# CHARACTERIZATION OF ANTI-ROTAVIRUS ACTIVITIES OF SAPONIN EXTRACTS FROM *QUILLAJA SAPONARIA*

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

August 2010

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Roner for giving me the opportunity to work in his lab. I will never forget his great mentorship. He is patient and is always there when we need him. I would like to thank Dr. Betran, Dr. Chippindale, Dr. Formanowicz, and Dr. Pritham for being on my committee and for the professional support on my dissertation. I would also like to thank the Biology department at UTA for giving me the teaching assistant position and giving me support in many different aspects. Special thanks go to Dr Badon for being a great Microbiology lab co-ordinator and for always being there for guidance and encouragement. I would have never be able to teach a class if it was not for her.

A big thank you goes to Melody Kiesling-Barrager and Alisa Moric in Dr Roner's lab, Christi Hull, Blaine Thompson, Briony Foster and many more in the Biology department.

Lastly, I would like to thank my family and friends for moral support, my parents, Tam Heng Keong and Leong Im Ha; my sisters, Vince and Fiona Tam; my friends, Huey Lin, Mae Tage, Chun Chan, Angela Tsang, Shirley Che, Grace Lei, Maggie Wan, Iny Chu, Susan Tsang, and many more. I would have never been able to make it through without everyone's support and care. Thank you everyone for believing in me and loving me.

June 4, 2010

ABSTRACT

CHARACTERIZATION OF ANTI-ROTAVIRUS ACTIVITIES

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The University of Texas at Arlington, 2010

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Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for over 500,000 adolescent deaths mostly in developing countries each year. Rotavirus-related deaths represent approximately 5% of all deaths in children younger than five years of age worldwide. Previous research has shown that *Quillaja* saponins demonstrate strong antiviral activity against 7 different viruses, including rotavirus, *in vitro*. *Quillaja* saponins, a natural, aqueous extract of triterpenoid saponins obtained from *Quillaja saponaria*, the Chilean Soap Bark tree that display strong adjuvant activity when used in vaccines. In this study, *Quillaja* saponins are stable in water and are approved by the US FDA for use as food additives. The proposed mechanism of *Quillaja* saponins is most likely through binding to membrane sterol of the epithelium of the host's small intestine and prevents rotavirus attachment. I proposed that the addition of *Quillaja* saponins to water supplies can inhibit rotavirus infection and thereby prevent diarrhea caused by rotavirus. This study evaluated the effect of *Quillaja* saponin against rotavirus induced diarrhea using mouse rhesus

rotavirus diarrhea model. Rhesus rotavirus was orally inoculated into newborn mice for 5 days. Toxicity testing showed that the $LD_{50}$ of *Quillaja* saponins is 0.65mg/ml for consecutive 5 days of treatment. Different titers of rotvirus were used to test for antiviral activity, $ED_{50}$ is at 0.22mg/ml over 5 consecutive days of virus challenge at 500 PFUs. Fractionation of the whole *Quillaja* saponin through FPLC showed difference in cytotoxicity and antiviral activity among fractions.

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# CHAPTER 1

## INTRODUCTION

Saponins are a class of chemical compounds found in a number of plant species (1) including soybean, peas, *Yucca schidigera*, and *Quillaja saponaria* (soapbark tree). Saponins are amphipathic glycosides (2), they contain both lipophilic and hydrophilic moieties. The unique property of saponins being both fat- and water-soluble has resulted in their use ranging from cholesterol reduction treatments, cancer treatments, antivirals, food additives and as foaming agents in soft drinks (3). Saponin-containing plants have been used for centuries as folk medicines to cure different symptoms like rheumatism, neuralgia, and against pains (3, 4).

The natural saponin extract this study explores is water extracted from *Quillaja saponaria*, commonly known as soapbark and is native to Peru and Chile (5). The extract is a complex mixture of triterpenoids, each built around a common quillaic acid. The crude extract obtained from commercial vendors has been estimated to contain fifty to sixty individual saponins with slightly different structures. All 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 (6).

Previous research has shown that *Quillaja* saponin extracts exhibit antiviral activity *in vitro* on a broad range of viruses including enveloped viruses such as vaccinia virus, Herpes simplex virus type 1, Varicella-zoster virus, HIV-1, and HIV-2. It was proposed that saponins disrupt cellular membrane proteins and/or virus receptors, preventing virus attachment to these cells, and thus inhibiting virus attachment and infection (7). It has been demonstrated that aqueous extracts of *Quillaja saponaria* can also prevent non-enveloped viruses such as reovirus and rotavirus from attaching to host cells and therefore preventing infection. At

concentrations as low as 0.0001% extract, over 99.5% of the cells were protected from infection for at least 16 hours (7).

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for over 500,000 adolescent deaths mostly in developing countries each year (8, 9, 10). Rotavirus-related deaths represent approximately 5% of all deaths in children younger than five years of age worldwide (11). Saponins are readily soluble in water and are approved by the FDA for inclusion in beverages intended for human consumption. The addition of saponins to existing water supplies offers a new intervention in the cycle of rotavirus infection. Our lab believes that saponins will “coat” the epithelium of the host’s small intestine and prevent attachment of rotavirus. This research dissertation provides *in vivo* data for the possibility of including saponin in drinking water to prevent infections of rotavirus.

To prepare for testing the hypothesis that saponins can prevent rotavirus-induced diarrhea in humans, testing was conducted using an animal model. The animal model used in this study was the BALB/c mouse (12). Newborn mice that are four to six days old are inoculated with Rhesus Rotavirus to induce diarrhea, an animal model commonly used to mimic rotavirus infections in humans to evaluate the antiviral activity of the saponin extract, newborn mice exposed to rotavirus were also orally inoculated with the saponin extracts in an attempt to prevent rotavirus induced diarrhea in these animals.

The goal of this study is to examine if *Quillaja* saponin has the same antiviral activity against rotavirus *in vivo* as we have demonstrated it possesses *in vitro* (7). There are three specific aims in this study: 1.) To evaluate the *in vivo* toxicity of the *Quillaja* saponin extracts at concentrations demonstrating biological activity. 2.) To evaluate if *Quillaja* saponin extracts fed to mice exposed to rotavirus are effective in preventing rotavirus induced diarrhea. 3.) To evaluate if purification of the anti-rotavirus activity demonstrated for the *Quillaja* saponin extracts can potentially reduce toxicity and possibly enhance the antiviral activity in the mouse diarrhea model to prepare for human testing.

# CHAPTER 2

# BACKGROUND

## 2.1 Advantages of Saponins

Saponins are widely distributed in the plant kingdom (1), and are present in soybeans, chick peas, peanuts, mung beans, spinach, garlic, potatoes, onions, asparagus, etc,. By 1972, Kofler listed 472 saponin-containing plants (13) and it is now known that over 90 plant families contain saponins. They are natural detergents that produce a stable foam when dissolved in water (14, 15, 16). The soap-like properties of saponins are traceable to their structure as amphipathic glycosides (2); they contain both lipophilic and hydrophilic moieties. Having both fat-soluble and water-soluble properties, saponins make excellent surfactants and have been used as soaps for hundreds of years. The unique properties of saponins have resulted in their wide range of applications from cholesterol lowering, antitumor, antiviral, antibacterial to their use as food additives and foaming agents in a number of popular soft drinks such as root beer (3). Saponin-containing plants have been used as folk medicines and in the food, veterinary and medical industries (3).

### *2.1.1 Antiviral activity*

Saponins from a variety of sources have demonstrated antiviral activity against DNA and RNA viruses, and enveloped and naked viruses. For example, the whole-plant extract of *Anagallis arvensis* (Primulaceae) was active against herpes simplex virus and poliovirus (17, 18) by inhibiting cytopathic action and reducing the virus population (3). It has been suggested that the saponins isolated are not virucidal and that the antiviral activity involves the inhibition of virus-host cell attachment (18). Several DNA and RNA viruses are inhibited by Glycyrrhizin with no effect on viral protein synthesis and replication of uninfected cells (3). Saikosaponins from roots of *Bupleurum falcatum* (Umbelliferate) have been shown to possess antiviral activity

against hepatitis B virus (19), human immunodeficiency virus (20), measles virus (21), influenza virus (22, 23), herpes simplex virus (21) and varicella-zoster virus (20). Viral inactivation by the saponin was suggested to involve interaction with membrane glycoprotein since there was no effect on poliovirus which lacks an envelope (21, 22).

For evaluation of *in vivo* antiviral activity, herpes simplex keratitis induced in rabbit eyes was used as a model (24). A saponin ointment preparation had almost the same effectiveness at treating the keratitis as many available drugs like adenine arabinoside and idoxuridine (24). However, the therapeutic index for the saponin was low and small increases in saponin concentration in the ointment gave toxic effects. It is believed that saponins offer more than one novel mechanisms of antiviral action, including interactions with viral envelopes leading to their destruction, interactions with host-cell membranes leading to a loss of virus binding sites and coating of cells to prevent virus binding (7, 24).

*2.1.2 Antimicrobial activity*

The antibacterial, antifungal, and antiprotozal activities of saponins have also been reported extensively. Many different species of the genus *Phytolacca* (Phytolaccaceae) contain saponins that show antifungal activity (25, 26). Two of the saponins, phytolaccosides B and E, isolated from the berries of *Phytolacca tetramera* Hauman (26) showed antifungal activity against the human pathogenic opportunistic fungi like *Trichophyton mentagrophytes.* Triterpenoid saponins from the seeds of *Chenopodium quinoa* Willd have been reported to have antifungal activity (27). One of the mechanisms by which saponins might exert antimicrobial activity is in the formation of complexes with sterols present in the membrane of the microorganisms. This would result in damage to the membrane and the consequent collapse of cells. (28, 29).

The anti- rumen protozoal activity of saponins has been well documented both in vitro (30, 31, 32) and in vivo (33, 34). Specifically, yucca saponins are as effective as the drug

metronidazole in killing tropozoites of *Giardia lamblia* in the intestine (35). By complexing with cholesterol in protozoal cell membranes, saponins cause damage to the integrity of the membrane, and cell lysis results (1, 36).The antiprotozoal activity requires the intact saponin structure with both nucleus and side chain present. Protozoal diseases in which part of the life cycle occurs in the gastrointestinal tract respond to the anti-protozoal activity of saponins.

Saponins also show antibacterial activity, although weaker compared to antifungal and antiprotozal activities. Some are weakly active against Gram positive bacteria but show no effect on Gram negative bacteria (37). Some saponins were shown to have antimicrobial activity on both prokaryotic and eukaryotic organisms, but only at low cell densities. The saponins did not inhibit microbial growth of dense populations. Four triterpenoid saponins isolated from *Hedyotis nudicaulis* were tested against *Bacillus subtilis* (38) and showed weak antibacterial activity. A jujubogenin saponin isolated from *Colubrina retusa* had antimycobacterial activity when tested against *Mycobacterium intracellulare* (39). The mode of action of the antibacterial activity of saponins is not yet clear. It has been noted that the aglycone (the non-sugar compound remaining after replacement of the glycosyl group from a glycoside by a hydrogen atom) part of the saponins are the determinant of antibacterial activity which suggested that saponins may be interacting in some way with the bacterial cell walls. (40, 41)

*2.1.3 Cholesterol lowering activity*

It is well established that saponins have cell membrane lytic action and facilitate changes in membrane fluidity as a result of affinity of the aglycone moiety for membrane sterols, particularly cholesterol (42, 43). When binding with cholesterol, saponins change the lipid environment of membrane proteins, including ion channels, transporters and receptors, causing secondary biochemical responses such as direct cytotoxicity, growth inhibitory effects against tumor cells and immune-modulatory effects from various saponin sources (40, 41).

*2.1.4 Antitumor activity*

Saponins appear to directly inhibit or kill cancer cells. Cancer cells generally possess more cholesterol-type compounds in their membranes than normal cells (44, 45). Saponins can bind cholesterol and thus interfere with cell growth and division. For example, soybean saponin inhibits colon tumor cell growth *in vitro* (46). Soybean saponin treated cells formed numerous cytoplasmic vesicles followed by deformation in the plasma and nuclear membrane, suggesting that saponins actively interact with cell membrane components, resulting in changes in intracellular morphology and cell membrane permeability. Another suggested mechanism for antitumor activity is through the suppression of glucose uptake in tumor cells (47). IH-901, a saponin isolated from geinseng showed antitumor activity against four human cancer cell lines and one cell line that is resistant to cisplatin (48).

*2.1.5 Other implications*

Research has investigated the benefits of adding saponin to poultry feed. Saponins are known to increase the permeability of intestinal mucosal cells and may facilitate the uptake of substances across a mucus layer. Saponin extracts from *Yucca schidigera*, for example, were found to boost chicken productivity, increase weight gain and reduce the risk of environmental contamination (49, 50, 51). *Yucca* extract reduces or eliminates the colonization of bacteria in the digestive and intestinal tracts of vertebrates, thereby creating a healthier mucosa and an overall healthier vertebrate (Nouveau Technologies, US Patent 5770204). Based on the study, saponin was safe and efficacious to be included in poultry diets. Multiple sections of duodenum, jejunum, cecum, colon, pancreas, lung and trachea show no morphological changes or significant alternations in any of the tissues along the intestinal tract in chickens given feed containing saponins (Nouveau Technologies, US Patent 5770204).

Saponin containing yucca extracts are currently used for ammonia and odor control in pig and poultry-raising facilities and in dog and cat foods. Yucca saponins bind to ammonia and other odiferous compounds and prevent them from being released into the air (1).

## 2.2 Quillaja Saponins

The saponin this study examines is water extracted from *Quillaja saponaria* a large evergreen tree with shiny, leathery leaves and a thick bark which is native to Peru and the arid region of Chile (5). Figure 2.3 shows a google earth reference on where the *Quillaja saponaria* are harvested from central Chile. Quillaja saponin extracts have been used as a foaming agent in soft drinks, such as root beer and cocktail mixes and as an emulsifier in other foods, such as baked goods, candies, frozen dairy products, etc.

Figure 2.1. *Quillaja saponaria* Molina

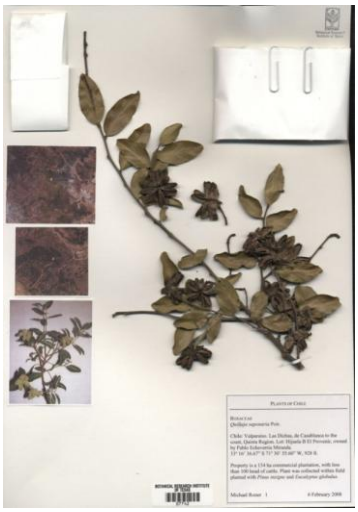


Figure 2.2. Voucher specimen. Voucher on file with BRIT of material used in this study.

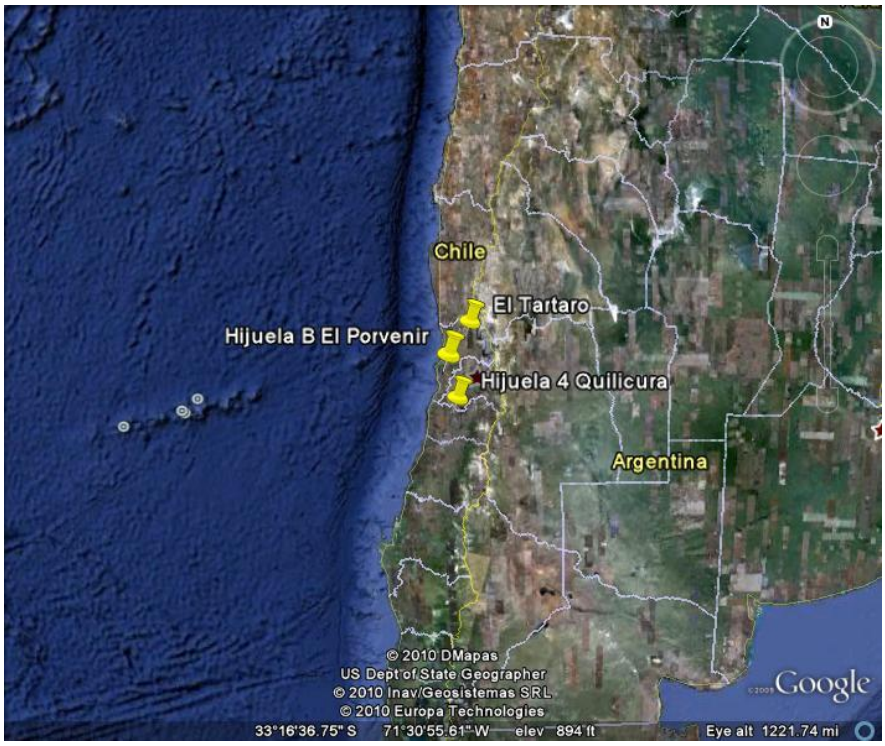



Figure 2.3. El Tartaro, Hijuela B El Porvenir, Hijuela 4 Quillicura in Chile, where *Quillaja saponaria* Molina are harvested.

*Quillaja* saponins are a natural, aqueous extract of triterpenoid saponins obtained from *Quillaja saponaria.* The extract is a complex mixture of triterpenoids, each built around a

common quillaic acid. The crude extract obtained from commercial vendors has been estimated to contain fifty to sixty components. Ultra Dry 100 Q is a standard saponin material prepared for human consumption, and has a saponin content of 65%; it has been approved by the FDA (under 21 CFR 172.510, FEMA GRAS number 2973. It is approved for 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. The *Quillaja* saponin that we used in this study is Vax Sap, a further purified medical grade material obtained following additional purification of the Ultra Dry 100 Q material. Vax Sap has a saponin content of over 90% and has been approved to be used as adjuvants in human vaccines. Figure 2.4 and 2.5 below show the HPLC profile of lots of Ultra Dry 100 Q and Vax Sap that were used in this study.

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 (6). 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 (24, 52).

It is likely that the true number of saponin variants would exceed 100 if all conformational isomers are considered (53). The saponins content and identification can be determined by RP-HPLC. 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 adequate to express the saponins content of the *Quillaja* saponins because they represent up to 90% of the total saponins present (54). Previous studies on these purified saponins showed that these related triterpenoid saponins have similar immune stimulating activities but significant differences in their toxicity (55). Saponins QS-7 and QS-21 showed no or very low toxicity in mice, while QS-18 being the highly toxic (56). Figure 2.6 shows the structure of *Quillaja* saponins. Table 2.1 shows side chains that have been previously identified

for the four major saponins QS-7, QS-17, QS-18 and QS-21 (52, 57, 58, 59, 60, 61, 62, 63, 64, 65).

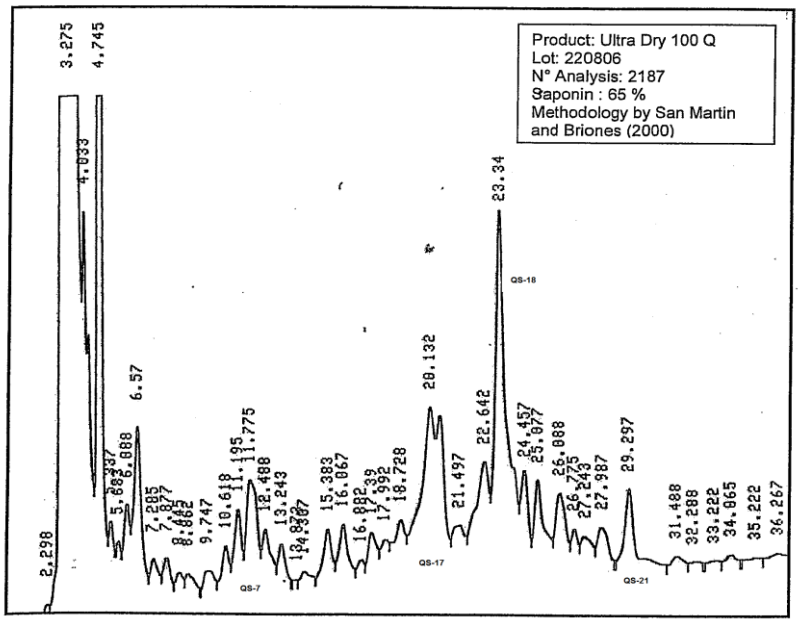



Figure 2.4. HPLC profile of Ultra Dry 100 Q. This HPLC profile of a crude saponin extract from *Quillaja saponaria* demonstrates the four major saponins found in a water extract. Image provided by Desert King International for the Ultra Dry 100 Q sample provided to us..

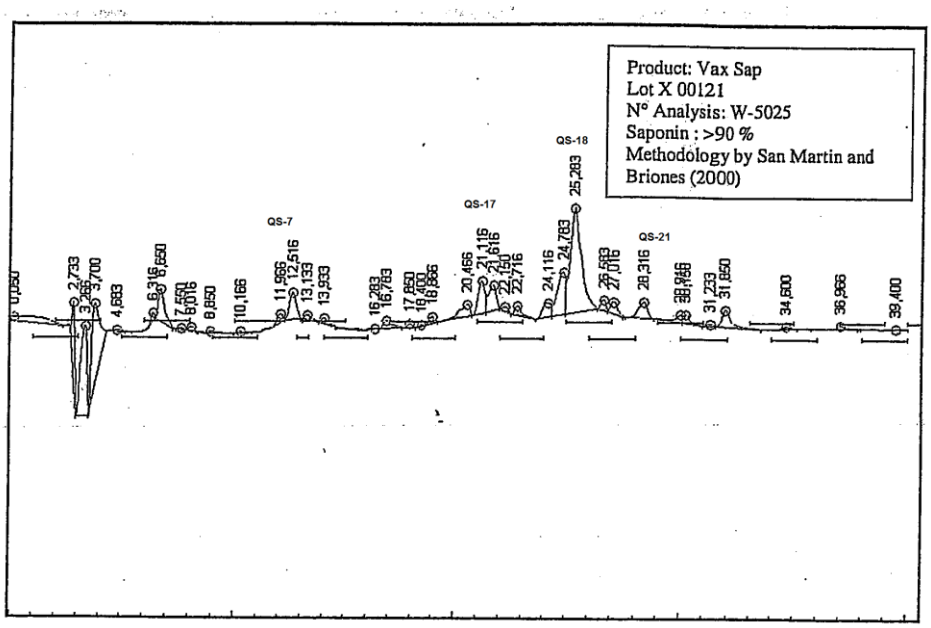



Figure 2.5. HPLC profile of Vax Sap. The purified extract of Ultra Dry 100 Q contains over 90% of saponin. Image provided by Desert King International for the Vax Sap sample provided to us.

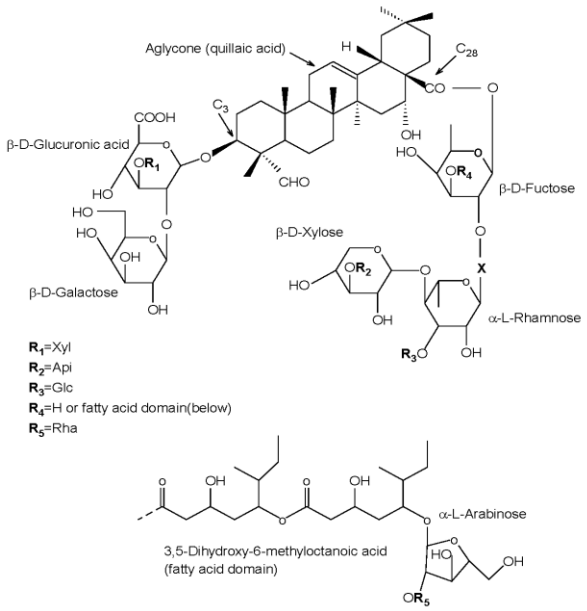



Figure 2.6. Molecular structures of the major saponins from *Quillaja saponaria* Molina. (Adapted from 52, 57, 58, 59, 60, 61, 62, 63, 64, 65).

Table 2.1. Side chains previously identified for the four major saponins QS-7, QS-17, QS-18 and QS-21.

| **Major Saponin peak** | **Molecular weight** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **$R_5$** |
|---|---|---|---|---|---|---|
| QS-7 | 1862 | ---C---- CH3<br>II<br>O | Apiofuranosyl-<br>Xylopyranosyl-<br>Glucopyranosyl-<br>Rhamnopyranosyl | Rhamno-<br>pyranosyl | Not found | Not found |
| QS-17 | 2296 | H | Apiofuranosyl-<br>Xylopyranosyl-<br>Glucopyranosyl-<br>Rhamnopyranosyl | Fatty acid domain-<br>Arabino-<br>furanosyl-<br>Rhamno-<br>pyranosyl | H | α-L-Rham*p* |
| QS-18 | 2150 | Fatty acid domain-<br>Arabino-<br>furanosyl | Apiofuranosyl-<br>Xylopyranosyl-<br>Glucopyranosyl-<br>Rhamnopyranosyl | H | H | H |
| QS-21 | 1988 | Fatty acid domain-<br>Arabino-<br>furanosyl | Rhamnopyranosyl<br>Apiofuranosyl-<br>Xylopyranosyl | H | H | H |

*2.2.1 Quillaja saponins as adjuvants*

*Quillaja* saponins have been shown to exhibit strong adjuvant activity that can enhance the effectiveness of vaccines (57, 58). *Quillaja* saponins can be used as antigen-presenting systems in the form of immunostimulating complex (ISCOMS). QS-21, the most active component of the extract forms soluble vaccine quality complexes with soluble antigens. The hydrophobic interactions consisting of cholesterol, phospholipid, and antigen of the *Quillaja* allow its function as an adjuvant (46, 66).

*2.2.2 Quillaja saponins antiviral activity*

Previous work done in our lab have demonstrated that *Quillaja* saponin extracts exhibit antiviral activity *in vitro* on a broad range of viruses including reovirus, rotavirus, vaccinia virus, herpes simplex virus type 1, varicella-zoster virus, HIV-1, and HIV-2 (See table 2.2 and 2.3, 7). For antiviral activity against rotavirus in specific, Ultra Dry 100 Q and Vax Sap prevent rhesus rotavirus infection of MA104 cells at concentrations of 1 mg/ml (w/v) or greater. In another study, our data demonstrates that treatment of cells with Quillaja extract for one hour 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 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 (unpublished data). It was 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 (7). *Quillaja* extract modifies the cells, preventing virus from attaching to these cells, and any virus that does manage to attach to cells and is internalized is reduced in its capacity to initiate and maintain an infection (7). Median cellular cytotoxicity concentration or $CCIC_{50}$ represented the concentration of the drug that results in the death of 50% (7). Effective Dose or $ED_{50}$ represented does of the drug that is effective at inactivating 50% of the treated virus (7).

Table 2.2 Cytotoxicity of Ultra Dry 100 Q and direct inactivation of viruses.

| Cells | $CCIC_{50}$ Loss of cell viability of 50% of treated cells after 96 hours Extract [mg/ml] | Virus | $ED_{50}$ Inactivation of 50% of virus within 1 hour Extract [mg/ml] |
|---|---|---|---|
| L929 | 1.0 +/- 0.1 | Reovirus | >1.0 +/- 0.1 |
| 143 | 0.9 +/- 0.1 | Vaccinia | 0.11 +/- 0.02 |
| Vero | 0.9 +/- 0.1 | HSV-1 | 0.09 +/- 0.01 |
| BS-C-1 | 0.9 +/- 0.1 | VZV | 0.10 +/- 0.01 |
| CEMx174 | 1.0 +/- 0.1 | HIV-1 | 0.15 +/- 0.02 |
| CEMx174 | 1.0 +/- 0.1 | HIV-2 | 0.14 +/- 0.02 |
| MA104 | 1.0 +/- 0.1 | RRota | >1.0 +/- 0.1 |

Table 2.3. Cytotoxicity of Vax Sap and direct inactivation of viruses.

| Cells | $CCIC_{50}$ Loss of cell viability of 50% of treated cells after 96 hours Extract [mg/ml] | Virus | $ED_{50}$ Inactivation of 50% of virus within 1 hour Extract [mg/ml] |
|---|---|---|---|
| L929 | 1.38 +/- 0.1 | Reovirus | >1.0 +/- 0.1 |
| 143 | 1.39 +/- 0.1 | Vaccinia | 0.08 +/- 0.02 |
| Vero | 1.50 +/- 0.1 | HSV-1 | 0.06 +/- 0.01 |
| BS-C-1 | 1.44 +/- 0.1 | VZV | 0.08 +/- 0.01 |
| CEMx174 | 1.48 +/- 0.1 | HIV-1 | 0.10 +/- 0.02 |
| CEMx174 | 1.37 +/- 0.1 | HIV-2 | 0.09+/- 0.02 |
| MA104 | 1.46 +/- 0.1 | RRota | >1.0 +/- 0.1 |

*2.2.3 Quillaja saponins as food additive*

Quillaja extracts may be used as foaming agents in soft drinks, and as emulsifier in other foods. The major food use is in soft drinks. In 2009, quillaja extracts are listed under Codex General Standard for Food additives (GSFA) for recommended maximum level of 50mg/kg in food group 14.1.4 Water-based flavored drinks, including “sport”, “energy” or “electrolyte” drinks and particulated drinks. Acceptable daily intake or ADI is a measure of the amount of a specific substance (usually a food additive, a residue of a veterinary drug or pesticide) in food or drinking water that can be ingested (orally) over a lifetime without an appreciable health risk (Joint FAO/WHO Expert Committee on Food Additives-JECFA). The

suggested ADI of quillaja extract is 5mg/kg body weight. Root Beer contains 100-150 grams per 1000 liters of syrup, frozen carbonated beverages contain 400 to 1000 grams per 1000 liters of syrup. The amount of quillaja ingested per eating occasion was estimated from container sizes, which range from 8 to 64 fluid ounces, corresponding to 24-1900 ml. These amounts correspond to 70-558 mg of quillaja or 23-186% of the current ADI per eating occasion (67).

## 2.3 Rotavirus

Rotaviruses are 70 nm icosahedral, nonenveloped viruses in the family Reoviridiae. The virus is composed of three protein shells, an outer capsid, an inner capsid, and an internal core, that surround the 11 segments of double stranded RNA (11). Each gene segment codes for a single protein except for gene segments 9 and 11 which encode for two proteins (68). There are seven structural proteins (VP1-7) which made up the three protein shells and six non-structural proteins (NSP1-6) (See Table 3 below, 69, 70). When two or more rotavirus strains infect the same cell, gene segments from the parental viruses reassort independently, producing reassortments of mixed parentage (11). Rotavirus consists of seven serogroups (serogroups A to G) differing in antigen composition but similar in morphology. 90% of infections in humans are caused by serogroup A. The antigen regions associated with serogroups A-C are on the VP7 structural protein located on the surface of the outer capsid. The vast majority of rotavirus infections of humans involve group A viruses (71), while serogroups D, E, F, and G are nonhuman animal pathogens.

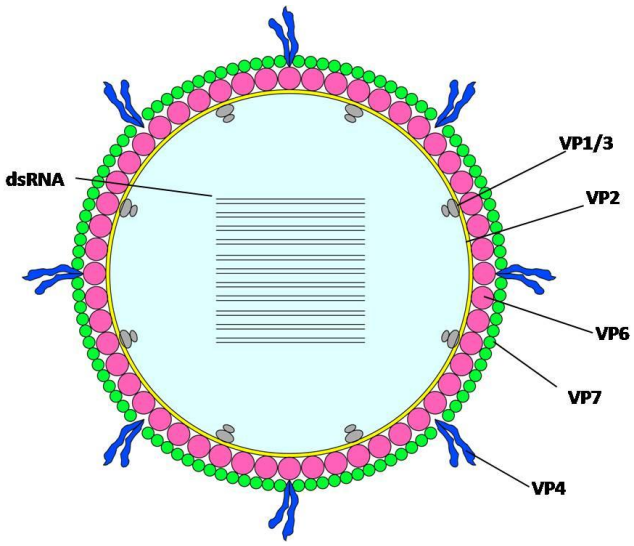



Figure 2.7. Structure of Rotavirus.

Table 2.4 Rotavirus genes and proteins (70).

| Gene Segment | Protein | Location | Function |
|---|---|---|---|
| 1 | VP1 | At the vertices of the core | RNA-dependent-RNA polymerase |
| 2 | VP2 | Forms inner shell of the core | Stimulates viral RNA replicase |
| 3 | VP3 | At the vertices of the core | Guanylyl transferase mRNA capping enzyme |
| 4 | VP4 | Surface spike | Cell attachment, virulence |
| 5 | NSP1 | Nonstructural | Not essential to virus growth |
| 6 | VP6 | Inner Capsid | Structural and species-specific antigen |
| 7 | NSP3 | Nonstructural | Enhances viral mRNA activity and shut offs cellular protein synthesis |
| 8 | NSP2 | Nonstructural | NTPase involved in RNA packaginig |
| 9 | $VP7^1$<br>$VP7^2$ | Surface | Structural and neutralization antigen |
| 10 | NSP4 | Nonstructural | Enterotoxin |
| 11 | NSP5<br>NSP6 | Nonstructural | ssRNA and dsRNA binding modulator of NSP2 |

*2.3.1 Rotavirus replication cycle*

Rotavirus replicates mainly in the gut and infect enterocytes of the villi of the small intestine (see Figure 2.8). The three protein shells make them resistant to the acidic pH of the stomach and the digestive enzyme in the gut (72). Virus enters cells by receptor mediated endocytosis (72). VP4 protein attaches to saliac acid to the target cell and the viral particle is

endocytosed into the cell and forms a vesicle known as endosome. Endosome fuses with lysosome to form endo-lysosome where the outer layer was shredded, leaving a double layered particle (DLP).

The RNA-dependent-RNA polymerase of the DLP functions as a transcriptase to synthesize the 11 viral plus-strand RNAs. The plus-strand RNAs are extruded from DLPs through channels that extend through both protein layers and translated to give rise to the structural and nonstructural proteins. The plus-strand RNAs also function as templates for the synthesis of the dsRNA genome segments. RNA replication occurs concurrently with the packaging of the genome segments into newly formed cores. Rotaviurs infection leads to the formation of viroplasms, which are inclusion body in a cell where viral replication and assembly occurs. It is within the viroplasms that minus strand synthesis occur along with core and DLP assembly. DLPs migrate to rough endoplasmic reticulum where they obtain their third layer. The progeny viruses are released from the cell upon lysis (70, 73).

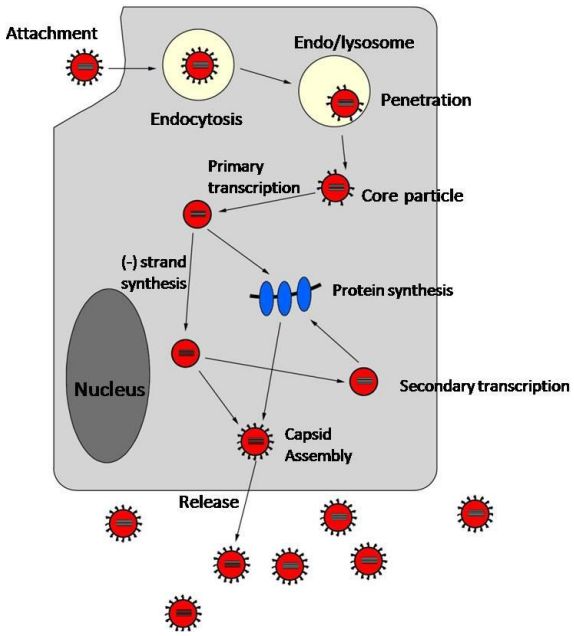



Figure 2.8. Rotavirus replication cycle.

*2.3.2 Rotavirus induced diarrhea*

Rotaviruses infect mature enterocytes in the mid and upper villous epithelium of the host's small intestine (74, 75). The virus has also been found in goblet cells, epithelial endocrine cells, and macrophages in the lamina propria (76, 77). Epithelial cells are killed and sloughed off, resulting in stunting of villi. The ensuing maladsorption contributes to the diarrhea typical symptomatic rotavirus infection (78).

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for about 600-850,000 children deaths mostly in developing countries like south Asia and sub-Saharan Africa every year (8, 9, 10). Rotavirus-related deaths represent approximately 5% of all deaths in children younger than five years of age worldwide (11). In United States, rotavirus gastroenteritis results in only 20 to 70 childhood deaths per year (79, 80). However, nearly every child in the United States is infected with rotavirus by five years of age, and most will develop gastroenteritis (11).

*2.3.3 Routes of transmission*

The incubation period of rotavirus infection may vary from 19 hours to two days (81). Rotaviruses are excreted in large number in the stools of acutely ill patients. Concentrations as high as $10^{10}$ rotavirus particles per gram of stool have been reported. Ingestion is still the only proven route of transmission (82), although a respiratory route has been suggested. Rotavirus can be transmitted due to ingestion of contaminated drinking water (83), and transmission from contaminated environmental surfaces (84). The presumed usual routes of secondary spread of rotavirus are direct fecal-oral inoculation. Secondary spread between parents and children with gastroenteritis has been observed to range from 32 to 70% (84).

*2.3.4 Infective dose of rotavirus*

The minimal infective dose of rotavirus has been studied in various models. A human rotavirus study in volunteers against human rotavirus (CJN strain) (84) suggested that the human 50% infectious dose was about ten FFU (focus-forming unit, a measurement of the

concentration of live virus in a given amount of fluid), and one FFU should infect ~ 25% of susceptible adult subjects. Studies performed in miniature swine piglets (45, 84, 86) showed that that minimal infective dose was frequently in the range from 1 to 100 PFUs (plaque-forming unit, a measurement of the number of particles capable of forming plaques per unit volume). Studies with heterologous system (simian rotavirus SA11 in newborn mice) suggested a (87, 88) higher minimal infective dose of 1000 PFUs.

*2.3.5 Research Significance*

Treatment of rotavirus infection is nonspecific and mainly involves rehydration of the patient. Rates of rotavirus illness among children in industrialized and resource-poor countries are similar, indicating that clean water supplies and good hygiene have little effect on virus transmission, and further improvements in water or hygiene are unlikely to prevent the disease (11). WHO recommends worldwide use of rotavirus vaccine for children to reduce the estimated half million deaths and two million hospitalizations from diarrhea caused by rotavirus each year (10). There are currently two rotavirus vaccines that have been proven successful in massive clinical trials, and RotaTeq has been approved for use in the US. However, the three doses of RotaTeq cost US$187.50, making it one of the most expensive vaccines currently available for children. We hypothesize that saponins offer the possibility to prevent rotavirus infection at a much reduced cost than vaccines.

## 2.4 Animal Model Used in this study

In this study, the inbred mouse strain (BALB/c) was used. BALB/c mice are one of the most commonly used inbred mice and have been inbred for more than 200 generations. Continual inbreeding produces mice that are genetically uniform, being homozygous at virtually all of their loci (89). The mouse offers several advantages not found in any other model system (90): 1.) Mice are well characterized and are used extensively in immunology and infectious disease studies. 2.) High quality reagents for detailed immunologic studies in the mouse are

available from many commercial sources. 3.) Commercial sources of rotavirus-free mice have been identified and numerous strains of inbred, out bred, and genetically altered mice are becoming available. 4.) Mice are inexpensive to maintain and large numbers of animals can be evaluated simultaneously. 5.) Inbred strains can be traced back to a single ancestral breeding pair, and are therefore genetically identical. Each inbred strain has a unique geneotype and consequently a unique phenotype (e.g. unique pattern of behavior, specific immune responses, susceptibility to certain diseases, etc.). 6.) A very well characterized model for human diarrhea exists employing the BALB/c mice.

In this study, we infected BALB/c newborn mice with rhesus rotavirus (RRV) (ATCC VR-954). The best animal model available for human rotavirus infections is the induction of diarrhea in newborn mice after exposure to rhesus rotavirus. The mouse model has been widely utilized to define the pathogenesis of rotavirus (88, 90, 91). Both homologous and heterologous rotaviruses have been shown to cause viremia in both newborn and adult mice (92).

Rotavirus induced diarrhea usually begins around 48 hours after infection and can persist up to one week (3). Infected suckling newborn mice have soft and yellow feces. In severe instances, the mice may be stunted, have dry scaly skin, or are virtually covered with fecal material (3). Morbidity is very high but mortality is usually low. Susceptibility to rotavirus depends on the age of the host and peaks between four and fourteen days of age (93, 94). Mice older than two weeks can still be infected but only a small number of enterocytes become infected, there is little replication of virus, and diarrhea does not occur. The exact reason for this age-related resistance to disease is unknown (95).

## 2.5 Cell line Used in this Study

Rhesus Monkey Kidney Epithelial MA104 (ATCC CRL-2378.1) cells were used to propagate the rhesus rotavirus. Sialic acid on the surface of target cells is crucial for rotavirus attachment and infection (96). Sialic acid present on MA104 makes this cell line highly

susceptible to rotavirus. Propagation and titration of rotavirus are performed in MA104 cells with the presence of trypsin. Proteolytic cleavage of the VP4 protein in the outer protein coat by trypsin is important to enhance viral infectivity. (97)

# CHAPTER 3

# METHODS AND MATERIALS

## 3.1 Maintaining Cells and Culturing Plates

### *3.1.1 Maintaining Cell Cultures*

MA104 cells were maintained in cell culture flasks to allow anchoring to the surface and incubate at 37˚C, 5% carbon dioxide. Cells were maintained in Minimal Essential Media (MEM) with 1% antibiotic containing penicillin and streptomycin (P/S) and 5% Fetal Bovine Serum (FBS) added to the MEM. Cells were split aseptically in a biological laminar flow hood when they reach confluency. For splitting cells, media was first discarded followed by rinsing in 1X Saline Sodium Citrate (SSC). Trypsin was then added to release the anchored cells from the surface of the cell culture flasks. Cells were then incubated at 37˚C for five minute and rocked to remove cells from the surface. The appropriate media was added to the flask and pipetted to mix the media and cells evenly. The amount of cells to be transferred to new cell culture flask depended upon how soon the cells would be needed. The media in the cell culture flasks were removed and replenished every three to four days for healthy growth of the cells.

### *3.1.2 Culturing Cells on Plates*

Six well plates were used for the experiments. The plates had to be set up at 75% confluency for the cells to survive the experimental period. A hemacytometer was used in the enumeration of cells. During splitting of cells, after cells had been trypsinized and the appropriate amount of media added, a small amount of the media (0.01 ml) was retrieved and loaded onto both grooves of the hemacytometer chamber. Each chamber contains nine large squares, the four squares at the corners and the center square were counted. Number of cells per ml was then calculated with the following formula:

$$\text{hemacytometer count} \times 2 \times 10^2$$

With the percentage of confluency and the number of cells per ml known, the following formula was then used to determine how much media was needed to add to the plates:

$$\frac{\text{the required number of cells per well}}{\text{the number of cells per ml}}$$

## 3.2 Growth of Rhesus Rotavirus

RRV was propagated in successive passages to generate large amount of virus. The goal was to propagate virus at MOI under 20 (Multiplicity Of Infection = ratio of infectious virus particles to cells). High MOI infections can result in large amount of Defective Interfering Particles (DIPs) that represent partial genomes packaged by complementation from intact genomes coinfected in the same cell.

### *3.2.1 First Passage Lysate*

Twenty-five $cm^3$ flasks were seeded with 1 x $10^6$ MA104 cells with five mls of MEM with 1% P/S and 5% FBS. The flasks were incubated overnight at 37 ˚C, 5% $CO_2$ environment. The MA104 cells were infected with RRV retrieved from the original stock. The media was removed and 0.1 ml of the RRV with 10% trypsin was used to infect the MA104 cells. The flask was then incubated for one hour at 37˚C and 5% $CO_2$ environment with rocking every 15 minutes to ensure even virus attachment. Five mls of MEM with 1% P/S and 5% FBS was added to the flask. The flask was incubated at 37˚C and 5% $CO_2$ environment until 75% of the cells were lysed. The cells were then sonicated and the lysate was transferred to 15 ml centrifuge tube and stored at -20˚C.

### *3.2.2 Second Passage Lysate*

Seventy-five $cm^3$ flasks were seeded with 4 x $10^6$ MA104 cells with ten mls of MEM with 1% P/S and 5% FBS. The flasks were incubated overnight at 37 ˚C, 5% $CO_2$ environment. The media was removed and 1ml of the first passage lysate with 10% trypsin was used to infect the cells. The flask was then incubated for one hour at 37˚C and 5% $CO_2$ environment with

rocking every 15 minutes to ensure even virus attachment. Ten mls of MEM with 1% P/S and 5% FBS was added to flask. The flask was incubated at 37˚C and 5% $CO_2$ environment until 75% of the cells were lysed. The cells were then sonicated and the lysate was transferred to 50 ml centrifuge tube and stored at -20˚C.

*3.2.3 Third Passage Lysate*

One hundred and fifty $cm^3$ flasks were seeded with 1 x $10^7$ MA104 cells with 25 mls of MEM with 1% P/S and 5% FBS. The flasks were incubated overnight at 37 ˚C, 5% $CO_2$ environment. The media was removed and half of the second passage lysate with 10% trypsin was used to infect the cells. The flask was then incubated for one hour at 37˚C and 5% $CO_2$ environment with rocking every 15 minutes to ensure even virus attachment. 25 mls of MEM with 1% P/S and 5% FBS was added to flask. The flask was incubated at 37˚C and 5% $CO_2$ environment until 75% of the cells were lysed. The cells were then sonicated and the lysate was transferred to 50 ml centrifuge tube and stored at -20˚C.

## 3.3 Preparation of Virus Stock

*3.3.1 Determining Titer of Virus*

A plaque assay was carried out to determine the titer of the RRV virus stock. Six well plates were seeded with 5 × $10^5$ MA104 cells/ well with two mls MEM with 1% antibiotic P/S and 5% FBS. The plates were incubated overnight at 37 ˚C and 5% $CO_2$ environment. After 24 hours, cells were infected with RRV. $10^{-1}$ to $10^{-6}$ dilutions were prepared. Third passage lysates stored at -20˚C were thawed out in 37˚C incubator. 100 µl of the lysates were taken out and were diluted in six serial dilution blanks containing 900 µl MEM with 1% P/S and 10% trypsin. The media in the six well plates were removed by the use of vacuum pump attached to a sterile pipette and pipette tips. Pipette tips were changed after removing media from each well to ensure sterility. 250 µl of each serial dilution was added to the corresponding labeled wells. The plate was then incubated for one hour at 37˚C and 5% $CO_2$ environment with rocking every 15

minutes to ensure even virus attachment. Two % noble agar was melted in the microwave and allowed to cool in the 42˚C incubator. TwoX MEM with 2% P/S and 10% FBS was allowed to warm up for not more than 40 minutes in the 42˚C incubator before the addition of overlay agar. To make the overlay agar, 5% trypsin was added to an equal amount of twoX MEM with 2% P/S and 10% FBS mixed with 2% noble agar. Two mls of overlay agar was added to each well slowly after one hour of incubator period. The agar was allowed to solidify and the plate was then transferred to 37˚C and 5% $CO_2$ environment for incubation. It took six days to complete the plaque essays for RRV. Two mls of overlay agar was added to each well every two days. Therefore, overlay agar was added on the first, third, and the fifth day, bringing a total of three overlay agar. On the last overlay agar, an addition of 2% neutral red was added to overlay mixture. The last overlay agar had to be added in the dark to prevent any direct light which may cause the neutral red to react with the cells and cause cell death. The plates were then wrapped in aluminum foil for incubation after solidification. Wells with 30-300 plaques were counted. The numbers of plaques were counted on the sixth day, and the number of plaque forming units per ml (PFU/ml) was determined with the following formula:

Plaque number x reciprocal of the dilution x reciprocal of the volume of virus added

*3.3.2 Dilution of Working Virus Stock*

After plaque assay was performed and the titer of the third passage lysate was determined, virus stocks of different titers were prepared. Third passage lysate was transferred to sterilized 1X Standard Saline Citrate (SSC) to yield the first desired concentration of virus stock, $1x10^6$ PFU/ml. From this virus stock, a serial dilution was performed by transferring five mls of the virus stock into 45 mls of SSC consecutively to yield three more virus stocks that were at titer of $1x10^5$, $1x10^4$, and $1x10^3$ PFU/ml respectively. All virus stocks were kept at -20°C for later use.

## 3.4 Maintaining and Breeding of Animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC, Protocol # A07.033) 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 (PHS) policy.

### *3.4.1 Maintenance of Animals*

Adult (seven weeks old) male and female Balb/c mice (see Appendix A for information on mice) were purchased from Charles River, Raleigh, NC and Harlan Laboratories, Livermore, CA for breeding. All mice were housed in polycarbonate cages with bedding made of corn cobs (Bed-o-Cobs, Maumee, OH) in an environmentally controlled animal facility. Environmental factors were closely monitored daily and maintained within the recommended range for the animals (Temperature 64-74°F, humidity 40-75%, and photo period 12 hours light/12 hours dark). Beddings and cages were changed once a week to ensure cleanliness. Mice were maintained on a standard diet (2019 Teklad Global Rodent diet, Harlan, Madison, WI) and water ad libitum. The diet contained a minimum 19% protein and 9% fat that met the nutrient requirements of gestation, lactation and early growth of rats and mice. Mice were acclimated one week at the facility before breeding pairs were set up.

### *3.4.2 Breeding of Animals*

One adult male and one adult female were housed together to set up breeding pairs after the 1 week acclimating period. Breeding pairs were left undisturbed for two weeks before the males were transferred to separate cages prior to the birth of newborns. Females are separated from males after breeding because females can become pregnant again in less than 24 hours after giving birth. Pregnancy should be avoided during lactating period because it can increase the rate of female cannibalism and neglect of the newborns. Litters where female parents become pregnant during nursing period were excluded from the data set to avoid other factors that may influence the interpretation of the data.

Cannibalism of newborns is a logical response for a lactating female with more newborns than she can feed; infanticide allows her to reduce her litter size to a sustainable level (69). Human interaction with newborns is another stress factor that tends to also increase mortality. Several means used to reduce cannibalism with varying degrees of success include addition of nesting material to cage, placing feed on the floor of cage, minimizing disturbance of female and newborns (98). All the suggested means to reduce cannibalism were executed. Human interaction was reduced as much as possible, limiting to treatment administration and record of weight.

*3.4.3 Animal Euthanasia*

Euthanasia of mice was achieved with the use of carbon dioxide inhalation. Death was induced as painlessly and quickly as possible. Euthanasia was carried out according to the recommendation of AVMA Guidelines on Euthanasia. Transparent chamber was used to allow ready visibility of the animals. Compressed $CO_2$ gas in cylinders was used because it allowed the inflow of gas to the chamber to be controlled. 100% carbon dioxide at the rate of 10-20% of the chamber volume per minute was used to optimize reduction in distress. After mice became unconscious, the flow rate was increased to minimize the time to death. Upon completion of the procedure, death was confirmed by noting mice's fixed and dilated pupils (see Appendix B for Guidelines for Euthanasia of Rodents).

## 3.5 Preparation of Saponin Extract

The *Quillaja* extract used was obtained from Desert King International (San Diego, CA). The material, Ultra Dry 100-Q, is the spray-dried purified aqueous extract of the Chilean soapbark tree (*Quillaja saponaria* Molina). Vax Sap is a further purified medical grade material obtained following addition purification of the Ultra Dry 100 Q material. The saponin stock was prepared by dissolving the dried material into sterilized SSC at a concentration of one gram of

Vax Sap per 100 milliliter to yield a 10 mg/ml stock. SSC was used instead of water because dissolving saponin in SSC gives a final pH of 7.2 to 7.4 while saponin in water has a pH of 6.5.

From this saponin stock, different concentrations of *Quillaja* saponin extract were prepared by transferring the appropriate volume to sterilized SSC to yield the desired concentration. All tested media were filtered sterilized with 0.2 µm Sterile Syringe Filter from Corning Inc. All saponin stocks were kept at room temperature.

## 3.6 Establishing the standard conditions for Rotavirus Induced Diarrhea

It was necessary to establish the standard conditions for the model system of rotavirus induced diarrhea in newborn mice inoculated with RRV. Previous research showed that minimal infective dose in heterologous system (simian rotavirus in mice) was 1000 PFUs. However, data presented in previous studies were generated in mice from single dose infection. I explored that a lower concentration of virus would cause a considerable rate of rotavirus induced diarrhea in this study because mice were orally inoculated with virus for five doses in five consecutive days and secondary infection could occur within litters among newborn mice. Four different titers of RRV ranging from $1x10^3$ to $1x10^6$ PFU/ml were used for oral inoculation. Experimental subjects were transferred to a separate facility where the same environmental conditions were maintained, this was done to ensure that the breeding pairs would not be infected or exposed to any RRV before the study took place.

### *3.6.1 Inoculation of Virus in Newborn Mice*

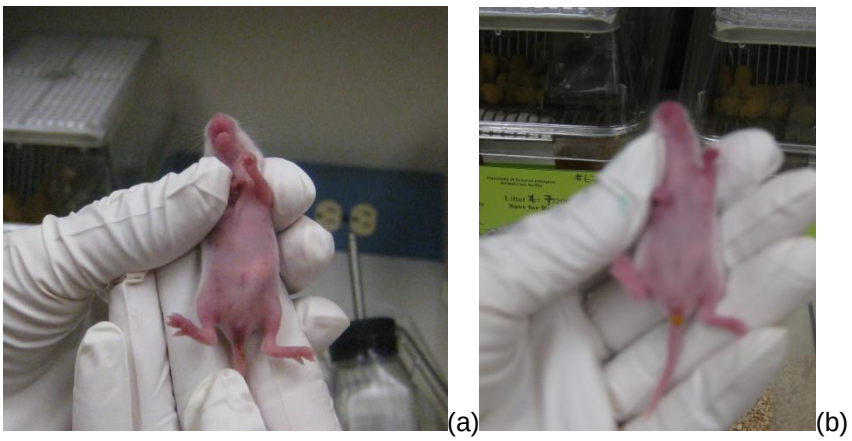
Newborn mice of four to six days old and at least three grams in weight were inoculated orally with Gilmont® Micrometer Syringes with 50 µl of rhesus rotavirus at different titers ranging from $10^3$ to $10^6$ PFU/ml which corresponds to 50 PFUs to 50,000 PFUs per mouse for five consecutive days. See Table 3.1 for corresponding PFUs. Treatment was given to newborn mice slowly to ensure full intake of the treatment and to prevent suffocation. Micrometer syringes were treated with bleach and sterilized in the autoclave before use every time.

Table 3.1. Total PFU for 50 ul at different virus titers.

| Virus Titer | Equivalent PFUs for 50ul |
|---|---|
| $1X10^3$ PFU/ml | 50 PFUs |
| $1X10^4$ PFU/ml | 500 PFUs |
| $1X10^5$ PFU/ml | 5,000 PFUs |
| $1X10^6$ PFU/ml | 50,000 PFUs |

*3.6.2 Observation of Rotavirus-Infected Newborn Mice*

Clinical signs of rotavirus induced diarrhea in young laboratory mice are generally limited to mice under 16 days of age, these animals present with watery, mustard-colored stools, lethargy, and distended abdomens. Newborn mice were observed for two months after rotavirus inoculation. Record of weight and stool observations were made at 24 hours intervals for the first ten days, and once every week until mice reach full sexual maturity (56 days). Stool samples were retrieved by gentle palpation of the abdomen. 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; 4, liquid yellow (12). Different categories of diarrhea reflect the amount of water lost during rotavirus infection. Category 3 and 4 were considered as diarrhea.



(a) (b)

Figure 3.1. Mouse diarrhea photo scoring system. Panel (a) as category 2 as liquid brown stool, and panel (b) shows liquid yellow category 4 stool.

## 3.7 Acute Toxicity Study of Saponin Extract

The purpose of this part of the study was to evaluate the toxicity of the saponin extract in newborn mice. Acute toxicity testing attempts to determine minimum lethal dosage and any important clinical signs that may attribute to high doses of the test substance (99). This objective can usually be achieved by treating from one to three groups of three to five mice at different doses (99). Experimental subjects were transferred to a separate facility where same environmental conditions were maintained.

### *3.7.1 Inoculation of Saponin Extract in Newborn Mice*

The purpose of this part of the study was to determine the lowest concentration of *Quillaja* saponin extract that is effective in preventing rotavirus induced diarrhea in newborn mice. The objective of short-term or subchronic studies was to describe and define the toxicity associated clinical signs of the treatment (99). Short-term studies range in duration of seven to ninety days, group sizes for these studies should fall within five to forty mice per dose group with daily clinical observation and weekly physical examination and body weights (99).

Newborn mice of four to six days old and at least three grams in weight were inoculated orally with Gilmont® Micrometer Syringes with 50 µl of saponin extract at different concentrations ranging from 0 to 10 mg/ml for seven consecutive days. Treatment was given to newborn mice slowly to ensure full intake of the treatment and to prevent suffocation. Micrometer syringes were treated with bleach and sterilized in the autoclave before use every time.

Table 3.2. Experiment design for saponin acute toxicity testing.

| Group | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|---|---|---|---|---|---|---|---|
| 1 | Water | Water | Water | Water | Water | Water | Water |
| 2 | Saponin | Saponin | Saponin | Saponin | Saponin | Saponin | Saponin |

In group 1, newborns were given water during the treatment period. This served as the control group to demonstrate the weight gain pattern and conditions of healthy newborn mice.

Group 2 explored the highest concentration of saponin that can be given to newborn mice without causing any side effects on growth.

*3.7.2 Observation of Saponin Treated Newborn Mice*

Newborn mice were observed for two months following treatment to ensure that saponin did not induce some type of sub-chronic condition as measured by a reduction in weight gain, growth or appearance. Record of weight was made at 24 hours intervals for the first ten days, and once every week until mice reach full sexual maturity (56 days).

## 3.8 Determining the Effect of Saponin Extract on Rotavirus Induced Diarrhea

The purpose of this part of the study was to determine the lowest concentration of *Quillaja* saponin extract that is effective in preventing rotavirus induced diarrhea in newborn mice. Based on previous research *in vitro*, pretreating of MA104 cells with saponin extract is more efficient in 'blocking' rotavirus infection than direct inactivation of the virus. From our rotavirus induced diarrhea model, newborn mice recovered from rhesus rotavirus infection within five days post beginning of treatment. In this study, newborn mice were pretreated with saponin extract for before rhesus rotavirus was inoculated for five consecutive days. Experimental subjects were transferred to a separate facility where same environmental conditions were maintained.

*3.8.1 Inoculation of Saponin Extract and Virus in Newborn Mice*

Newborn mice of four to six days old and at least three grams in weight were inoculated orally with Gilmont® Micrometer Syringes with 50 µl of treatment for seven consecutive days. Table 3.3 showed the experimental design. From previous *in vitro* studies, pretreatment of MA104 cells with saponin extract at concentration as low as 0.0001 mg/ml were able to completely block rhesus rotavirus binding. Group 1 served as the rotavirus induced model. 50µl of water was given to newborn mice for two days and rotavirus in 50 µl of water was given to newborn for the following five days. Group 2 served as the testing group. 50 µl of saponin at

different concentration was given to newborn mice for two days and rotavirus mixed in 50 µl of saponin was given to newborn mice for the following five days.

Table 3.3. Experiment design for saponin and rotavirus interaction.

| Group | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|---|---|---|---|---|---|---|---|
| 1 | Water | Water | Water<br>Rotavirus | Water<br>Rotavirus | Water<br>Rotavirus | Water<br>Rotavirus | Water<br>Rotavirus |
| 2 | Saponin | Saponin | Saponin<br>Rotavirus | Saponin<br>Rotavirus | Saponin<br>Rotavirus | Saponin<br>Rotavirus | Saponin<br>Rotavirus |

*3.8.2 Observation of Experimental Subjects*

Newborn mice were observed for two months following treatment to ensure that inoculation of saponin and rotavirus did not induce any sub-chronic condition as measured by a reduction in weight gain, growth or appearance. Record of weight and stool observations were made at 24 hours intervals for the first ten days, and once every week until mice reach full sexual maturity (56 days). Stool samples were retrieved and evaluated as mentioned in 3*.6.2.*

## 3.9 Testing Different Parameters

After selecting the concentration of saponin extract that provided the greatest antiviral activity against rotavirus in mice, different conditions were tested to generate a profile for how long the saponin extract could provide protection against rotavirus infection in mice.

*3.9.1 Inoculation of Saponin Extract and Virus in Newborn Mice*

Newborn mice of four to six days old and at least three grams in weight were inoculated orally with Gilmont® Micrometer Syringes with 50 µl of treatment for seven consecutive days. Table 3.4 showed the experimental scheme for testing different parameters. Group 1 was the initial condition that we used in *3.8*. Group 2 represented newborn mice that were pre-treated with saponin extract for one day before rotavirus was inoculated. Group 3 represented newborn mice that were not pre-treated with saponin extract before rotavirus was inoculated. Group 4 represented newborn mice that were pre-treated with saponin extract but no saponin was given when rotavirus was inoculated.

Table 3.4. Experiment design for testing different parameters of saponin and rotavirus interaction.

| Group | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|---|---|---|---|---|---|---|---|
| 1 | Saponin | Saponin | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus |
| 2 | | Saponin | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus |
| 3 | | | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus | Saponin Rotavirus |
| 4 | Saponin | Saponin | Rotavirus | Rotavirus | Rotavirus | Rotavirus | Rotavirus |

*3.9.2 Observation of Experimental Subjects*

Newborn mice were observed for two months following treatment to ensure that inoculation of saponin and rotavirus did not induce any sub-chronic condition as measured by a reduction in weight gain, growth or appearance. Record of weight and stool observations were made at 24 hours intervals for the first ten days, and once every week until mice reach full sexual maturity (56 days). Stool samples were retrieved and evaluated as mentioned in *3.6.2.*

## 3.10 Long Term Observation of Experimental Subjects

The objective of the chronic toxicity studies was to evaluate the potential for saponins to impart long term health impacts on treated animals, duration of observation lasts from about 26 weeks to as long as two years (99).

*3.10.1 90 days Observation Period*

Ten mice from each tested condition were randomly selected to continue the chronic toxicity study. Individuals from each tested condition, (1) control group, those whom were given water for seven days, (2) acute toxicity group, those whom were given saponin extract for seven days, (3) rotavirus induced diarrhea, those whom were given rotavirus for 5 days, and (4) testing group – those whom were given saponin extract for two days and saponin extract with rotavirus for five days were kept for long term observation of 90 days after sexual maturity (56 days) to look for chronic damage to the experimental subjects. To check for signs of chronic

damage, individuals were monitored daily and weighed every month to ensure there was no major reduction in weight.

*3.10.2 Breeding of Experimental Subjects*

After the 90 day observation period, breeding pairs were setup for each testing condition. Experimental subjects were allowed to breed to ensure reasonable litter size and healthy offspring were achieved after breeding. Newborn offspring were observed for two months until mice reached full sexual maturity. Mice were monitored daily and weighed once a week during the two months period.

## 3.11 Fractionation of Saponin Extract

Saponin extract was fractionated with ion exchange chromatography (IEC). Fractionated extracts were tested in an effort to identify the optimum compositions for reduced toxicity and effective antiviral activity.

*3.11.1 FPLC fractionation of the Saponin Extract*

Whole saponin extract was separated into three fractions with Fast Protein Liquid Chromatography (FPLC) under conditions listed in Appendix C. Dialysis of fractions was performed in cellulose membrane dialysis bags and the sealed bags were placed in 1XSSC for forty eight hours. Dialyzed fractions were filter sterilized with a 0.2 µm Sterile Syringe Filter from Corning Inc. The saponin concentration of fractions was achieved from drying down 300 µl of the fractions and measuring the dried weight. Fractions were then analyzed with High Performance Liquid Chromatography (HPLC). The HPLC profiles of fractions were generated under the conditions listed in Appendix C for comparison with the whole saponin extract.

*3.11.2 Cytotoxicity Assay*

MA104 cells were seeded in 24-wells plate at 75% confluency with 500µl of MEM with 5% FBS and 1% P/S per well and incubated overnight at 37˚C and 5% $CO_2$ environment. Fractions collected following FPLC were diluted in MEM containing 5% FBS and 1% P/S to

produce the desired concentrations. For initial testing, ten-fold serial dilutions were prepared, beginning at initial concentration of 1 mg/ml and diluted in MEM with 5% FBS and 1% P/S. The diluted fractions were then added to each well at a volume of 500 µl and allowed to incubate for 96 hours at 37˚C and 5% $CO_2$ environment.

After 96 hours of incubation, the media was removed with the vacuum pump attached to a sterile pipette. Staining solution consist of 10% trypan blue and 90% MEM with 1% P/S was added to each well and incubated at 37˚C and 5% $CO_2$ environment for five minutes. The living cells reject the dye while the dead cells were stained blue. To determine viability, the number of dead (dyed – blue) cells and the living (not dyed – clear) were counted under a microscope, a total of 500 cells were counted in each well.

To determine the critical concentration toxic to cells, further testing was carried out after the initial result was obtained. Two-fold dilutions were prepared. Incubation and staining procedures was the same as discussed above.

*3.11.3 Plaque Reduction Assay*

A plaque reduction assay was used to test the ability of saponin fractions to directly inhibit rhesus rotavirus replication. Virus-induced cell death causes a plaque in a cell monolayer. In short, $1x10^6$ PFU of rhesus rotavirus was incubated with or without the addition of saponin fractions. A standard plaque assay was performed and results were compared to measure the effectiveness of the compound tested in reducing plaque formation. After the toxicity assay, plaque assays were carried out to measure the antiviral activity of the saponin fractions against rhesus rotavirus in MA104 cells. Plaque assays were performed as mentioned in *3.3.1*. Saponin extract or fractions were added to the dilution blanks during initial infection as well as to the agar overlays. Plaque assays were performed with original saponin extract, saponin fractions, and without saponin which served as the control. Results were compared to control (without saponin) to calculate plaque reduction, a sensitive measure of antiviral activity.

*3.11.4 Effect of Fractions on Rotavirus Induced Diarrhea*

Newborn mice of four to six days old and at least three grams in weight were inoculated orally with Gilmont® Micrometer Syringes with 50 ul of treatment daily for seven consecutive days. Newborn mice were divided into three groups where each group was pretreated with 50 µl of one of the fractions for two days and rotavirus mixed in 50 µl of fractions for the following five days. Records of weight and stool observations were made at 24 hours intervals for the first ten days. Stool samples were retrieved and evaluated as mentioned in 3*.6.2.*

## 3.12 Statistical Analysis

Repeated measures Analysis of Variance (ANOVA) was used to analyze the differences in weight of the newborn mice among treatments and control (100, 111). With repeated measures ANOVA, weight data recorded at days 7, 14, 21, 28, 35, 42, and 56 (weekly) were used to test the null hypothesis that there was no difference in weight of newborn mice among treatments and control. In all cases, a *P* value of less than 0.05 was used to reject the null hypothesis of no significant treatment effect in weight among groups. The initial weight recorded for each animal at day one was subtracted from weight data before analysis to exclude the possibility that differences in weight of the starting newborn mice among treatments and control did not contribute to the results account for the difference in weight among individuals when treatment began.

Rotavirus induced diarrhea was evaluated based on stool consistency as discussed in *3.6.2*. Individuals with no diarrhea 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 (101). Significance was determined at a *P* value of less than 0.05.

Evaluation of average weight recorded and diarrhea score was performed only among individuals that survived the whole 56 days observation period. Individuals that died during this period was reported in the mortality tables for each set of the experiment.

ANOVA was used to analyze the *in vitro* data of different fractions collected from FPLC. Results from cytotoxicity assays and plaque reduction assays were compared among each other. Tukey's post hoc test was used for pairwise comparison. Significance was determined at a *P* value of less than 0.05.

Analysis of data was done with SYSTAT statistical analysis software version 13.0 for Windows (Carnes Software International).

# CHAPTER 4

# RESULTS

## 4.1 Titer of Rhesus Rotavirus and Preparation of Working Virus Stock

One hundred-fifty ml of third passage lysate was harvested as discussed in section *3.2*. Plaque assays in triplicates were carried out to determine the titer of the rhesus rotavirus. Wells with 30-300 plaques were counted. The dilution with the appropriate number of plaques for evaluation was within $10^{-5}$ dilutions (Table 4.1).

Table 4.1. Number of Plaques counted in wells with $10^{-5}$ dilution.

| | Plate 1 | Plate 2 | Plate 3 | Avg ± std. error |
|---|---|---|---|---|
| Number of Plaques | 42 | 31 | 36 | 36.333±3.181 |

The number of PFU/ml was determined with the following formula: Plaque number x reciprocal dilution x reciprocal volume of virus added which equaled to 36.333 x $1/10^{-5}$ x1/0.25 ml = $1.453x10^7$ PFU/ml. Ten mls of the working virus stocks with titer of $1x10^6$, $10^5$, $10^4$, and $10^3$ PFU/ml were prepared from the original stock.

## 4.2 Development of Rotavirus Induced Diarrhea

The development of rotavirus induced diarrhea in newborn mice inoculated with different amounts of rhesus rotavirus was determined by orally introducing virus for five consecutive days. Four different titers of rhesus rotavirus ranging from $1x10^3$ to $1x10^6$ PFU/ml were prepared for oral inoculation. Inborn mice were given 50 µl of rhesus rotavirus at different titers (Table 3.1). Records of weight and stool observations were made at 24 hours intervals for the first ten days, and once every week until mice reached full sexual maturity (56 days). Stool samples were evaluated on a five point scale as mentioned in *3.6.2*. Different categories of

diarrhea reflect the amount of water lost during rotavirus infection. Category 3 and 4 were considered as proof of diarrhea in the mice.

Although previous research showed that the minimal infective dose in a heterologous system (simian rotavirus in mice) was 1000 PFUs, the data presented was generated in mice from single dose infection that a lower concentration of virus would cause a considerable rate of rotavirus induced diarrhea in this study because mice were orally inoculated with virus for five doses in five consecutive days and secondary infection could occur within the litter among newborn mice through fecal-oral transmission. Four different amounts of RRV ranging from 50 to 50,000 PFUs were prepared for oral inoculation.

Over 85% of the newborn mice orally inoculated with the two highest concentrations of RRV, 50,000 PFUs and 5,000 PFUs; over 75% of the newborn mice inoculated with 500 PFUs of RRV develop diarrhea over the five days inoculation period; while only 33% of the newborn mice orally inoculated with 50 PFUs of RRV developed diarrhea (Table 4.2). When compared the average diarrhea score among newborn mice orally inoculated with RRV with different concentration, only newborn mice inoculated at the lowest concentration (500 PFUs) was significantly lower than the other groups (Table 4.3). This indicated that there is a threshold to the amount of virus required to trigger a significant amount of the inoculated individuals to develop rotavirus induced diarrhea in mice and the threshold lie between 50 to 500 PFUs. In this study, I was not able to induce 100% of the inoculated newborn to develop diarrhea due to experimental difficulties. Newborn mice were orally inoculated with RRV and there was chance that the treated individual did not ingest the given amount and therefore no diarrhea was induced.

Repeated measures ANOVA of weight among mice inoculated with different titers of rotavirus showed that there was significant different among groups (Table 4.5). Comparison of weight among groups was done with weight increased in mice from the beginning of treatment to eliminate the possibility that differences in initial weight would obscure the result of the

ANOVA. Repeated measures ANOVA of weight was then performed between different titers against control which show no significant difference among weight except between 50,000 PFUs against control (Table 4.4). Average weight recorded for mice that were orally inoculated with RRV at 50,000 PFUs was significantly higher than that recorded for the control group (Table 4.5). This indicated that there is no significant short term health impact on mice inoculated with different titers of virus.

Throughout the 56 day observation periods, newborn mice inoculated with RRV only developed diarrhea within the first week post beginning of treatment. Rotavirus induced diarrhea began as early as 24 hours post treatment. Rate of rotavirus induced diarrhea within litter usually increased after one of the newborn developed diarrhea which may indicate the possibility of secondary infection. Newborn mice usually recovered within two to three days. No diarrhea was observed beyond five days post inoculation of RRV.

Table 4.2. Rate of rotavirus induced diarrhea in mice with different titers of RRV.

| Amount of Rotavirus (PFUs) | Number of mice | | | % that developed diarrhea |
|---|---|---|---|---|
| | Tested | Developed Diarrhea | No Diarrhea | |
| 50,000 | 27 | 24 | 3 | 88.889 |
| 5,000 | 33 | 29 | 4 | 87.879 |
| 500 | 41 | 31 | 10 | 75.610 |
| 50 | 12 | 4 | 8 | 33.333 |

Table 4.3. Pairwise comparison of mouse diarrhea score with standard error in newborn mice inoculated with different titers of RRV.

| Amount of Rotavirus PFUs | 50,000 (0.889±0.062) | 5,000 (0.879±0.057) | 500 (0.756±0.070) |
|---|---|---|---|
| 50 (0.333±0.142) | **<0.001** | **<0.001** | **0.012** |
| 500 (0.732±0.070) | 0.120 | 0.120 | |
| 5,000 (0.879±0.057) | 0.904 | | |

Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold

Table 4.4. Average weight gained from beginning of treatment for mice inoculated with different titers of RRV.

| Days Post treatment | RRV | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 50,000 PFUs | | 5,000 PFUs | | 500 PFUs | | 50 PFUs | | | |
| | Avg. wt. | Std. Err. | Avg. wt. | Std. Err. | Avg. wt. | Std. Err. | Avg. wt. | Std. Err. | Avg. wt. | Std. Err. |
| 2 | 0.506 | 0.035 | 2.277 | 1.611 | 0.500 | 0.034 | 0.458 | 0.042 | 0.409 | 0.034 |
| 3 | 0.926 | 0.061 | 1.222 | 0.044 | 1.049 | 0.045 | 1.041 | 0.054 | 0.960 | 0.057 |
| 4 | 1.528 | 0.073 | 1.647 | 0.063 | 1.643 | 0.074 | 1.640 | 0.074 | 1.410 | 0.080 |
| 5 | 2.006 | 0.107 | 2.243 | 0.084 | 2.302 | 0.105 | 2.123 | 0.096 | 2.021 | 0.084 |
| 6 | 2.373 | 0.119 | 2.812 | 0.104 | 2.890 | 0.149 | 2.651 | 0.105 | 2.552 | 0.100 |
| 7 | 2.830 | 0.192 | 3.454 | 0.131 | 3.452 | 0.159 | 3.130 | 0.114 | 3.073 | 0.117 |
| 8 | 3.226 | 0.252 | 4.116 | 0.152 | 3.873 | 0.166 | 3.679 | 0.172 | 3.595 | 0.119 |
| 9 | 3.524 | 0.319 | 4.696 | 0.152 | 4.395 | 0.192 | 4.164 | 0.182 | 4.037 | 0.112 |
| 10 | 4.106 | 0.325 | 5.186 | 0.146 | 4.790 | 0.186 | 4.587 | 0.213 | 4.571 | 0.128 |
| 14 | 4.831 | 0.294 | 6.728 | 0.204 | 5.407 | 0.150 | 5.262 | 0.257 | 5.773 | 0.142 |
| 21 | 7.892 | 0.411 | 9.247 | 0.184 | 8.433 | 0.162 | 8.215 | 0.307 | 8.142 | 0.239 |
| 28 | 11.968 | 0.622 | 12.554 | 0.391 | 13.530 | 0.244 | 12.124 | 0.410 | 11.893 | 0.341 |
| 35 | 14.257 | 0.448 | 14.301 | 0.292 | 15.274 | 0.156 | 13.092 | 0.547 | 15.014 | 0.260 |
| 42 | 14.930 | 0.364 | 15.288 | 0.301 | 15.691 | 0.199 | 14.714 | 0.414 | 15.738 | 0.302 |
| 56 | 16.779 | 0.322 | 17.702 | 0.445 | 18.447 | 0.395 | 17.506 | 0.608 | 17.514 | 0.302 |

Table 4.5. Repeated measures ANOVA of weight among mice inoculated with different titers of RRV.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 5 Levels | | | | | |
| Treatment: | Control | 50,000 PFUs | 5,000 PFUs | 500 PFUs | 50 PFUs |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 238.432 | 4 | 59.608 | 4.020 | **0.004** |
| Error | 1927.603 | 130 | 14.828 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | 50,000 PFUs | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 218.412 | 1 | 218.412 | 30.916 | **<0.001** |
| Error | 339.107 | 48 | 7.065 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | 5,000 PFUs | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 16.666 | 1 | 16.666 | 2.525 | 0.117 |
| Error | 415.742 | 63 | 6.599 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | 500 PFUs | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 71.529 | 1 | 71.529 | 3.391 | 0.070 |
| Error | 1476.695 | 70 | 21.096 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | 50 PFU s | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 10.833 | 1 | 10.833 | 1.170 | 0.286 |
| Error | 388.986 | 42 | 9.262 | | |

## 4.3 Acute Toxicity Testing of Saponin Extract

The toxicity of saponin extracts on newborn mice was evaluated at different concentrations ranging from 0 to 10 mg/ml for seven consecutive days. Records of weight were made at 24 hours intervals for the first ten days, and once every week until the mice reached full sexual maturity (56 days). Individuals orally inoculated with 0.75 mg/ml or more had a mortality rate of higher than 50% (Table 4.6). With linear regression, dosage that killed more than 50% of the population ($LD_{50}$) was calculated from the toxicity testing. The $LD_{50}$ of *Quillaja* saponin for seven consecutive days was 0.65 mg/ml and this concentration would serve as the maximum

concentration for further testing. Repeated measures ANOVA of weight was performed among mice inoculated with different saponin concentration with less than 50% mortality rate and control. There was no significant difference in weight among mice inoculated with different saponin concentration and control (Table 4.8), indicated that there was no significant short term health impact on mice that survived the saponin treatment.

The average weight of newborn mice at the beginning of treatment was about three grams, Treatment of 50 µl at 0.75 mg/ml is equivalent to 7.5 µg of saponin extract. The average weight of newborn mice at the beginning of treatment was about three grams, this is equivalent to 2.5 mg/kg of body weight in human. WHO recommend ADI of 5 mg/kg body weight for human; this suggests that newborn mice are more sensitive to saponin treatment than human.

Table 4.6. Mortality rate of newborn mice inoculated with different saponin concentration.

| Saponin concentration [mg/ml] | Number of Mice | | | Mortality rate (Death/100 mice) |
|---|---|---|---|---|
| | tested | survived | died | |
| 0 | 33 | 32 | 1 | 3.030 |
| 10 | 8 | 0 | 8 | 100.000 |
| 7.5 | 9 | 2 | 7 | 77.800 |
| 5 | 9 | 3 | 6 | 66.667 |
| 1 | 14 | 7 | 7 | 50.000 |
| 0.75 | 15 | 2 | 13 | 86.667 |
| 0.5 | 26 | 18 | 8 | 30.769 |
| 0.3 | 43 | 39 | 4 | 9.302 |
| 0.25 | 16 | 15 | 1 | 6.667 |
| 0.1 | 13 | 13 | 0 | 0.000 |

Table 4.7. Average weight gained from beginning of treatment for mice inoculated with different saponin concentration.

| Days Post Treatment | Saponin Concentration [mg/ml] | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | | 0.3 | | 0.25 | | 0.1 | | | |
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 2 | 0.547 | 0.050 | 0.660 | 0.037 | 0.642 | 0.044 | 0.626 | 0.064 | 0.409 | 0.034 |
| 3 | 1.113 | 0.069 | 1.326 | 0.064 | 1.144 | 0.077 | 1.227 | 0.090 | 0.960 | 0.057 |
| 4 | 1.728 | 0.109 | 1.875 | 0.077 | 1.767 | 0.098 | 2.056 | 0.122 | 1.410 | 0.080 |
| 5 | 2.240 | 0.148 | 2.394 | 0.127 | 2.466 | 0.122 | 2.651 | 0.129 | 2.021 | 0.084 |
| 6 | 2.854 | 0.177 | 3.076 | 0.099 | 3.244 | 0.168 | 3.134 | 0.206 | 2.552 | 0.100 |
| 7 | 3.384 | 0.187 | 3.510 | 0.109 | 3.714 | 0.235 | 3.503 | 0.237 | 3.073 | 0.117 |
| 8 | 3.320 | 0.384 | 3.898 | 0.133 | 4.366 | 0.183 | 3.990 | 0.217 | 3.595 | 0.119 |
| 9 | 3.873 | 0.167 | 4.101 | 0.265 | 4.899 | 0.195 | 4.586 | 0.238 | 4.037 | 0.112 |
| 10 | 4.320 | 0.199 | 4.961 | 0.151 | 5.361 | 0.211 | 5.279 | 0.439 | 4.571 | 0.128 |
| 14 | 6.572 | 0.253 | 6.006 | 0.158 | 6.211 | 0.231 | 5.977 | 0.531 | 5.773 | 0.142 |
| 21 | 9.808 | 0.167 | 9.197 | 0.233 | 9.594 | 0.349 | 9.137 | 0.350 | 8.142 | 0.239 |
| 28 | 14.673 | 0.269 | 13.167 | 0.205 | 12.457 | 0.521 | 13.078 | 0.732 | 11.893 | 0.341 |
| 35 | 16.273 | 0.301 | 14.518 | 0.214 | 15.045 | 0.448 | 15.333 | 0.614 | 15.014 | 0.260 |
| 42 | 17.385 | 0.356 | 15.337 | 0.248 | 16.435 | 0.441 | 16.514 | 0.608 | 15.738 | 0.302 |
| 56 | 20.108 | 0.431 | 18.091 | 0.390 | 18.982 | 0.498 | 16.800 | 0.883 | 17.514 | 0.302 |

Table 4.8. Repeated measures ANOVA of weight among different saponin concentrations and control.

| Variables: 5 Levels (Saponin Concentration) | | | | | |
|---|---|---|---|---|---|
| Treatment: | Control (0) | 0.1 mg/ml | 0.25 mg/ml | 0.3 mg/ml | 0.5 mg/ml |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 90.939 | 4 | 22.735 | 1.429 | 0.229 |
| Error | 17234.231 | 109 | 15.910 | | |

## 4.4 Impact of Saponin Extract on Rotavirus Induced Diarrhea

The impact of saponin extract on rotavirus induced diarrhea in newborn mice was tested at different concentrations for seven consecutive days. As discussed in *2.2.2*, pretreatment of MA104 cells with saponin extract at concentration as low as 0.0001 mg/ml were able to completely block rhesus rotavirus binding *in vitro*. Newborn mice were pretreated with saponin extract for two days, followed by inoculation of different titers of rhesus rotavirus mixed with saponin extract for five days. Records of weight and stool observations were made at 24 hours intervals for the first ten days, and once every week until mice reach full sexual maturity (56 days). Stool samples were evaluated on a five point scale as mentioned in *2.6.2*. Different

categories of diarrhea reflect the amount of water lost during rotavirus infection. Animals demonstrating category 3 and 4 diarrhea were considered as positive for diarrhea.

*4.4.1 Effect of Saponin Extract on 50,000 PFUs RRV*

Newborn mice were pretreated with saponin extract for two days, followed by inoculation of 50,000 PFUs RRV mixed with saponin extract for five days. Table 4.9 shows the summary of treatment at 50,000 PFUs.

Table 4.9. Summary of treatments at 50,000 PFUs.

| | Treatment Period | | | | | | |
|---|---|---|---|---|---|---|---|
|  | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Control | ← Water → | | | | | | |
| Rotavirus Induced Group | ← Water → | | ← 50,000 PFUs RRV → | | | | |
| Testing Groups | ← Saponin → | | | | | | |
| | | | ← 50,000 PFUs RRV → | | | | |

Newborn mice treated with saponin concentration at 0.5 mg/ml or higher had a mortality rate of over 60%, and 37.5% at saponin concentration of 0.25 mg/ml (Table 4.10). Individuals that developed rotavirus induced diarrhea treated with saponin concentration at 0.6 mg/ml against 50,000 PFUs RRV was reduced from 88.9% to 28.5%, while it was only reduced to 70% for mice that were treated at lower concentration of saponin of 0.5 mg/ml and 0.25mg/ml (Table 4.11). It was only at 0.6 mg/ml of saponin treatment that the reduction in diarrhea was significant (Table 4.12), indicating that a high concentration of saponin was required to block RRV infection at 50,000 PFUs. Treating newborn mice with a higher concentration of saponin result in more cells being “coated” by the saponin treatment, blocking most RRV access to cell membrane, and hence reducing rotavirus induced diarrhea. Repeated measures ANOVA of weight show that there was significant difference among groups (Table 4.14). Repeated measures ANOVA of weight between different saponin concentrations with 50,000 PFUs RRV against control show no significant difference among weight except between group that was

inoculated with 50,000 PFUs RRV (*P* value < 0.001) and group inoculated with saponin concentration at 0.5 mg/ml (*P* value = 0.025). The average weight recorded for mice of two groups are significantly higher than that recorded for the control group, indicating that there was no significant short term health impact on mice treated with saponin and RRV (Table 4.13).

Table 4.10. Mortality rate of newborn mice pretreated with saponin extract for two days.

| Treatment | Number of Mice | | | Mortality rate (Death/100 mice) |
|---|---|---|---|---|
| | Tested | Survived | Died | |
| Control – water for seven days | 33 | 32 | 1 | 3.030 |
| 50,000 PFUs RRV for five days | 27 | 27 | 0 | 0.000 |
| Saponin at 0.6 mg/ml for two days, Saponin at 0.6 mg/ml with 50,000 PFUs RRV for five days | 39 | 14 | 25 | 64.103 |
| Saponin at 0.5 mg/ml for two days, Saponin at 0.6 mg/ml with 50,000 PFUs RRV for five days | 68 | 27 | 41 | 60.294 |
| Saponin at 0.25 mg/ml for two days, Saponin at 0.025 mg/ml with 50,000 PFUs RRV for five days | 16 | 10 | 6 | 37.500 |

Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

Table 4.11. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days.

| Treatment | Number of Mice | | | % that developed diarrhea |
|---|---|---|---|---|
| | Tested | Diarrhea | No Diarrhea | |
| 50,000 PFUs RRV | 27 | 24 | 3 | 88.889 |
| Saponin at 0.6 mg/ml with RRV | 14 | 4 | 10 | 28.571 |
| Saponin at 0.5 mg/ml with RRV | 27 | 19 | 8 | 70.370 |
| Saponin at 0.25 mg/ml with RRV | 10 | 7 | 3 | 70.000 |

Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

Table 4.12. Mouse diarrhea score with standard error in newborn mice pretreated with saponin extract for two days.

| Treatment | Saponin at 0.6 mg/ml with RRV | Saponin at 0.5 mg/ml with RRV | Saponin at 0.25 mg/ml with RRV |
|---|---|---|---|
| | 0.286±0.125 | 0.704±0.088 | 0.727±0.135 |
| 50,000 PFUs RRV at for 5 days | 0.889±0.062 | 0.889±0.062 | 0.889±0.062 |
| *P* value | **<0.001** | 0.094 | 0.221 |

Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold.

Table 4.13. Average weight gained from beginning of treatment for mice pretreated with saponin extract for two days.

| Days Post Treatment | Treatment | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (a) | | (b) | | (c) | | (d) | | | |
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 2 | 0.547 | 0.050 | 0.347 | 0.124 | 0.527 | 0.074 | 0.596 | 0.077 | 0.409 | 0.034 |
| 3 | 1.113 | 0.069 | 0.936 | 0.158 | 1.045 | 0.102 | 1.147 | 0.089 | 0.960 | 0.057 |
| 4 | 1.728 | 0.109 | 1.448 | 0.203 | 1.598 | 0.126 | 1.782 | 0.114 | 1.410 | 0.080 |
| 5 | 2.240 | 0.148 | 2.086 | 0.232 | 2.184 | 0.135 | 2.529 | 0.192 | 2.021 | 0.084 |
| 6 | 2.854 | 0.177 | 2.588 | 0.237 | 2.791 | 0.148 | 3.084 | 0.191 | 2.552 | 0.100 |
| 7 | 3.384 | 0.187 | 3.204 | 0.216 | 3.306 | 0.156 | 3.605 | 0.242 | 3.073 | 0.117 |
| 8 | 3.320 | 0.384 | 3.694 | 0.278 | 3.877 | 0.181 | 3.965 | 0.289 | 3.595 | 0.119 |
| 9 | 3.873 | 0.167 | 4.148 | 0.314 | 4.504 | 0.230 | 4.673 | 0.617 | 4.037 | 0.112 |
| 10 | 4.320 | 0.199 | 4.431 | 0.352 | 5.691 | 0.207 | 4.919 | 0.845 | 4.571 | 0.128 |
| 14 | 6.572 | 0.253 | 4.354 | 0.447 | 5.270 | 0.342 | 4.713 | 0.469 | 5.773 | 0.142 |
| 21 | 9.808 | 0.167 | 7.280 | 0.722 | 8.015 | 0.513 | 8.693 | 0.556 | 8.142 | 0.239 |
| 28 | 14.673 | 0.269 | 11.793 | 1.022 | 12.979 | 0.674 | 12.658 | 0.415 | 11.893 | 0.341 |
| 35 | 16.273 | 0.301 | 14.367 | 0.882 | 16.182 | 0.541 | 14.260 | 0.575 | 15.014 | 0.260 |
| 42 | 17.385 | 0.356 | 15.371 | 0.939 | 17.878 | 0.543 | 15.311 | 0.563 | 15.738 | 0.302 |
| 56 | 20.108 | 0.431 | 17.526 | 1.435 | 20.422 | 0.770 | 17.367 | 0.680 | 17.514 | 0.302 |

Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Treatment (a): 50,000 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.5 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV.

Table 4.14. Repeated measures ANOVA of weight among mice pretreated with saponin extract for two days.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 5 Levels | | | | | |
| Treatment: | Control | (a) | (b) | (c) | (d) |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 374.979 | 4 | 93.745 | 5.325 | **0.001** |
| Error | 1637.290 | 93 | 17.605 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (a) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 218.412 | 1 | 218.412 | 30.916 | **<0.001** |
| Error | 339.107 | 48 | 7.065 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (b) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 14.699 | 1 | 14.499 | 0.896 | 0.349 |
| Error | 721.536 | 44 | 16.399 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (c) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 93.420 | 1 | 93.420 | 5.324 | **0.025** |
| Error | 645.687 | 54 | 17.546 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (d) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 0.317 | 1 | 0.317 | 0.039 | 0.844 |
| Error | 322.101 | 40 | 8.053 | | |

Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Treatment (a): 50,000 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.5 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV. *P* values indicating significant differences at the 5% level are in bold.

*4.4.2 Effect of Saponin Extract on 5,000 PFUs RRV*

Newborn mice were pretreated with saponin extract for two days, followed by inoculation of 5,000 PFUs RRV mixed with saponin extract for five days. Table 4.15 shows the summary of treatment at 5,000 PFUs.

Table 4.15. Summary of treatments at 5,000 PFUs.

| | Treatment Period | | | | | | |
|---|---|---|---|---|---|---|---|
|  | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Control | ← Water → | | | | | | |
| Rotavirus Induced Group | ← Water → | | ← 5,000 PFUs RRV → | | | | |
| Testing Groups | ← Saponin → | | | | | | |
| | | | ← 5,000 PFUs RRV → | | | | |

The evaluation of saponin against RRV at 50,000 PFUs showed that the rotavirus induced diarrhea was only significantly reduced at high concentration (0.6 mg/ml) of saponin. However, mortality of newborn mice at concentration effective against RRV infection was also high. Saponin concentration and RRV titers were both reduced to test the effect. At saponin concentration of 0.3 mg/ml against 5,000 PFUs, the mortality rate was greatly reduced to 6.6% with the change in treatment parameters (Table 4.16). However, with reduction of saponin concentration to 0.3 mg/ml, individuals that developed rotavirus induced diarrhea were only reduced from 87.8% to 57.1% (Table 4.17). Lowering the concentration of saponin treatment results in less cell being “coated” by saponin, blockage of RRV infection was less efficient compared to higher concentration, and therefore, more individuals developed rotavirus induced diarrhea. Repeated measures ANOVA of weight among groups with 5,000 PFUs RRV, saponin treatment with RRV inoculation, against control show no significant difference among weight (Table 4.19) indicating that there was no significant short term health impact on mice treated with saponin and RRV (Table 4.18).

Table 4.16. Mortality rate of newborn mice pretreated with saponin extract for two days.

| Treatment | Number of Mice | | | Mortality rate (Death/100 mice) |
|---|---|---|---|---|
| | Tested | Survived | Died | |
| Control – water | 33 | 32 | 1 | 3.030 |
| 5,000 PFUs RRV | 33 | 33 | 0 | 0.000 |
| Saponin at 0.3 mg/ml with RRV | 30 | 28 | 2 | 6.667 |

Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days.

Table 4.17. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days

| Treatment | Number of Mice | | | % that developed diarrhea |
|---|---|---|---|---|
| | Tested | Diarrhea | No Diarrhea | |
| 5,000 PFUs RRV | 33 | 29 | 4 | 87.879 |
| Saponin at 0.3 mg/ml with RRV | 28 | 16 | 12 | 57.143 |

Pretreatment was . followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days.

Table 4.18. Mouse diarrhea score with standard error in newborn mice pretreated with saponin extract for two days.

| Treatment | Saponin at 0.3 mg/ml with RRV |
|---|---|
| | 0.571±0.095 |
| 5,000 PFUs RRV | 0.879±0.057 |
| *P* value | **0.007** |

Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days. Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold.

Table 4.19. Average weight gained from beginning of treatment for mice inoculated pretreated with saponin extract for two days.

| Days Post treatment | Treatment (a) Avg. Wt. | Treatment (a) Std. Err. | Treatment (b) Avg. Wt. | Treatment (b) Std. Err. | Control Avg. Wt. | Control Std. Err. |
|---|---|---|---|---|---|---|
| 2 | 0.660 | 0.037 | 0.686 | 0.081 | 0.409 | 0.034 |
| 3 | 1.326 | 0.064 | 1.403 | 0.123 | 0.960 | 0.057 |
| 4 | 1.875 | 0.077 | 2.011 | 0.152 | 1.410 | 0.080 |
| 5 | 2.394 | 0.127 | 2.807 | 0.168 | 2.021 | 0.084 |
| 6 | 3.076 | 0.099 | 3.456 | 0.191 | 2.552 | 0.100 |
| 7 | 3.510 | 0.109 | 4.113 | 0.199 | 3.073 | 0.117 |
| 8 | 3.898 | 0.133 | 4.775 | 0.225 | 3.595 | 0.119 |
| 9 | 4.101 | 0.265 | 5.252 | 0.230 | 4.037 | 0.112 |
| 10 | 4.961 | 0.151 | 5.715 | 0.263 | 4.571 | 0.128 |
| 14 | 6.006 | 0.158 | 6.534 | 0.243 | 5.773 | 0.142 |
| 21 | 9.197 | 0.233 | 9.036 | 0.259 | 8.142 | 0.239 |
| 28 | 13.167 | 0.205 | 12.431 | 0.385 | 11.893 | 0.341 |
| 35 | 14.518 | 0.214 | 14.316 | 0.339 | 15.014 | 0.260 |
| 42 | 15.337 | 0.248 | 15.054 | 0.379 | 15.738 | 0.302 |
| 56 | 18.091 | 0.390 | 18.193 | 0.463 | 17.514 | 0.302 |

Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days. Control: water. Treatment (a): 5,000 PFUs RRV. Treatment (b) saponin at 0.3 mg/ml with RRV.

Table 4.20. Repeated measures ANOVA of weight among mice inoculated pretreated with saponin extract for two days.

| Variables: 3 Levels | | | | | |
|---|---|---|---|---|---|
| Treatment: | Control | (a) | (b) | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 20.492 | 2 | 10.246 | 1.190 | 0.309 |
| Error | 774.962 | 90 | 8.611 | | |

Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days. Control: water. Treatment (a): 5,000 PFUs RRV. Treatment (b) saponin at 0.3 mg/ml with RRV.

*4.4.3 Effect of Saponin Extract on 500 PFUs RRV*

Newborn mice were pretreated with saponin extract for two days, followed by inoculation of 500 PFUs RRV mixed with saponin extract for five days. Table 4.21 shows the summary of treatment at 500 PFUs.

Table 4.21. Summary of treatments at 500 PFUs.

| | Treatment Period | | | | | | |
|---|---|---|---|---|---|---|---|
|  | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Control | ← Water → | | | | | | |
| Rotavirus Induced Group | ← Water → | | ← 500 PFUs RRV → | | | | |
| Testing Groups | ← Saponin → | | | | | | |
| | | | ← 500 PFUs RRV → | | | | |

The evaluation of saponin against RRV at 50,000 PFUs and 5,000 PFUs showed that there was a tradeoff between inhibition of RRV infection and mortality. Oral inoculation of saponin at high concentration results in “coating” effect of cells and blocks RRV infection. The higher the concentration of saponin inoculated, the more cells are “coated”, and hence the less number of individual develop rotavirus induced diarrhea. However, high concentration of saponin also results in disruption of cell membrane environment, which can result in cells death, and death in the animal.

At 500 PFUs RRV, newborn mice treated with saponin concentration at 0.6 mg/ml had a mortality rate of 54.5%; it was greatly reduced to 11.9% and 6.9% when saponin concentration was reduced to 0.3 mg/ml and 0.25 mg/ml, respectively (Table 4.22). Individuals that developed rotavirus induced diarrhea treated with saponin against 500 PFUs RRV were significantly reduced from 75.6% to 11.1%, 9.5%, and 27.9% when treated at concentration of 0.6 mg/ml, 0.3 mg/ml, and 0.25 mg/ml, respectively (Table 4.23). The optimum concentration of saponin that I explored during this experiment was 0.3 mg/ml because it was able to reduce the rotavirus induced diarrhea significantly with a low mortality rate, unlike what happened at 0.6 mg/ml of saponin (Table 4.23). Diarrhea score increased when saponin treatment was reduced from 0.3 mg/ml to 0.25 mg/ml, indicating that lowering the concentration treatment further would not help with inhibiting RRV infection. The lower the concentration of saponin treatment, the

less cells would be “coated” for protection. With linear regression, dosage that effectively decreased the number of individuals that developed rotavirus induced diarrhea by 50% ($ED_{50}$) was calculated. The $ED_{50}$ of *Quillaja* saponin against rotavirus induced diarrhea at 500 PFUs over a seven days period was 0.22 mg/ml.

Repeated measures ANOVA of weight show that there was significant difference among groups (Table 4.26). Repeated measures ANOVA of weight between different saponin concentrations with 500 PFUs RRV against control show no significant difference among weight except between group that was inoculated with saponin concentration at 0.25 mg/ml (*P* value = 0.023). The average weight recorded was significantly higher than that recorded for the control group (Table 4.25), indicating that there was no significant short term health impact on mice that survived the saponin treatment.

Table 4.22. Mortality rate of newborn mice pretreated with saponin extract for two days. Followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

| Treatment | Number of Mice | | | Mortality rate (Death/100 mice) |
|---|---|---|---|---|
| | Tested | Survived | Died | |
| Control – water | 33 | 32 | 1 | 3.030 |
| 500 PFUs RRV | 41 | 41 | 0 | 0.000 |
| Saponin at 0.6 mg/ml with RRV | 44 | 20 | 24 | 54.545 |
| Saponin at 0.3 mg/ml with RRV | 47 | 42 | 5 | 11.905 |
| Saponin at 0.25 mg/ml with RRV | 46 | 43 | 3 | 6.977 |

.Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

Table 4.23. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days.

| Treatment | Number of Mice | | | % that developed diarrhea |
|---|---|---|---|---|
| | Tested | Diarrhea | No Diarrhea | |
| 500 PFUs RRV | 41 | 31 | 10 | 75.610 |
| Saponin at 0.6 mg/ml with RRV | 20 | 2 | 18 | 11.111 |
| Saponin at 0.3 mg/ml with RRV | 42 | 4 | 38 | 9.524 |
| Saponin at 0.25 mg/ml with RRV | 43 | 12 | 31 | 27.907 |

Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

Table 4.24. Mouse diarrhea score with standard error in newborn mice pretreated with saponin extract for two days.

| Treatment | Saponin at 0.6 mg/ml with RRV | Saponin at 0.3 mg/ml with RRV | Saponin at 0.25 mg/ml with RRV |
|---|---|---|---|
| | 0.1±0.049 | 0.095±0.046 | 0.286±0.069 |
| 500 PFUs RRV | 0.732±0.070 | 0.732±0.070 | 0.732±0.070 |
| *P* value | **<0.001** | **<0.001** | **<0.001** |

Pretreatment was . followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold.

Table 4.25. Average weight gained from beginning of treatment for mice inoculated pretreated with saponin extract for two days.

| Days Post Treatment | Treatment | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (a) | | (b) | | (c) | | (d) | | | |
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 2 | 0.642 | 0.044 | 0.248 | 0.101 | 0.593 | 0.050 | 0.460 | 0.037 | 0.409 | 0.034 |
| 3 | 1.144 | 0.077 | 0.845 | 0.160 | 1.094 | 0.116 | 1.105 | 0.042 | 0.960 | 0.057 |
| 4 | 1.767 | 0.098 | 1.063 | 0.313 | 1.708 | 0.058 | 1.714 | 0.045 | 1.410 | 0.080 |
| 5 | 2.466 | 0.122 | 1.672 | 0.360 | 2.390 | 0.066 | 2.306 | 0.095 | 2.021 | 0.084 |
| 6 | 3.244 | 0.168 | 2.536 | 0.233 | 2.997 | 0.085 | 3.149 | 0.074 | 2.552 | 0.100 |
| 7 | 3.714 | 0.235 | 3.437 | 0.298 | 3.671 | 0.096 | 3.779 | 0.081 | 3.073 | 0.117 |
| 8 | 4.366 | 0.183 | 3.845 | 0.326 | 4.126 | 0.113 | 4.254 | 0.085 | 3.595 | 0.119 |
| 9 | 4.899 | 0.195 | 4.410 | 0.365 | 4.471 | 0.117 | 4.623 | 0.097 | 4.037 | 0.112 |
| 10 | 5.361 | 0.211 | 5.047 | 0.404 | 4.864 | 0.127 | 5.092 | 0.128 | 4.571 | 0.128 |
| 14 | 6.211 | 0.231 | 6.083 | 0.375 | 5.414 | 0.176 | 6.056 | 0.167 | 5.773 | 0.142 |
| 21 | 9.594 | 0.349 | 10.244 | 0.615 | 8.870 | 0.361 | 9.294 | 0.157 | 8.142 | 0.239 |
| 28 | 12.457 | 0.521 | 14.143 | 0.500 | 13.232 | 0.319 | 13.241 | 0.195 | 11.893 | 0.341 |
| 35 | 15.045 | 0.448 | 16.409 | 0.431 | 14.956 | 0.268 | 16.282 | 0.214 | 15.014 | 0.260 |
| 42 | 16.435 | 0.441 | 16.737 | 0.450 | 16.585 | 0.267 | 17.154 | 0.303 | 15.738 | 0.302 |
| 56 | 18.982 | 0.498 | 18.318 | 0.332 | 19.160 | 0.676 | 18.779 | 0.307 | 17.514 | 0.302 |

Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Control: water. Treatment (a): 500 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.3 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV.

Table 4.26. Repeated measures ANOVA of weight among mice inoculated pretreated with saponin extract for two days.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 5 Levels | | | | | |
| Treatment: | Control | (a) | (b) | (c) | (d) |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 181.304 | 4 | 45.326 | 2.733 | **0.031** |
| Error | 2919.159 | 176 | 16.586 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (a) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 76.494 | 1 | 76.494 | 3.634 | 0.061 |
| Error | 1473.547 | 70 | 21.051 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (b) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 114.434 | 1 | 114.434 | 9.805 | **0.003** |
| Error | 583.566 | 50 | 11.671 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (c) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 59.496 | 1 | 59.496 | 4.644 | **0.034** |
| Error | 948.000 | 74 | 12.811 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (d) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 148.377 | 1 | 148.377 | 18.334 | **<0.001** |
| Error | 606.974 | 75 | 8.093 | | |

Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Control: water. Treatment (a): 500 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.3 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV. *P* values indicating significant differences at the 5% level are in bold.

## 4.5 Testing of Different Virus Exposure/Saponin Treatment Parameters

From section 4.4 (Impact of Saponin Extract on Rotavirus Induced Diarrhea), the data demonstrate that 0.3 mg/ml of saponin extract provided the greatest protection against rotavirus induced diarrhea when mice were exposed to 500s PFU. Different conditions were tested to generate a profile on how long the saponin extract can provide protection against rotavirus infection in mice. Table 4.27 shows the summary of treatment. Group 1 represented group

pretreated with saponin at 0.3 mg/ml for two days, followed by saponin at 0.3 mg/ml with 500 PFUs rhesus rotavirus for five days. Group 2 represented group pretreated with saponin at 0.3 mg/ml for one day, followed by saponin at 0.3 mg/ml with 500 PFUs RRV for five days. Group 3 represented group with no pretreatment of saponin extract, followed by saponin at 0.3 mg/ml with 500 PFUs RRV for five days. Group 4 represented group pretreated with saponin at 0.3 mg/ml for two days, followed by 500 PFUs RRV for five days.

Table 4.27. Summary of treatments for parameter testing.

| | Treatment Period | | | | | | |
|---|---|---|---|---|---|---|---|
|  | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
| Group 1 | ← Saponin → | | | | | | |
| | | | ← 500 PFUs RRV → | | | | |
| Group 2 | | ← Saponin → | | | | | |
| | | | ← 500 PFUs RRV → | | | | |
| Group 3 | | | ← Saponin → | | | | |
| | | | ← 500 PFUs RRV → | | | | |
| Group 4 | ← Saponin → | | | | | | |
| | | | ← 500 PFUs RRV → | | | | |

The number of individuals that developed rotavirus induced diarrhea increased with the decrease in pretreating time of saponin. It increased from 9.5% to 23% when there was no pretreatment of saponin (Table 4.28). However, the increase was not significantly different (Table 4.29). It was expected that the longer the pretreatment time, the better chance that cells are “coated” with saponin, and hence blocking RRV infection. This observation was opposite than expected, indicating that saponin “coats” cells efficiently in a short amount of time. Rotavirus induced diarrhea score increased significantly when saponin was only given to newborn mice for two days before inoculation of 500 PFUs RRV when compared to group that was pretreated and continued treated during inoculation of the virus (Table 4.28 and Table 4.29). One important observation was noted that newborn mice pretreated with saponin extract for two days only developed rotavirus induced diarrhea towards the end of the virus inoculation (at day six or seven). This showed that saponin extract did “block” RRV infection for 48 to 72

hours after extract was given to newborn mice. The difference in duration of pretreatment (0 to two days) of saponin extract did not have an effect on the antiviral activity of saponin against rotavirus induced diarrhea among groups. Repeated measures ANOVA of weight (Table 4.31) shows that there was significant difference among groups (*P* value = 0.040). Repeated measures ANOVA of weight between different parameter settings and control show no significant difference among weight except between group that was pretreated with saponin for two days followed by inoculation with 500 PFUs RRV (*P* value = 0.035), it was significantly higher than that recorded for the control group (Table 4.30), indicating that there was no significant short term health effect on the individuals.

Table 4.28. Rate of rotavirus induced diarrhea in newborn mice.

| Treatment | Number of Mice | | | % that developed diarrhea |
|---|---|---|---|---|
| | Tested | Diarrhea | No Diarrhea | |
| 500 PFUs RRV | 41 | 31 | 10 | 75.610 |
| Pretreatment with saponin for two days | 42 | 4 | 38 | 9.524 |
| Pretreatment with saponin for one day | 39 | 7 | 32 | 17.949 |
| No pretreatment with saponin | 30 | 7 | 23 | 23.333 |
| Pretreatment with saponin for two days, no saponin given after pretreatment | 33 | 14 | 19 | 42.424 |

Different parameters tested at 0.03 mg/ml saponin extract with 500 PFUs RRV.

Table 4.29. Mouse diarrhea score with standard error in mice.

| Treatment | Pretreatment with saponin for one day s | No pretreatment with saponin | Pretreatment with saponin for two days, no saponin given after pretreatment |
|---|---|---|---|
| | 0.179±0.060 | 0.233±0.066 | 0.424±0.077 |
| Pretreatment with saponin for two days | 0.095±0.046 | 0.095±0.046 | 0.095±0.046 |
| *P* value | 0.272 | 0.111 | **0.001** |

Different parameters test at 0.3 mg/ml saponin extract with 500 PFUs RRV. Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold.

Table 4.30. Average weight gained from beginning of treatment for mice.

| Days Post Treatment | Treatment (a) Avg. Wt. | (a) Std. Err. | (b) Avg. Wt. | (b) Std. Err. | (c) Avg. Wt. | (c) Std. Err. | (d) Avg. Wt. | (d) Std. Err. | Control Avg. Wt. | Control Std. Err. |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.642 | 0.044 | 0.593 | 0.050 | 0.587 | 0.017 | 0.590 | 0.027 | 0.409 | 0.034 |
| 3 | 1.144 | 0.077 | 1.094 | 0.116 | 1.119 | 0.058 | 1.183 | 0.061 | 0.960 | 0.057 |
| 4 | 1.767 | 0.098 | 1.708 | 0.058 | 1.640 | 0.070 | 1.630 | 0.093 | 1.410 | 0.080 |
| 5 | 2.466 | 0.122 | 2.390 | 0.066 | 2.123 | 0.093 | 2.078 | 0.115 | 2.021 | 0.084 |
| 6 | 3.244 | 0.168 | 2.997 | 0.085 | 2.646 | 0.103 | 2.652 | 0.115 | 2.552 | 0.100 |
| 7 | 3.714 | 0.235 | 3.671 | 0.096 | 3.258 | 0.120 | 3.106 | 0.127 | 3.073 | 0.117 |
| 8 | 4.366 | 0.183 | 4.126 | 0.113 | 3.695 | 0.124 | 5.583 | 2.018 | 3.595 | 0.119 |
| 9 | 4.899 | 0.195 | 4.471 | 0.117 | 4.088 | 0.132 | 4.195 | 0.180 | 4.037 | 0.112 |
| 10 | 5.361 | 0.211 | 4.864 | 0.127 | 4.515 | 0.145 | 4.752 | 0.200 | 4.571 | 0.128 |
| 14 | 6.211 | 0.231 | 5.414 | 0.176 | 5.321 | 0.232 | 5.590 | 0.275 | 5.773 | 0.142 |
| 21 | 9.594 | 0.349 | 8.870 | 0.361 | 7.848 | 0.290 | 8.552 | 0.435 | 8.142 | 0.239 |
| 28 | 12.457 | 0.521 | 13.232 | 0.319 | 11.228 | 0.439 | 13.345 | 0.540 | 11.893 | 0.341 |
| 35 | 15.045 | 0.448 | 14.956 | 0.268 | 13.542 | 0.516 | 14.501 | 0.560 | 15.014 | 0.260 |
| 42 | 16.435 | 0.441 | 16.585 | 0.267 | 15.123 | 0.503 | 15.761 | 0.491 | 15.738 | 0.302 |
| 56 | 18.982 | 0.498 | 19.160 | 0.676 | 17.293 | 0.559 | 17.579 | 0.507 | 17.514 | 0.302 |

Different parameters tested at 0.3 mg/ml saponin extract with 500 PFUs RRV. Treatment (a): pretreatment of saponin for two days. Treatment (b): pretreatment of saponin for one day. Treatment (c): no pretreatment of saponin. Treatment (d): pretreatment of saponin for two days followed by RRV inoculation.

Table 4.31. Repeated measures ANOVA of weight among mice.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 4 Levels | | | | | |
| Treatment: | Control | (a) | (b) | (c) | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 165.201 | 3 | 55.067 | 2.858 | **0.040** |
| Error | 2505.108 | 130 | 19.270 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (a) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 31.343 | 1 | 31.343 | 1.718 | 0.194 |
| Error | 1259.018 | 69 | 18.247 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (b) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 3.671 | 1 | 3.671 | 0.209 | 0.649 |
| Error | 1054.024 | 60 | 17.567 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (c) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 47.734 | 1 | 47.734 | 4.653 | **0.035** |
| Error | 615.503 | 60 | 10.258 | | |

Different parameters tested at 0.3 mg/ml saponin extract with 500 PFUs RRV. Treatment (a): pretreatment of saponin for two days. Treatment (b): pretreatment of saponin for one day. Treatment (c): no pretreatment of saponin. Treatment (d): pretreatment of saponin for two days followed by RRV inoculation. *P* values indicating significant differences at the 5% level are in bold.

## 4.6 Long Term Observation of Experimental Subjects

The objective of chronic toxicity studies is to refine the description of the toxicity associated with the doses of a test substance, duration of observation lasts from about 26 weeks to as long as two years (40). To check for signs of chronic damage, individuals were weighed every month to ensure there was no major reduction in weight and reasonable litter size and healthy offspring were achieved after breeding. Chronic toxicity studies were carried out after the 56 days period.

*4.6.1 90 days observation period*

Ten mice from each tested condition were randomly selected to continue the chronic toxicity study. Tested conditions included a.) control group that were inoculated with water for seven days; b.) acute toxicity group that were inoculated with 0.3 mg/ml saponin extract for seven days, c.) rotavirus induced diarrhea group that were inoculated with 500 PFU rhesus rotavirus for five days; and d.) testing group that were pretreated with 0.3 mg/ml saponin extract for two days, followed saponin extraction at 0.3 mg/ml with 500 PFUs rhesus rotavirus for five days. Repeated measures ANOVA of weight recorded for mice over the 90 days observation period showed no significant difference (*P* value = 0.222) in weight among groups indicating that there is no chronic effect on mice treated with different conditions (Table 4.33).

Table 4.32. Average weight recorded for mice over 90 days observation period.

| Days post 56 days observation | Acute Toxicity Group | | Rotavirus Induced Diarrhea Group | | Testing Group | | Control | |
|---|---|---|---|---|---|---|---|---|
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 0 | 25.053 | 1.932 | 21.234 | 0.873 | 22.938 | 0.764 | 21.269 | 1.163 |
| 30 | 25.998 | 1.849 | 24.915 | 0.882 | 25.054 | 1.255 | 23.956 | 0.803 |
| 60 | 30.253 | 1.784 | 29.080 | 1.296 | 27.820 | 1.738 | 25.176 | 0.624 |
| 90 | 31.923 | 1.526 | 28.792 | 1.444 | 27.198 | 1.874 | 27.705 | 0.853 |

Control group were inoculated with water for seven days; Acute toxicity group were inoculated with 0.3 mg/ml saponin extract for seven days; Rotavirus induced diarrhea group were inoculated with 500 PFUs RRV for five days; Testing group were pretreated with 0.3 mg/ml saponin extract for two days, followed saponin extraction at 0.3 mg/ml with 500 PFUs RRV for five days.

Table 4.33. Repeated measures ANOVA of weight among mice over 90 days observation period.

| Variables: 4 Levels | | | | | |
|---|---|---|---|---|---|
| Treatment: | | (b) | (c) | (d) | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 298.658 | 3 | 99.553 | 1.537 | 0.222 |
| Error | 2332.292 | 36 | 64.786 | | |

Treatment (a) represented control group; Treatment (b) represented acute toxicity group; Treatment (c) represented rotavirus induced diarrhea group; Treatment (d) represented testing group. *P* values indicating significant differences at the 5% level are in bold.

*4.6.2 Offspring of Experimental Subjects*

After the 90 days observation period, breeding pairs were setup for each tested condition. Experimental subjects were allowed to breed to ensure reasonable litter size and healthy offspring were achieved after breeding. Newborn offspring were observed for two months until mice reach full sexual maturity. Mice were monitored daily and weighed once a week during the two months period. Repeated measures ANOVA of weight recorded for mice over the 90 days observation period showed that there was no significant difference (*P* value = 0.570) in weight among offspring of different treatment groups, indicating that the saponin treatment has no long term impact on reproductive ability or chronic effect on offspring (Table 4.35).

Table 4.34. Average weight recorded for mice for offspring over the two months observation period.

| Days | Acute Toxicity Group | | Rotavirus Induced Diarrhea Group | | Testing Group | | Control | |
|---|---|---|---|---|---|---|---|---|
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 7 | 1.384 | 0.035 | 1.582 | 0.060 | 1.338 | 0.040 | 1.264 | 0.019 |
| 14 | 4.901 | 0.141 | 4.150 | 0.106 | 3.642 | 0.115 | 4.071 | 0.104 |
| 21 | 7.950 | 0.203 | 8.046 | 0.200 | 7.142 | 0.194 | 8.168 | 0.218 |
| 28 | 9.454 | 0.186 | 9.167 | 0.248 | 8.946 | 0.220 | 9.363 | 0.266 |
| 35 | 12.945 | 0.349 | 12.957 | 0.474 | 13.302 | 0.256 | 13.175 | 0.262 |
| 42 | 15.817 | 0.369 | 16.370 | 0.479 | 16.839 | 0.391 | 16.314 | 0.394 |
| 56 | 16.846 | 0.415 | 18.235 | 0.505 | 18.037 | 0.262 | 18.892 | 0.257 |

Table 4.35. Repeated measures ANOVA of weight for offspring over the two months observation period.

| Variables: 4 Levels | | | | | |
|---|---|---|---|---|---|
| Treatment: | Control | (a) | (b) | (c) | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 28.621 | 3 | 9.540 | 0.782 | 0.507 |
| Error | 1073.842 | 88 | 12.203 | | |

Control represented offspring of control group; Treatment (a) represented offspring of acute toxicity group; Treatment (b) represented offspring of rotavirus induced diarrhea group; Treatment (c) represented offspring of testing group. *P* values indicating significant differences at the 5% level are in bold.

## 4.7 Fractionation of Saponin Extract

Whole saponin extract was separated into three fractions with Fast Protein Liquid Chromatography (FPLC) as discussed in section *3.12.1*. Figure 4.1 shows the FPLC profile, Fraction A was collected between six to nine minutes, Fraction B was collected between twenty to twenty-five minutes, Fraction C was collected between twenty six to thirty minutes.

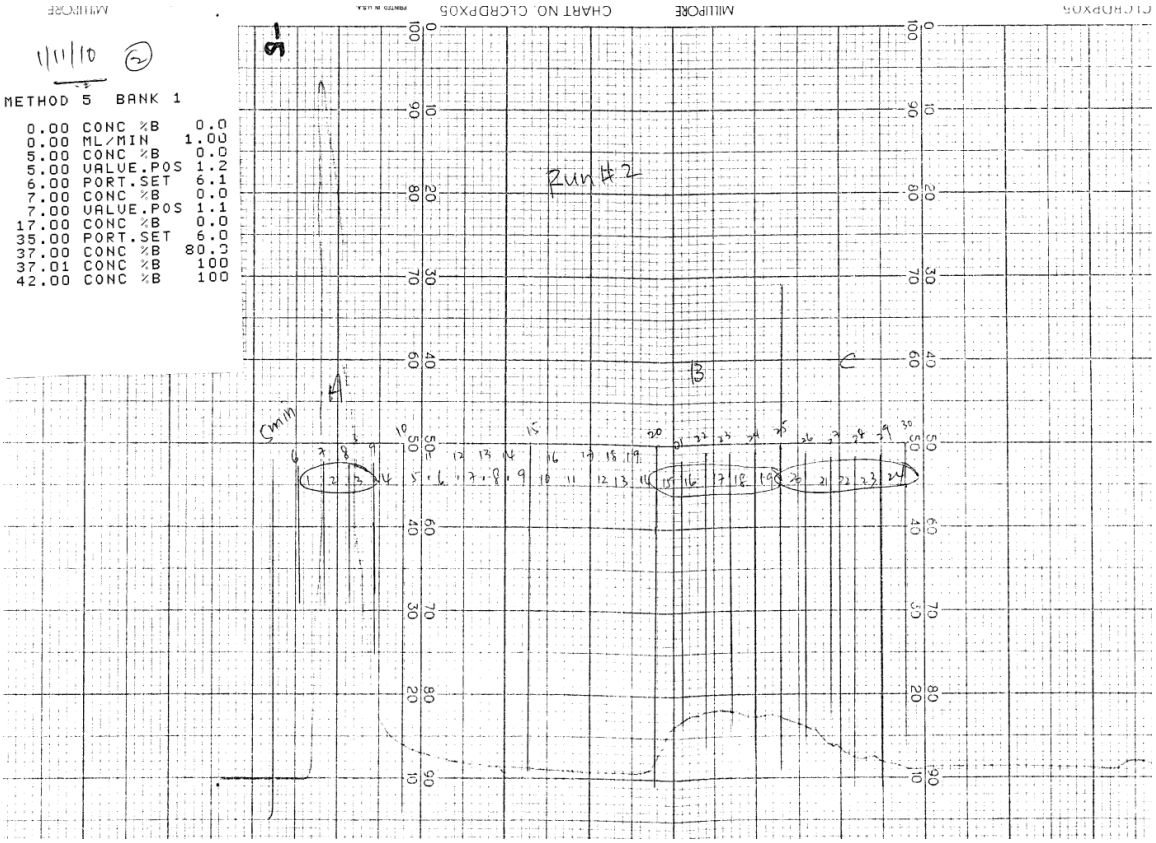



Figure 4.1. FPLC profile of whole saponin extract.

Fractions were then analyzed with High Performance Liquid Chromatography (HPLC). The HPLC profile of whole saponin extract (Figure 4.2) shows majority of the saponin variants eluted out in the first five minutes. Profile of Fraction A (Figure 4.3) shows major peaks at three and four minutes and at 20 minutes. Fraction B and C (Figure 4.4 and Figure 4.5) have similar profiles where majority of the saponin variants eluted out between 16 to 22 minutes. Fraction B

and C were collected within the same peak during FPLC, as expected the two fractions have similar HPLC profiles.

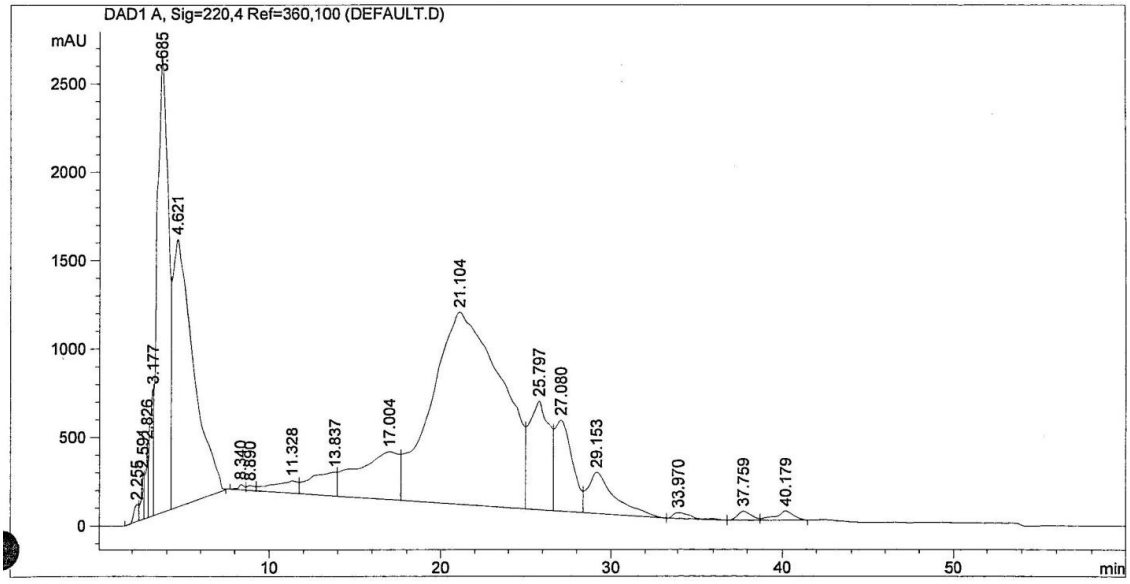



Figure 4.2. HPLC profile of whole saponin extract.

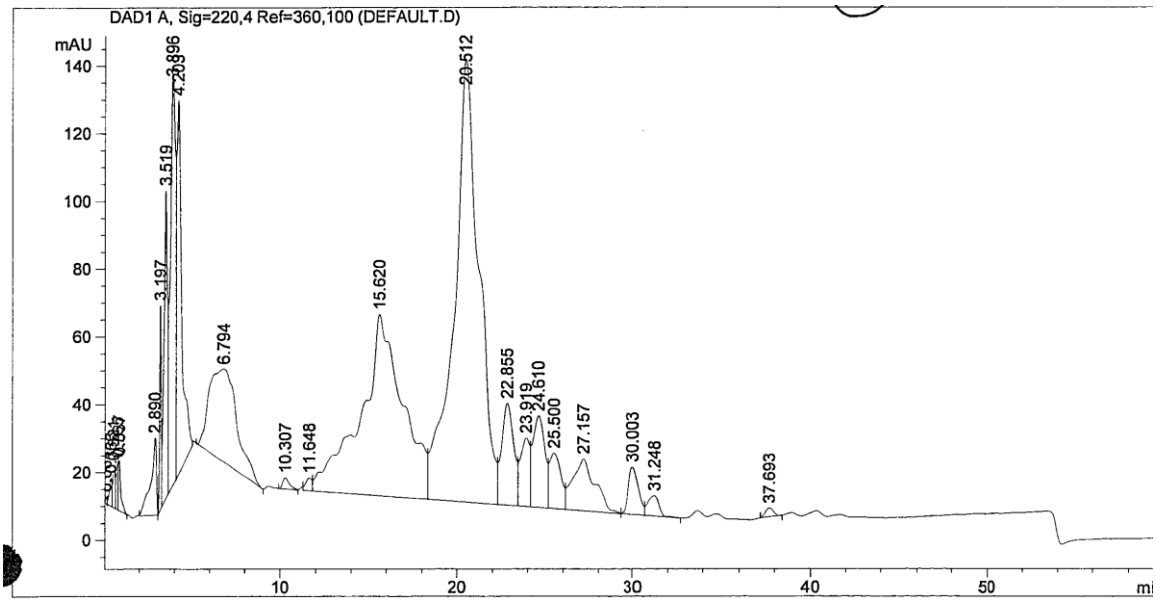



Figure 4.3. HPLC profile of Fraction A.

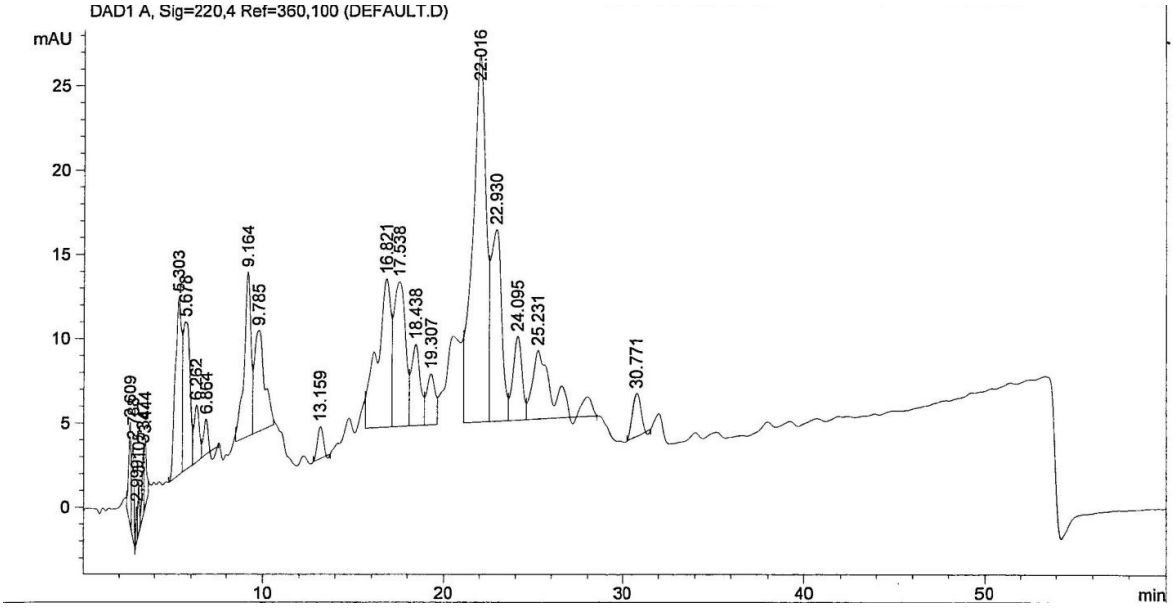



Figure 4.4. HPLC profile of Fraction B.

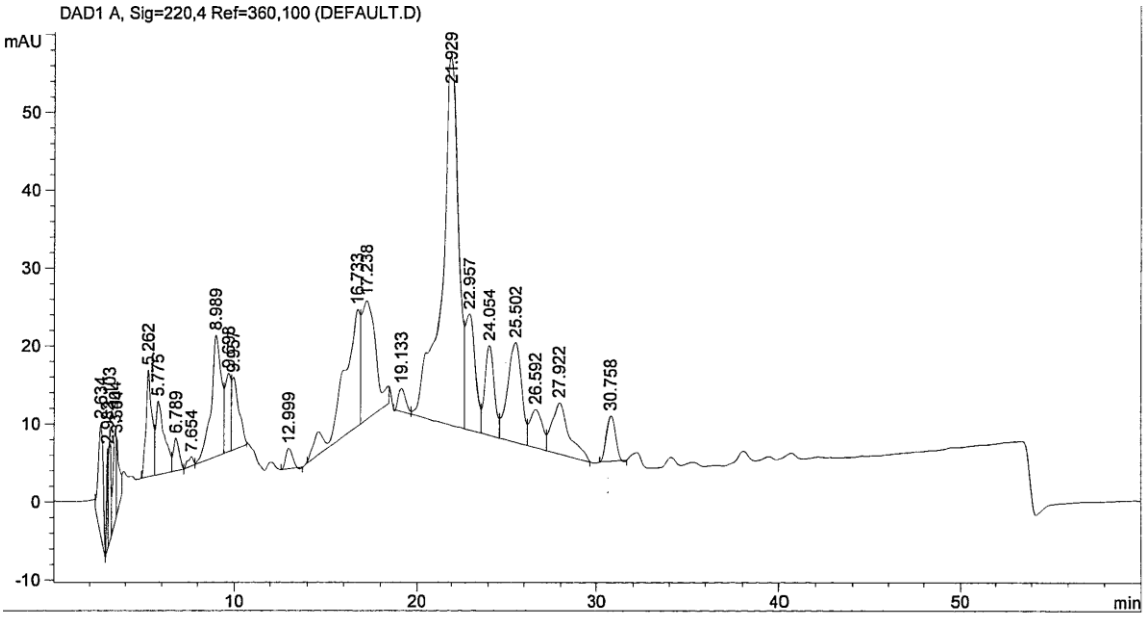



Figure 4.5. HPLC profile of Fraction C.

*4.7.1 Cytotoxicity Assay*

MA104 cells were seeded and incubate overnight, fractions were added to MEM containing 5% FBS and 1% P/S. For initial testing, ten-fold serial dilutions were prepared;

beginning at initial concentration of one mg/ml. Plates were incubated for 96 hours. To determine viability, the number of dead and living cells were counted under a microscope, a total of 500 cells were counted in each well. It was shown that concentration of saponin fractions at 0.1 mg/ml or higher results in complete cell death, while less than 20 cells out of 500 cells counted were dead at 0.01 mg/ml of saponin fractions (Table 4.36).

Table 4.36. Initial cytotoxicity assay for different fractions.

| Concentration (mg/ml) | Fraction A | Fraction B | Fraction C | Whole Saponin | Control |
|---|---|---|---|---|---|
| 1 | All dead | All dead | All dead | All dead | 19±3.181 |
| 0.1 | All dead | All dead | All dead | All dead | |
| 0.01 | 13.67±5.840 | 13.33±4.409 | 8±1.154 | 18±2.081 | |

. The number of dead cells out of 500 cells counted.

To determine the critical concentration toxic to cells, further dilutions were prepared between 0.1 mg/ml and 0.01 mg/ml. All three fractions show higher toxicity when compared to the whole saponin (Table 4.37). Despite Fraction B and C looked almost identical in the HPLC profile, there was indeed difference in cytotoxicity with Fraction C being the most toxic out of all the fractions tested (Table 4.37). ANOVA showed that there was significant difference in cytotoxicty among fractions and whole saponin at 0.1 mg/ml (Table 4.38), but Tukey's post hoc test shows that there was no significant difference between the cytotoxicity assay for different fractions at 0.01 mg/ml when compared to control (Table 4.39). Further studies on antivirial activities of different fractions against RRV were performed at concentration of 0.01 mg/ml – this included plaque reduction assay and inoculation of newborn mice

Table 4.37. Further cytotoxicity assay for different fractions.

| Concentration (mg/ml) | Fraction A | Fraction B | Fraction C | Whole Saponin | Control |
|---|---|---|---|---|---|
| 0.1 | All dead | All dead | All dead | All dead | 10.5±0.645 |
| 0.075 | All dead | All dead | All dead | All dead | |
| 0.05 | All dead | All dead | All dead | 89.75±5.779 | |
| 0.025 | 209±8.684 | 56±3.391 | All dead | 35.75±6.115 | |
| 0.01 | 11.75±3.326 | 8.5±2.661 | 8.5±2.175 | 8.25±1.109 | |

The number of dead cells out of 500 cells counted.

Table 4.38. ANOVA for cytotoxicity assay for different fractions at 0.01 mg/ml and control.

| Variables: 5 Levels | | | | | |
|---|---|---|---|---|---|
| Treatment: | Control | Fraction A | Fraction B | Fraction C | Whole Saponin |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 292.700 | 4 | 73.17 | 3.730 | 0.027 |
| Error | 294.250 | 15 | 19.617 | | |

Table 4.39. Tukey's post hoc test for cytotoxicity assay.

| Treatment | Treatment | Difference | *P* value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| A | B | 3.250 | 0.834 | -6.421 | 12.921 |
| A | C | -7.000 | 0.220 | -16.671 | 2.671 |
| A | WholeSaponin | 3.500 | 0.795 | -6.171 | 13.171 |
| **A** | **Control** | **1.250** | **0.994** | **-8.421** | **10.921** |
| B | C | -10.250 | 0.035 | 19.921 | -0.579 |
| B | WholeSaponin | 0.250 | 1.000 | -9.421 | 9.921 |
| **B** | **Control** | **-2.000** | **0.966** | **-11.671** | **7.671** |
| C | WholeSaponin | 10.500 | 0.030 | 0.829 | 20.171 |
| **C** | **Control** | **8.250** | **0.113** | **-1.421** | **17.921** |
| **WholeSaponin** | **Control** | **2.250** | **0.949** | **-7.421** | **11.921** |

Data for different fractions at 0.01 mg/ml and control. *P* values indicating significant differences at the 5% level are in bold.

*4.7.2 Plaque Reduction Assay*

From the previous section, different fractions displayed same cytotoxicity as the control at 0.01 mg/ml. Plaque reduction assay was carried out to measure the antiviral activity of the saponin fractions against rhesus rotavirus in MA104 cells at this concentration. 0.01 mg/ml of saponin fractions were added when serial dilutions of RRV were prepared. Plaque reduction was calculated by comparing virus titers between the control (where no saponin was added) and different saponin fractions at concentration of 0.01 mg/ml. For whole saponin (original extract before fractionation), virus titer was reduced 35 folds from $5.57x10^7$ to $1.58x10^6$. Out of the three fractions, fraction C has the greatest antiviral activity where it had a plaque reduction of five folds (Table 4.40). Same interesting result was observed between Fraction B and C. Despite the similarity of HPLC profile, Fraction C was able to reduce plaque formation more efficiently than Fraction B (5.6 folds vs. 1.7 folds). Combinations of different saponin fractions were also evaluated. Fraction A and fraction C has the greatest antiviral activity of eight folds of

plaque reduction (Table 4.40). Combining all three fractions served as a positive control to test if any critical activities were lost during fractionation. Fraction A+B+C gave 22 folds reduction in plaque formation, which was much greater than any individual or combination of fractions. However, antiviral activity was still lower compared to whole saponin (35 folds) which could imply that some critical activities were lost during fractionation.

Table 4.40. Plaque reduction assays for different saponin fractions at 0.01 mg/ml.

| Saponin | Virus Titer (± std. error) | Plaque Reduction (compared to control) |
|---|---|---|
| Control (no saponin) | $5.57 \pm 0.628 \times 10^7$ | N/A |
| Whole saponin | $1.58 \pm 0.375 \times 10^6$ | 35.3 folds |
| Fraction A | $1.48 \pm 0.174 \times 10^7$ | 3.7 folds |
| Fraction B | $3.20 \pm 0.384 \times 10^7$ | 1.7 folds |
| Fraction C | $9.86 \pm 0.208 \times 10^6$ | 5.6 folds |
| Fraction A+B | $1.81 \pm 0.370 \times 10^7$ | 3.1 folds |
| Fraction B+C | $1.18 \pm 0.173 \times 10^7$ | 4.7 folds |
| Fraction A+C | $6.68 \pm 1.372 \times 10^6$ | 8.3 folds |
| Fraction A+B+C | $2.49 \pm 0.245 \times 10^6$ | 22.3 folds |

*4.7.3 Effect of Saponin Fractions on 500 PFUs RRV*

Newborn mice were pretreated with saponin fractions at 0.01 mg/ml for two days, followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days. Repeated measures ANOVA of weight of mice inoculated with different saponin fractions at 0.01 mg/ml for seven days and control (Table 4.42) shows that there was significant difference among groups ($P$ value < 0.001). Repeated measures ANOVA of weight between control and fractions A and B show no significant difference in weight. However, mice inoculated with fraction C and whole saponin for seven days weighed significantly higher than that recorded for the control group (Table 4.41). At 0.01 mg/ml, whole saponin extract reduced rotavirus induced diarrhea from 75% to 45% when compared to mice inoculated with 500 PFUs RRV (Table 4.43). Interesting, Fractions A and C worked just as efficiently as whole saponin at concentration of 0.01 mg/ml, and reduced rotavirus induced diarrhea from 75% to 50% and 46% respectively at 500 PFUs rotavirus. Fraction B only reduced the diarrhea from 75% to 72%. This agrees with *in vitro* data where Fraction B has the least antiviral activity out of three fractions

and whole saponin during plaque reduction assays. However, rotavirus induced diarrhea score was not significantly different (Table 4.44) when compared between whole saponin with different saponin fractions against 500 PFU RRV. Repeated measures ANOVA of weight (Table 4.46) show that there was significant difference among groups (*P* value < 0.001). Repeated measures ANOVA of weight between different saponin fractions and control show significant difference among weight except between group that was treated with fraction C (*P* value = 0.378), it was significantly higher than that recorded for the control group (Table 4.45).

Although neither reduction in cytoxicity or increase in antiviral activity was achieved through this fractionation method. The fact that Fraction B and C are so similar in HPLC profile, yet displaying very different effect on cells and RRV infections, differences that lie between components in Fraction B and C would definitely be helpful to hypothesize the compounds critical to cytotoxicity and antiviral activity.

Table 4.41. Average weight recorded for mice inoculated with different saponin fractions at 0.01 mg/ml.

| Days Post Treatment | Treatment | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Fraction A | | Fraction B | | Fraction C | | Saponin | | | |
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 1 | 3.369 | 0.214 | 3.321 | 0.117 | 3.538 | 0.114 | 3.229 | 0.171 | 2.674 | 0.112 |
| 2 | 4.026 | 0.249 | 4.150 | 0.141 | 4.229 | 0.150 | 4.014 | 0.182 | 3.084 | 0.134 |
| 3 | 4.595 | 0.262 | 4.966 | 0.142 | 4.896 | 0.162 | 4.673 | 0.177 | 3.635 | 0.159 |
| 4 | 5.335 | 0.290 | 5.565 | 0.152 | 5.742 | 0.204 | 5.451 | 0.175 | 4.085 | 0.177 |
| 5 | 5.995 | 0.306 | 6.175 | 0.199 | 6.527 | 0.220 | 6.111 | 0.219 | 4.700 | 0.186 |
| 6 | 7.021 | 0.347 | 6.570 | 0.193 | 7.341 | 0.274 | 6.955 | 0.262 | 5.230 | 0.194 |
| 7 | 7.545 | 0.342 | 6.952 | 0.326 | 7.871 | 0.294 | 7.649 | 0.283 | 5.752 | 0.199 |
| 8 | 7.775 | 0.354 | 7.384 | 0.282 | 8.568 | 0.344 | 8.309 | 0.297 | 6.273 | 0.196 |
| 9 | 8.459 | 0.328 | 7.935 | 0.294 | 9.072 | 0.360 | 9.065 | 0.306 | 6.716 | 0.192 |
| 10 | 8.955 | 0.337 | 8.193 | 0.289 | 9.463 | 0.382 | 9.652 | 0.393 | 7.250 | 0.204 |

Table 4.42. Repeated measures ANOVA of weight among mice.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 5 Levels | | | | | |
| Treatment: | Control | Saponin | Fraction A | Fraction B | Fraction C |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 4.160 | 4 | 1.040 | 6.933 | **<0.001** |
| Error | 10.650 | 71 | 0.150 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | Saponin | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 3.119 | 1 | 3.119 | 40.208 | **<0.001** |
| Error | 3.180 | 41 | 0.078 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | Fraction A | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 0.037 | 1 | 0.037 | 0.194 | 0.662 |
| Error | 7.752 | 41 | 0.189 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | Fraction B | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 0.082 | 1 | 0.082 | 1.276 | 0.265 |
| Error | 2.643 | 41 | 0.064 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | Fraction C | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 1.667 | 1 | 1.667 | 25.714 | **<0.001** |
| Error | 2.659 | 41 | 0.065 | | |

For mice inoculated with different saponin fractions at 0.01 mg/ml. *P* values indicating significant differences at the 5% level are in bold.

Table 4.43. Rate of rotavirus induced diarrhea in newborn mice.

| Treatment | Number of Mice | | | |
|---|---|---|---|---|
| | Tested | Diarrhea | No Diarrhea | % that developed diarrhea |
| 500 PFUs RRV for five days | 41 | 31 | 10 | 75.610 |
| Whole saponin with 500 PFUs RRV | 11 | 5 | 6 | 45.455 |
| Fraction A with 500 PFUs RRV | 10 | 5 | 10 | 50.000 |
| Fraction B with 500 PFUs RRV | 11 | 8 | 3 | 72.727 |
| Fraction C with 500 PFUs RRV | 15 | 7 | 8 | 46.667 |

Mice were pretreated with saponin fractions at 0.01 mg/ml for two days, followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days.

Table 4.44. Mouse diarrhea score with standard error in newborn mice.

| Treatment | Fraction A | Fraction B | Fraction C |
|---|---|---|---|
| | 0.5±0.167 | 0.727±0.141 | 0.467±0.133 |
| Whole saponin | 0.455±0.157 | 0.455±0.157 | 0.455±0.157 |
| *P* value | 0.839 | 0.204 | 0.952 |

Mice were pretreated with saponin fractions at 0.01 mg/ml for two days, followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days. Diarrhea was compared using a twopoint scale (0, no diarrhea; 1, with diarrhea). Statistical analysis was done by Mann-Whitney nonparametric test. *P* values indicating significant differences at the 5% level are in bold.

Table 4.45. Average weight recorded for newborn mice.

| Days Post Treatment | Treatment | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (a) | | (b) | | (c) | | (d) | | | |
| | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. | Avg. Wt. | Std. Err. |
| 1 | 3.210 | 0.160 | 3.314 | 0.152 | 3.367 | 0.102 | 2.855 | 0.092 | 2.674 | 0.112 |
| 2 | 3.920 | 0.186 | 3.957 | 0.173 | 4.103 | 0.128 | 3.580 | 0.103 | 3.084 | 0.134 |
| 3 | 4.439 | 0.229 | 4.506 | 0.211 | 4.873 | 0.117 | 4.054 | 0.120 | 3.635 | 0.159 |
| 4 | 5.166 | 0.273 | 5.209 | 0.211 | 5.749 | 0.128 | 4.750 | 0.144 | 4.085 | 0.177 |
| 5 | 5.903 | 0.301 | 5.935 | 0.221 | 6.560 | 0.161 | 5.305 | 0.137 | 4.700 | 0.186 |
| 6 | 6.657 | 0.385 | 6.746 | 0.247 | 7.443 | 0.194 | 5.903 | 0.160 | 5.230 | 0.194 |
| 7 | 7.137 | 0.515 | 7.387 | 0.266 | 8.144 | 0.245 | 6.209 | 0.278 | 5.752 | 0.199 |
| 8 | 7.865 | 0.532 | 7.980 | 0.314 | 8.965 | 0.228 | 6.781 | 0.278 | 6.273 | 0.196 |
| 9 | 8.438 | 0.575 | 8.591 | 0.353 | 9.736 | 0.252 | 7.286 | 0.288 | 6.716 | 0.192 |
| 10 | 8.864 | 0.679 | 9.070 | 0.394 | 10.431 | 0.314 | 7.663 | 0.307 | 7.250 | 0.204 |

Mice were pretreated with saponin fractions at 0.01 mg/ml for two days, followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days. Control represented group treated with water for seven days. Treatment (a) represented group treated with whole saponin. Treatment (b) represented group treated with Fraction A. Treatment (c) treated with Fraction B. Treatment (d) treated with Fraction C.

Table 4.46. Repeated measures ANOVA of weight among mice.

| | | | | | |
|---|---|---|---|---|---|
| Variables: 5 Levels | | | | | |
| Treatment: | Control | (a) | (b) | (c) | (d) |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 6.466 | 4 | 1.616 | 12.358 | **<0.001** |
| Error | 9.658 | 74 | 0.131 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (a) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 1.066 | 1 | 1.066 | 7.836 | **0.008** |
| Error | 5.577 | 41 | 0.136 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (b) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 1.189 | 1 | 1.189 | 13.881 | **0.001** |
| Error | 3.425 | 40 | 0.086 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | (c) | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 5.651 | 1 | 5.651 | 89.163 | **0.000** |
| Error | 2.599 | 41 | 0.063 | | |
| Variables: 2 Levels | | | | | |
| Treatment: | Control | Fraction C | | | |
| Source | SS | df | Mean Squares | F-Ratio | *P* value |
| Treatment | 0.064 | 1 | 0.064 | 0.792 | 0.378 |
| Error | 3.641 | 45 | 0.081 | | |

Mice were pretreated with saponin fractions at 0.01 mg/ml for two days, followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days. Control represented group treated with water for seven days. Treatment (a) represented group treated with whole saponin. Treatment (b) represented group treated with Fraction A. Treatment (c) treated with Fraction B. Treatment (d) treated with Fraction C.

# CHAPTER 5

# DISCUSSION

Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for about 600-850,000 deaths, representing approximately 5% of all deaths in children younger than five years of age worldwide (11). In this study, we evaluated the antiviral activity of a natural extract, *Quillaja* saponin against rotavirus in a mouse model. The model uses newborn BALB/c mice that when exposed to rhesus rotavirus, develop diarrhea within 1 to 5 days. Previous studies have demonstrated that in a heterlogous system (viral infection established in host with virus isolated from another species, for examples, infecting newborn mice with simian rotavirus SA11) (87, 88) a higher infective dose of 1000 PFUs are required compared to one to 100 PFUs in a homologous system (viral infection established in host with virus isolated from same species, for example, infecting newborn mice with mouse rotavirus, also called Epizootic Diarrhea of Infant Mice or EDIM). I was able to establish the heterlogous system of infecting newborn mice with RRV here at UTA with half of PFUs, over 70% of the newborn mice developed diarrhea when inoculated with 50µl of $1x10^4$ PFU/ml (which is equivalent to 500 total PFUs). The amount of virus required to induce diarrhea in our study is lower than published reports employing a single virus exposure protocol. To better model the natural exposure of children to rotavirus, the result of fecal-contaminated water supplies, I 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 might occur in a family between brothers and sisters.

*Quillaja* saponins 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. The $CCIC_{50}$ values of different cell lines were at 0.9-1 mg/ml. 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 50 µl of saponin extract at a concentration of 0.5 mg/ml for seven consecutive days had a mortality rate of less than 50% ($LD_{50} \geq 0.5$ mg/ml). Growth of newborn mice was unaffected at 0.5 mg/ml or less compared to the control. Long term observation and breeding of 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 with saponin began. Exposures were initiated using 50 µl of extract at a concentration of 0.5 mg/ml saponin extract. This exposure is equivalent to 1.67 mg/kg body weight. The Food and Agricultural Organization of the United Nation and World Health Organization suggested that the Acceptable Daily Intake (ADI) of quillaja extract is 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 occasion. Based on these current human exposures it appears that human exhibit a higher tolerance for saponin extract than mice.

Saponins from a variety of sources have demonstrated antiviral activity against viruses of different types including herpes simplex virus, poliovirus, vaccinia virus, potato virus X, and others (3, 17, 18, 19, 20, 21, 46). It was suggested by some researchers that the antiviral activity was not due to a direct virucidal effect but appeared to involve an inhibition of virus-host cell attachment. *Quillaja* extract has been previously shown to exhibit antiviral activity against seven different viruses *in vitro* (7). In this study, exploring for antiviral activity against rotavirus, I found that *Quillaja* saponins prevent rhesus rotavirus infection of MA104 cells at concentrations

of 1mg/ml (w/v) or greater and that the treatment of cells with *Quillaja* extract for one hour renders them resistant to rotavirus infection at concentrations as low as 0.0001 mg/ml.

Based on previous research *in vitro* (7), pretreating of MA104 cells with saponin extract is more efficient in 'blocking' rotavirus infection than direct inactivation of the virus.

Using our rotavirus induced diarrhea model, newborn mice recovered from RRV infection within five days post beginning of treatment. In this study, newborn mice were pretreated with saponin extract before rhesus rotavirus was inoculated for five consecutive days. At 0.3 mg/ml of saponin extract, rotavirus induced diarrhea was significantly reduced from 79% to 11% when mice were exposed to 500 PFUs RRV for five consecutive days. However rotavirus induced diarrhea was only reduced from 88% to 57% when mice were exposed to 5,000 PFU. When mice were exposed to 50,000 PFUs of RRV, induced diarrhea was only reduced when mice were treated with twice as much saponin extract at 0.6 mg/ml. It was 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 (7). The saponin extract may cause a reversible modification of the cell membrane or modification of the cellular endocytosis process. This observation can be explained by the possibility that not all cells are being "treated/coated/modified" by the saponin extract at low concentration (0.3 mg/ml), hence at higher inoculums (5,000 PFUs), RRV can still establish an infection and induce diarrhea in over half of the individuals. And at even higher inoculums (50,000 PFUs), a higher concentration (0.6 mg/ml) of saponin extract would be needed to "block" virus infection. However, saponin extract is quite toxic to the newborns when the concentration is doubled to 0.6 mg/ml, therefore attempts in purification of the saponin extract to reduce toxicity would be important.

In this study, the initial experimental design employed pretreating newborn mice with saponin extract for 48 hours before being inoculated with RRV. Rotavirus induced diarrhea was most efficiently inhibited at 500 PFUs of RRV with 0.3 mg/ml of saponin extract. With the same

rotavirus and saponin extract concentrations, different parameters were tested where newborn mice were pretreated one day or not at all. The results showed that pretreatment were not important and saponin extract given at the same time when RRV was inoculated was sufficient to block rotavirus infection. This may be critically important when applying these findings to children. Another group of newborn mice were given saponin extract only for 48 hours before RRV was inoculated. 42% of mice treated under this condition developed diarrhea, but it should be noted that diarrhea developed much later in these animals, five to six days, versus what is normally seen when diarrhea develops two to three days after exposure to virus. It appears from these observations that the saponin extract was able to "block" RRV infection for 48 to 72 hours after administration.

For a potential therapeutic agent, it is important to calculate the therapeutic index which is a comparison of the amount of a compound that causes the therapeutic effect to the amount that causes death. A therapeutic index is the lethal dose of a drug for 50% of the population ($LD_{50}$) divided by the minimum effective dose for 50% of the population ($ED_{50}$). In this study, administration of 0.75 mg/ml of saponin extract killed ≥ 85% of the newborn mice, and 0.5 mg/ml killed 30%. With linear regression, this gives saponin extract a $LD_{50}$ of 0.65 mg/ml in newborn mice over a seven day period. At 500 PFUs of rhesus rotavirus, 0.03 mg/ml of saponin extract was able to reduced diarrhea from 79% to 9% (equivalent to 88% reduction in diarrhea rate) while 0.25 mg/100ml of saponin extract was able to reduced diarrhea from 79% to 27% (equivalent to 65% reduction in diarrhea rate). With linear regression, this gives saponin extract $ED_{50}$ of 0.22 mg/ml over a seven day period. In this sense, saponin extract gives a therapeutic index of 2.95 at 500 PFUs of RRV over a seven days period. Purification to reduce toxicity should greatly improve the potential use for saponin extract as therapeutic agent.

To explore the possibility of reducing the cytoxicity but retaining the antiviral activity, the saponin extract was fractionated through Fast Performance Liquid Chromatography. Cytotoxicity assays showed that the toxicity was not reduced in any individual fraction compared

to the whole extract. Using a plaque reduction assay and *in vivo* testing of fractions in mice, the fractions also showed less antiviral activity when compared to whole extract. Individual saponins were not separated effectively but grouped when using ion exchange chromatography because of the tendency of saponins to form mixed micelles (61). It is possible that individual saponins make retain antiviral activity and demonstrate reduced toxicity; it is also likely that the antiviral activity is due to a "collection" of individual saponins and fractionation will simply reduce the activity. Effective separation of individual saponins requires the use of solvent/water systems that solubilize the amphiphilic saponins as monomers so that the formation of mixed micelles does not interfere with separation. This approach has been attempted, but we were unable to obtain sufficient amounts of individual fractions to test. A more concentrated sample or a preparative reverse phase chromatography system that allows large volume injection should be used for further research. Previous studies on saponin extract showed that triterpenoid saponins have similar immune stimulating activities but significant differences in their toxicity (55). Saponins QS-7 and QS-21 showed no or very low toxicity in mice, while QS-18 being the highly toxic (56). Further studies will address if the same is true for antiviral activity.

One of the issues that we ran into throughout this study was to accurately estimate the mortality rate of newborn mice from saponin extract administration. Cannibalism of newborns in mice is a fairly common response for a lactating female with more newborns than she can feed and may lead to infanticide that allows her to reduce litter size to a sustainable level (98). Human interaction with newborns, environmental factors like temperature of researcher's hand, noise in the laboratory can trigger stress in females that tends to also increase mortality (61). Little is known about what triggers infanticide and factors may vary among different females. In addition, administration of treatment in a volume of 50 µl in a three grams newborn mouse is equivalent to 170 ml in a newborn baby of three kg. Administration of the treatment in newborn mice can result in forcing water down the trachea and "drown" the subject. All suggested means to minimize disturbance and slow administration of treatment have been executed but

occasional casualties were inevitable and difficult to estimate and explain. Most of the time, it was difficult to estimate the number of mice died from experimental errors, cannibalism, or from the treatment because many times newborn mice were eaten by the mother and no trace of the body could be found to infer on the death cause.

Alternative animal models with larger animals or animals that have a longer susceptibility period can avoid casualties that may obscure the data. Different animal models like swine piglet or rabbit to test the effect of saponin extract on rhesus rotavirus. Gnotobiotic piglets closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity. Gnotobiotic piglets are susceptible until at least six weeks of age to infection and disease with several human rotavirus strains (102, 103). 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 (58). Rabbit model can also be maintained at a modest cost compared with large animal models. With these animal models, 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

*Quillaja* saponin can block virus infection either by disrupting cell membrane environment and/or virus envelope and hence, inhibit attachment. There is strong evidence that saponin extract is able to “block” rotavirus infection by coating target cells and reduce rotavirus induced diarrhea in newborn mice. Future research that measures virus shedding and antibody production in newborn mice will give more insights in to what extend 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 have been “vaccinated” during this treatment

and will remain resistant to future challenges by rotavirus. This will be very important in humans. Should saponin treatment greatly reduce rotavirus induced diarrhea in individuals while allowing a small amount of virus for infection that trigger an immune response. In this way, antibodies are produced to protect individual from future challenges by rotavirus while symptom (diarrhea) is inhibited or significantly reduced.

Previous research also shows the effect of saponin extract on other viruses (7). Saponin extract works in inhibiting host cell-virus attachment. Efficient direct inactivation of enveloped viruses like HIV, Herpes simplex virus was observed at low concentration of saponin extract *in vitro*. Saponin extract definitely has the potential as one of the antiviral agents.

There is no specific treatment of rotavirus infection besides rehydration of the infected individuals. Although the 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. It has been shown that oral administration of saponin with water is able to reduce rotavirus induced diarrhea in mice. The extract is cheap to produce, soluble in water, pH stable from pH 2 to 11, and is already approved as food additive. I believe that saponin has great potential to offer the opportunity to prevent rotavirus infection in humans at a much reduced cost compared to the current vaccination program available.

# APPENDIX A

## INFORMATION ON ANIMAL SUBJECT: MOUSE

Scientific Name: *Mus musculus*

Class: Mammalia, Order: Rodentia

Body Temperature: 99 degrees F (36.5-38 degrees C)

Respiration Rate: 163 BPM

Weight: Adult Male 25 - 50 gm, Adult Female 25 - 50 gm, Newborn 1.0 gm

Water Consumption: 15 ml/100 gm/day

Food Consumption: 15 gm/100 gm/day

Sexual Maturity: Male 7 weeks, Female 7 weeks

Estrus Cycle: 4 - 5 days

Gestation period: 19 - 21 days

Life Span: 2 years

Avg. Litter size: 5 - 10 pups

Breeding Life: Male 8 months, Female 8 months

Age at weaning: 21 days

Recommended Temperature: 64.4-78.8 degrees F (18-26 degrees C)

Relative Humidity: 4 0 - 7 0 %

Identification Methods: ear tags, ear punch, notch

Photo Period (light/dark): 12/12

Housing Requirements: floor area/animal = <10gms – 6 sq. in., 10-15 gms – 8 sq. in., 15-25 gms – 12 sq. in, >25 gms – 15 sq. in.

Bleeding Methods: orbital sinus, cardiac puncture in anesthetized animal (terminal procedure)

Anesthesia Methods: ketamine/xylazine, isoflurene, barbiturates pentobarbital 5 mg/100 gm body weight IP

Euthanasia Methods: OD barbiturates, CO2, cervical dislocation

Problem areas & diseases: grooming, teeth (malocclusion), cannibalism, pinworms, mouse pox - ectromelia virus, sendai, mouse hepatitis virus, mycoplasmosis

APPENDIX B

GUIDELINES FOR EUTHANASIA OF RODENTS USING CARBON DIOXIDE

Guidelines are retrieved from NIH. Rodents must be euthanized by trained personnel using appropriate technique, equipment and agents. This is necessary to ensure a painless death that satisfies research requirements. Death should be induced as painlessly and quickly as possible. Upon completion of the procedure, death must be confirmed by an appropriate method, such as ascertaining cardiac and respiratory arrest or noting an animal's fixed and dilated pupils (1). Euthanasia should not be performed in the animal room. The euthanasia method must be appropriate to the species, approved in the animal study proposal and conform to the most recent Report of the AVMA Panel on Euthanasia (2). CO2 inhalation is the most common method of euthanasia used at NIH for mice, rats, guinea pigs and hamsters. A few important aspects of this procedure are:

1. The euthanasia chamber should allow ready visibility of the animals. Do not overcrowd the chamber: all animals in the chamber must be able to make normal postural adjustments.
2. Compressed CO2 gas in cylinders is the only recommended source of carbon dioxide as it allows the inflow of gas to the induction chamber to be controlled. Without pre-charging the chamber, place the animal(s) in the chamber and introduce 100% carbon dioxide at the rate of 10-20% of the chamber volume per minute so as to optimize reduction in distress. (For a 10-liter volume chamber, use a flow rate of approximately 1-2 liter(s) per minute.) After the animals become unconscious, the flow rate can be increased to minimize the time to death. Sudden exposure of conscious animals to carbon dioxide concentrations of 70% or greater has been shown to be distressful (3).
3. Animals should be left in the container until clinical death has been ensured. Unintended recovery must be prevented by the use of appropriate CO2 concentrations and exposure times or by other means.
4. Neonatal animals (up to 10 days of age) are resistant to the effects of CO2, therefore, alternative methods are recommended (4). Carbon dioxide may be used for narcosis of neonatal animals provided it is followed by another method of euthanasia (e.g. decapitation

using sharp blades). Keeping neonates warm during CO2 exposure may decrease the time to death (5).

5. If an animal is not dead following CO2 exposure, another approved method of euthanasia (e.g. decapitation) must be added while the animal is under CO2 narcosis to assure death. Please refer to Appendixes 1 and 2 of the Report of the AVMA Panel on Euthanasia (2) for additional recommended methods.

References

1. NIH Guide for Grants and Contracts. 7/17/2002, notice: OD-02-062. [http://grants.nih.gov/grants/guide/notice-files/NOT-OD-02-062.html]

2. AVMA Panel on Euthanasia. 2000 Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc 2001, 218:669-696. [http://www.avma.org/resources/euthanasia.pdf]

3. Danneman PJ, Stein S, Walshaw SO. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab Anim Sci 1997, 47:376-385.

4. Guidelines for the Euthanasia of Rodent Feti and Neonates. NIH Animal Research Advisory Committee, 2004 [http://oacu.od.nih.gov/ARAC/euthmous.pdf]

5. Klaunberg BA, O'Malley J, Clark T, Davis JA. Euthanasia of Mouse Fetuses and Neonates. Contemp Top Lab Anim Sc 2004, 43:(5) 29-34.

# APPENDIX C

## FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

FPLC Conditions:

Column: Mono Q column (50 x 5 mm)

Column Temperature: room temperature

Solvent A: 50 mM NaCl, 10 mM TrisCl in HPLC-grade water

Solvent B: 1 M NaCl, 10 mM TrisCl in HPLC-grade water

Gradient:

| Time (min) | % solvent A | % solvent B |
|---|---|---|
| 0 | 100 | 0 |
| 30 | 20 | 80 |
| 35 | 0 | 100 |

Flow rate: 1 ml/min

Detection wavelength: 220 nm

Injection volume: 600 µl

## HIGH PERFOMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC Conditions:

Column: Vydac214TP54 (4.6x250mm length, 5µm pore)

Column Temperature: 40°C

Solvent A: 0.15% trifluoroacetic acid in HPLC-grade water

Solvent B: 0.15% trifluoroacetic acid in HPLC-grade acetonitrile

Gradient:

| Time (min) | % solvent A | % solvent B |
|---|---|---|
| 0 | 70 | 30 |
| 40 | 55 | 45 |
| 45 | 70 | 30 |

Flow rate: 1 ml/min

Detection wavelength: 220 nm

Injection volume: 100 µl

APPENDIX D

GRAPHS DEMOSTRATION FOR MOTALITY RATE IN MICE WITH DIFFERENT TREATMENTS

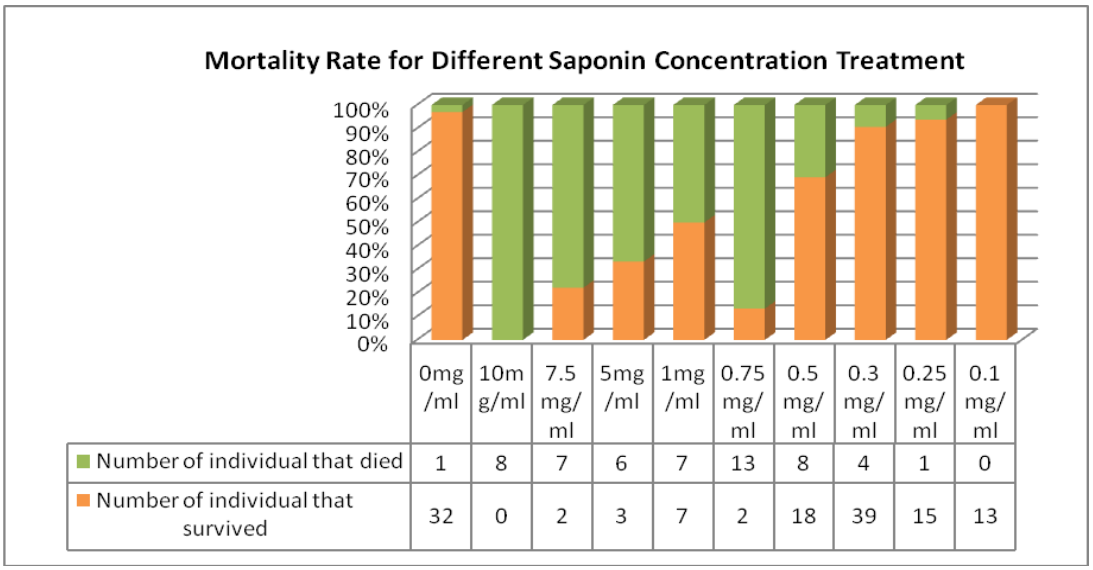



Figure D.1. Mortality rate of newborn mice inoculated with different saponin concentration.

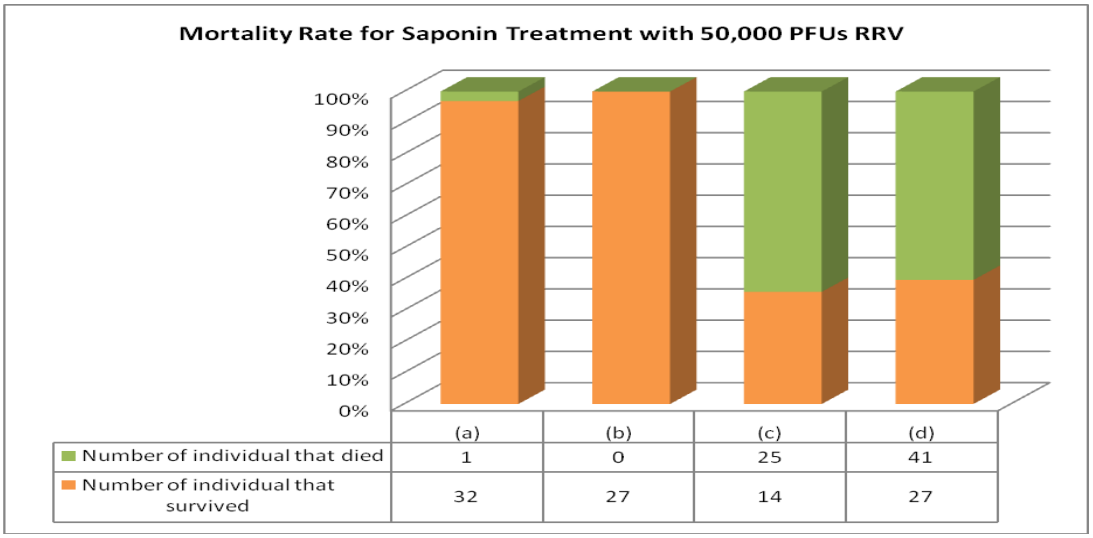



Figure D.2. Mortality rate of newborn mice pretreated with saponin extract for two days. , Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Control: inoculated with water. Treatment (a): 50,000 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.5 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV.

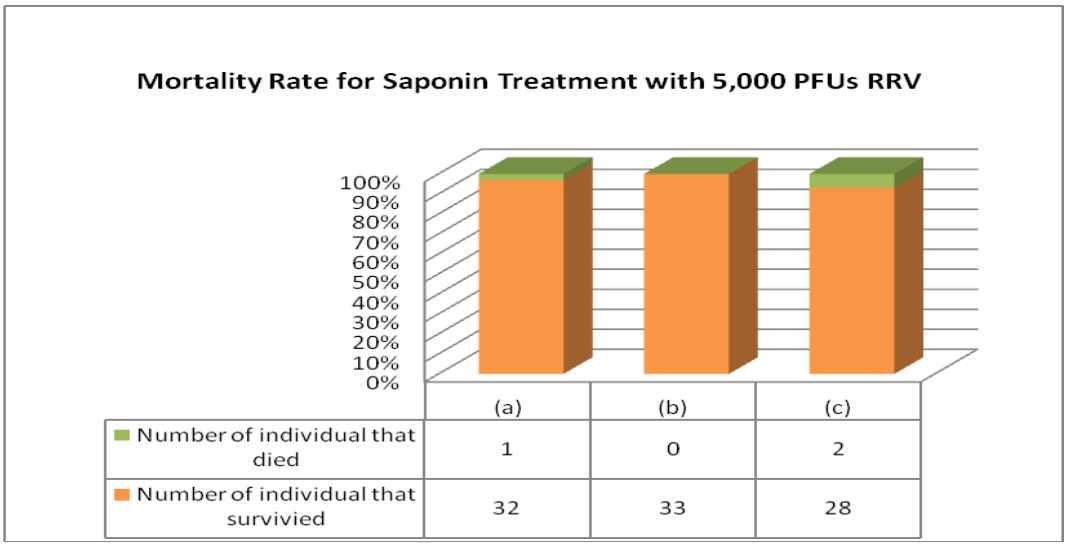



Figure D.3. Mortality rate of newborn mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml. Control: inoculated with water. Treatment (a): 5,000 PFUs RRV. Treatment (b): saponin at 0.3 mg/ml with RRV.

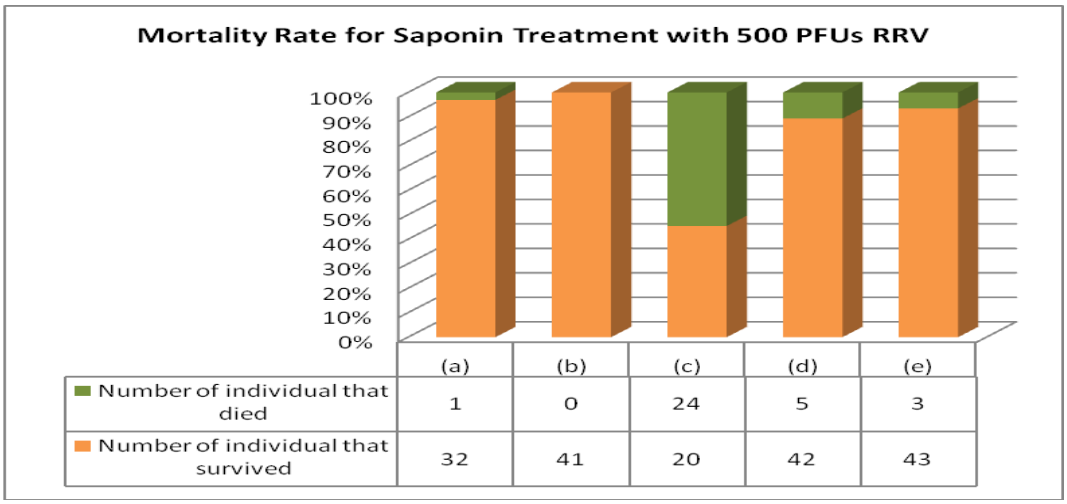



Figure D.4. Mortality rate of newborn mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Control: inoculated with water. Treatment (a): inoculated with 500 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.3 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV.

# APPENDIX E

## GRAPHS DEMOSTRATION FOR DIARRHEA RATE IN MICE WITH DIFFERENT TREATMENTS

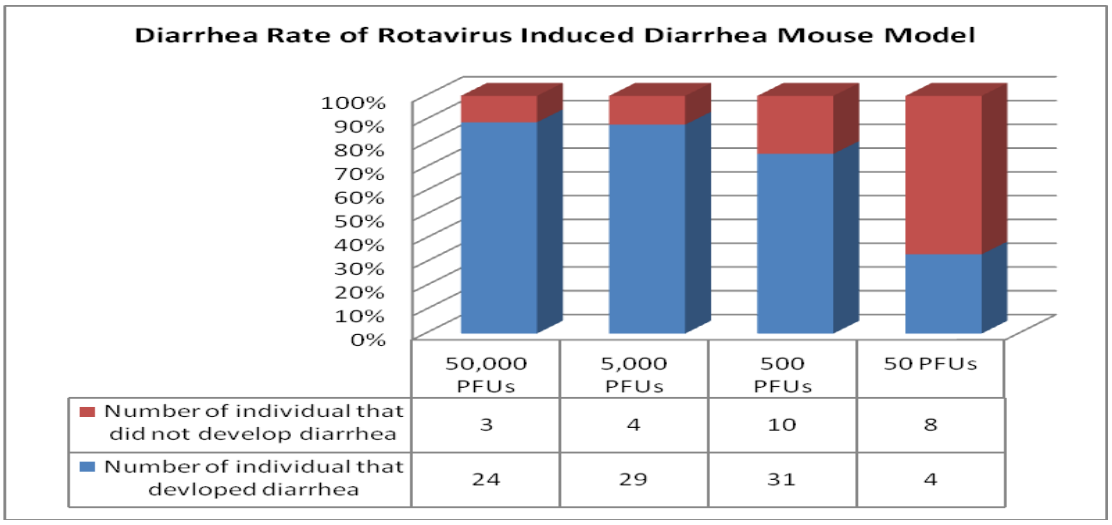



Figure E.1. Rate of rotavirus induced diarrhea in mice with different titers of RRV.

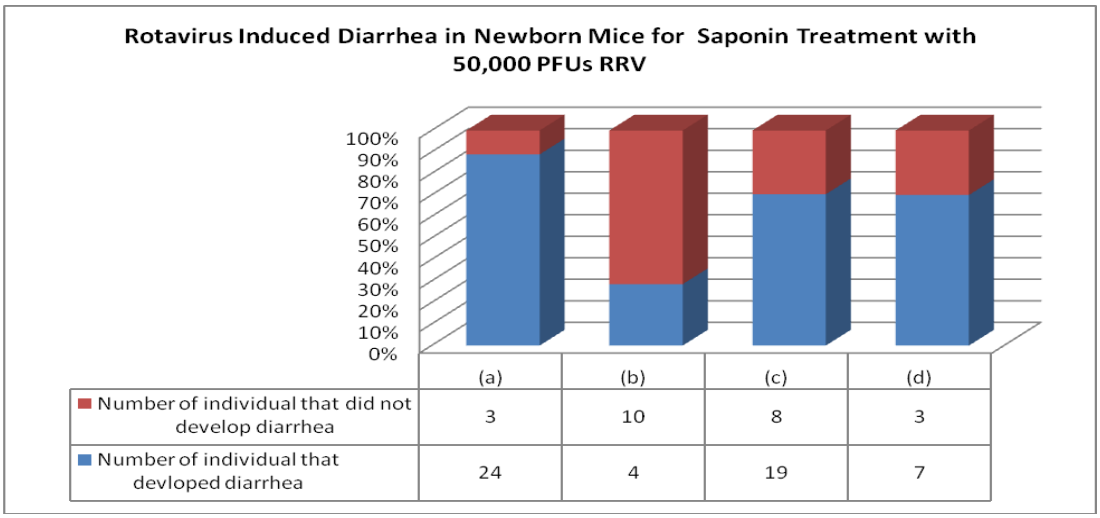



Figure E.2. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Treatment (a): 50,000 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.5 mg/ml with RRV. Treatment (d): saponin at 0.25 mg/ml with RRV.

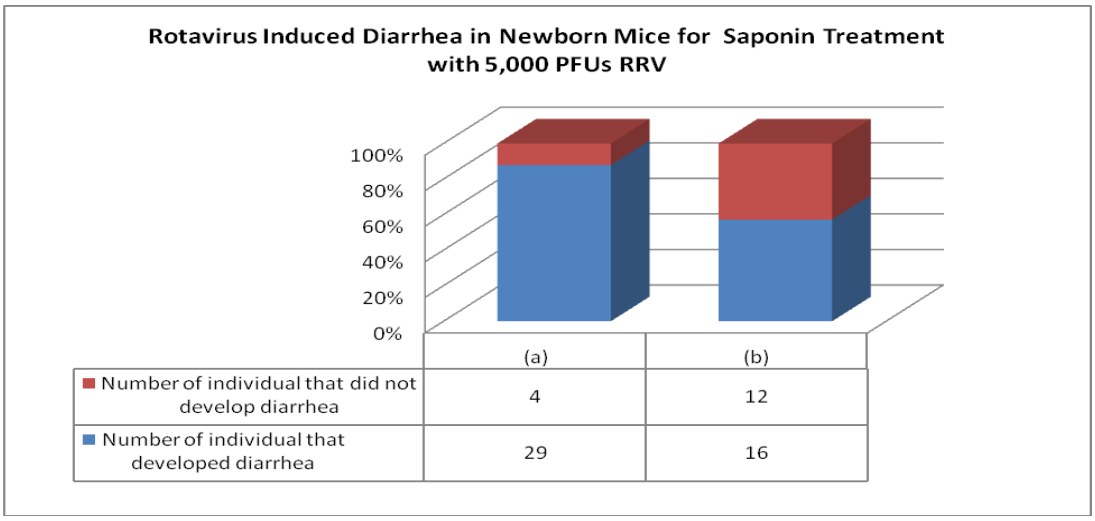



Figure E.3. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 5,000 PFUs RRV with saponin extract at 0.3 mg/ml. Treatment (a): 5,000 PFUs RRV. Treatment (b): saponin at 0.3 mg/ml with RRV.

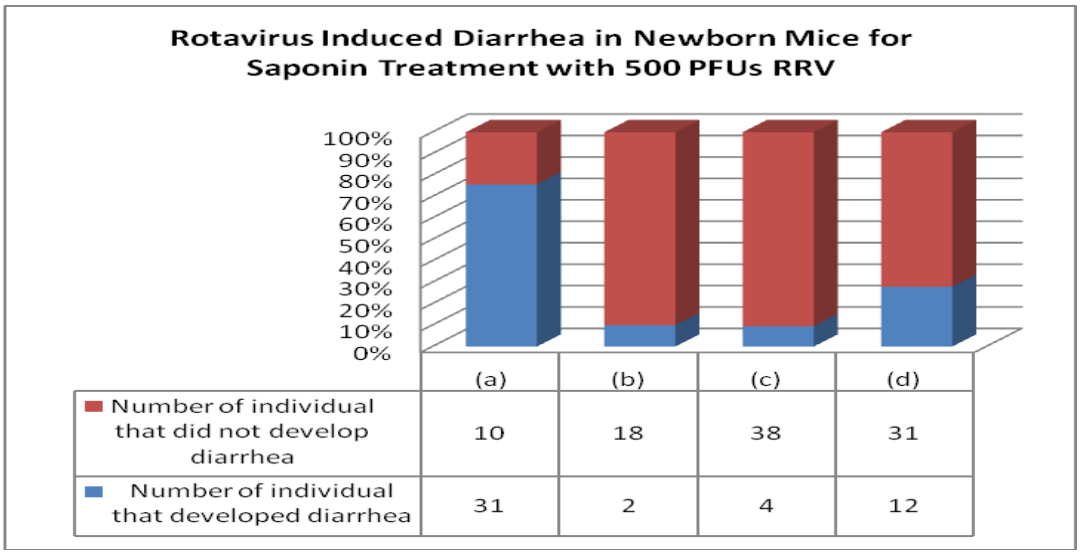



Figure E.4. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days. Treatment (a): 500 PFUs RRV. Treatment (b): saponin at 0.6 mg/ml with RRV. Treatment (c): saponin at 0.3 mg/ml with RRV Treatment (d): saponin at 0.25 mg/ml with RRV.

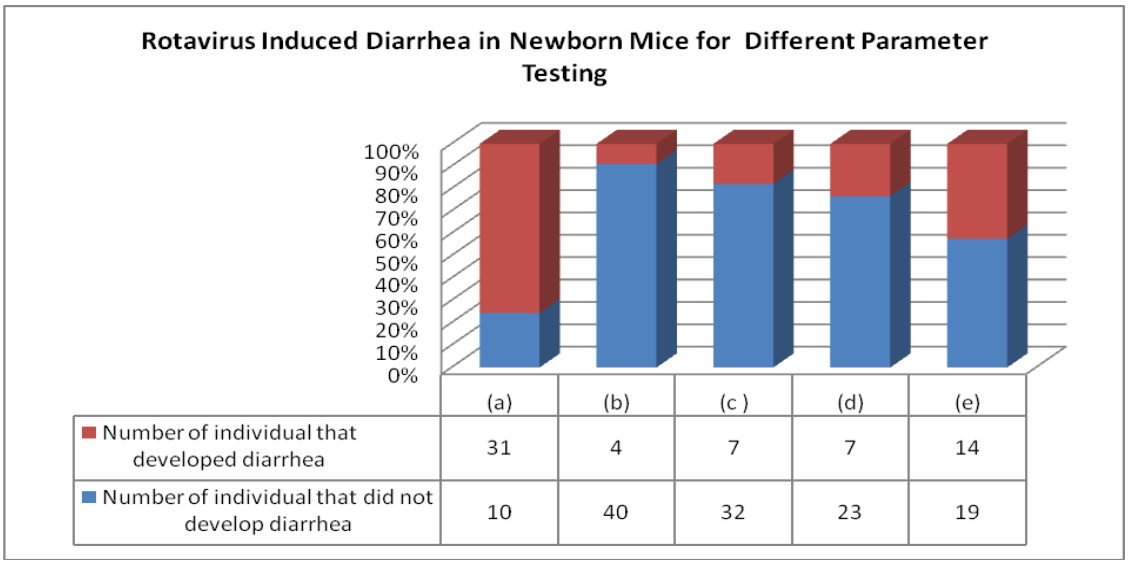



Figure E.5. Rate of rotavirus induced diarrhea in newborn mice. Different parameters tested at 0.3 mg/ml saponin extract with 500 PFUs RRV. Treatment (a): 500 PFUs RRV. Treatment (b): pretreatment of saponin for two days with RRV. Treatment (c): pretreatment of saponin for one day with RRV. Treatment (d): no pretreatment of saponin. Treatment (e): pretreatment of saponin for two days followed by RRV.

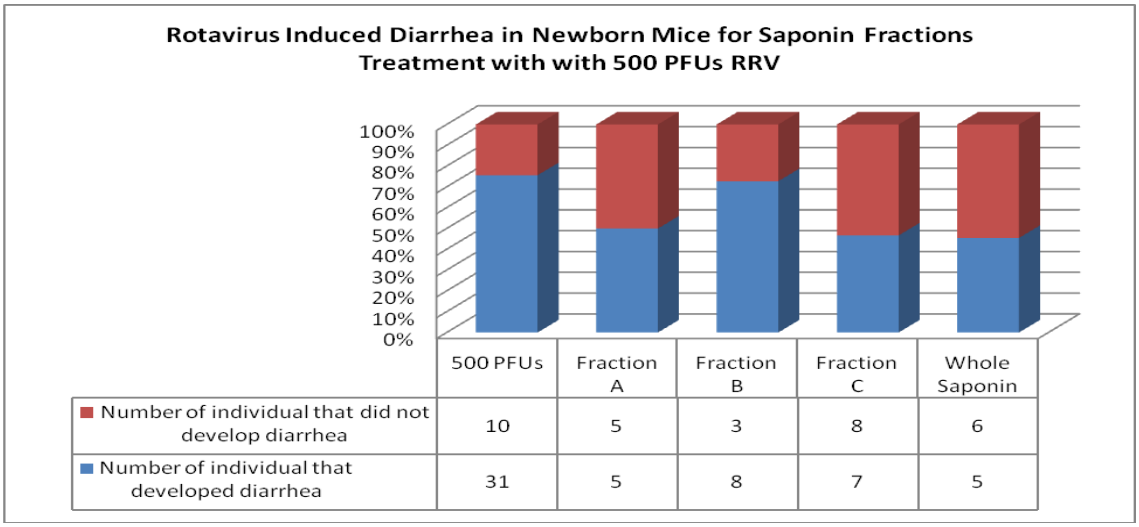



Figure E.6. Rate of rotavirus induced diarrhea in newborn mice pretreated with saponin fractions at 0.01 mg/ml for two days. Pretreatment was followed by inoculation of 500 PFUs RRV mixed with saponin fractions at 0.01 mg/ml for five days.

# APPENDIX F

## GRAPHS DEMOSTRATION FOR WEIGHT GAINED IN MICE WITH DIFFERENT TREATMENTS

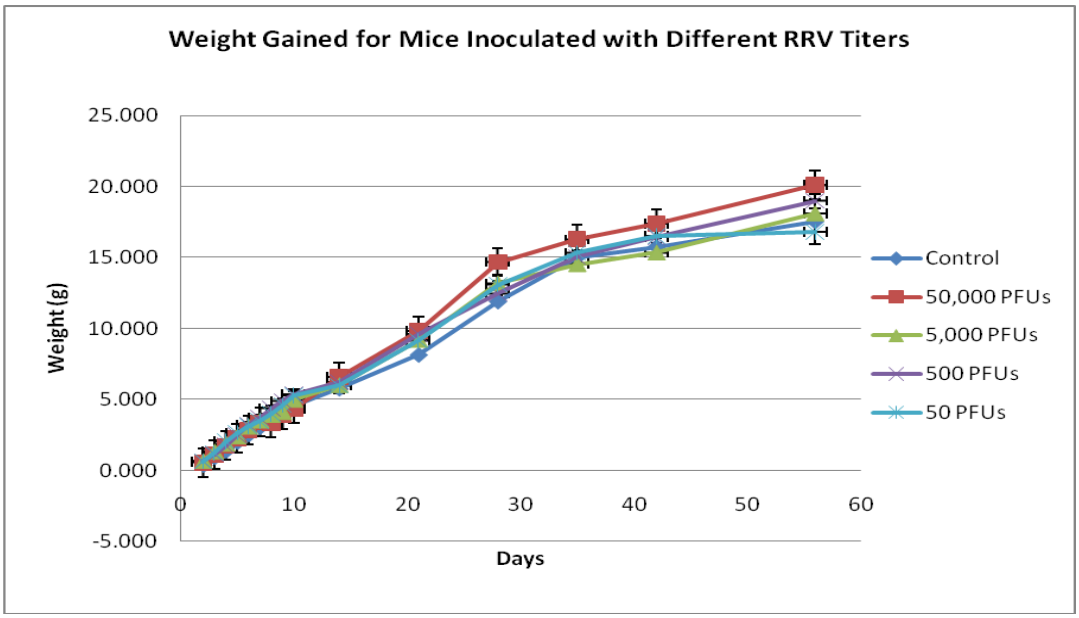



Figure F.1. Average weight gained for mice inoculated with different RRV titers.

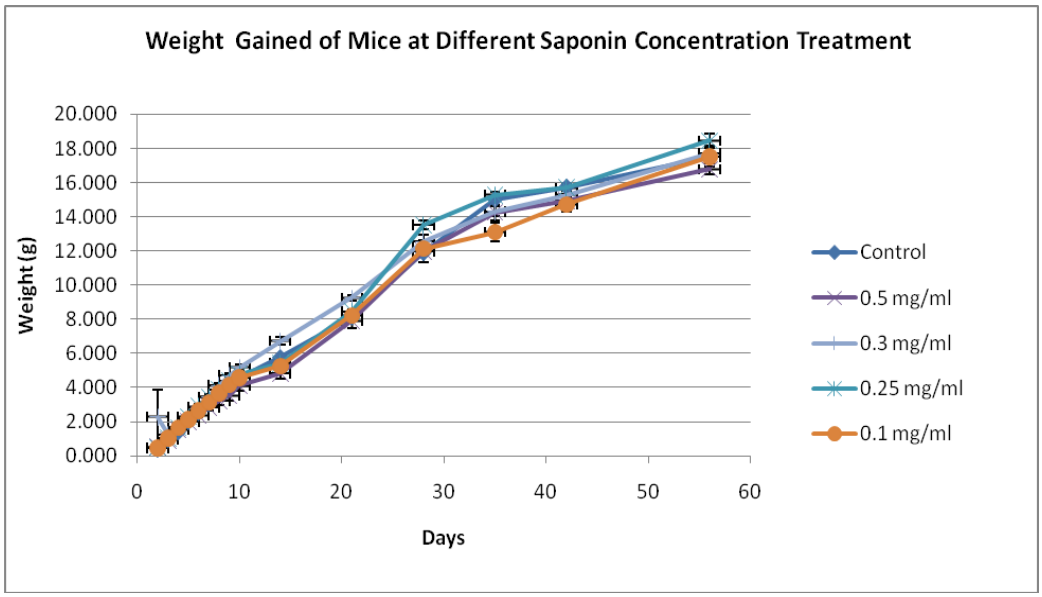



Figure F.2. Average weight gained for mice inoculated with different saponin concentration.

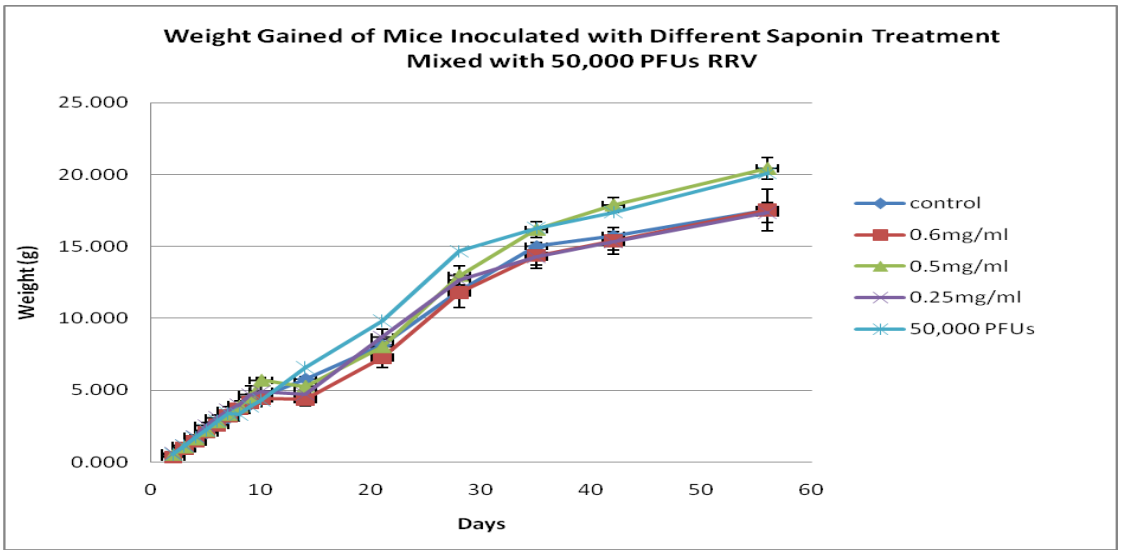



Figure F.3. Average weight gained for mice pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 50,000 PFUs RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

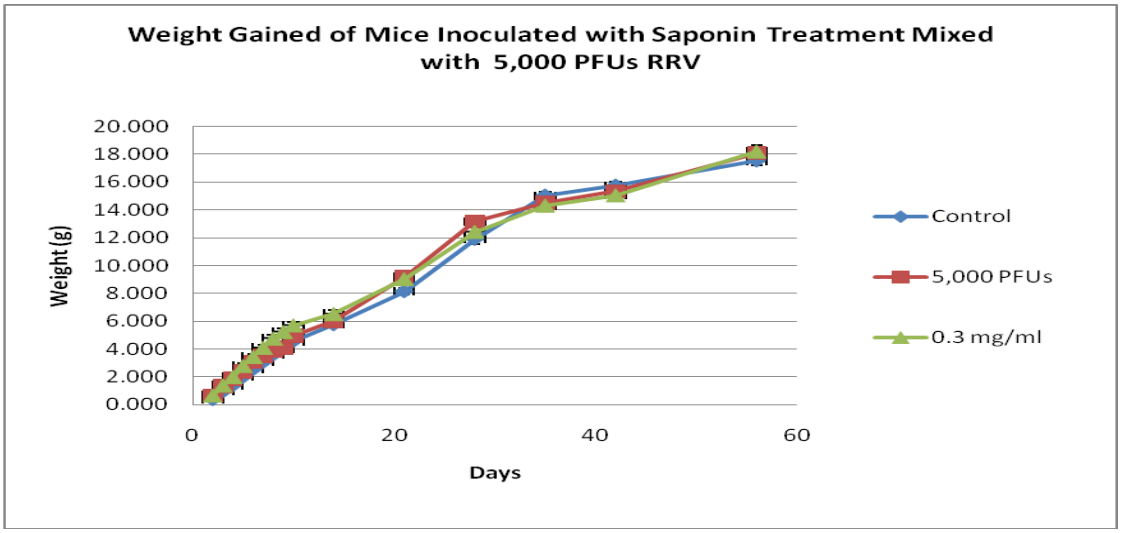



Figure F.4. Average weight gained for mice inoculated pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 5,000 PFUs RRV mixed with saponin extract at 0.3 mg/ml for five days.

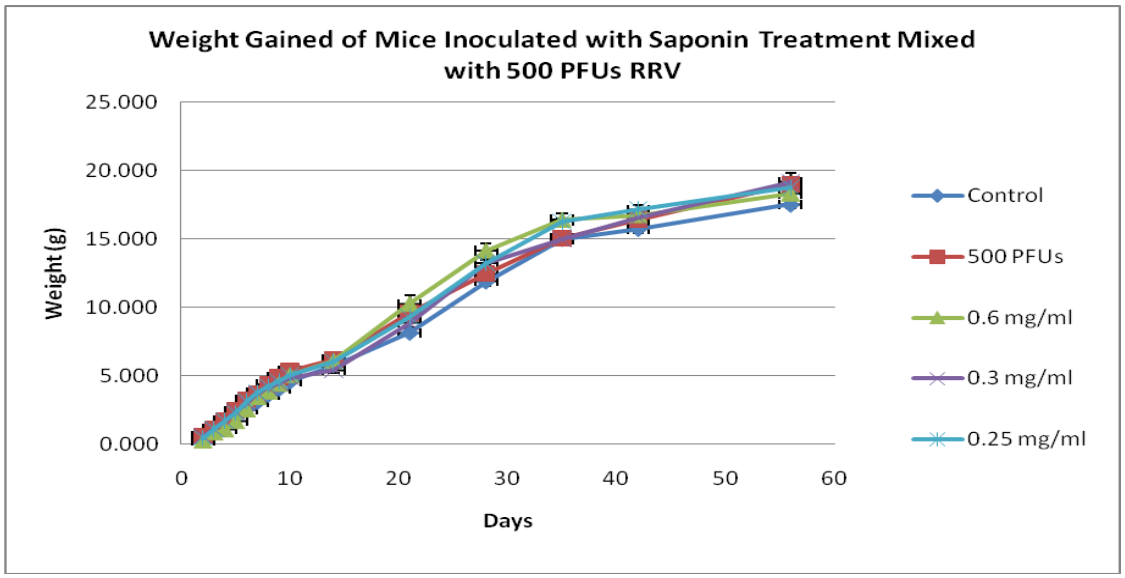



Figure F.5. Average weight gained for mice inoculated pretreated with saponin extract for two days. Pretreatment was followed by inoculation of 500 PFU RRV mixed with saponin extract at different concentration ranging from 0 to 0.6 mg/ml for five days.

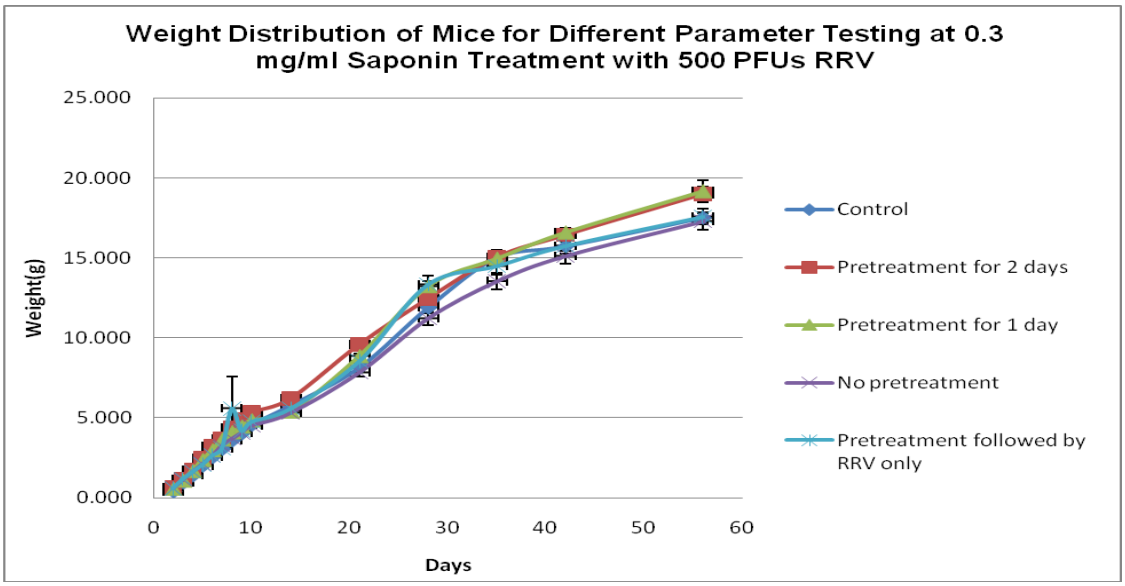



Figure F.6. Average weight gained for mice for different parameter testing at 0.3 mg/ml saponin extract with 500 PFUs RRV.

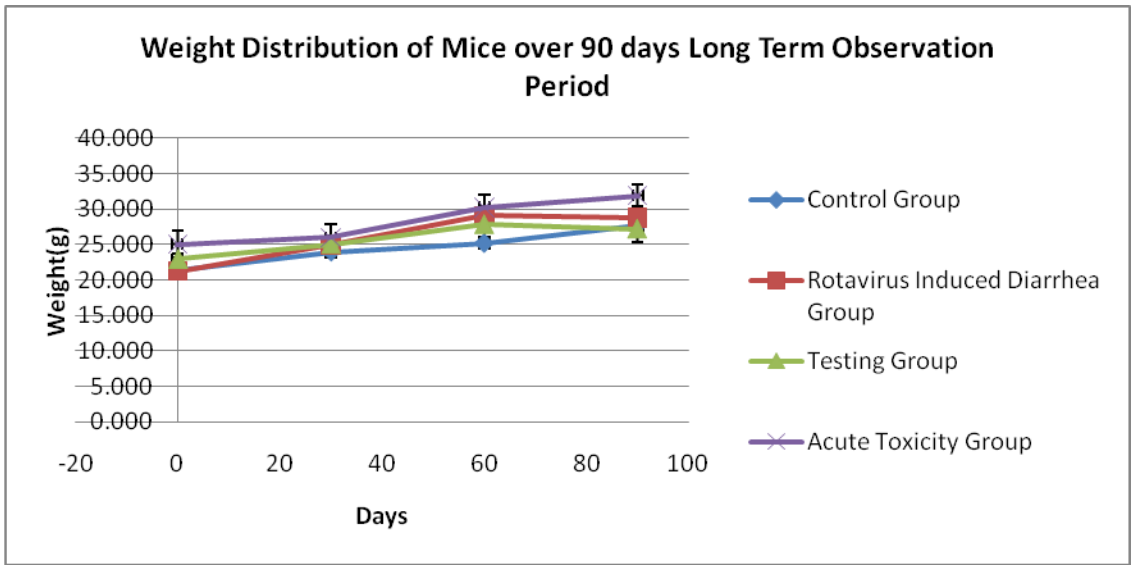



Figure F.7. Average weight recorded for mice over 90 days observation period.

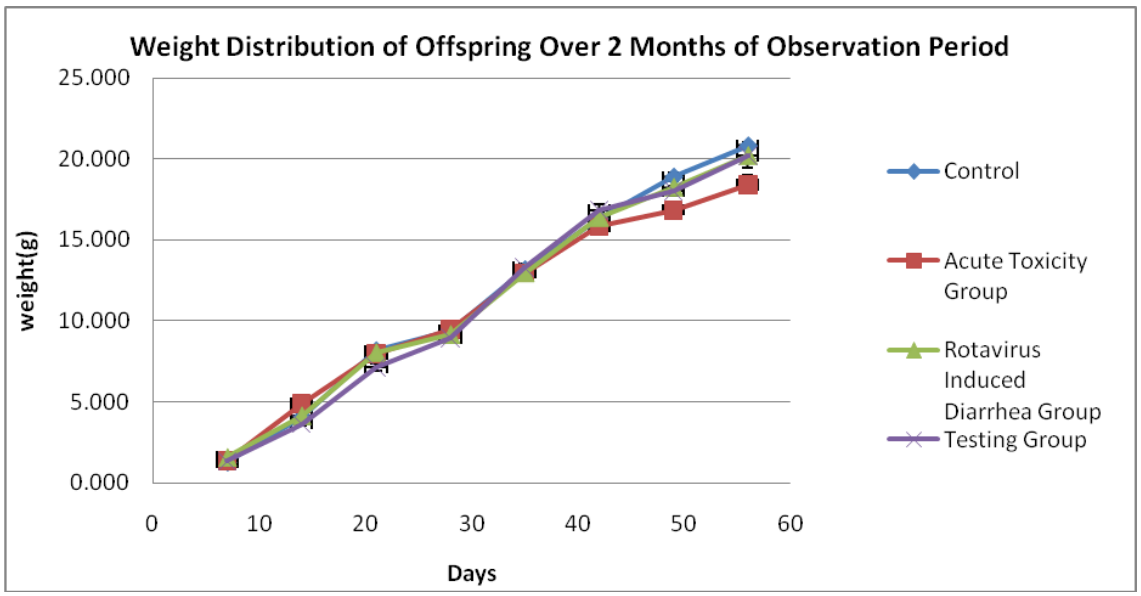



Figure F.8. Average weight recorded for mice for offspring over the two months observation period.

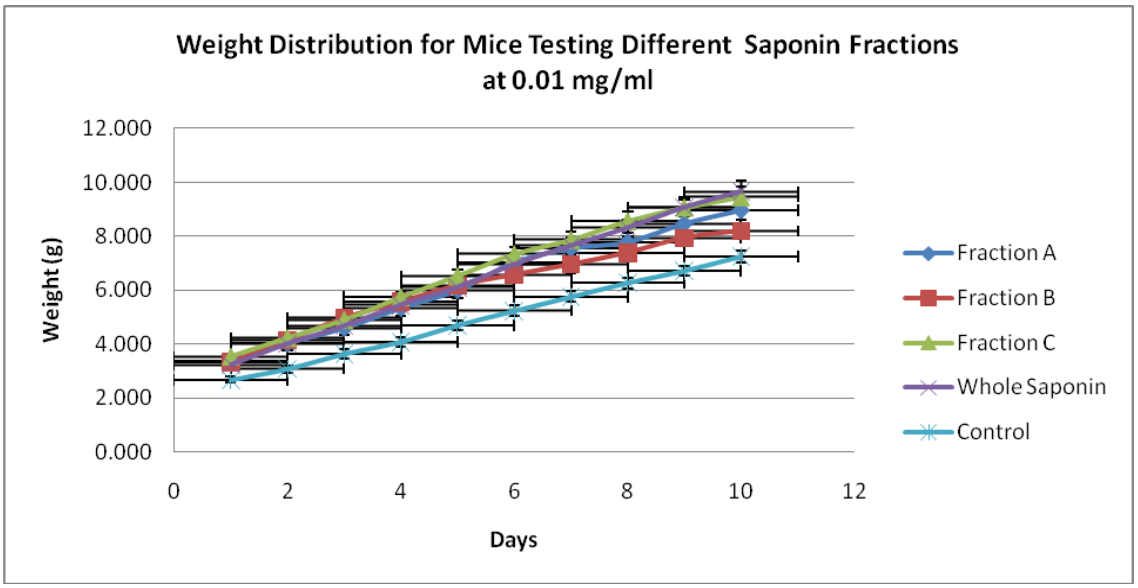



Figure F.9. Average weight recorded for mice inoculated with different saponin fractions at 0.01 mg/ml.

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## BIOGRAPHICAL INFORMATION

Ka Ian graduated from high school in Macau, China and started college at Abliene Christian University in 2000. She later transferred to the University of Texas at Arlington with a transfer scholarship in 2001. Ka Ian finished her bachelor degree of Science in Biology in Summer 2003 when she was 19. After graduating from her bachelor degree, Ka Ian entered the master program in Biology where she joined Dr. Michael Roner's Molecular Virology lab and graduate TESOL program in Linguistic at UTA. Her master thesis focused on analysis of genes controlling the oncolytic ability in reovirus with the use of ten groups of Reovirus temperature sensitive mutants. In August 2006, Ka Ian successfully completed both program requirements and graduated with her Master of Science in Biology. The same year, Ka Ian continued to pursue her PhD in Quantitative Biology under Dr. Roner's supervision. Her dissertation work focuses on exploring the antiviral activity of a natural aqueous extract retrived from the bark of *Quillaja* Saponaria. She has demonstrated that this natural product possesses antiviral activity against rotavirus both in vitro and in vivo with the use of a mouse rhesus rotavirus diarrhea model. Ka Ian worked part time at UTA as a graduate teaching assistant for the Microbiology lab. After one year, she was assigned to be the Lead GTA for the lab where she co-ordinated the labs, trained and supervised over 30 undergraduate students each semester. Ka Ian was also appointed to be the instructor for Biology Summer Enrichment Programs for high school students at UTA from 2004 to 2010. In addition, Ka Ian was awarded a scholarship from the Macau Government for Outstanding Researchers Foundation from 2005 – 2008. In August 2010, Ka Ian received her PhD in Quantitative Biology. She would like to work in industry, preferably the pharmaceutical industry. She wants to use knowledge in Microbiology, especially viruses, to find new ways to fight infectious disease around the globe.