillaja extract has not been shown to induce an interferon response, but we were unable to detect a response in our treated cells (data not shown). We demonstrated that only 0.25% of the added virus was able to attach to Quillaja extract-treated cells. This low percentage should result in at least 0.125% of the cells displaying active viral infections based on our results with untreated cells. We found less than 0.005% of the cells producing infectious virus.
Maintenance of virus protection following removal of Quillaja extract

We were also interested in the length of time that cells would remain resistant to virus attachment/infection after the Quillaja extract was removed. To examine this, cells (1 x 10⁶) were treated with Quillaja extract as indicated previously for 60 min. The Quillaja extract-containing medium was removed, the monolayers were washed three times and fresh MEM with 10% FBS was added. Immediately (t=0) or after 1, 2, 4, 8, 12, 16 or 24 h, 1 x 10⁶ p.f.u. or i.u. of each virus was added to the appropriate cell line. As described previously, the virus inoculum was removed after 1 h and, 6 h later, the cells were harvested and plated onto new cell monolayers or with uninfected cells (HIV-1 and HIV-2) as described for the infectious centre assay or infectious unit assay. As shown in Fig. 4, cells treated with the smallest amounts of Quillaja extract (0.001 or 0.0001 mg ml⁻¹) remain resistant to infection by each of the six viruses tested for at least 16 h after the Quillaja extract is removed. Twenty-four hours after treatment with Quillaja extract, the six cell lines tested returned to near normal in terms of virus susceptibility and virus infection rates were nearly identical to those of untreated cells, indicating no long-term alteration of the cells following treatment with Quillaja extract.

DISCUSSION

Natural, aqueous extracts of Quillaja saponaria Molina, the Chilean soapbark tree, contain a number of physiologically active triterpenoid saponins that have antiviral activity. Our results demonstrate that a Quillaja extract can disrupt viral envelopes and capsid proteins at concentrations 10-fold below cytotoxic levels. Additionally, following treatment of cells with this extract, a ‘block’ of the host cells occurs that prevents virus attachment and reduces virus spread to uninfected cells from cells that do manage to become infected, at concentrations 100- to 1000-fold below cytotoxic levels. The Quillaja extract used here is only cytotoxic at high concentrations (> 1 mg ml⁻¹) and is currently approved for use in food and beverages by the FDA (under CFR 172.510, FEMA number 2973 NON-GMO) and is allowed for use in organic foods (under N.O.P. 205.605). Based on the results presented here, we believe that this Quillaja extract can readily be combined with existing spermicidal agents and delivery systems to produce a product that is both spermicidal and virucidal, blocking virus attachment to cells in the treated area. The extract is well suited to such an application as it is pH stable, from pH 2 to 11, it is already approved as a food additive, it foams easily, is inexpensive and readily available and is soluble in water. The Quillaja extract is very effective at blocking virus attachment to cells in the treated area.

Fig. 3. Pretreatment of cells with Quillaja extract blocks virus infection of these cells, demonstrated using infectious centre assays on Quillaja extract-treated cells. The ability of reovirus, vaccinia virus, VZV, HSV-1, HIV-1 and HIV-2 to infect cells pretreated with Quillaja extract was tested. The results are shown as the number of cells demonstrating active viral infection (plaques) when plated onto uninfected cell monolayers 1 h after virus addition. See Methods for details. Results for controls without Quillaja extract are plotted as solid lines; dotted lines indicate results with the amount of Quillaja extract listed as follows. Virus/cell combinations are shown as: □, reovirus/L929, 0.001 mg ml⁻¹; △, vaccinia virus/143, 0.001 mg ml⁻¹; ○, VZV/BS-C-1, 0.001 mg ml⁻¹; ◊, HSV-1/Vero, 0.0001 mg ml⁻¹; ×, HIV-1/CEMx174, 0.0001 mg ml⁻¹; ★, HIV-2/CEMx174, 0.0001 mg ml⁻¹.

Fig. 4. Loss of virus protection following removal of Quillaja extract, demonstrated using infectious centre assays. The ability of reovirus, vaccinia virus, VZV, HSV-1, HIV-1 and HIV-2 to infect cells pretreated with Quillaja extract was tested. Cells were treated with Quillaja extract for 1 h and then the monolayers were washed and fresh medium without Quillaja extract was added (t=0). After 1, 2, 4, 8, 12, 16 and 24 h, virus was added to these cells. The results are shown as the number of cells demonstrating active viral infection (plaques) when plated onto uninfected cell monolayers 1 h after virus addition. See Methods for details. Results for controls without Quillaja extract are plotted as solid lines; dotted lines indicate results with the amount of Quillaja extract listed as follows. Virus/cell combinations are shown as: □, reovirus/L929, 0.001 mg ml⁻¹; △, vaccinia virus/143, 0.001 mg ml⁻¹; ○, VZV/BS-C-1, 0.001 mg ml⁻¹; ◊, HSV-1/Vero, 0.0001 mg ml⁻¹; ×, HIV-1/CEMx174, 0.0001 mg ml⁻¹; ★, HIV-2/CEMx174, 0.0001 mg ml⁻¹.
HIV-1 and HIV-2 infection of cells at concentrations as low as 0.0001 mg ml\(^{-1}\). This blocking activity is active for cells in the presence of the Quillaja extract and for up to 16 h after its removal, making it an excellent candidate to prevent the spread of sexually transmitted viruses when applied prior to exposure.

Other saponin extracts have demonstrated similar activity. The antiviral activity of a triterpene saponin isolated from *Anagallis arvensis* (Primulaceae) was studied in *vitro* against several viruses including HSV-1 and poliovirus type 2 (Amoros *et al.*, 1987). These authors demonstrated that the antiviral activity was not due to a virucidal effect but appeared to involve an inhibition of virus–host cell attachment. The antiviral effects of triterpene glycosides and monoterpenic glycosides were demonstrated by their ability to prevent viral activation of Raji cells by Epstein–Barr virus binding (Tokuda *et al.*, 1988).

We selected these six viruses because they cover a broad range of virus structures, cell receptors utilized, membrane transport and cellular localization. Based on our results, the most likely mechanism of action of the extract is through disruption of cellular membrane proteins and/or virus receptors, preventing virus infection of these cells. In support of this conclusion, we present a brief summary of what is known about the molecular events that control attachment of each of these viruses to their host cells.

Reovirus is a dsRNA, non-enveloped virus that utilizes cellular receptors containing sialic acid and normally uses endocytosis into vesicles to gain access to the cell cytoplasm. Within the cytoplasm, the infecting particle is not broken down but is activated as a transcription machine, producing large amounts of mRNA in infected cells (Tyler *et al.*, 2001). Reovirus is a model virus for rotavirus, a virus that, when ingested via contaminated water, results in diarrhoea and approximately 500,000 deaths worldwide each year (Parashar *et al.*, 2006). Reovirus was more resistant to direct inactivation by the extract than the enveloped viruses, but was just as sensitive to being ‘blocked’ from infecting extract-treated cells.

Vaccinia virus is the vaccine strain for smallpox virus. The virus is enveloped and enters the cell via membrane fusion. No unique viral attachment protein or cell-surface receptors have yet been identified. Cell-surface heparan sulfate and chondroitin sulfate act as primary receptors and help vaccinia virus to adsorb to cells (Smith & Law, 2004). The viral genome is dsDNA but, unlike most dsDNA viruses, it replicates in the cytoplasm of infected cells. Treatment of vaccinia virus with a number of proteases increases the likelihood that it will infect a cell. This virus served as a model to test whether saponins might have a similar enhancement effect. This was not what we found, however; we only measured a reduction in infectivity at high extract concentrations and no enhancement was seen with any of the viruses.

HSV-1 is a neurotropic dsDNA virus that initially replicates in epithelial cells of the oral cavity. Amplified progeny virus enters sensory neurons and is transported to the nuclei located in the trigeminal ganglion, where a latent infection is established (Burkhart, 2005). The entry of HSV into cells begins with binding of viral glycoproteins gB or gC to heparan sulfate proteoglycans on the cell surface. After this binding, a third glycoprotein, gD, binds one of its receptors. The gD–receptor binding triggers fusion of the virus envelope with the cell membrane. Once fused, viral capsid and some tegument proteins are released into the cytoplasm of the cell. The cellular receptors for gD are very diverse in nature. They come from three different classes of cell-surface receptors: (i) HVEM, the first known gD receptor, is a member of the tumour necrosis factor receptor family, (ii) two others (nectin-1 and -2) are members of the immunoglobulin superfamily related to the poivovirus receptor and (iii) some unique sites in heparan sulfate resulting from the action of specific 3-O-sulfotransferases also produce HSV-1-specific receptor. Interaction of gD with any one of those receptors triggers the membrane fusion reaction, which, by an unknown mechanism, also requires a concerted action of three other envelope glycoproteins, gB and gH–gL (O’Donnell *et al.*, 2006; Scanlan *et al.*, 2003; Tiwari *et al.*, 2004, 2005; Valyi-Nagy *et al.*, 2004; Xia *et al.*, 2002; Xu *et al.*, 2005). The Quillaja extract may disrupt any of these glycoproteins, resulting in a ‘block’ of virus infection. Future work will examine the attachment process in detail following Quillaja extract treatment.

VZV is transmitted as airborne virus particles shed from the skin of an infected person. The new host breathes in the virus, which enters the mucous membrane in the respiratory tract and begins to spread without its envelope from cell to cell. The virus invades T cells of the blood, and these T cells carry the virus to the skin. There, the virus can reactivate its envelope, because the top layer of the skin lacks the endosomal pathway that removes glycoproteins from the envelope (Cole & Grose, 2003). Studies with VZV have revealed that oligosaccharides derived from glycoproteins of the VZV envelope contain mannose 6-phosphate (Man6P) (Gabel *et al.*, 1989); moreover, Man6P and other phosphorylated monosaccharides protect cells from the cytopathic effect of VZV. Treatment of cells with chloroquine, which reduces the expression of the cation-independent Man6P receptor (MPRCl) at cell surfaces, also protects against infection by VZV. Electron microscopic observations have revealed that enveloped virions are associated with MPRCl at the cell surface and that newly enveloped virions are incorporated into MPRCl-containing vesicles in the Golgi network (Gershon *et al.*, 1994). Such a mechanism may also influence the infection of cells by HSV; HSV gD acquires Man6P residues and binds to Man6P receptors (Brunetti *et al.*, 1994). Binding of either VZV or HSV to a Man6P receptor, however, has yet to be demonstrated. This virus was selected because of its reliance on cell-to-cell spread to remain in its host. We found that cells treated with
saponins did not support the replication of this virus via spread within cell cultures; the *Quillaja* extract reduced the spread of this virus and resulted in a reduced number of plaques compared with mock-treated cells.

CD4 is a primary and necessary receptor for HIV-1, HIV-2 and SIV (Dalgleish et al., 1984; Klatzman et al., 1984). Several groups have shown that CCR5 is a necessary co-receptor for monocytotropic (M-tropic) HIV-1 isolates (Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). Additionally, the chemokine receptor CXCR4 (fusin) was described as being the co-receptor used by T cell-tropic (T-tropic) HIV isolates (Feng et al., 1996). M-tropic HIV-1 isolates are classically those viruses that are most easily propagated in macrophage cultures and are unable to infect T cell lines (i.e. immortalized T cells), but are able easily to infect primary T cells from peripheral blood samples. Conversely, T-tropic HIV-1 isolates have classically been identified as being those that are easily propagated in T cell lines (i.e. immortalized T cells), but are able easily to propagate in macrophage cultures and are unable to infect T cell lines (i.e. immortalized T cells), but are able easily to infect primary T cells from peripheral blood samples. T-tropic HIV-1 isolates mainly infect activated lines and grow poorly in macrophages, but are also able to infect primary T cells from peripheral blood samples. T-tropic HIV-1 isolates have classically been identified as being those that are easily propagated in T cell lines and grow poorly in macrophages, but are also able easily to infect primary T cells from peripheral blood samples. T-tropic HIV-1 isolates mainly infect activated peripheral blood CD4 T cells and cell lines and use CXCR4 for entry into the CD4+ target cell. M-tropic isolates are able to infect CD4 T cells, monocytes and macrophages and depend on the use of CCR5 and CD4 for viral entry. Using transfected cell lines, other chemokine receptors, such as CCR3, CCR2, CCR8, CCR9, STRL33 ('Bonzo'), Gpr15 ('Bob'), Gpr1, APJ and ChemR23, have been identified and shown to be used for entry by certain HIV isolates (Deng et al., 1997; Liao et al., 1997). APJ may represent a relevant co-receptor within the central nervous system.

Although several cellular proteins are shared and required for infection by two or more of the tested viruses, no common feature exists among all the viruses. HIV-1 and HIV-2 use mechanisms quite distinct from the other viruses. The *Quillaja* extract we tested does not act directly to inactivate either the enveloped or the non-enveloped viruses that we tested until the concentration reaches 1000 to 10 000 times the concentration required to protect cells from virus infection. A 2-log reduction in viral infectivity was seen with the enveloped viruses when the *Quillaja* extract concentration was increased from 0.1 to 1 mg ml \(^{-1}\), and an additional 2-log reduction was seen when the concentration was increased to 10 mg ml \(^{-1}\). For the enveloped reovirus, this effect was seen at 10-fold higher concentrations of *Quillaja* extract. At concentrations of extract only sufficient to protect cells from virus infection and not to inactivate the viruses, the interaction between the virus and the cell is the most likely site of inhibition. We postulate that the *Quillaja* extract modifies the cells, preventing virus from attaching to these cells, and any virus that does manage to attach to cells and is internalized is reduced in its capacity to initiate and maintain an infection. This may be due to a reversible modification of the cell membrane or to modification of the cellular endocytosis process. We demonstrate that the *Quillaja* extract-treated cells remain modified for 16 h and that, by 24 h, they return to normal and are readily infected at rates equivalent to pretreatment levels. Saponins have been reported to produce a marked decrease of microsomal enzyme activities (Chang et al., 1985; Choi et al., 2002; Larocca & Ledeen, 1993). Future research will address these questions.

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**REFERENCES**


