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Characterization of *in vivo* anti-rotavirus activities of saponin extracts from *Quillaja saponaria* Molina

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ABSTRACT

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide with approximately 300,000 pre-adolescent deaths each year. *Quillaja* saponins are a natural aqueous extract obtained from the Chilean soapbark tree. The extract is approved for use in humans by the FDA for use in beverages as a food addictive. We have demonstrated that *Quillaja* extracts have strong antiviral activities *in vitro* against six different viruses. In this study, we evaluated the *in vivo* antiviral activity of these extracts against rhesus rotavirus (RRV) using a mouse model. We established that at a dosage of 0.015 mg/mouse of saponin extract, RRV induced diarrhea can be significantly reduced from 79% to 11% when mice are exposed to 500 plaque-forming-units (PFU) for each of five consecutive days. Additionally, while a reduction of RRV induced diarrhea depended both on the concentration of virus introduced and on the amount of *Quillaja* extract given to each mouse, the severity and interval of diarrhea under a variety of conditions tested, in all the treated mice were greatly reduced when compared to those that did not receive the *Quillaja* extracts. Mechanistically, there is strong evidence that the *Quillaja* extracts are able to "block" rotavirus infection by inhibiting virus–host attachment through disruption of cellular membrane proteins and/or virus receptors. We believe that *Quillaja* extracts have promise as antivirals to reduce rotavirus infection and the severity of the disease in humans.

1. Introduction

Saponins are amphipathic glycosides found in over 500 plant species (Cheeke, 1996) including the Chilean soapbark tree *Quillaja saponaria* (Guo and Kenne, 2000b). Saponins have been exploited as natural detergents due to their ability to produce stable foams when dissolved in water (Acebes et al., 1998; Lamri et al., 1988; Tarade and Sinhai, 2005). Having both fat-soluble and water-soluble properties, saponins make excellent surfactants and have been used as soaps for hundreds of years. Additionally, it has been known for many years that saponins form insoluble complexes with cholesterol (Kersten et al., 1991; Roner et al., 2010; Schnurr et al., 2005; Taverna et al., 2004). Interactions of saponins with cholesterol and other sterols account for their many biological effects. The unique properties of saponins have resulted in their wide range of applications from cholesterol lowering (Anderson and Major, 2002; Harwood et al., 1993), antitumor (Lee et al., 1999; Rao and Sung, 1995), antiviral (Amoros et al., 1987, 1988; Hostettmann and Marston, 1995), antimicrobial (Escalante et al., 2002; Klita et al., 1996; Lu and Jorgensen, 1987) and their use as food additives and foaming agents in soft drinks (Hostettmann and Marston, 1995).

Saponins from a variety of sources have demonstrated antiviral activity against both naked and enveloped, DNA and RNA viruses (Amoros et al., 1987, 1988; Hostettmann and Marston, 1995). It has been suggested that the saponins are not directly virucidal but that the antiviral activity involves an inhibition of virus–host cell interaction and subsequent attachment (Amoros et al., 1988; Roner et al., 2007; Roner et al., 2010). Viral inactivation by saponins has also been suggested to involve interactions with membrane glycoproteins following the observation that no direct virucidal effect is seen on the nonenveloped poliovirus (Rao and Sinsheimer, 1974; Ushio and Abe, 1992). It is believed that saponins offer more than one novel mechanism 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 (Apers et al., 2001).

The antibacterial, antifungal, and antiprotozoal activities of saponins have also been reported extensively. Two saponins, phytolaccosides B and E, isolated from the berries of *Phytolacca tetramera* Hauman (Escalante et al., 2002) demonstrate antifungal activity against the human pathogenic opportunistic fungi *Trichophyton mentagrophytes*. The anti-rumen protozoal activity of saponins

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has been well documented both *in vitro* (Klita et al., 1996; Lu and Jorgensen, 1987) and *in vivo* (Makkar et al., 1998; Wang et al., 1998). Mechanistically it is proposed that saponins exert antimicrobial activity through the formation of complexes with sterols, especially cholesterol, present in the membrane of the microorganisms. This would most likely result in damage to the membrane and the subsequent collapse of the cells (Barile et al., 2007; Morrissey and Osbourn, 1999).

A number of studies, dating as far back as 1958, have explored the benefits of adding saponin to the mammalian diet (Afrose et al., 2009, 2010a,b; Ali et al., 2009; Baloyi et al., 2001; Benchaar et al., 2008; Cho et al., 2009; Duffy et al., 2001; Holtshausen et al., 2009; Ilsley et al., 2005; Kang et al., 2008; Karu et al., 2007; Kim et al., 2005; Raju et al., 2004). It is well documented that saponins facilitate changes in membrane fluidity and lipid environment of membrane proteins, including ion channels transporters and receptors (Bangham et al., 1962; Tiwari et al., 2008). Saponin extracts are currently used as dietary supplements for lowering cholesterol, they are believed to work by binding to and preventing the absorption of cholesterol (Waterhouse, 2002). Saponins also are known to increase the permeability of intestinal mucosal cells and may facilitate the uptake of substances across a mucus layer (Gee and Johnson, 1988; Gee et al., 1996).

The saponin that this study examines is a natural aqueous extract from the bark of *Quillaja saponaria*, which is a large evergreen tree native to Peru and the arid region of Chile (Leung, 1980). *Quillaja* saponin extracts are commercially prepared and used as a foaming agent in soft drinks and as emulsifier in other foods.

The crude extract obtained from commercial vendors has been estimated to contain fifty to sixty triterpenoids. Ultra Dry 100-Q, marketed by Desert King International, is a standard saponin material prepared for human consumption, and has a saponin content of 65%. The *Quillaja* saponin that we used in this study is Vax Sap, a further purified vaccine grade material obtained following additional purification of Ultra Dry 100-Q material, which has a saponin content of over 90%. We have demonstrated that both the standard and the vaccine grade *Quillaja* saponins exhibit strong antiviral activity against Rhesus Rotavirus (RRV) in cell culture (Roner et al., 2010).

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide and is estimated to be responsible for approximately 300,000 pre-adolescent deaths mostly in developing countries each year (Moon et al., 2010; Parashar et al., 2006; WHO, 2007). Rotavirus-related deaths represent approximately 5% of all deaths in children younger than five years of age worldwide (Dennehy, 2008). Two live oral vaccines, Rotarix and RotaTeq have shown great efficacy in reducing rotavirus related hospitalization and death among young children in developing countries (Ruiz-Palacios et al., 2006; Vesikari et al., 2004), but not in most developing countries in Asia and Africa (Moon et al., 2010). This current study is a continuous work that builds on the previous work performed with Ultra Dry 100 Q and Vax Sap (Roner et al., 2007, 2010). This study was conducted in Balb/c mice that were inoculated with Rhesus Rotavirus (RRV) and treated with *Quillaja* saponins to investigate the anti-rotavirus activity of saponins *in vivo*. We demonstrate that saponins can prevent rotavirus-induced diarrhea when included in the diet of newborn mice.

2. Material and methods

2.1. Virus and cell lines

The virus used in this study was rhesus rotavirus (RRV) (ATCCVR-954). The adherent monkey kidney cell line MA-104 clone 1 (ATCC CRL-2378) was used to support the growth of RRV. Briefly, MA-104 cells were propagated in monolayer cultures using minimal essential medium (MEM) with Earles' salts, supplemented with 10% fetal bovine serum (FBS). Cells were passaged using conventional procedures with 0.05% trypsin. A standard plaque assay was used to determine the titer of RRV stocks (Albert and Bishop, 1984; Wyatt et al., 1983).

2.2. Quillaja extract preparation

The *Quillaja* extracts used were obtained from Desert King International (San Diego, CA) (Roner et al., 2010). Ultra Dry 100-Q is a spray dried purified aqueous extract of the Chilean soapbark tree. The material for this study, Vax Sap, is a purified medical grade material obtained by addition purification of the Ultra Dry 100-Q. In the water-extracted saponin preparations, the identified structures have terpene quillaic acid with either a di- or one of the two tri-saccharides attached to C-3 and several oligosaccharides at C-28 (Guo and Kenne, 2000b). Different types of saponins have different levels of oxidation around the quillaic acid skeleton based on the types, location, and number of sugars and acyl moieties, respectively (Apers et al., 2001; van Setten and van de Werken, 1996). It is likely that the true number of saponin variants present in these extracts would exceed 100 if all conformational isomers are considered (Cox et al., 1998). The saponins content and identification can be determined by RP-HPLC (Fleck et al., 2006; Guo and Kenne, 2000a; Kensil et al., 1991; Marciani et al., 2003; Pham et al., 2006). At least 22 peaks (designated QS-1 to QS-22) can be distinguished. However, identification and quantification of four major saponins, QS-7, QS-17, QS-18 and QS-21, are generally adequate to express the saponins content of the *Quillaja* saponins as they represent up to 90% of the total saponins present (Kuznesof et al., 2005). Previous studies on these purified saponins showed that these related triterpenoid saponins have similar immune stimulating activities but significant differences in their toxicity (Marciani et al., 2003). Saponins QS-7 and QS-21 showed no or very low toxicity in mice, with QS-18 being the most toxic (Kensil et al., 1991).

2.3. Maintenance and breeding of animals

Adult Balb/c mice of seven weeks old were obtained commercially (Charles River, Wilmington, MA and Harlan Laboratories, Livermore, CA). Male and female mice were housed together to set up breeding pairs after a one week acclimating period. Females were separated from males prior to the birth of newborns. This study was approved by the UTA Institutional Animal Care and Use Committee (IACUC, Protocol # A07.33) which determined that this study was consistent with the recommendations in *The Guide for the Care and Use of Animals,* the Animal Welfare Regulations, and the Public Health Service policy. Newborn mice were kept for at least eight weeks. Weight was recorded every day for the first seven days and once every week until they reached sexual maturity to determine if oral treatment with saponins would have an impact on growth.

2.4. Quillaja extracts acute toxicity

Acute toxicity of *Quillaja* extract in newborn mice was determined by orally inoculating mice that are four to six days old and at least three grams in weight with 50 μl of saponin extract at concentration ranging from 0 to 0.5 mg/mouse for seven consecutive days. Newborn mice were observed for two months following treatment to explore if *Quillaja* saponin induced a sub-chronic condition. The mortality rate of newborn mice at different saponin concentrations was recorded to calculate the LD_{50} of *Quillaja* saponin of newborn mice over treatment for seven consecutive days.

2.5. RRV induced diarrhea in newborn mice

The incidence of RRV induced diarrhea in newborn mice was determined by orally inoculating mice with RRV at different infective doses for five consecutive days. Stool consistency was evaluated on a five point scale as followed: 0, normal, solid and black; 1, soft brown; 2, liquid brown; 3, soft yellow; and 4, liquid yellow (Shaw et al., 1995). The different categories of diarrhea reflect the amount of water lost during rotavirus infection. For this study, mice demonstrating level 3 or 4 stools were considered positive for diarrhea. Severity of diarrhea is reported as diarrhea score ranging from 3.0 to 4.0, where level 3 was less severe and level 4 was most severe.

2.6. Effectiveness of Quillaja extract on RRV induced diarrhea

The effectiveness of *Quillaja* extract on rotavirus induced diarrhea was determined by pretreating newborn mice with *Quillaja* extract for two days, followed by administration of RRV with *Quillaja* extract for five days. Stool consistency was evaluated as discussed above.

After selecting the concentration of saponin extract that provided the greatest antiviral activity against RRV in newborn mice, additional treatment parameters were explored to generate additional information such as how long the *Quillaja* extract could provide protection against RRV infection in newborn mice. Different durations of pretreatment with *Quillaja* saponins were tested to determine if pretreatments were critical to provide protection against RRV infection.

2.7. Quillaja extracts chronic toxicity

Ten individuals from each treatment protocol were randomly selected to become part of the chronic toxicity study. The tested conditions included: (1) the control group, inoculated with water for seven days; (2) the acute toxicity group, inoculated with *Quillaja* extracts for seven days; (3) the RRV induced diarrhea group, inoculated with RRV for five days; and (4) the testing group, inoculated with *Quillaja* extracts and RRV for seven days. The objective of this part of the study was to evaluate the potential for *Quillaja* extract to impart long term health impacts on treated individuals (Gad, 2007). Selected individuals were kept for an additional three months post sexual maturity to rule out the possibility of chronic damage due to ingestion of saponin. Individuals were then allowed to breed to evaluate litter size and health of the newborns.

2.8. Fractionation of Quillaja extract

The *Quillaja* extract was separated into three fractions with Fast Protein Liquid Chromatography (FPLC). The *Quillaja* extract was applied to a Mono Q column equilibrated with Buffer A (50 mM NaCl, 10 mM Tris in HPLC-grade water); extracts were eluted with a linear gradient of 50 mM–1 M NaCl of Buffer A over 35 min. This was followed by dialysis of fractions in cellulose membranes (Fisher Scientific, #21-152-10, 3500 MWCO, 10 Å) against 1× saline sodium citrate, pH 7.4 (SSC) at 4 °C for 48 h. Fractions were then analyzed with Reverse Phase High Performance Liquid Chromatography (RP-HPLC) (FAO/WHO, 2005) for comparison to the whole *Quillaja* extract.

Individual fractions were examined using a cytotoxicity assay performed in MA104 cells as described previously (Roner et al., 2010). Briefly, cytotoxicity was measured by counting 500 cells in individual wells using the trypan blue-dye exclusion procedure (Altman et al., 1993; Sarma et al., 2000). Median cellular cytotoxicity concentration was calculated and reported as $CCIC_{50}$. The direct virucidal effect of the fractions was measured by standard plaque-reduction assay, i.e. plaque assay of RRV was performed in the presence or absence of *Quillaja* extract and fractions. The fractions acute toxicity and effect of individual fractions on RRV induced diarrhea were also performed in newborn mice by oral administration over seven consecutive days as discussed earlier for comparison to the whole *Quillaja* extract.

2.9. Statistical analysis

Differences in weight of the newborn mice among treatments and controls were analyzed with Repeated measures Analysis of Variance (ANOVA) (Ferrante et al., 2002). RRV induced diarrhea was evaluated based on stool consistency as discussed previously. Individuals with no diarrhea over the 5 days were assigned a value of 0, and individuals with diarrhea were assigned a value of 1. Diarrhea scores were analyzed categorically by the Mann–Whitney U test (Rollo et al., 2005). ANOVA was used to analyze the *in vitro* data of different fractions collected following FPLC. Statistical analysis was done with SYSTAT statistical analysis software (Carnes Software International), and a P value of $\leqslant 0.05$ was considered significant.

3. Results and discussion

3.1. Acute toxicity of Quillaja extract in newborn mice

The potential toxicity of ingesting *Quillaja* extract to newborn mice was evaluated at concentrations ranging from 0 to 0.5 mg/mouse per day, for seven consecutive days. As summarized in Table 1, newborn mice orally inoculated with 0.0375 mg/mouse or more had a mortality rate of greater than 50%. Using linear regression, the LD_{50} of the *Quillaja* extract was 0.0325 mg/mouse over seven daily inoculations. Weights of newborn mice were recorded (Fig. 1) throughout the seven inoculations and until they reach sexual maturity at 56 days. The data was analyzed using repeated measures ANOVA of weight among the newborn mice inoculated with different *Quillaja* extract concentrations with less than 50% mortality. The analysis revealed no significant difference ($P = 0.229$) in weight indicating that there was no significant short term health impact on mice that survived the saponin treatment. Newborn mice inoculated with high concentrations of saponin extract died within 24–36 h post inoculation. We suspected that high concentrations of saponin extracts drastically change the membrane environment of the gastrointestinal tract, causing cells to burst. For animals that survived, reduced weight gain was observed, and they were significantly smaller compared to the control.

Table 1
Mortality rate of newborn mice orally inoculated with *Quillaja* extract.

Saponin concentration (mg/mouse)	Number of mice			Mortality rate (death/100 mice)
	Tested	Survived	Died	
0	33	32	1	3.0
0.005	13	13	0	0.0
0.0125	16	15	1	6.7
0.015	43	39	4	9.3
0.025	26	18	8	30.8
0.0375	15	2	13	86.7
0.05	14	7	7	50.0
0.25	9	3	6	66.7
0.375	9	2	7	77.8
0.5	8	0	8	100.0

To reduce animal numbers, treatment conditions resulting in high mortality were repeated with two liters only.

3.2. RRV induced diarrhea

The development of RRV induced diarrhea in newborn mice inoculated with different quantities of RRV was determined by orally introducing RRV each day for five consecutive days, ranging in amount from 50 to 50,000 plaque forming units (PFU). The data in Table 2 illustrate that more than 85% of the newborn mice developed diarrhea when inoculated with 50,000 or 5000 PFU of RRV for five consecutive daily inoculations. Additionally, 75% of the newborn mice developed diarrhea when inoculated with 500 PFU of RRV, while a significantly lower number ($P \leqslant 0.001$) of newborn mice, 33%, developed diarrhea when inoculated with only 50 PFU. A trend was seen for a corresponding decrease in the severity and duration of diarrhea in newborn mice developing diarrhea (level 3 and 4 only) decreased with reductions in the level of virus inoculation.

Throughout the 56 day observation period, diarrhea was only detected within the first week post initial inoculation of RRV. The RRV induced diarrhea began as early as 24 h post inoculation. The rate of RRV induced diarrhea within a litter usually increased after one of the newborns developed diarrhea which may indicate the possibility of secondary infection. All the mice recovered within two to three days. No diarrhea was observed beyond five days post RRV inoculation.

3.3. Impact of Quillaja extract on RRV induced diarrhea

The impact of the saponin extract on RRV induced diarrhea in newborn mice was tested at different extract concentrations for seven consecutive days. Pretreatment of MA104 cells with *Quillaja* extract at concentrations as low as 0.0001 mg/ml were able to completely block RRV binding *in vitro* (Roner et al., 2010). To explore the anti-rotavirus activity of *Quillaja* extract *in vivo*, newborn mice were pretreated with *Quillaja* extract for two days, followed by inoculation with various amounts of RRV together with *Quillaja* extract, for a treatment period of five consecutive days.

At 50,000 PFU' per mouse, the number of newborn mice that developed RRV induced diarrhea was significantly reduced from 88.9% to 28.5% when they were given *Quillaja* extract at 0.03 mg/mouse (Fig. 2a). The incidence of diarrhea was only reduced to 70% when mice were treated with 0.025 and 0.0125 mg/mouse indicating that a quantity of *Quillaja* extract greater than 0.03 mg/mouse was required to block RRV infection in mice receiving 50,000 PFU, a very high dose of virus. However at this extract concentration, the mortality rate was also high. Newborn mice treated with *Quillaja* extracts at 0.025 mg/mouse or higher had a mortality rate of over 60%, and 37.5% at 0.0125 mg/mouse (Fig. 3a). Upon reducing the virus inoculum to 5000 PFU' per mouse, RRV induced diarrhea was reduced from 87.8% to 57.1% when also treated with *Quillaja* extract at 0.015 mg/mouse (Fig. 2b). The mortality rate, on the other hand, was greatly reduced to 6.6% at this lower extract concentration (Fig. 3b). Further reducing the virus challenge to 500 PFU' per mouse still induced significant diarrhea and allowed the beneficial effects of the extract to be better measured. The RRV induced diarrhea under these conditions was reduced from 75.6% to 11.1%, 9.5%, and 27.9% when mice were treated with *Quillaja* extract at 0.03, 0.015, and 0.0125 mg/mouse, respectively (Fig. 2c). The mortality rate was at 54.5% when treated with *Quillaja* extract at 0.03 mg/mouse, while it was reduced to 11.9% and 6.9% when *Quillaja* extract was lowered to 0.015 and 0.0125 mg/mouse, respectively (Fig. 3c) while still protecting the animals from RRV induced diarrhea.

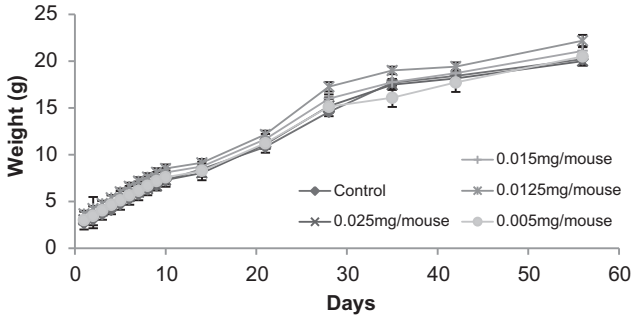

Fig. 1. Weight gained for newborn mice orally inoculated with *Quillaja* extract at different concentrations for seven consecutive days. Each data point represented 13 individuals or more.

Oral inoculation of *Quillaja* extract most likely results in a "coating" effect of cells and blocks RRV infection in treated mice. The higher the concentration of *Quillaja* extract inoculated, the more cells are "coated", and the better protected an animal is against RRV induced diarrhea. However, at very high concentrations, *Quillaja* extracts can also cause a disruption of the cell membrane environment, which can result in cell death, and eventually death of the animal. The optimum concentration of *Quillaja* extracts that we observed during this experiment was 0.015 mg/mouse. At the concentration the extract was able to reduce the RRV induced diarrhea significantly with little impact on the health of the treated animals. The rate of RRV induced diarrhea increased when the amount of the *Quillaja* extracts used to protect the mice was reduced from 0.015 to 0.0125 mg/mouse, indicating that we had reached the lower limit and reducing the treatment amount further might reduce the mortality but would also increase the RRV induced diarrhea. Using linear regression, we determined that the dosage required to effectively reduce the RRV induced diarrhea by 50% (ED_{50}) was 0.011 mg/mouse when the mice were challenged with 500 PFU' of RRV each day for a period of 5 days.

Weight gains in newborn mice were recorded from the beginning of the treatment until sexual maturity was reached. For all groups treated with different amounts of RRV and concentrations of *Quillaja* extracts, no signification reduction in weight was recorded when compared to the control group on which only water was given orally (Fig. 4). In fact, for some of the groups that were treated at low concentrations of extract, these mice actually gained significantly more weight when compared to the control group. These results indicate that there were no signification short term health impacts on mice that survived the treatment.

Table 2
RRV induced diarrhea in mice.

Amount of rotavirus (PFU)	Number of mice			% that developed diarrhea	Severity (diarrhea score ± s.d.)	Duration (days ± s.d.)
	Tested	Developed diarrhea	No diarrhea			
50,000	27	24	3	88.9	3.8 ± 0.2	2.8 ± 0.8
5000	33	29	4	87.9	3.3 ± 0.4	1.5 ± 0.6
500	41	31	10	75.6	3.3 ± 0.4	1.5 ± 0.6
50	12	4	8	33.3	3.1 ± 0.2	2.0 ± 0.8

To reduce animal numbers, treatment conditions not producing diarrhea were repeated with three liters only.

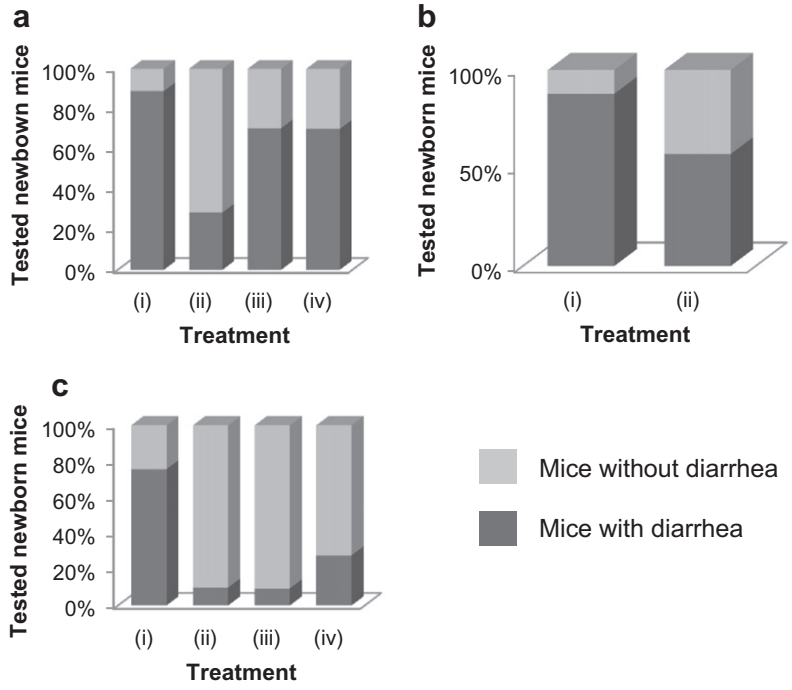

Fig. 2. RRV induced diarrhea in newborn mice orally inoculated with *Quillaja* extracts at different concentration and RRV at (a) 50,000 PFU: (i) 0 mg/mouse, (ii) 0.03 mg/mouse, (iii) 0.025 mg/mouse, and (iv) 0.0125 mg/mouse; (b) 5000 PFU: (i) 0 mg/mouse and (ii) 0.015 mg/mouse; and (c) 500 PFU: (i) 0 mg/mouse, (ii) 0.03 mg/mouse, (iii) 0.015 mg/mouse, and (iv) 0.0125 mg/mouse.

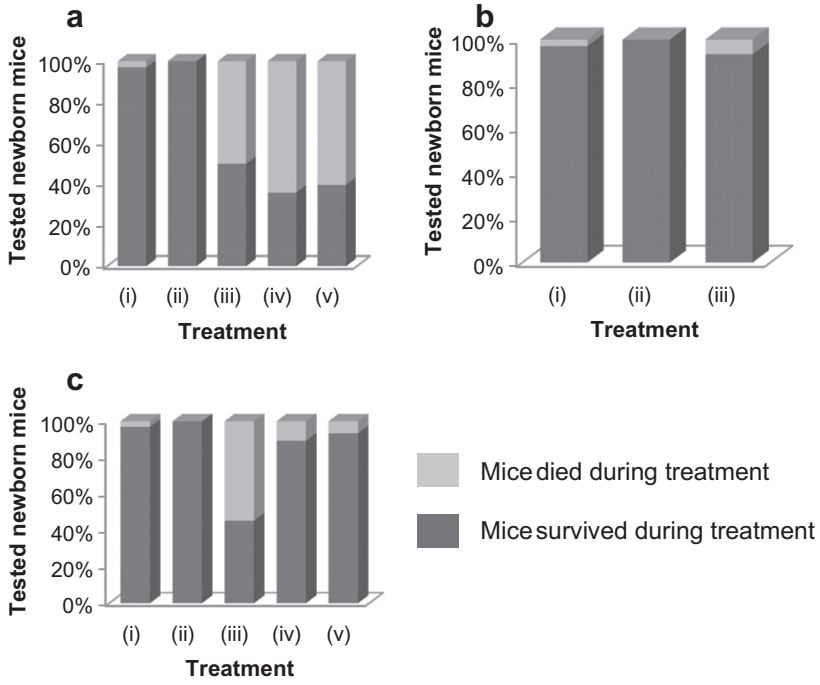

Fig. 3. Mortality in newborn mice orally inoculated with *Quillaja* extracts at different concentration and RRV at (a) 50,000 PFU: (i) control (only water), (ii) 0 mg/mouse, (iii) 0.03 mg/mouse, (iv) 0.025 mg/mouse, and (v) 0.0125 mg/mouse; (b) 5000 PFU: (i) control (only water), (ii) 0 mg/mouse and (ii) 0.015 mg/mouse; and (c) 500 PFU: (i) control(only water), (ii) 0 mg/mouse, (iii) 0.03 mg/mouse, (iv) 0.015 mg/mouse, and (v) 0.0125 mg/mouse.

At 0.015 mg/mouse, *Quillaja* extracts demonstrated the greatest protection against RRV induced diarrhea when mice were exposed to 500 PFU of virus. Different parameter conditions were tested to investigate if the duration of pretreatment with *Quillaja* extracts before exposure to RRV would have an effect on reducing the diarrhea rate. The number of newborn mice that developed RRV induced diarrhea increased with the decrease in pretreating time of *Quillaja* extracts (Table 3). Diarrhea increased from 9.5% for two days of pretreatment to 23% for no pretreatment. Although a trend was observed, this increase was not statistically significant (P value = 0.111). On the other hand, RRV induced diarrhea in the mice was significantly increased from 9.5% to 42.4% when only pretreat-

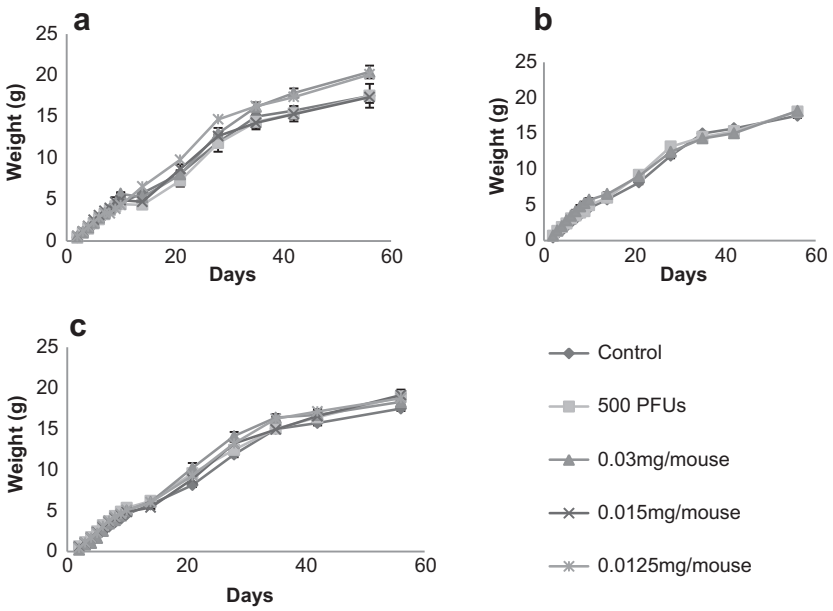

Fig. 4. Weight gain in newborn mice orally inoculated with *Quillaja* extracts at different concentration and RRV at (a) 50,000 PFU, (b) 5000 PFU, and (c) 500 PFU. Each data point represents 25 individuals or more.

Table 3
RRV induced diarrhea at a treatment concentration of 0.015 mg/mouse *Quillaja* extracts with 500 PFU RRV.

Treatment	Number of mice			% that developed diarrhea	Severity (diarrhea score ± s.d.)	Duration (days ± s.d.)
	Tested	Developed diarrhea	No diarrhea			
500 PFU RRV	41	31	10	75.6	3.3 ± 0.4	1.5 ± 0.6
Pretreatment with *Quillaja* extracts for two days	42	4	38	9.5	3.0 ± 0.2	1.3 ± 0.5
Pretreatment with *Quillaja* extracts for one day	39	7	32	17.9	3.14 ± 0.3	1.3 ± 0.4
No pretreatment of *Quillaja* extracts	30	7	23	23.3	3.11 ± 0.2	1.2 ± 0.5
Pretreatment with *Quillaja* extracts for two days, no *Quillaja* extracts given after pretreatment	33	14	19	42.4	3.6 ± 0.4	1.5 ± 0.7

ment of *Quillaja* extracts was administered and not continued while they received the RRV. One additionally important observation was noted in mice treated in this manner. Pretreated with extract but not present at the time of virus exposure, these mice only developed RRV induced diarrhea towards the end of the virus inoculation (at day six or seven), indicating that *Quillaja* extracts given during the pretreatment period, were active and did "block" RRV infection for 48–72 h after the pretreatment but during virus challenge. This observation concurred with our earlier *in vitro* findings that cell lines returned to near normal in terms of virus susceptibility and virus infections rates were nearly identical to those of untreated cells following treatment with the extract. These findings indicate that no long term alteration and/or protection of the cells following treatment with *Quillaja* extracts occurs but demonstrates that saponin does not need to be present at the same time as virus exposure. These findings are critically important when applying to children. Although pretreatment is not required to prevent RRV induced diarrhea, it serves as a possible preventative measure. A reduction in the severity of the diarrhea is most likely all that will be needed to prevent death in children.

3.4. Quillaja extracts chronic toxicity

For the chronic studies, ten mice from each tested condition were randomly selected to continue for 90 days observation post sexual maturity (Fig. 5). These individuals were also allowed to breed and offspring were kept until sexually matured. There was no significant difference in weight among groups over both the 90 days observation (P value = 0.222) and also among the offspring (P value = 0.507). Litter sizes of five to eight were commonly observed and ranged from one group of two and one group of eleven. These findings indicated that there was no long term impact on treated individuals weight gain, reproductive ability or chronic effect on the offspring following exposure to the extract and/or RRV.

3.5. Fractionation of Quillaja extracts

Whole *Quillaja* extracts were separated into three fractions using FPLC as described. Fraction A was collected between six to nine minutes, fraction B was collected between twenty to twenty-five minutes and fraction C was collected between twenty-six to thirty minutes. The HPLC profile of whole *Quillaja* extracts (Fig. 6a) demonstrates that a majority of the saponin variants eluted out in the first five minutes. Fraction A (Fig. 6b) showed major peaks at three and four minutes and at 20 min. Fraction B and C (Fig. 6c and d) had similar profiles where a majority of the saponin variants eluted out between 16 and 22 min. Fractions B and C were collected within the same peak during FPLC, and as expected the two fractions have similar HPLC profiles.

The cytotoxicity of fractions was evaluated in MA104 cells over 96 h. To determine viability, the number of dead and living cells were counted under a microscope, a total of 500 cells were counted

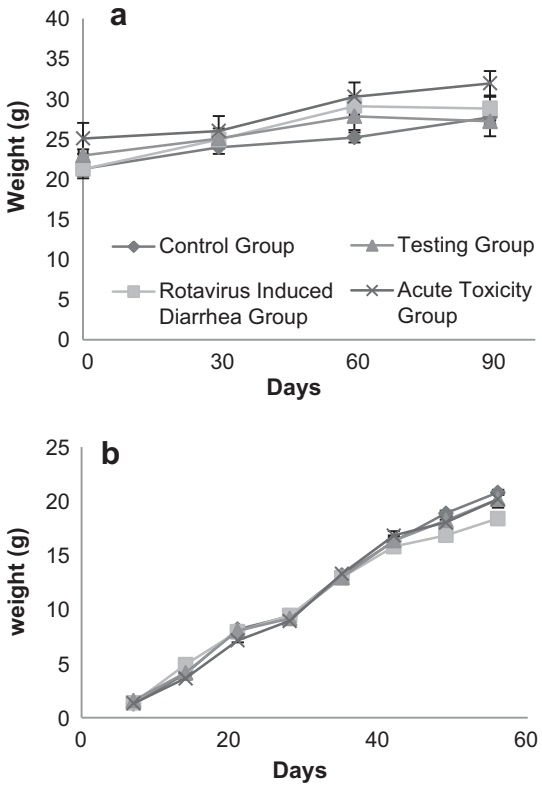

Fig. 5. Chronic study of *Quillaja* extracts inoculated in newborn mice. (a) 90 days observation post sexual maturity, (b) offsprings of treated newborn mice. Each data point represents 10 individuals.

in each well. The results demonstrate that at a concentration 0.1 mg/ml or higher results in complete cell death, while less than 4% of the cells are dead at 0.01 mg/ml. To determine the critical concentration toxic to cells, further dilutions were prepared between 0.1 mg/ml and 0.01 mg/ml. All three FPLC fractions showed higher toxicity when compared to the whole *Quillaja* extracts. Despite fractions B and C looking almost identical in the HPLC profile, there was indeed a significant difference in cytotoxicity with fraction C being the most toxic out of all fractions tested.

Each of the three fractions displayed similar cytotoxicity as the control at 0.01 mg/ml. A plaque reduction assay was carried out to measure the antiviral activity of the *Quillaja* fractions against RRV in MA104 cells at this concentration. For whole *Quillaja* extracts, the virus infectivity was reduced 35-fold from 5.57×10^7 to 1.58×10^6 (Table 4). Out of the three fractions, fraction C had the greatest antiviral activity, reducing the virus infectivity fivefold. An interesting result was observed between fractions B and C. Despite the similarity of their HPLC profiles, fraction C reduced the virus infectivity more efficiently than fraction B (5.6-fold versus 1.7-fold, respectively). Combinations of the fractions were also evaluated. Fractions A and C had the greatest antiviral activity with an eightfold reduction in virus infectivity. Combining all three fractions served as a positive control to test if any critical activities were possibly lost duration the fractionation process. Fractions A + B + C regained the ability to reduce virus infectivity by 22-fold versus 35-fold for the starting material. No other combination of the three fractions was able to restore significant antiviral activity indicating that some critical activities were lost during fractionation.

The activity of the saponin fractions against RRV induced diarrhea in newborn mice was also evaluated. At 0.0005 mg/mouse, whole *Quillaja* extracts reduced RRV induced diarrhea from 75% to 45% (Table 5), and fractions A and C had similar antiviral activities at this concentration. Fraction B, on the other hand, only reduced RRV induced diarrhea from 75% to 72%. This agreed with *in vitro* data where fraction B had the least antiviral activity out of three fractions using a plaque reduction assay. None of the treated groups had a significant reduction in weight when compared to control (Fig. 7).

3.6. Saponins as a new microbiocide to prevent diarrhea

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for approximately 600–850,000 deaths each year. This represents approximately 5% of all deaths in children younger than five years of age worldwide (Dennehy, 2008). In this study, we evaluated the antiviral activity of a natural extract, *Quillaja* saponin against RRV in a mouse model.

The model uses newborn Balb/c mice that when exposed to RRV, develop diarrhea within 1–5 days. Previous studies have demonstrated that in a heterologous system, infecting newborn mice with simian rotavirus SA11 (Feng et al., 1994; Offit et al., 1984), requires a higher infective dose of 1000 PFU compared to one to 100 PFU in a homologous system. We were able to establish a heterologous system of infecting newborn mice with RRV requiring half of the inoculum with over 70% of the newborn mice developing diarrhea when inoculated with 50 μl of 1×10^4 PFU/ml (which is equivalent to 500 total PFU/mouse). To better model the natural exposure of children to rotavirus, the result of fecal-contaminated water supplies, foods and direct contact, we used a protocol where the mice were exposed/challenged daily for five consecutive days and where secondary infections could occur within each litter among newborn mice through fecal–oral transmission. This was an attempt to mimic what would likely occur in a family between brothers and sisters, or children in school or daycare or areas of poor sanitation. As a result of this challenge protocol, the amount of virus required to induce diarrhea in our study is as we would predict, lower than most published reports employing a single virus exposure scenario.

The *Quillaja* saponins that we used are a natural, aqueous extract of triterpenoid saponins obtained from *Quillaja saponaria* Molina, the Chilean soapbark tree. The extract is a complex mixture of triterpenoids, each built around a common quillaic acid. The *Quillaja* extract is currently approved for use in food and beverages by the FDA (under 21 CFR 172.510, FEMA GRAS number 2973) and is approved for its use in the European Union in water-based non-alcoholic drinks, (under code E 999. Current CAS number: 68990-67-0) to be used as food addictive. This surmounts a major hurdle for the application of our findings to humans. The toxicity of saponin extracts was evaluated *in vivo* in this study using a mouse model with Balb/c mice. Newborn mice orally inoculated with the saponin extract at a concentration of 0.015 mg/mouse for each of seven consecutive days had a mortality rate of less than 10% ($LD_{50} \leqslant 0.025$ mg/mouse). Growth of newborn mice was unaffected at concentrations as high as 0.025 mg/mouse or less compared to the control. Long term observations and breeding among the treated individuals demonstrated that the saponin extract does not exert any chronic damage when inoculated during the infancy period.

Newborn mice weighed on average about three grams when treatments began. Exposures were initiated using 0.025 mg/mouse saponin extract, equivalent to 1.67 mg/kg body weight. The Food and Agricultural Organization of the United Nation and World Health Organization suggests that the Acceptable Daily Intake (ADI) of *Quillaja* extract is between zero to five mg/kg body weight and it was shown from the WHO statistics that it is not uncommon for individuals to ingest up to 186% of the current ADI per eating

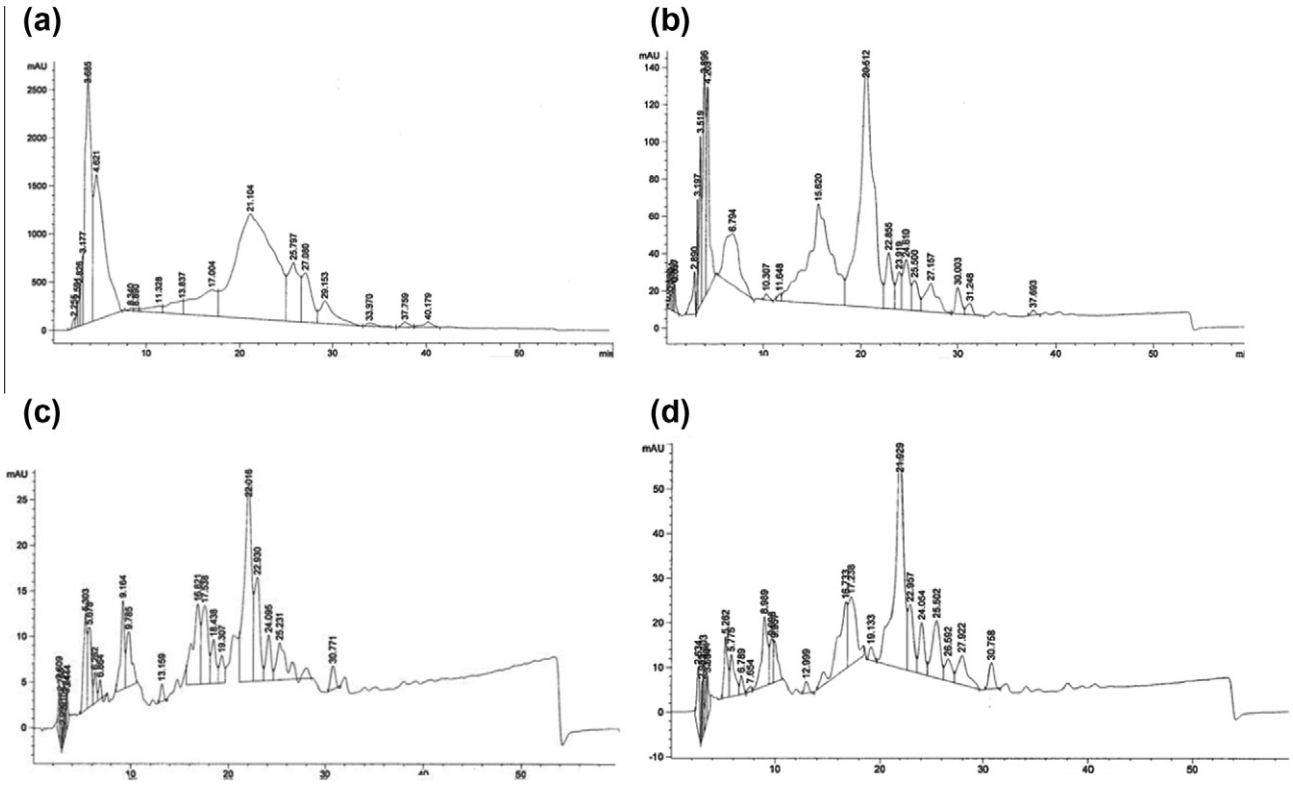

Fig. 6. HPLC profiles of: (a) whole *Quillaja* extracts, (b) fraction A, (c) fraction B, and (d) fraction C.

Table 4
Plaque reduction assays for extract fractions at a concentration of 0.01 mg/ml.

Saponin	Virus titer (± std. error)	Plaque reduction (compared to control)
Control (no saponin)	$5.6 \pm 0.6 \times 10^7$	N/A
Whole saponin	$1.6 \pm 0.4 \times 10^6$	35.3-fold
Fraction A	$1.5 \pm 0.2 \times 10^7$	3.7-fold
Fraction B	$3.2 \pm 0.4 \times 10^7$	1.7-fold
Fraction C	$9.9 \pm 0.2 \times 10^6$	5.6-fold
Fraction A + B	$1.8 \pm 0.4 \times 10^7$	3.1-fold
Fraction B + C	$1.2 \pm 0.2 \times 10^7$	4.7-fold
Fraction A + C	$6.7 \pm 1.4 \times 10^6$	8.3-fold
Fraction A + B + C	$2.5 \pm 0.2 \times 10^6$	22.3-fold

occasion. Based on these current human exposures it appears that human's exhibit a much higher tolerance for saponin extract than mice. Again this adds additional support for using these extracts to prevent infections and diarrheal diseases in humans.

Using our RRV induced diarrhea model, newborn mice recovered from RRV infection within five days post initiation of treatment. In this study, newborn mice were pretreated with saponin extract before RRV was inoculated for five consecutive days. We have proposed that 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 (Roner et al., 2007, 2010). The *Quillaja* extracts may cause a reversible modification of the cell membrane or modification of the cellular endocytosis process. Our *in vivo* results can be explained by the possibility that not all cells are being "treated/coated/modified" by the saponin extract at the low concentration (0.015 mg/mouse), hence at higher inocula (5000 PFU), RRV can still establish an infection and induce diarrhea in over half of the individuals. And at even higher inocula (50,000 PFU), a higher concentration (0.03 mg/mouse) of saponin extract would be needed to "block" virus infection, which is what we find. However, the saponin extract is quite toxic to the newborn mice when the concentration is doubled to 0.03 mg/mouse, therefore attempts in purification of the saponin extracts to reduce toxicity would be important. It is important to note that although the incidence of diarrhea was only reduced by 50% or so in some treatments, the severity and interval of the diarrhea in the animals was greatly reduced when compared to animals that did not receive the saponin-containing extract. No animals ever died as a result of the RRV-induced diarrhea. The animals that died did so within 24–36 h of receiving the saponin extract, most likely due to toxicity.

For a potential therapeutic agent, it is important to calculate the therapeutic index. In this study, administration of 0.0375 mg/mouse of saponin extract killed $\geqslant$85% of the newborn mice, and 0.025 mg/mouse killed 30%. Using linear regression, this yields an LD_{50} of 0.0325 mg/mouse per day in newborn mice over a seven day period. At 500 PFU of RRV, 0.015 mg/mouse of saponin extract was able to reduce the diarrhea by 88% while 0.0125 mg/mouse of saponin extract reduced the diarrhea by 65%. Using linear regression, this yields an ED_{50} of 0.011 mg/mouse over a seven day period. Under these conditions, *Quillaja* extracts demonstrate a therapeutic index of 2.95 at 500 PFU of RRV over a seven days period. Purification to reduce toxicity should greatly improve the potential use for saponin extract as therapeutic agent.

One of the issues that we ran into throughout this study were the difficulties in accurately estimating the mortality rate of newborn mice from *Quillaja* extracts administration. Cannibalism of newborns was commonly seen in larger litters (Gandelman and Simon, 1978). Not much is known about what triggers infanticide and factors may vary among different females (Fox, 2007). In addition, administration of treatment in a volume of 50 μl in a three gram newborn mouse is equivalent to 170 ml in a newborn baby of three kilogram. Attempts at reducing this volume were met with numerous technical problems and greatly reduced the accuracy

Table 5
Antiviral activity of extract fractions at a treatment concentration of 0.005 mg/mouse.

Treatment	Number of mice			% that developed diarrhea	Severity (diarrhea score ± s.d.)	Duration (days ± s.d.)
	Tested	Developed diarrhea	No diarrhea			
500 PFUs RRV	41	31	10	75.6	3.3 ± 0.4	1.5 ± 0.6
Whole saponin with 500 PFU RRV	11	5	6	45.5	3.2 ± 0.3	1.6 ± 0.54
Fraction A with 500 PFU RRV	10	5	10	50.0	3.2 ± 0.3	1.5 ± 0.8
Fraction B with 500 PFU RRV	11	8	3	72.7	3.5 ± 0.5	1.2 ± 0.5
Fraction C with 500 PFU RRV	15	7	8	46.7	3.1 ± 0.2	1.5 ± 0.8

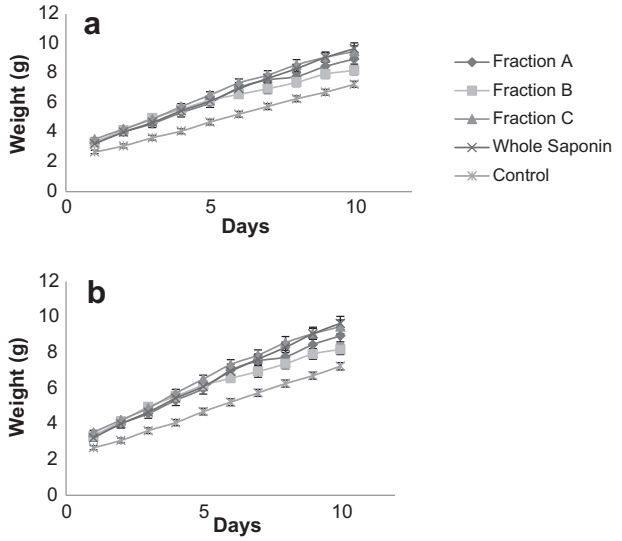

Fig. 7. Weight gained for newborn mice orally inoculated with (a) *Quillaja* fractions, and (b) *Quillaja* fractions with 500 PFU RRV. Each data point represent at 10 individuals or more.

and reproducibility of the inoculations. Administration of the treatment in newborn mice can result in forcing water down the trachea and can inadvertently "drown" the subject. All suggested means to minimize disturbance and slow administration of treatment were executed but occasional casualties were inevitable and difficult to estimate and explain. Future studies in progress will use larger animals, rabbits as test subjects.

Alternative animal models with larger animals or animals that have a longer susceptibility period can avoid casualties that may obscure the data. Gnotobiotic piglets are susceptible until at least six weeks of age to infection and disease with several human rotaviruses (Saif et al., 1996; Schaller et al., 1992), closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity. Rabbits remained susceptible to rotavirus infection to at least 16 weeks of age which make infections of rabbits more analogous to natural infections in children in whom the majority of severe infections occur between six months and two years of age (Conner et al., 1988). The longer susceptibility period can delay the treatment until the animals can feed on their own where saponin extract can be mixed in water; and/or saponin extract can be mixed in milk and given to animals in metal bottles. Administration of treatment can also be accomplished easier due to the larger size of the animal.

There is strong evidence that saponin extract is able to "block" rotavirus infection by coating target cells and hence reduce rotavirus induced diarrhea in newborn mice. Future research that measures virus shedding and antibody production in newborn mice and rabbits will give more insights into what extend the saponin extract can work against rotavirus infection in humans.

Equally important is the immune response to rotavirus in these treated animals. Future studies will explore the possibility that these mice and rabbits have been "vaccinated" during this treatment and will hopefully remain resistant to future challenges by rotavirus. This will be very important in humans. It is hoped that the saponin treatment while greatly reducing rotavirus induced diarrhea in individuals will allow a small amount of virus for infection that will trigger an immune response but greatly reduce the severity of the diarrhea. In this way, antibodies are produced to protect individual from future challenges by rotavirus while the symptoms (diarrhea) are inhibited or significantly reduced.

There is no specific treatment of rotavirus infection except for rehydration of the infected individuals. Although WHO recommends use of rotavirus vaccine worldwide to reduce rotavirus related death in children, the cost of the two current rotavirus vaccines is fairly high. We have shown that oral administration of saponin with water is able to reduce rotavirus induced diarrhea in mice. The extract is a renewable natural product, inexpensive to produce, soluble in water, pH stable from pH 2 to 11 and is already approved for use in humans as food addictive. We believe that saponins have great potential to prevent severe rotavirus infections in humans at a very affordable cost.

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