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Prevention of rotavirus infections *in vitro* with aqueous extracts of *Quillaja Saponaria* Molina

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Abstract

Background—Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for over 300,000 childhood deaths every year, mostly in developing countries. Rotavirus-related deaths represent approximately 5% of all deaths in children younger than 5 years of age worldwide. Saponins are readily soluble in water and are approved by the US FDA for inclusion in beverages intended for human consumption. The addition of saponins to existing water supplies offers a new form of intervention into the cycle of rotavirus infection. We believe that saponins will 'coat' the epithelium of the host's small intestine and prevent attachment of rotavirus.

Discussion—This experiment provides *in vitro* data for the possibility of including saponin in drinking water to prevent infections of rotavirus. We demonstrate that microgram amounts of extract, while exhibiting no cell cytotoxicity or direct virucidal activity, prevent rotavirus from infecting its host cells. In addition, the presence of residual amounts of extract continue to block viral infection and render cells resistant to infection for at least 16 h after the removal of the extract from the cell culture media.

Conclusion—We demonstrate that two *Quillaja* extracts possess strong antiviral activity at concentrations more than 1000-fold lower than concentrations exhibiting cell cytotoxicity. Extract concentrations as high as 1000 μg/ml are not cytotoxic, but concentrations as low as 1.0 μg/ml are able to block rotavirus and reovirus attachment and infection.

Saponins are natural detergents that form stable foams [1–4]. They contain a lipophilic nucleus and one or more side chains of hydrophilic carbo hydrate. Thus, the intact saponin molecule is a surfactant, with both fat- and water-soluble moieties. It has been known for many years that saponins form insoluble complexes with cholesterol [5–7]. Interactions of saponins with cholesterol and other sterols account for many of their biological effects, particularly those involving membrane activity. It was demonstrated years ago that dietary saponin reduces blood cholesterol level [8–13]. This effect is a result of the saponins binding to cholesterol excreted in bile, thus inhibiting entero hepatic cholesterol recycling.

In a similar manner, saponins demonstrate antiprotozoan activity by complexing with cholesterol in protozoan cell membranes, causing damage to the integrity of the membrane, and cell lysis. This has been well demonstrated with rumen protozoa [14–22]. The antiprotozoal (cholesterol-binding) activity requires the intact saponin structure with both nucleus and side chain present. Protozoan diseases, in which part of the life cycle occurs in the GI tract, respond to the antiprotozoan activity of saponins. Yucca saponins are as

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effective as the drug metronidazole in killing tropozoites of *Giardia lamblia* in the intestine [23].

Saponins have been suggested to have additional health benefits. According to work by Waterhouse [24], drinking red wine helps lower cholesterol and red wines contain approximately the same amount of saponin as they do resveratrol [25–28,101]. However, while resveratrol is thought to block cholesterol oxidation by its antioxidant action, saponins are believed to work by binding to and preventing the absorption of cholesterol, he says. He also mentioned that saponins are known to affect inflammation pathways, an effect that could have implications in heart disease and cancer, according to published studies.

Triterpenoid saponins from other sources such as *Maesa lanceolata*, *Maesa chisia* and *Maesa indica* have also been reported to exhibit antiviral activity against Ranikhet disease virus, vaccinia virus and herpes simplex virus. Some saponins have also been shown to exhibit direct virucidal mechanisms of action, including destruction of viral envelopes and interaction with host cell membranes, leading to the loss of viral binding sites [29–36].

Natural, aqueous extracts of the Chilean soap bark tree (*Quillaja Saponaria* Molina) contain a number of physiologically active triterpenoid saponins [37]. These saponins have been shown to exhibit strong adjuvant activity that has been exploited for use in animal and human vaccines [38–44]. *Quillaja* extracts have strong immune-enhancing activity that may lead to a reduction in virus infection *in vivo*. We have demonstrated saponin antiviral activity in cell culture in the absence of an immune system. A large amount of research exists on saponin enhancement of the immune system and direct interaction with viral and cellular membranes. Extensive literature searches reveal that this mechanism has not been explored in detail. Saponins offer the possibility of a new virucidal mechanism of action, interaction with viral envelopes leading to their destruction and/or interaction with host cell membranes leading to a loss of viral binding sites [45]. We have previously demonstrated the activity of Ultra Dry 100 Q against reovirus [46] and we have included reovirus in this study to permit comparisons to be made between the earlier study and this current study reporting strong antiviral activity against a related virus, rotavirus. This current study presents new findings of antiviral activity against rotavirus, the leading single cause of severe diarrhea among infants and young children, and one of several viruses that cause infections commonly known as ‘stomach flu’, despite having no relation to influenza. This new study builds on the previous work with the saponin extract Ultra Dry 100 Q and explores the antiviral activity of the highly purified saponin extract, Vax Sap, against both of these viruses.

Key Terms

Saponins

Diverse class of natural surfactants, or detergents, found in many plants, but which are most abundant in the desert plants Yucca and *Quillaja*. Extracts from these plants are commonly used as foaming agents for beverages such as root beer.

***Quillaja saponaria* Molina**

Evergreen tree growing to 18 by 6 m at a slow rate, known commonly as the soap bark tree. It has a long history of medicinal use with the Andean people who used it especially as a treatment for various chest problems.

Ultra Dry 100 Q

Water extract from *Quillaja saponaria* Molina in powder form. It contains mainly triterpenic saponins larger than 65%.

Reovirus

Any one of three ubiquitous, double-stranded RNA viruses found in the respiratory and alimentary tracts of both healthy and sick people. Reoviruses have been implicated in some cases of upper respiratory tract disease and infantile gastroenteritis. Reo indicates respiratory enteric orphan.

Rotavirus

Discovered in 1973 and taking its name from its wheel-like appearance (rota means wheel in latin). Rotavirus is a double-stranded RNA virus in the family Reoviridae that causes diarrhea in the young of many species, including the human gastroenteritis viruses that cause infant diarrhea. Also called gastroenteritis virus type B.

Vax sap

Highly purified *Quillaja saponaria* Molina water extract in powder form. It contains mainly triterpenic saponins greater than 90%.

Experimental

■ Virus & cell lines

The viruses used in this study were reovirus sero-type 3 (ST3) strain Dearing (MRV-3DE) [47] and rhesus rotavirus (MMU 18006) (RRV-ATCC VR-954). Mouse L929 fibroblasts (ATCC CCL-1) were used to support the growth of reovirus and the Vervent monkey kidney cell line MA-104 clone 1 (ATCC CRL-2378.1) to support the growth of RR virus. All cell lines were propagated in monolayer cultures using minimal essential medium (MEM) with Earles' salts, supplemented with 10% fetal bovine serum (FBS). Cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures using trypsin 0.05% with EDTA 0.02%.

■ *Quillaja* extract preparation

The *Quillaja* extract used was obtained from Desert King International, San Diego, CA, USA. The material, Ultra Dry 100-Q is the spray-dried purified aqueous extract of the Chilean Soap bark tree (*Quillaja Saponaria* Molina), consisting of larger than 65% saponins and 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/100 ml and filter sterilized to yield a 10 mg/ml stock. From this working stock, material was transferred to MEM with 10% FBS to yield the indicated final concentrations, and the media added to the cell cultures.

The extract is a complex mixture of triterpenoids (saponins) [48–50]. It has been established that these terpenes are built around a common quillaic acid, which is decorated with oligo saccharides at C3 and in most cases C28. The differences in the members of this family arise in 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 fuctose – 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). See Figures 1 & 2 for the general structure of the *Quillaja* saponins.

An additional extract was also tested, Vax Sap. This material is a further purification of the original Ultra Dry 100-Q material with a saponin content of greater than 90% and approval for use as an adjuvant in human vaccines.

The existence of many different saponins, which vary in their chemical or biological activities, makes the characterization of *Quillaja* extracts difficult. The variable content of the individual saponins in the extracts also contributes to the difficulty of characterizing them. Additional characterization requires identification of individual saponins to assess the quality, purity and toxicity of extract, a process we are now engaged in. For most studies, researchers have relied on the identification and quantification of the major saponins, QS-7, QS-17, QS-18 and QS-21, because they represent up to 90% of total saponin content in most extract preparations. The saponin content and identification of unpurified, semi-purified and highly purified *Quillaja* extracts can be performed by reverse-phase HPLC; we have included the profiles from the extracts we used in this study in Figures 1 & 2. At least 22 peaks (denominated QS-1 to QS-22) are separable. The individual components can be identified by retention time on a Vydac C4 HPLC column; we have compiled data from a number of researchers and included this in Figure 2 [38,40,42,43,49,51–55].

■ *Quillaja* extract cytotoxicity

Quillaja extract cytotoxicity was determined by plating the indicated cells at a concentration of 5×10^5 cells per well (six-well plate) in MEM with 10% FBS and incubating the plates at 37°C in 5% CO_2 for approximately 24 h, or until the cells divided to yield 1×10^6 cells per well. At this time, the medium was removed and replaced with MEM with 10% FBS, the indicated *Quillaja* extract concentration and the cells 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 six-well plate, 500 cells/well and using the trypan blue-dye exclusion procedure [56]. 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 by the initializations $CCIC_{50}$.

■ Direct virucidal effect of *Quillaja* extract

The ability of the *Quillaja* extract to directly inactivate reovirus or rhesus rotavirus was examined by a standard plaque-reduction assay. Briefly, 1×10^6 plaque-forming units (PFUs) of each virus were incubated in the presence or absence of *Quillaja* extract ranging from 0 to 10 mg/ml. Viruses were suspended in 200 μl of *Quillaja* extract-containing MEM with 1% fetal bovine serum for 1 h at 37°C. Virus was pelleted at 100,000 × g for 15 min, re-suspended in fresh MEM with 1% FBS, repelleted and the process repeated three times to remove any remaining *Quillaja* extract. After final centrifugation the virus was resuspended in 200 μl MEM with 1% FBS and virus infectivity assayed by plaque assay or infectious units as described. Each assay was performed in triplicate.

■ Virus attachment to cells

The ability of these viruses to attach to their host cells was determined by adding 1×10^6 PFUs to 1×10^6 of the appropriate host cells in six-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 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 six-well plate. Assays were preformed in triplicate to generate nine measurements per time point. We selected this experimental design because it permitted us to follow the role of both unattached virus and virus able to attach 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 prevented it from attaching to the cell monolayers as we had hypothesized. For virus that did attach, we examined the cells to see if 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-labeled virus (^{35}S-methionine/cysteine) to cells and measuring cell-associated radioactivity. Briefly, radioisotope-labeled virus was prepared, infecting the indicated host cell line at a multiplicity of infection of 20 in the presence of ^{35}S-methionine/cysteine (20 uCi/ml) for 48 h and the viruses extracted from cell debris as described [57]. The ability of each virus to attach to their host cells was determined by adding 1×10^6 CPM of each virus to 1×10^6 of the appropriate host cells in six-well plates in MEM with 10% FBS. After 5, 10, 15, 20, 30, 45 or 60 min the virus-containing media was removed and the cells washed three times with 1 ml of fresh MEM with 10% FBS. The cells were then harvested and the radioactivity (bound virus) determined by liquid scintillation counting of triplicate samples. The amount of virus bound is expressed as a percentage of the total input 1×10^6 CPM.

■ Infectious center assay to measure cells infected following treatment with *Quillaja* extract

The ability of each virus to infect its host cells following *Quillaja* extract treatment was determined by incubating 1×10^6 of the appropriate host cells in six-well plates in MEM with 10% FBS supplemented with the indicated concentration of*Quillaja* extract. Following incubation for 1 h at 37°C, 5% CO_2, the *Quillaja* extract-containing medium was removed, the cells washed three times with MEM without extract, and the cells infected by adding 1×10^6 PFUs of the indicated virus to duplicate wells of the appropriate host cells in six-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 washed with 1 ml fresh MEM with 10% FBS. After washing three times to remove unbound virus the cells were harvested into 1 ml of MEM without serum, pipetted forcefully to generate a single cell suspension and serial tenfold dilutions prepared. The diluted cell suspensions were plated onto cell monolayers as described for the standard viral plaque 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 which will infect cells of the cell monolayers and generate plaques. Assays were preformed 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’.

■ Infectious center 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 center assay as described earlier. Cells (1×10^6) were treated with *Quillaja* extract as previously described for 1 h. The *Quillaja* extract-containing medium was removed, the cells washed three times and fresh MEM with 10% FBS added. Immediately (time 0) or after 1, 2, 4, 8, 12, 16 or 24 h, the cells were infected by adding 1×10^6 PFUs of the indicated virus to duplicate wells of the appropriate host cells in six-well plates in MEM with 10% FBS. After an additional 60 min the virus-containing medium was removed and the cells washed with 1 ml of fresh MEM with 10% FBS. The remaining procedure was identical as described for the infectious center assay previous.

■ Reovirus & rhesus rotavirus plaque assays

L929 or MA-104 cells were grown to confluency in six-well plates in MEM with 10% FBS. The medium was removed and the cells infected with either reovirus or rhesus rotavirus at serial tenfold dilutions ranging from 1×10^6 to 10 PFUs per well in 250 μl of MEM without serum. After 60 min the medium was removed and replaced with 2 ml of MEM with 5% FBS and noble agar 1%. After incubation at 37°C in 5% CO_2 for a total of 96 h the cells were stained with neutral red, 0.1%, and the plaques counted [58]. Assays were preformed in triplicate.

Results & discussion

■ Highest concentration of *Quillaja* extract tolerated by cell cultures

As summarized in Table 1, the growth of L929 and MA-104 cells was unaffected by concentrations of 0.1 mg/ml for the Ultra Dry 100 Q and 1 mg/ml for the VaxSap extract in MEM with 10% FBS. The $CCIC_{50}$ values for these cells were tenfold higher at 1 mg/ml for the Ultra Dry 100 Q and 9.5 mg/ml for the Vax Sap extract. Cells maintained in this *Quillaja* extract-containing medium continued to divide during the 96 h of 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 1.0 and 9.5 mg/ml. We have previously demonstrated the activity of Ultra Dry 100 Q against reovirus [46] and we have included reovirus in this study to permit comparisons to be made to the newly reported antiviral activity against a related virus, rotavirus. This study presents new findings and evaluates the antiviral activity of the highly purified saponin extract, VaxSap, against both of these viruses.

■ Direct inactivation of viruses by *Quillaja* extracts

Also summarized in Table 1 are the results of tests to examine the ability of *Quillaja* extract to directly inactivate both of these viruses at concentrations from 0 to 9.5 mg/ml. The results are presented as the ED_{50}s, 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 at *Quillaja* extract concentrations of 1.0 mg/ml or less we measured no reduction in virus infectivity. If the *Quillaja* extract does demonstrate direct antiviral activity it would be only at concentrations higher than those cytotoxic for the cell lines themselves and, thus, would prevent treatment at such a high concentration.

■ Can *Quillaja* extracts be used to treat cells & reduce infection efficiency?

To determine if *Quillaja* extracts treated cells were now resistant to virus attachment, we treated cells with *Quillaja* extracts, Ultra Dry 100 Q or Vax Sap for 1 h, replaced the media with extract-free media and then measured the amount of virus that could bind to treated or untreated cells within an additional 60 min. We tested both *Quillaja* extracts at concentrations ranging from the highest that demonstrated no cell cytotoxicity (0.1 mg/ml) across a 4-log reduction to 0.00001 mg/ml. The ability of each virus to attach to its host cells following treatment of the cells was determined as described. We measured virus binding using two methods, the first an assay for infectious virus, the second an assay measuring binding of radiolabeled virus to the treated cells. The infectious assay measures the removal (binding to cells) of virus from the media during the 5– 60 min test period. Summarized in Figures 3–6, panels A and B are the results of the infectious virus assay, with panels C and D showing the binding of radiolabeled virus. The data demonstrate that treatment of cells with *Quillaja* extract renders them resistant to virus infection even in the absence of continuous *Quillaja* extract in the media. As demonstrated for reovirus and rotavirus, Ultra Dry 100 Q extract at concentrations as low as 0.001 mg/ml and as low as 0.0001 mg/ml for

the Vax Sap extract were able to completely block virus binding as measured using both the infectious virus and radiolabeled virus binding assays.

As demonstrated in Figures 3–6, within 45 min after virus addition to untreated cells, more than 95% of each of the two viruses tested attached to their host cells (were removed from the test media) in the absence of either *Quillaja* extract. Following treatment with noncytotoxic amounts of either *Quillaja* extract (0.001–0.1 mg/ml), less than 0.25% of each of the two viruses were able to attach to their host cells after 60 min and remained free and infectious in the cell culture media. The treated cells demonstrated no cytotoxicity, yet remained highly resistant to virus binding during both of the virus binding assays. Greater than 99.75% of the original 1×10^6 PFUs of added infectious virus remained free and infectious in the supernatant and less than 1% of the radiolabeled virus was able to bind to these cells. It should be noted that the infectious assay measures 'loss' of virus from the test media during the incubation period. This is because the test medium is removed, the cells washed to remove unbound virus and the combined rinse media tested for infectious virus. If any residual *Quillaja* extract is acting on the viruses directly and rendering them noninfectious, then they would not be detected in the plaque assay of the media preformed at the end of the treatment period. This is not what we found; viruses added to cells treated with either *Quillaja* extract do not absorb to cells but remain 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 radiolabeled virus assay in combination with the infectious virus assay support our hypothesis that the *Quillaja* extracts prevent virus binding/association to treated cells.

■ *Quillaja* extract treated cells do not demonstrate active viral 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 center assay. This method, as described in the materials and methods, involves recovering treated and potentially infected cells and plating them onto new cell monolayers to assay for infectious virus produced and released by these treated cells. This assay permits 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, then the progeny virus would infect cells of the new monolayer and generate a plaque. As shown in Figure 7, allowing virus to attach to untreated cells for 60 min followed by incubation for an additional 6 h, results in both virus/host cell systems demonstrating active viral infections in 70–90% of the cells. Following infection of cells pretreated with the lowest active amounts of *Quillaja* extract (0.001 or 0.0001 mg/ml), less than 0.005% of each of the virus/host cells displayed active viral infections. *Quillaja* extract treatment appears to alter the cells and suppress virus replication even for the rare event when 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 virus added 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 that less than 0.005% of the cells produce infectious virus.

■ Maintenance of virus protection following *Quillaja* extract removal

We were also interested in the length of time cells would remain resistant to virus attachment/infection after the *Quillaja* extract was removed. To examine this, cells (1×10^6) were treated with *Quillaja* extract as previously indicated for 60 min. The *Quillaja* extract-

containing medium was removed, the monolayers washed three times and fresh MEM with 10% FBS was added. Immediately (time 0), or after 1, 2, 4, 8, 12, 16 or 24 h, 1×10^6 PFUs or IUs of each virus were added to the appropriate cell line. As described previously, the virus inoculum was removed after 1 h, and 6 h later cells harvested and plated onto new cell monolayers as described for the infectious center assay. As demonstrated in Figure 8, cells treated with the lowest amounts of either *Quillaja* extract (0.001 or 0.0001 mg/ml) remain resistant to infection by each of the viruses tested for at least 16 h after the *Quillaja* extract is removed. A total of 24 h after treatment with *Quillaja* extract, the two cell lines tested returned to near normal with regard to virus susceptibility and virus infection were rates nearly identical to that of untreated cells indicating no long term alteration of the cells following *Quillaja* extract treatment.

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 herpes simplex virus type 1 and poliovirus type 2. The authors demonstrated that the antiviral activity was not due to a virucidal effect but appeared to involve inhibition of virus–host cell attachment [59]. The antiviral effects of triterpene glycosides and monoterpene glycosides were demonstrated by their ability to prevent viral activation of Raji cells by Epstein-Barr virus binding [60].

Conclusion

Reovirus is a double stranded RNA(dsRNA), nonenveloped 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 the infected cells [61]. Rotavirus is also a dsRNA virus but, unlike its mostly benign cousin, reovirus, is a virus that when ingested via contaminated water results in diarrhea and approximately 300,000 deaths worldwide each year [62–64].

We hypothesized that natural, aqueous extracts of *Quillaja Saponaria* Molina, the Chilean soap bark tree, contain a number of physiologically active triterpenoid saponins that have antiviral activity. Our results demonstrate that a *Quillaja* extract does not disrupt viral envelopes and capsid proteins at concentrations of ten- (Ultra Dry 100 Q) to 100-fold (Vax-Sap) below cytotoxic levels. Additionally, following treatment of cells with this extract a ‘block’ prevents virus attachment and reduces virus spread to uninfected cells from cells that do manage to become infected, at concentrations 100- to 10,000-fold below the cytotoxic levels. *Quillaja Saponaria* Molina, which we are currently using, is only cytotoxic at high concentrations (>1 mg/ml), and is currently approved for use in food and beverages by the US FDA (under CFR 172.510, FEMA number 2973 NON-GMO) and is allowed for use in organic foods (under N.O.P 205.605).

One question for which a simple answer does not yet exist surrounds the mechanism by which saponins might ‘coat’ cells to prevent virus attachment and subsequent infection. We demonstrated that two viruses that use different viral attachment proteins are both blocked from binding to saponin-treated cells. Reovirus uses the junction adhesion molecule [65] for attachment to cells and rota-virus uses a generalized sialic acid or possibly the $\alpha_2\beta_1$ or $\alpha_V\beta_3$ integrins [66]. A mixture of saponin in water or cell culture media forms a colloidal emulsion, which, when applied to cells, reduces the surface tension. Saponins are also known to interact with cell membranes due to the affinity of the aglycone moiety for membrane sterols, particularly cholesterol [67]. Since removal of cholesterol leads to an increase in membrane fluidity, conformational changes that ATPases undergo during their transport cycle may be facilitated. Membrane fluidity controls the enzyme activity of

biological membranes and plays an important role in ion transport, therefore the ability of saponins to affect this parameter may explain their impact on the availability of functional receptors for virus binding. Additional impacts on cell membranes include the ability to open large Ca-dependent conductance channels causing membrane hyperpolarization [67], suppression of electrical activity and relaxation of smooth muscle [68]. On the other hand, there are also reports of the ability of saponins to block membrane ion channels on neurons and human neutrophils [69]. These interactions are complex and may involve different mechanisms, but together suggest major yet reversible changes to the cell membranes of saponin-treated cells, which would result in reduced protein–protein interactions at the membrane surface that would likely reduce the ability of a virus to dock and attach to these cells.

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Future perspective

At extract concentrations only sufficient to protect cells from virus infection and not 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 the virus from attaching to these cells. 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 the *Quillaja* extract-treated cells remain modified for 16 h and 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 [70–72]. Future research will address these questions.

Yucca and *Quillaja* extracts are natural phytochemicals currently approved by the FDA for use in humans and that are present in a number of foods, beverages and herbal products. In addition to the novel antiviral activity we report here, research from has suggested that the consumption of saponin-rich herbs by the Masai tribe's people in East Africa may be responsible for their very low serum cholesterol levels. These people consume a diet very high in animal products, cholesterol and saturated fat yet maintain very low serum cholesterol levels.

Yucca schidigera and *Quillaja saponaria* are packed with phytochemicals that have the potential to improve human health, nutrition and battle infectious diseases. Researchers are just beginning to explore and gain an understanding of the many biological effects of steroidal and triterpenoid saponins, and their potentials for improving human health. The future medical applications of saponins remain to be discovered.

Executive summary

Antiviral activity of Ultra Dry 100 Q

■ We demonstrate that a water extract from the soap bark tree (*Quillaja saponaria* Molina) is equally effective at preventing rotavirus infection of cells in culture as we have previously demonstrated for reovirus. This has major implications for human health as rotavirus, unlike reovirus, infects the human bowels and is the most common cause of severe diarrhea among infants and children throughout the world,

being responsible for the death of approximately 300,000 children worldwide annually.

■ The Ultra Dry 100 Q extract demonstrated low cytotoxicity (0.1 mg/ml) against cell lines used to support the replication of both rotavirus and reovirus.

■ The presence of the Ultra Dry 100 Q extract in cell culture media was very effective at preventing both rotavirus and reovirus from attaching to cells, thereby blocking infection.

■ The presence of the Ultra Dry 100 Q extract in cell culture media was also very effective at reducing the spread of both rotavirus and from any infected cell to the surrounding cells.

■ A single exposure to the Ultra Dry 100 Q extract in cell culture media was very effective at preventing both rotavirus and reovirus from attaching to cells for at least 16 h.

Antiviral activity of Vax Sap

■ In preparation for testing in animals and eventually in humans, a purified preparation derived from the Ultra Dry 100 Q, Vax Sap was tested for activity against both rotavirus and reovirus *in vitro*. As demonstrated by the HPLC profiles in Figure 1, the Vax Sap material contains the same saponins found in the parent Ultra Dry 100 Q but greatly reduced amounts of the contaminants present in the early elution times.

■ The Vax Sap only 10% as toxic and was active at concentrations approximately tenfold lower than the Ultra Dry 100 Q.

Antiviral properties of saponin containing extracts of Quillaja saponaria *Molina*

■ The extracts do not have direct virucidal activity against nonenveloped viruses.

■ The extracts can prevent virus binding to cells.

■ The extracts can prevent virus spread from infected to uninfected cells.

■ The ‘protective coating’ of cells by the extract lasts for at least 16 h.

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Financial & competing interests disclosure

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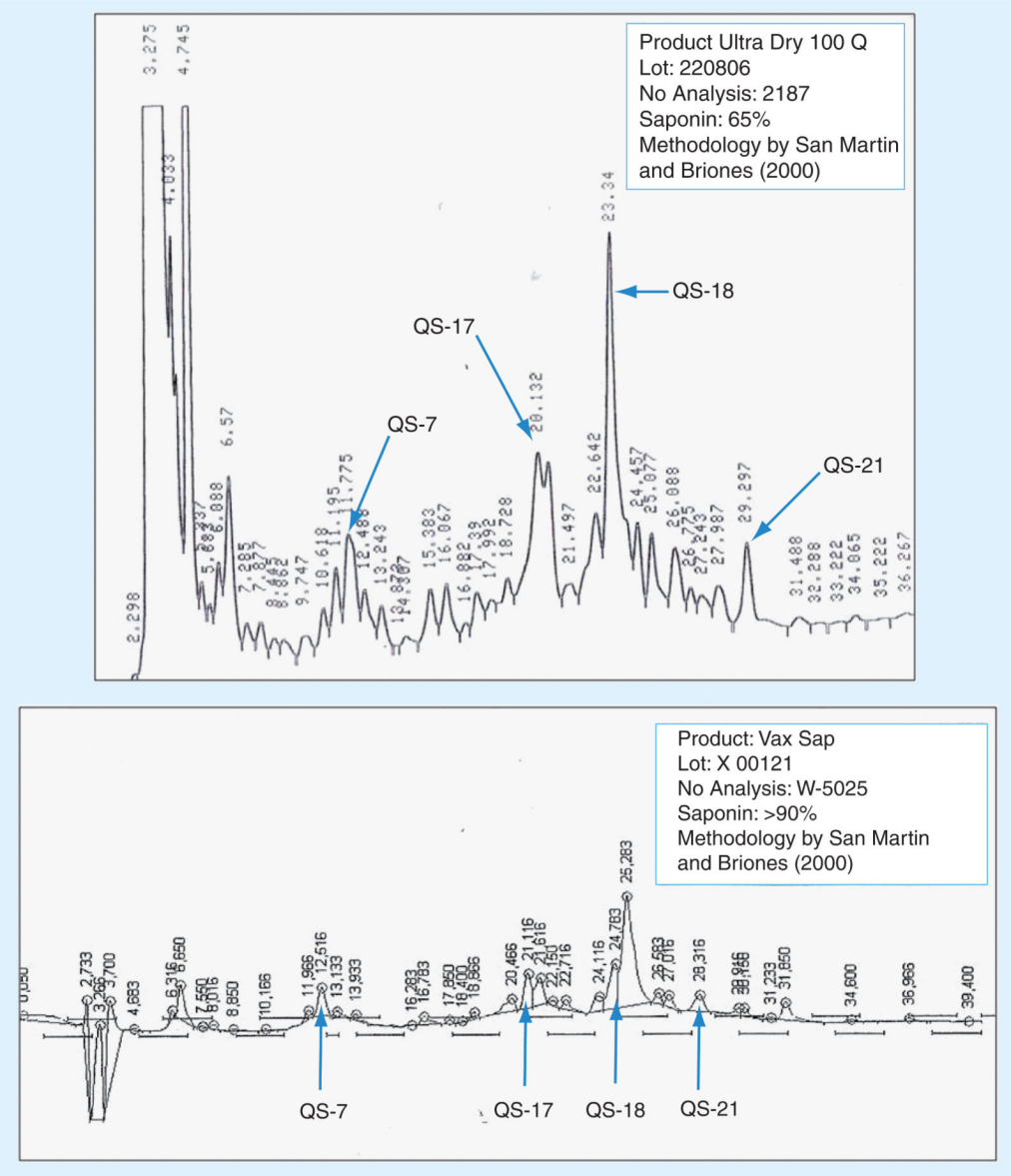
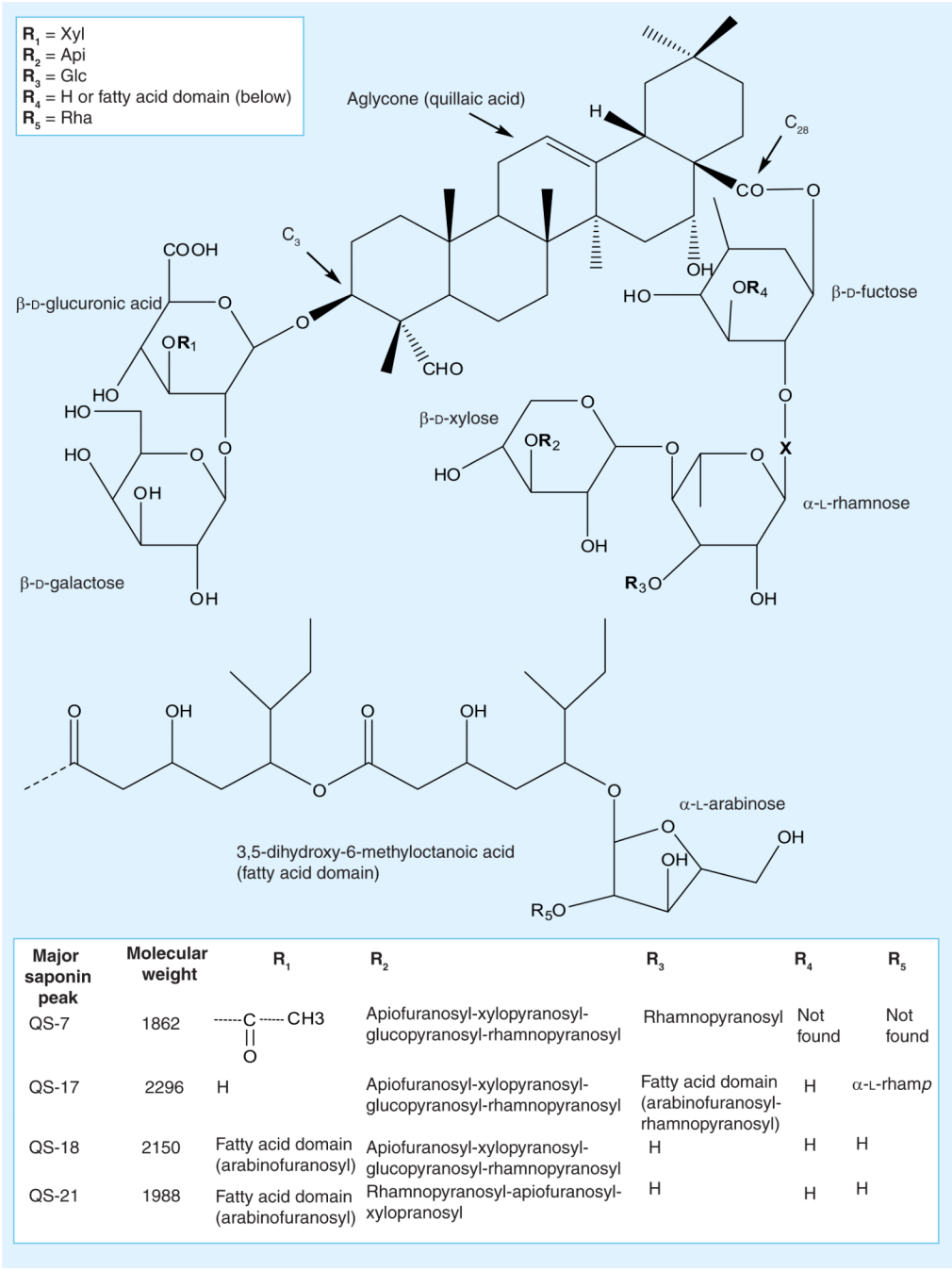


Figure 1. HPLC analysis of Ultra Dry 100 Q and Vax Sap water extracts used in this study
Adapted with permission from [38,40,42,43,49,51–55].



Major saponin peak	Molecular weight	R_1	R_2	R_3	R_4	R_5
QS-7	1862	------C(=O)----- CH3	Apiofuranosyl-xylopyranosyl-glucopyranosyl-rhamnopyranosyl	Rhamnopyranosyl	Not found	Not found
QS-17	2296	H	Apiofuranosyl-xylopyranosyl-glucopyranosyl-rhamnopyranosyl	Fatty acid domain (arabinofuranosyl-rhamnopyranosyl)	H	α-L-rhamp
QS-18	2150	Fatty acid domain (arabinofuranosyl)	Apiofuranosyl-xylopyranosyl-glucopyranosyl-rhamnopyranosyl	H	H	H
QS-21	1988	Fatty acid domain (arabinofuranosyl)	Rhamnopyranosyl-apiofuranosyl-xylopranosyl	H	H	H

Figure 2. Major saponins from *Quillaja saponaria* Molina

Api: Arabinose; Glc: Glucose; Rha: Rhamnose; Xyl: Xylose. Adapted with permission from [38,40,42,43,49,51–55].

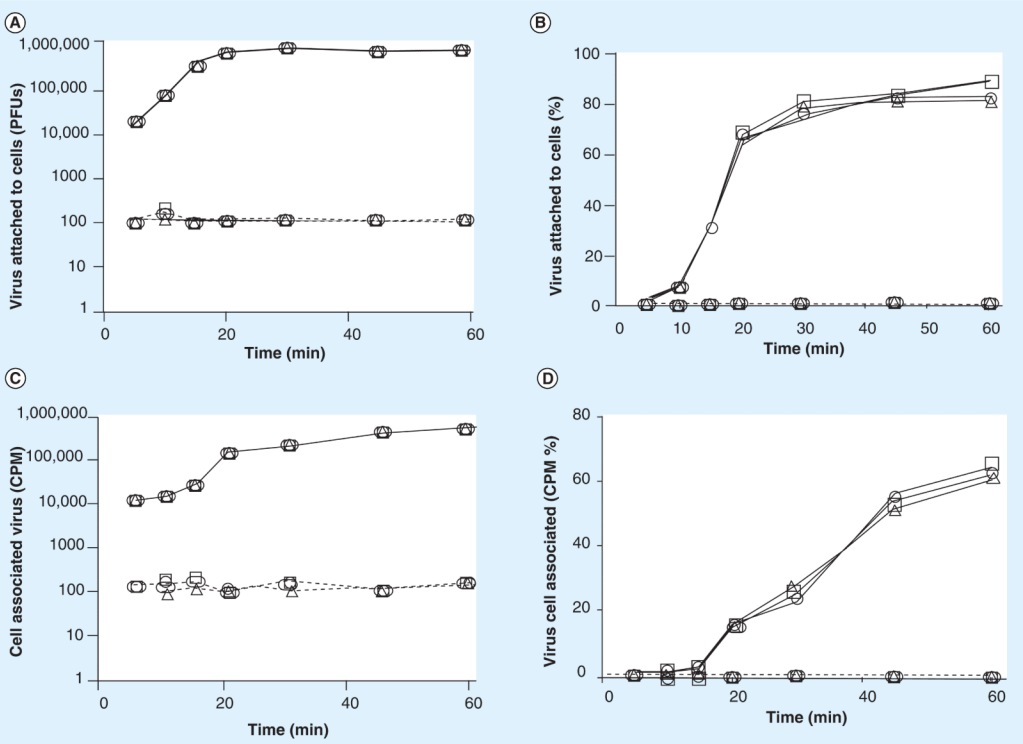


Figure 3. Treatment of cells with Ultra Dry 100 Q extract blocks reovirus cell attachment

The ability of reovirus to attach to cells pretreated with Ultra Dry 100 Q extract was tested. The results are represented as **(A)** the number of virus PFUs, **(B)** percentage of total added virus, **(C)** the amount of radioisotope-labeled virus and **(D)** the percentage of radiolabeled virus bound to the cell monolayers (1×10^6 cells) within 1 h. See materials and methods for details. The data are plotted as —□— (Ultra Dry 100 Q extract = 0 mg/ml), ----△---- (Ultra Dry 100 Q extract = 0.1 mg/ml), ---○--- (Ultra Dry 100 Q extract = 0.01 mg/ml), ---□---- (Ultra Dry 100 Q extract = 0.001 mg/ml), —△— (Ultra Dry 100 Q extract = 0.0001 mg/ml), —○— (Ultra Dry 100 Q extract = 0.00001 mg/ml). These data are new but generated by repeating experiments previously published [47]. The experiments were repeated and the data are included to permit a direct comparison to the new data presented for both reovirus and rotavirus in Figures 3 -7. PFU: Plaque-forming unit.

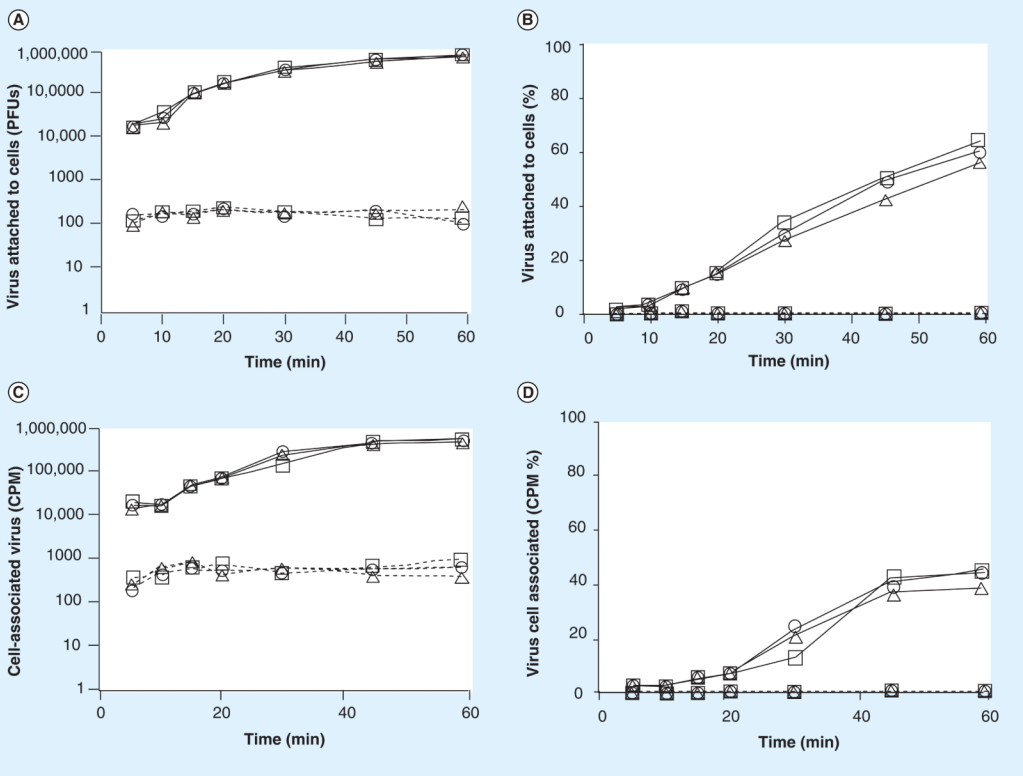


Figure 4. Treatment of cells with Ultra Dry 100 Q blocks rotavirus cell attachment

The ability of rotavirus to attach to cells pretreated with Vax Sap was tested. The results are represented as **(A)** the number of virus PFUs, **(B)** percentage of total added virus, **(C)** the amount of radioisotope labeled virus and **(D)** the percentage of radiolabeled virus bound to the cell monolayers (1×10^6 cells) within 1 h. See materials and methods for details. The data are plotted as —□— (Vax Sap extract = 0 mg/ml), ----△---- (Vax Sap extract = 0.1 mg/ml), ---○--- (Vax Sap extract = 0.01 mg/ml), ----□---- (Vax Sap extract = 0.001 mg/ml), —△— (Vax Sap extract = 0.0001 mg/ml), —○— (Vax Sap extract = 0.00001 mg/ml). PFU: Plaque-forming unit.

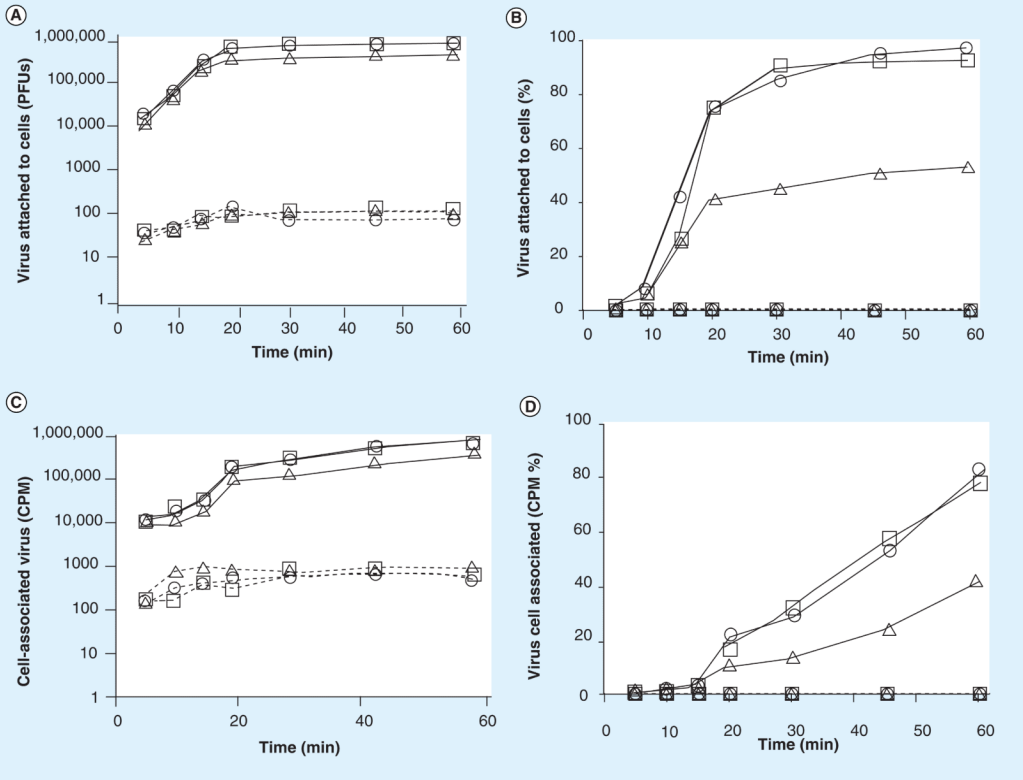


Figure 5. Treatment of cells with Vax Sap blocks reovirus cell attachment

The ability rotavirus to attach to cells pretreated with Vax Sap was tested. The results are represented as the **(A)** number of virus PFUs, **(B)** percentage of total added virus, **(C)** the amount of radioisotope labeled virus and **(D)** the percentage of radiolabeled virus bound to the cell monolayers (1×10^6 cells) within 1 h. See materials and methods for details. The data are plotted as —□— (Vax Sap extract = 0 mg/ml), ----△---- (Vax Sap extract = 0.1 mg/ml), ---○--- (Vax Sap extract = 0.01 mg/ml), ----□---- (Vax Sap extract = 0.001 mg/ml), —△— (Vax Sap extract = 0.0001 mg/ml), —○— (Vax Sap extract = 0.00001 mg/ml). PFU: Plaque-forming unit.

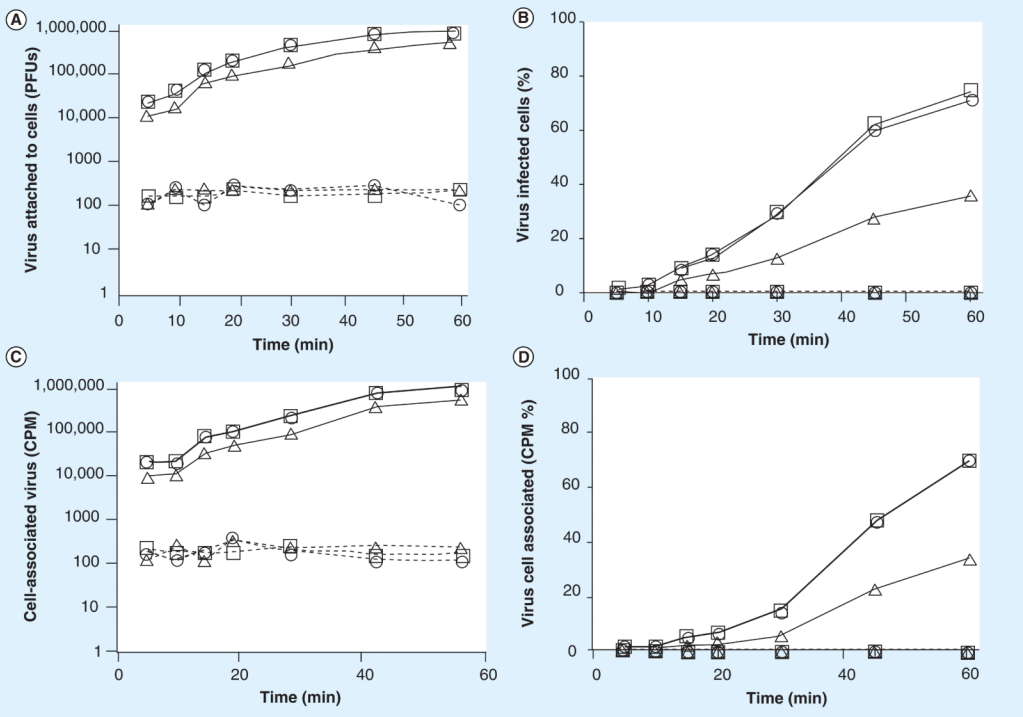


Figure 6. Treatment of cells with Vax Sap blocks rotavirus cell attachment

The ability of rotavirus to attach to cells pretreated with Vax Sap was tested. The results are represented as **(A)** the number of virus PFUs, **(B)** percentage of total added virus, **(C)** the amount of radioisotope labeled virus and **(D)** the percentage of radiolabeled virus bound to the cell monolayers (1×10^6 cells) within 1 h. See materials and methods for details. The data are plotted as —□— (Vax Sap extract = 0 mg/ml), ----△---- (Vax Sap extract = 0.1 mg/ml), ---○--- (Vax Sap extract = 0.01 mg/ml), ----□---- (Vax Sap extract = 0.001 mg/ml), —△— (Vax Sap extract = 0.0001 mg/ml), —○— (Vax Sap extract = 0.00001 mg/ml). PFU: Plaque-forming unit.

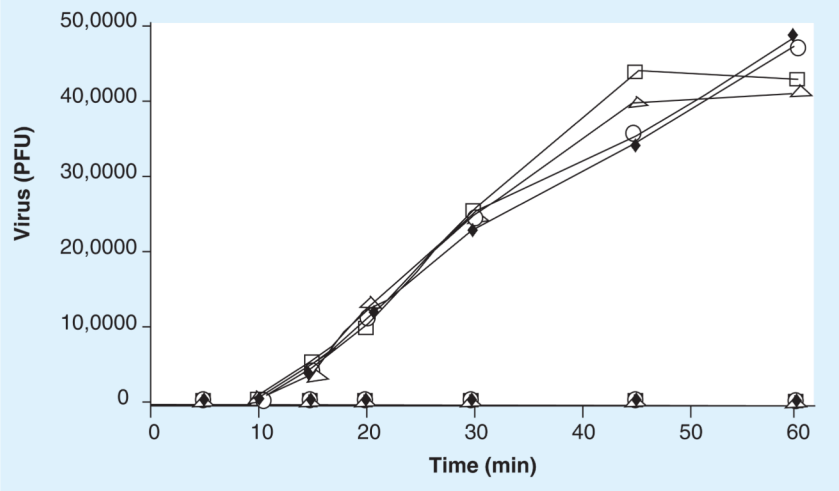


Figure 7. Pretreatment of cells with Ultra Dry 100 Q or Vax Sap extract blocks virus infection of these cells

Infectious center assays on extract-treated cells demonstrating active viral infection. The ability of reovirus or rotavirus to infect cells pretreated with either *Quillaja* extract was tested. The results are represented as the number of cells demonstrating active viral infection (plaques) when infected within 1 h by immediately plating the infected cells onto uninfected cell monolayers. See materials and methods for details. The data are plotted as reovirus/L929 —□— (Ultra Dry 100 Q = 0 mg/ml), reovirus/L929 - - - □- - - (Ultra Dry 100 Q = 0.001 mg/ml), reovirus/L929 —Δ— (Vax Sap = 0 mg/ml), reovirus/L929 - - - Δ- - - (Vax Sap = 0.0001 mg/ml), rotavirus/MA104 —◆— (Ultra Dry 100 Q = 0 mg/ml), rotavirus/MA104 - - - ◆- - - (Ultra Dry 100 Q = 0.001 mg/ml), rotavirus/MA104 —0— (Vax Sap = 0 mg/ml), rotavirus/MA104 - - -0- - - (Vax Sap = 0.0001 mg/ml). These data from the treatment of reovirus with Ultra Dry 100 Q are new but generated by repeating experiments previously published [47]. The experiments were repeated and the data are included to permit a direct comparison to the new data presented for reovirus treated with Vax Sap and rotavirus treated with either Ultra Dry 100 Q or Vax Sap. PFU: Plaque-forming unit.

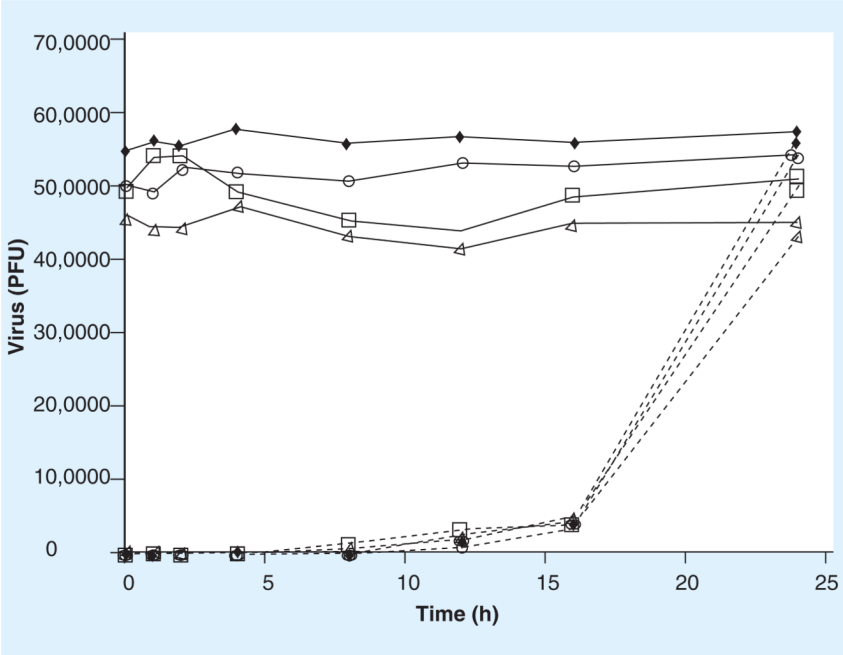


Figure 8. Loss of virus protection following Ultra Dry 100 Q or Vax Sap extract removal

Infectious center assays on demonstrating the loss of virus protection following *Quillaja* extract removal. The ability of reovirus or rotavirus to infect cells pretreated with *Quillaja* extract was tested. Cells were treated with *Quillaja* extract for 1 h and then the monolayers washed and fresh media added without *Quillaja* extract. This represents time 0. After 1, 2, 4, 8, 12, 16 and 24 h virus was added to these cells. The results are represented as the number of cells demonstrating active viral infection (plaques) when plated onto uninfected cell monolayers 1 h after virus addition. See materials and methods for details. These data are plotted as reovirus/L929 —□— (Ultra Dry 100 Q = 0 mg/ml), reovirus/L929 - - - □- - - (Ultra Dry 100 Q = 0.001 mg/ml), reovirus/L929 —△— (Vax Sap = 0 mg/ml), reovirus/L929 - - - △- - - (Vax Sap = 0.0001 mg/ml), rotavirus/MA104 —◆— (Ultra Dry 100 Q = 0 mg/ml), rotavirus/MA104 - - - ◆- - -(Ultra Dry 100 Q = 0.001 mg/ml), rotavirus/MA104 —0— (Vax Sap = 0 mg/ml), rotavirus/MA104 - - -0- - - (Vax Sap = 0.0001 mg/ml). These data from the treatment of reovirus with Ultra Dry 100 Q are new but generated by repeating experiments previously published [47]. The experiments were repeated and the data is included to permit a direct comparison to the new data presented for reovirus treated with Vax Sap and rotavirus treated with either Ultra Dry 100 Q or Vax Sap. PFU: Plaque-forming unit.

Table 1

Cytotoxicity of extracts and direct inactivation of viruses.

Cells	$CCIC_{50}$†		Virus	ED_{50}‡	
	Ultra Dry 100 Q extract (mg/ml)	*Vax Sap extract (mg/ml)*		*Ultra Dry 100 Q extract (mg/ml)*	*Vax Sap extract (mg/ml)*
L929	1.0 +/- 0.1	9.5 +/- 0.1	Reovirus	>1.0 +/- 0.1	>9.5 +/- 0.1
MA-104	1.0 +/- 0.1	9.5 +/- 0.5	Rhesus rotavirus	>1.0 +/- 0.1	>9.5 +/- 0.5

The results are presented as means of four experiments using triplicate samples in each experiment. See materials and methods for conditions for the assays. These data from the treatment of reovirus with Ultra Dry 100 Q are new but generated by repeating experiments previously published [47]. *The experiments were repeated and the data are included to permit a direct comparison to the new data presented for reovirus treated with Vax Sap and rotavirus treated with either Ultra Dry 100 Q or Vax Sap.*

† Loss of cell viability of 50% of treated cells after 96 h.

‡ Inactivation of 50% of virus within 1 h.