SAPONINS AS AGENTS PREVENTING INFECTION CAUSED BY COMMON WATERBORNE PATHOGENS

by

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Abstract

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Waterborne diseases are the leading cause of deaths globally, estimated to result in more than 3.4 million deaths and approximately 4 billion cases of diarrhea annually according to the World Health Organization (WHO). The United Nations estimates that 4000 children die each day as a result of water-related disease caused by enteric pathogens of bacterial and viral origin that reside in water sources contaminated by raw sewage. Natural compounds from plants have been the source of a number of antimicrobials drugs. Saponins are a diverse group of glycosides that are widely distributed within the plant kingdom and have an equally broad range of biological properties. Saponin extracts may offer a new microbiocide for the prevention of diarrheal disease caused by enteric viruses and bacteria. In this study, I characterized the antiviral and antibacterial activity of aqueous extracts from *Tribulus terrestris*, *Yucca schidigera* and *Quillaja saponaria*, all of which contain saponins in the range of 12% to 90%. The *T. terrestris* and *Y. schidigera* saponin-containing extracts were examined for their *in vitro* antiviral activity against two viruses: rhesus rotavirus (RRV) and reovirus serotype 3 (ST3) strain Dearing (MRV-3DE). Saponins were added during the infection, prior the infection and post-infection to characterize the antiviral activity. I have found that pre-treatment or post-treatment of cells alone was not sufficient to block virus infection and

subsequent replication. However, co-treatment of cells with *T. terrestris* or *Y. schidigera* extract was able to reduce both rhesus rotavirus and reovirus infectivity. Saponin-containing extracts from the *T. terrestris* demonstrated a more robust activity, 93% inhibition of reovirus and 90% inhibition of rotavirus infectivity. The antibacterial activity of *T. terrestris*, *Y. schidigera and Q. saponaria* saponin extracts were examined against the following water and food-borne pathogens: *Escherichia coli* 0157:H7, *Yersinia enterocolitica*, *Listeria monocytogenes*, two strains of *Salmonella enterica* serovar Typhimurium, *Shigella flexneri* and *Vibrio cholerae*. The saponin extracts tested in this study did not exhibit a growth inhibitory activity against any of the bacterial strains tested. This study also explored the ability of saponin extracts to inhibit adhesion and invasion of cultured HeLa cells by these bacteria. The individual saponin extract was added to the HeLa cells simultaneously with or prior to infection to test for extract activity. The natural saponin extracts tested did not significantly reduce adhesion of individual bacteria to the HeLa cells, while presence of the *Y. schidigera* extract prior to and during the infection resulted in a reduction of invasion by the bacteria tested. The presence of the *Y. schidigera* extract during the infection had the strongest antibacterial effect.

This study also explored two possible mechanisms of antimicrobial activity of saponin extracts. Numerous biological properties of saponins have been ascribed to their action on cellular membranes. Building on previous findings that certain saponin molecules have an affinity for cell membrane cholesterol, I examined saponin treated cells for changes in cholesterol levels during conditions under which antimicrobial activity was measured. The results suggest that the pre-treatment of cells with extracts from *Y. schidigera* or *Q. saponaria* can modulate cellular membrane cholesterol levels. In this study, I also investigated whether saponin extracts can modulate activity of the Ca^{2+} ion channels. The results demonstrate that the treatment of cells for 6 or 24 hours with

individual saponin extracts does not produce a change in the intracellular concentration of Ca^{2+} ions. Taken together, *T. terrestris* saponin extract has antiviral activity that warrants further testing in animals and eventually people, while the *Y. schidigera* saponin extract was effective in preventing the intracellular entry of invasive pathogens used in this study the activity does not look as promising for use in animals and eventually people. My results suggest that the protective effect of both *T. terrestris* and *Y. schidigera* saponin extracts may be due to modulation of plasma membrane cholesterol leading to disruption in the cell membrane organization.

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Chapter 1

Introduction

Diarrheal disease is the second leading cause of death among children under the age of five. The World Health Organization (WHO) estimates that 1.1 billion people lack clean, safe drinking water and 2.4 billion do not have improved sanitation [1]. The reason for the high annual morbidity and mortality rates from diarrheal disease are the lack of safe, potable water and sanitation. Additional factors likely play a role, particularly the high incidence of other infectious diseases in developing countries [2]. Even though the sanitation infrastructure and water legislation has reduced the number of waterborne infection in developing countries, they still occur, resulting in up to 19 million cases of diarrheal disease annually [3].

I believe saponins can be used to reduce diarrheal disease caused by unclear water. Saponins are uniquely suited to such an application. Saponins are high molecular weight glycosides that are present in a diversity of plants and some marine organisms. Structurally, they have a hydrophilic sugar moiety linked to a hydrophobic aglycone skeleton. Saponins can be classified into steroidal or triterpenoid saponins based on the structure of the aglycone part of the molecule [4]. Due to the presence of a lipid soluble aglycone and water soluble sugar chain(s) they have surfactant properties. Saponins possess immense structural variation due to modifications in the aglycone structure and its subsequent combination with different sugar chains, along with the composition of those sugar chains. These structural variations can contribute to numerous biological properties ranging from antiviral, antifungal, adjuvant as well as antitumor activities all of which have been reported for various saponins [4]. It has been shown that saponin-rich water extracts from *Q. saponaria* are effective at blocking infections of mammalian cells

in vitro against a broad range of viruses, including enveloped and non-enveloped as well as DNA and RNA viruses [5, 6]. *Q. saponaria* extracts have been shown to completely block rotavirus infections *in vitro* and *in vivo* [7, 8].

Here, I explore the hypothesis that saponin extracts have the ability to modify mammalian cells, particularly the cell membrane, thereby preventing viruses and bacteria from binding and initiating an infection. Saponins are known to interact with the cholesterol in the cell membrane and depending on the structural features of an individual saponin, may have additional interactions with the cell membrane [9]. Ultimately saponin interactions with cell membranes could lead to a modification of lipids in the cell membrane which would affect the ability of viruses and bacteria to interact with these modified membranes. The ability of viruses and bacteria to interact with the host cell membrane is critical to the ability of the microorganisms to infect and subsequently cause disease in a host.

In this study, there were three specific aims. The first aim was to examine if the saponins from two never before tested plant sources: *T. terrestris* and *Y. schidigera* have similar antiviral activity against rotavirus and reovirus *in vitro* as has been seen with the *Q. saponaria* saponin extract. The second aim was to examine and quantify any antibacterial activity these plant extracts might have against selected bacterial species that are commonly associated with waterborne diarrhea. Specifically I used: *V. cholerae*, *S. flexneri*, *S. enterica*, *L. monocytogenes, E. coli* O157:H7 and *Y. enterocolitica*. The third aim was to explore several possible mechanisms of saponin antimicrobial activity by examining interactions that the saponin extracts may have with cellular membranes. The third aim was accomplished by measuring changes in Ca^{2+} ion fluxes and cholesterol changes when cells are treated with saponin containing extracts.

Chapter 2

Background

2.1 Advantages of Saponins

Saponins are a diverse group of secondary metabolites, widely distributed in the plant kingdom [4, 10, 11]. Plants have an ability to synthesize a variety of low molecular weight secondary metabolites, which are thought to play a role in protecting the plant from abiotic and biotic environmental challenges. Collectively, plant secondary metabolites encompass a diverse array of chemical compounds including alkaloids, flavonoids, isoflavonids, phenolics, saponins and many others. Flavonoids are thought to function in protecting plants against UV radiation and likely play a role in the reproductive success of plants [12]. Whereas, saponins are thought to protect plants against various pathogen attacks [13]. Besides having a beneficial effect on the plant, secondary metabolites have been of interest as a source of pharmaceuticals for a long time, and as an example, morphine, an alkaloid, is isolated from the opium poppy, *Papaver somniferum*.

One feature distinguishing saponins from other secondary metabolites are their surfactant properties; in water they form soap-like foams when shaken. Their name reflects these surfactant properties and *sapo* in Latin stands for soap. Due to these soap like properties of saponins, they are sought after for use in household detergents [4]. Additionally, saponins from a variety of plant sources have been shown to have pharmacological properties and are the major components of many plant-derived drugs and folk remedies [14]. The amphiphilic nature of saponins has enabled a wide range of applications such as a dietary supplement for cholesterol lowering, as an adjuvant for vaccines, as an antiviral, as an antimicrobial, as an anticancer drug and when included in animals feeds as a growth stimulatory supplement [15-19].

The cholesterol lowering activity of saponins is a well sought out pharmacological property of these compounds. The first observations made dating back to the 1960's demonstrated that saponins can form complexes with cholesterol. A number of studies have observed that when saponins are added to the diet of experimental animals there is a significant decrease in plasma cholesterol levels [14, 20]. This cholesterol lowering property of saponins has also been observed in human studies [20-22]. It is thought that saponins form complexes with bile and/or cholesterol, therefore preventing absorption of cholesterol by the small intestine of the gastrointestinal tract.

Saponins also have the ability to enhance both humoral and cell mediated immune responses to antigens. They have been shown to increase antigen-specific antibody production as well as to induce a strong cytotoxic T lymphocyte response when they are added to a vaccine [23-25]. A complex of cholesterol, saponin and antigen known as an immunostimulating complex or ISCOM can induce both a humoral and cellular immune response in animal models [26]. Besides modulating the adaptive arm of the immune system, saponins have been found to modulate innate immune responses as well [27]. *Q. saponaria* saponin extract is currently used in animal vaccines including one for rabies, as an adjuvant [28, 29].

A number of studies have demonstrated an antiviral activity of saponins against both DNA and RNA viruses [6, 8, 30]. One specific example is a saponin isolated from *Analgallis arvensis*, which has been found to have inhibitory activity against both herpes simplex virus and poliovirus by reducing the virus production in cells pre-treated with saponin [6]. It has been suggested that the antiviral activity of saponins is not due to a direct virucidal effect on the virus itself, but that antiviral activity involves an inhibition of the virus-host cell attachment process [8].

The antimicrobial activities of saponins have also been reported on extensively. As an example, alfalfa root saponins included in the feed, have been shown to reduce the protozoa population in the gastrointestinal tract of sheep [31]. A *Y. schidigera* saponin extract is very effective in reducing protozoa in the gastrointestinal tract of ruminants leading to significant ammonia reduction [32]. A major source of ammonia in ruminants is bacterial protein degradation which results from ingestion of bacteria by protozoa in the gut of ruminants [32, 33]. Currently *Y. schidigera* and *Q. saponaria* saponin extracts are used as additives in animal feed for odor reduction [32]. Eight saponins isolated from *T. terrestris* have reported antifungal activity against various species of yeast. The mechanism is thought to be inhibiting hyphae formation and destruction of cell membranes [34]. Some *Y. schidigera* saponins have demonstrated antimicrobial activity against *Bacillus pasteurii*, a prokaryote and *Saccharomyces cerevisiae* a eukaryote but activity was only seen when the microorganisms were present at a low cell density [35]. The reported studies on the antibacterial activity of saponins is somewhat-inconsistent. Saponins extracted from *Colubrina retusa* have been reported to show growth inhibitory activity when tested against *Mycobacterium intracellulare* [36]. While, *Q. saponaria* and *Y. schidigera* saponin extracts from a number of commercial sources have been reported to possess growth stimulatory activity when tested on *Escherichia coli* K-12 [37].

Another interesting characteristic of certain saponins is their antitumor activity. Several saponins exhibit antitumor activity in various cancer cell lines by induction of apoptosis. Continued research on saponins from numerous plant sources as possible cancer treatment agents, has generated considerable interest [15, 38].

Some saponins appear to possess a growth promoting activity. When *Q. saponaria* saponin extract was tested on vertebrae embryonic development using zebrafish as a model, it was found that the addition of saponins had a growth promoting

effect on the zebrafish embryo [39]. The authors suggested that the observed accelerated embryonic development was due to increased absorption of nutrients across the membrane possibly due to a saponin induction of membrane permeabilization. Other studies have found that saponins can increase permeability of the intestinal mucosal cells inducing passive transport of substances across mucus layer [40].

2.2 Saponins Used in This Study

Saponins consist a sugar moiety linked to an aglycone skeleton and depending on the nature of the aglycone skeleton, can be classified as either steroidal or triterpenoid saponins [4, 27, 32]. The steroidal saponins contain an aglycone region of the molecule generally with 27 carbons, typically a six-ring structure, while in some cases the hydroxyl group at C-26 can be involved in a glycosidic linkage making the aglycone structure pentacyclic (Figure 2.1 A, B). The triterpenoid saponins are more abundant in nature and contain 30 carbons arranged in a pentacyclic structure (Figure 2.1 C). Oligosaccharides attached to the aglycone skeleton of either type of saponin are often composed of two to eleven monosaccharides of glucose, galactose or rhamnose. One or two, linear or branched, oligosaccharides are generally attached to the aglycone skeleton of the saponin molecule [41]. The saponin extracts used in the study are water extracts obtained from *Q. saponaria*, *T. terrestris* or *Y. schidigera*. Each of the extracts contain a mixture of individual saponins each with slightly different saponin structure. While the *Q. saponaria* extract contain saponins with triterpenoid aglycone skeleton, extracts from *Y. schidigera* and *T. terrestris* contain a mixture of saponins with a steroidal aglycone skeleton.

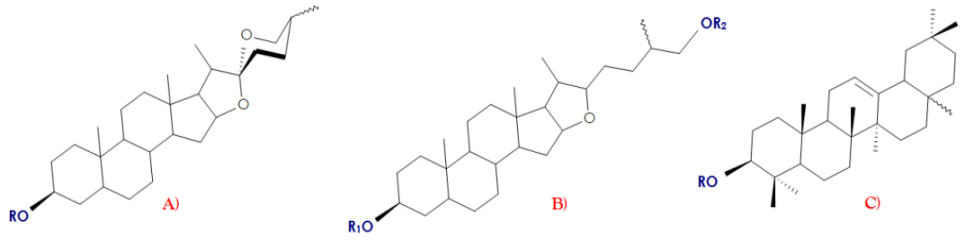


Figure 2.1. Saponin molecule aglycone skeleton. A) Steroidal-spirostane B) Steroidal-furostane C) Triterpenoid. R-oligosaccharide (Adapted from [42]).

2.2.1 *Q. saponaria* saponin extract

Q. saponaria is an evergreen tree with leathery leaves and thick bark, native to central Chile. The *Q. saponaria* extract is obtained by water extraction of the inner bark of the *Q. saponaria* Molina tree. The crude extract is further purified and known as Ultra Dry 100-Q extract with a 65% saponin content determined by HPLC analysis. Ultra Dry 100-Q is approved by the FDA (under 21 CFR 172.510, FEMA GRAS number 2973) and it is used in various soft drinks as a foaming agent [43]. *Q. saponaria* saponin extracts are known to contain additional components such as polyphenols and tannins.

The *Q. saponaria* extract used in this study is Vax Sap, a further purified medical grade material obtained following additional purification of the Ultra Dry 100-Q material and contains over 90% saponin content by HPLC analysis and a significant reduction of the polyphenols and tannins. Vax Sap has already been approved as an adjuvant for certain veterinary vaccines including rabies [23]. The Vax Sap extract contains a mixture of triterpenoid saponins. Each of the saponins in the extract contains an aglycone skeleton known as quillaic acid with branched oligosaccharides attached to it. The oligosaccharides are commonly attached to the C-3 and C-28 position on the aglycone skeleton [43].

2.2.2 *Y. schidigera* saponin extract

Y. schidigera is a plant belonging to the *Agavaceae* family, native to the southwestern United States and Mexico [44]. Two products, a powder and a liquid extract, obtained from the trunk of the *Y. schidigera* are available commercially. *Y. schidigera* extract is obtained through mechanical squeezing and further concentrated by evaporation. The *Y. schidigera* extract was used in this study. The reported concentration of saponins in the extract I used was 12%.

The main commercial application of the *Y. schidigera* extract is as a feed additive to improve both the growth and health of ruminants, specifically pigs and poultry [32]. Reported studies on the *Y. schidigera* extract suggest its usefulness for various applications. As an example, a butanol extract of *Y. schidigera* was found to be effective in killing *Giardia lamblia* trophozoites *in vitro* [45].

2.2.3 *T. terrestris* saponin extract

T. terrestris is an annual herb commonly known as puncture vine in the family *Zygophyllaceae*, native to the Mediterranean region but now widely distributed across the globe. Spirostanol and furostanol steroidal saponins are characteristic features of the plant [34]. More than 50 steroidal saponins have been isolated from this plant to date [46].

There is a substantial amount of research on the saponins of *T. terrestris* effectiveness in male and female libido disorders and infertility. Some studies center on its cardiovascular, cytotoxic or antimicrobial activities [34].

T. terrestris is sold in Europe and United States as a food supplement, mainly under the application to improve sports performance. In our study, commercial *T. terrestris* saponin extract was purchased from General Nutrition Center (GNC). The reported concentration of saponins in the extract I used was 45%.

2.3 Action of Saponins on Cellular Membrane

Multiple biological properties of saponins have been ascribed to their action on membranes [41, 47, 48]. Early studies of saponin interactions with membranes demonstrated that saponins have membrane lytic activity [49]. The lytic action of saponins was attributed to the interaction of aglycone with membrane cholesterol. The evidence of saponin affinity for membrane cholesterol comes from a study by Bangham and Horne (1962) where the authors demonstrated that membrane treatment with saponin leads to pores in the membrane [50]. Additional studies have demonstrated that the interaction of saponin with the cell membrane is more complex than just the introduction of pores. Certain saponins such as ophiopogonins can cause haemagglutination and not lysis of human red blood cells [51]. Digitonin is a saponin obtained from the plant *Digitalis purpurea* and it was found to form complexes with cholesterol in the cell membrane leading to cholesterol-free domains in the membrane [52]. Other studies have found that insertion of the aglycone region of the saponin is independent of cholesterol presence in the membrane if the saponin contains two sugar chains at C-3 and C-28 [53]. Also, gingenoside, a triterpenoid saponin present in *Panax* species (ginseng) was found to interact with the polar heads of either phospholipids or cholesterol in the cell membrane via their hydroxyl (OH) groups, whereas their hydrophobic aglycone could intercalate into the hydrophobic region of the lipid bilayer of the cell membrane. [54]. Regardless of the interaction, saponin treatment of cell membranes does alter the membranes, potentially altering the interaction of microbes with these treated membranes.

A few studies exploring the action of saponins on membranes have examined changes in conductance across the cell membrane [55]. Gingenoside was found to inhibit

Ca^{2+} influx through voltage-gated Ca^{2+} channels similar to the effect produced by steroid hormones such as estrogen [54]. Also, a study by Gogelein and Huby (1984) demonstrated that digitonin induces channel-like fluctuations in both cholesterol-free and cholesterol containing membranes with the effect being greater in the cholesterol-containing membranes [9]. Another study by Ladha et al. (1999) found that Avenacin A-1, a type of saponin found in oat plants, has the ability to increase conductivity across artificial membranes when cholesterol is present in those membranes [52].

Saponins also have the ability to affect cell membrane fluidity. As an example, saponins can increase ATPase activity possibly through an interaction with cholesterol in the membrane, displacing it from the immediate environment of ATPases. Removal of cholesterol from the cell membrane leads to an increase in the membrane fluidity which can facilitate conformational changes that ATPases undergo during their transport activity [4]. The ability of saponins to affect membrane fluidity may alter ion transport and activity of membrane proteins and enzymes which ultimately may explain the mechanism of action of saponins on cellular functions [54].

2.4 Importance of Membrane Organization in Virus or Bacteria Interaction with Cell

Attachment of a virus or bacteria to the host cell is generally the first step in the infection process [56-58]. In addition, all viruses must cross membrane barriers to gain access to the cytoplasm of their host cells, some bacteria also have to invade the host cell.

The initial virus interaction with the cell is similar for both enveloped and non-enveloped viruses and requires an interaction and attachment of viral surface proteins with host cell surface receptors (proteins, carbohydrates or lipids) [59]. Binding to host

cell receptor(s) is often specific and will generally induce cell signaling leading to the virus entry into the cell.

Bacteria have evolved various tools to adhere to their host cells. The initial interactions between bacteria and host cell are often nonspecific (hydrophobic or electrostatic interactions), and the structures that mediate these interactions are found on the outer surface of bacteria such as pilli or lipopolysaccharide (LPS) [56]. Along with pilli or LPS bacteria can utilize a variety of adhesion factors, such as the bacterial outer proteins or the bacterially encoded type III secretion system [60]. It is thought that bacteria use some or most of these adhesion factors to interact with their host cell.

Both bacteria and viruses have been found to utilize lipid rafts to interact with a host cell [61, 62]. Lipid rafts are ordered domains in the cell membrane enriched in cholesterol, sphinoglipids and certain proteins [63]. Lipid rafts, also known as membrane domains are important in various cellular events such as membrane signaling and trafficking [64]. It is thought that these membrane domains cluster into larger platforms upon activation of their proteins by pathogens. Clustering of membrane domains allows additional membrane proteins to be recruited, facilitating protein-protein interaction and eventual membrane uptake of a pathogen [63, 65].

Several studies suggest that viruses enter the cell by interacting with cell surface receptors located in the lipid rafts [66-71]. Cell membrane proteins implicated in rotavirus entry are either located in the lipid rafts or could be recruited to them [72]. Cell entry of bacteria such as *Listeria* requires integrity of membrane micro-domains or lipid rafts and cholesterol depletion inhibits this process [73, 74]. The initial interaction of *S. flexneri* with a host cell requires binding of the cell surface receptor CD44 located in lipid rafts. During *S. flexneri* entry to the host cell the lipid rafts proteins cluster around the bacteria [74].

Membrane lipid composition and structure can affect the kinetics and efficiency of virus and bacteria attachment and entry [59]. Many studies suggest that overall organization of plasma membrane is critical for the efficient orchestration of cell signaling during bacterial or viral cell attachment and entry [61, 73, 75]. Disruption of lipid rafts by treating cells with various chemical compounds known to deplete the membrane cholesterol can inhibit virus and bacteria entry, including poliovirus, ebola, rotavirus, herpes simplex, *Shigella* and *Listeria* [67, 68].

2.5 Importance of Ca^{2+} Signaling in Cellular Processes

The calcium ion is an important intracellular secondary messenger, involved in number of cellular processes [76]. Mammalian cells regulate the concentration of Ca^{2+} ions in the cytoplasm and a ten-fold rise in the free intracellular Ca^{2+} levels can affect many cellular processes such as membrane excitability or signaling from the cell membrane. Additionally, a number of plasma membrane proteins when activated can mobilize Ca^{2+} ions, affecting the cell function in a number of ways [77].

Activation of Ca^{2+} signaling pathways can be triggered by entry of many viruses and bacteria [78-80]. Herpes simplex virus 1 (HSV-1) triggers release of Ca^{2+} ions which seems to play crucial role in virus entry [79]. Also exposure of macrophage-like cells to *Listeria* results in a rapid rise in Ca^{2+} levels [81]. *Salmonella* also induces changes in Ca^{2+} ion flux during its entry into the cell [82].

2.6 Proposed Mechanisms of Saponins in the Plant Extract on Cellular Membrane

This study examines two hypotheses that may help to explain the action of plant derived saponin-rich extracts on the cellular membranes (Figure 2.2). The first hypothesis states that *Y. schidigera*, *T. terrestris* and *Q. saponaria* saponins in the extracts can

interact with the cell membrane cholesterol affecting the cell membrane proteins availability to bacteria or viruses. The second hypothesis is that *Y. schidigera*, *T. terrestris* and *Q. saponaria* saponins in the extracts can open Ca^{2+} dependent conductance channels that leads to improper functioning of targeted cell membrane proteins thus preventing bacteria or viruses from initiating an infection of these treated cells.

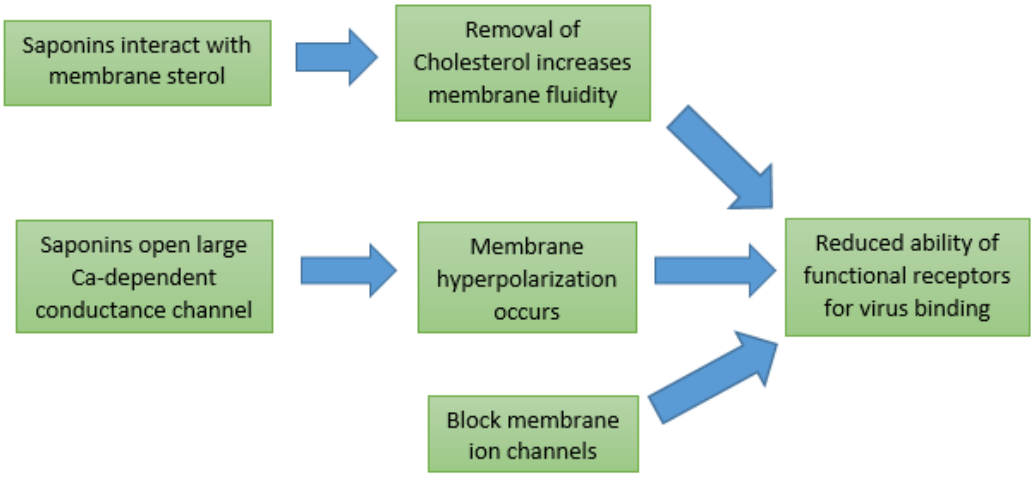


Figure 2.2. Proposed mechanism of saponin action on cellular membranes

2.7 Pathogens Used in This Study

2.7.1 Rotavirus

The human rotavirus was first discovered in 1973 in Australia and simultaneously in the United States by Dr. Albert Kapikian, nearly a decade after the first animal rotaviruses were visualized [83]. Rotavirus is a 100 nm, non-enveloped, icosahedral virus of the *Rotavirus* genus in the *Reoviridae* family. Rotavirus particles are composed of three concentric proteins layers or capsids that surround the viral genome of 11 segments of double-stranded RNA (dsRNA) [84]. Rotavirus genome segments code for

six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six nonstructural (NSP1-NSP6) proteins (Figure 2.3). The rotavirus genome segments are monocistronic and code for one protein with the exception of genome segment 11, which in some rotavirus strains codes for two proteins: NSP5 and NSP6 [85].

Rotavirus is classified into seven serogroups (A-G) based on antigenic variation of middle capsid protein or VP6. Rotavirus A, B, and C serogroups are known to infect humans and various animals, whereas serogroups D-H have only been identified in animals, mostly birds [86]. Additional classification of rotavirus into serotypes is based on antigenic variation in outer capsid proteins, namely VP4 and VP7. The protein VP4 determines G serotypes while VP7 determines P serotypes. To date, there are 28 P serotypes and 15 G serotypes identified, generating a large repertoire of rotavirus serotype variation [84].

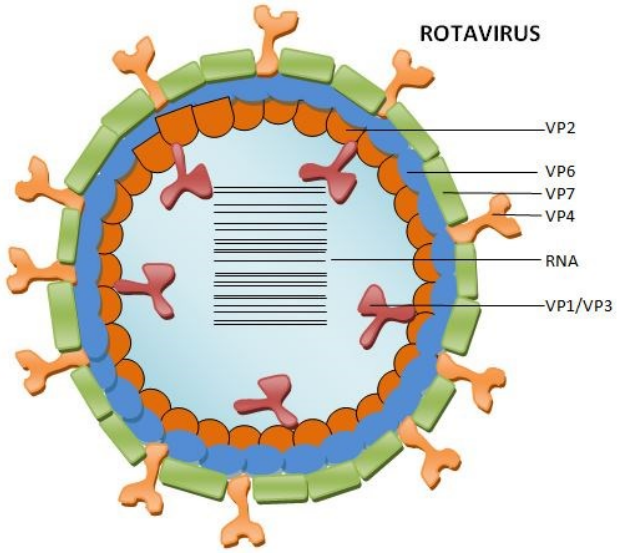


Figure 2.3. Rotavirus structure

2.7.1.1 Rotavirus Entry

In vivo, rotavirus binds and infects differentiated enterocytes of the tip of the intestinal villi of the jejunum and ileum of the small intestine and replicates in the cell

cytoplasm. One of the interesting features of rotaviruses is the need for proteolytic cleavage of its outer capsid protein VP4 into two fragments known as VP5 and VP8 that remain associated with virion prior to the virus attachment to cellular receptors. Protein subunits, VP5 and VP8 will then initiate binding of rotavirus to the enterocyte and subsequent entry into cell cytoplasm [85].

2.7.1.2 Rotavirus Replication Cycle

During or shortly after cell entry, rotavirus loses its outer layer capsid, yielding a double layer particle (DLP). Only the DLP is transcriptionally active and when activated extrudes messenger ribonucleic acid (mRNA) into the cytoplasm for translation by host ribosomes. Two viral encoded proteins: NSP2 and NSP5 form a viroplasm in the cell by accumulating around the DLPs. Viroplasm serve as a platform for viral mRNAs accumulation and capsid protein assembly. Concurrently, accumulating mRNA will serve as a template for the synthesis of the dsRNA viral genome. Once the DLP with its genome assembles within the viroplasm it will migrate to the rough endoplasmic reticulum (ER) obtaining third capsid layer. The progeny virus is then released following lysis of the cell.

2.7.2 Reovirus

Reovirus is the prototype member of the *Reoviridae* family of viruses. Reovirus is a non-enveloped, 85 nm diameter icosahedral virus with only two protein concentric layers or capsids as opposed to the three found in rotaviruses. The inner reovirus capsid surrounds the 10 segments of the dsRNA genome. Genome segments of reovirus are classified according to size into 3 large (L), 3 medium (M), and 4 small (S) segments. The L segments encode lambda (λ) proteins while M segments encode mu (μ) and S segments encode sigma (σ) proteins. Each genome segment encodes a single protein with the exception of the S1 segment which encodes two polypeptides, σ1 and σ1s. The

virus is composed of 8 structural proteins forming the inner and outer capsid and 3 nonstructural proteins found only in virus-infected cells [87].

2.7.2.1 Reovirus Entry

Reovirus can infect a variety of cells *in vivo* and *in vitro*. The mouse fibroblast cell line, L929 is the preferred cell line for reovirus infection *in vitro*. Prior to reaching the cytoplasm where the virus replicates, the viral σ1 protein interacts with a host cell surface receptor, sialic acid and junction adhesion molecule (JAM) to initiate a sequence of events leading to reovirus uptake via clathrin-mediated endocytosis [88, 89].

2.7.2.2 Reovirus Replication Cycle

During the entry process, reovirus losses its outer capsid layer generating a core particle which are transcriptionally active extruding mRNAs into the cytoplasm [90]. The mRNAs are capped and non-polyadenylated each corresponding to a single gene segment and translated by host ribosomes. The mRNAs also serve as templates for negative sense strand synthesis, generating segments of genomic dsRNAs [91]. Two reovirus nonstructural proteins (σNS, μNS,) and one structural protein (μ2) form viral inclusion bodies where virus assembly occurs. During the final stages of virus assembly, the outer capsid proteins are assembled with a core particle forming an infectious virus particle and completing the reovirus replication cycle.

2.7.3 *Vibrio cholerae*

Vibrio cholerae is a gram-negative, facultative pathogen, and the causative agent of cholera, severe watery diarrhea [92]. *V. cholerae* has two stages in its life cycle: the human small intestine and the aquatic environment [93]. Currently, the organism is classified into 206 serogroups based upon differences in the sugar composition of the O antigen of its lipopolysaccharide (LPS) but only strains belonging to O1 and O139 serogroups cause disease in humans. Pathogenic strains of *V. cholerae* poses several

key virulence factors. The first is cholera toxin (CT), which is the main factor responsible for massive secretion of chloride and water in the lumen of the intestine. The second is the toxin co-regulated pilus (TCP), a colonization factor, primarily responsible for aggregating bacterial cells together on the surface of the host intestinal epithelium which helps against shearing forces acting on the bacteria in the small intestine [94-96].

2.7.3.1 *Vibrio cholerae* Pathogenesis

Infection starts with the ingestion of food or water contaminated with as few as 10^3 *V. cholerae* organisms [97]. Bacteria that survive the gastric acidity of the host's stomach typically will penetrate the mucus layer of the small intestine and adhere to the intestinal epithelial cells. The virulence factor TCP is one of most important colonization factors possessed by this bacterium, but it does not seem to be involved in direct binding to the intestinal epithelium, but rather seems to facilitate bacteria-bacteria interactions on the surface of the epithelium [96]. One of the outer membrane proteins U (OmpU) has been implicated in adherence of *V. cholerae* to the intestinal cells *in vitro* [95]. Recently, protein, N-acetylglucosamine (GlcNAc) has been implicated in *V. cholerae* adhesion to human intestinal cells and it appears to bind mucins present in the mucosal layer of the gastrointestinal tract [98]. Upon *V. cholerae* colonization, there is CT production which leads to fluid loss of up to 1 liter per hour and up to 20 liter per day in adults [94]. Due to large water loss mediated by the action of CT, death can occur within 12 hours of the appearance first symptoms.

2.7.4 *Salmonella enterica*

Salmonella enterica is a gram-negative facultative pathogen and the causative agent of food or water-borne diarrheal disease known as salmonellosis [99]. The range of clinical manifestations of salmonellosis include watery diarrhea to severe bacteremia.

2.7.4.1 *Salmonella enterica* Pathogenesis

Salmonella enterica infection is initiated when the organism is ingested via contaminated food or water. The organism must penetrate the mucus layer lining the small intestinal epithelium before it can adhere to the epithelium. *Salmonella* uses fimbriae to attach to the intestinal epithelial cells [100]. Shortly after attachment, there is rearrangement of the cell cytoskeleton and appearance of membrane ruffles around the bacteria. Following appearance of membrane ruffles, *Salmonella* is taken up by the host cell in a process similar to macropinocytosis [101]. Once inside the cell, *Salmonella* reside in a modified phagosomic vesicle known as a salmonella containing vacuole (SCV), in which the bacterium replicates [101]. One interesting feature of *Salmonella* is the presence of two type III protein secretion systems also known as “molecular syringe” or “injectisome”. A type III protein secretion systems serves to translocate various bacterial effector proteins directly to the host cytosol where effector proteins can have many functions such as facilitating bacterial uptake or survival in the host cell [102].

2.7.5 *Escherichia coli* O157:H7

Escherichia coli O157:H7 is a gram-negative, facultative organism that causes gastrointestinal disease ranging from watery or bloody diarrhea to hemorrhagic colitis. A small subset of individuals that develop hemorrhagic colitis can also develop hemolytic uremic syndrome characterized by hemolytic anemia and kidney failure [103]. Cattle are the main reservoir for *E. coli* O157:H7 and infections in cattle typically result in asymptomatic disease [104, 105]. Transmission to humans is commonly via ingestion of uncooked meat contaminated with this bacterium. However, other modes of transmission are possible and have been reported, including ingestion of contaminated water or vegetables and via direct contact with animals at petting zoos [106].

2.7.5.1 *Escherichia coli* O157:H7 Pathogenesis

Escherichia coli O157:H7 has a low infectious dose with as few as 1-100 colony forming units/ml (CFU/ml) are sufficient to initiate an infection in humans [107]. The initial adhesion of *E. coli* O157:H7 to the large intestinal epithelium is not a well understood process. There are several bacterially expressed factors such as fimbriae, flagella and outer membrane protein A (OmpA) that have been implicated in the initial adherence process. However, only the adhesion factor outer membrane protein known as intimin has been demonstrated to play a role in the intestinal colonization of bacteria *in vivo* [105, 108]. The intimin protein binds its receptor, Tir, located in the host cell membrane. Interestingly, Tir is manufactured by the *E. coli* O157:H7 and translocated into the host cell membrane via a type III protein secretion system. When the successful colonization of the large intestine by *E. coli* O157:H7 is established, the bacterium starts synthesizing and releasing shiga-toxin into the lumen of the intestine. Shiga toxin is an AB subunit toxin and its B subunit binds to a receptor on the cell surface, allowing effective translocation of the A subunit of the toxin via receptor-mediated endocytosis. Enzymatic activity of the shiga toxin prevents host cell protein synthesis, resulting in the death of a cell [105].

2.7.6 *Shigella flexneri*

Shigella microorganisms are gram-negative, facultative anaerobes that cause an intestinal infection or shigellosis which results in a mild watery diarrhea to severe inflammatory bloody diarrhea. The genus *Shigella* has four species, and each species is cable of causing shigellosis in humans [109]. *Shigella sonnei* is responsible for majority of the cases of shigellosis, followed by *S. flexneri* [110]. As opposed to other water and food-borne enteric pathogens such as *Salmonella and E. coli,* humans and occasionally primates are the only natural host for this pathogen [111].

2.7.6.1 *Shigella flexneri* Pathogenesis

Shigella flexneri is capable of causing shigellosis when orally ingested in a very low numbers, approximately 10-100 bacteria are sufficient to cause infection in healthy individuals [112, 113]. The current model of *S. flexneri* pathogenesis includes: attachment of the bacteria to the epithelial cells of the large intestine, uptake by the microfold (M) cells in the intestine, replication within the cell cytosol and subsequent spread to adjacent cells via actin based motility [114]. Several factors have been implicated in the adhesion of *S. flexneri* to colonic epithelium such as LPS and two outer membrane proteins (OspE1 and OspE2). However it is not well understood whether there is an adherence of bacteria to the apical surface of epithelial cells prior to its uptake by the M cells [112, 115].

This bacterium also utilizes a type III protein secretion system, through which it translocates various effector proteins. Proteins that are translocated will interact with the host cell cytoskeleton machinery leading to bacterial uptake by the cell [115]. *Shigella flexneri* is capable of not only replicating within the cellular cytoplasm, but also has the ability move in the cell cytoplasm via a polymerized actin tail [111].

2.7.7 *Yersinia enterocolitica*

Yersinia enterocolitica is a gram-negative pathogen, and a frequent cause of gastrointestinal infections or yersiniosis. Yersiniosis ranges from mild to severe diarrhea, mesenteric lymphadenitis and in some individuals abscess in the spleen or liver can occur [116]. The first human cases of yersiniosis were observed in the 1930s [117], however in 1970s owing to better detection methods the organism was recognized worldwide as a common cause of gastroenteritis [117].

Yersinia enterocolitica is a ubiquitous organism, found in the soil, water and animals, with over 50 different serotypes identified. Currently serotypes: O:3, O:4, 32,

O:5, 27, O:6, 30, O:6, 31, O:8, O:9, and O:21 are frequently isolated from humans with yersiniosis. The main reservoir of human pathogenic *Y. enterocolitica* serotypes are pigs [117]. In pigs the bacteria is frequently isolated from the tonsils and is carried asymptomatically [116]. *Yersinia enterocolitica* is mainly transmitted via contaminated food or water and occasionally direct human to human or animal to human transmission occurs.

2.7.7.1 *Yersinia enterocolitica* Pathogenesis

The pathogenesis of *Y. enterocolitica* includes: oral ingestion of bacteria, attachment to the distal small intestinal epithelium, followed by colonization of Peyer's patches and in some cases dissemination of the infection into mesenteric lymph nodes or other extra-intestinal sites [118, 119]. The pathogenesis of *Y. enterocolitica* depends on several virulence factors encoded either chromosomally or on a plasmid. Chromosomally encoded adhesion factor or invasin binds the beta 1 chain integrin on the surface of M cells which are located among the small intestinal epithelial cells. Binding of invasin to the beta 1 chain integrin induces bacterial uptake. There are also two additional adhesion factors known as attachment invasion locus (Ail) and yersinia adhesion A (YadA). These adhesion factors promote adhesion to extra-intestinal infection sites [120].

This bacterium encodes a type III protein secretion system which promotes translocation of at least 6 effector proteins known as yersinia outer proteins or Yops [121]. The type III protein secretion system and its effectors are not involved in colonization and invasion of the intestinal epithelial cells but seem to function in counteracting several components of the host innate immune responses, thereby delaying clearance of the infection [122].

2.7.8 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, facultative anaerobe and the causative agent of gastrointestinal infection or listeriosis, resulting in a range of symptoms from mild gastroenteritis to severe invasive disease in immunocompromised individuals, pregnant women and newborns [123]. *Listeria monocytogenes* is an unusual pathogen, being able to cross three different tight barriers in the host: intestinal, blood-brain and feto-placental [124].

Listeria monocytogenes was first isolated in 1920s from humans, but it was not until 1980 that it was recognized as a foodborne pathogen [125]. This bacterium is an ubiquitous pathogen that can thrive in various environments including the soil, water and wild and domestic animals [124, 126]. Out of the eight species in the *Listeria* genus only *L. monocytogenes* is pathogenic to humans [125]. The main mode of transmission of *L. monocytogenes* to humans is via contaminated food, mainly lunch meat and dairy products.

2.7.8.1 *Listeria monocytogenes* Pathogenesis

Listeria monocytogenes has the ability to invade and replicate in a number of cell types [127]. The virulence factors possessed by *L. monocytogenes* allow this pathogen to disseminate via blood or lymph to other tissues [127]. However, the gastrointestinal tract is a primary site of invasion for this bacterium.

Evidence from *in vitro* studies using polarized intestinal epithelial cells suggest that the bacterial surface protein (In1A) binds to the host cell adhesion receptor located at adherent junctions in the basolateral region of the intestinal cell. *Listeria monocytogenes*, *S. flexneri* and rotavirus, all bind to cell surface receptors that are located on the basolateral side of the intestinal epithelial cells, and it remains controversial how this occurs *in vivo* since those receptors are not exposed to the apical side. Recent studies

suggest that continuous death and renewal of the intestinal epithelial cells may allow for transient exposure of those receptors to the apical side [128].

Once the *L. monocytogenes* is taken up by the cell, it remains in a membrane bound vacuole for a short time. Listeriolysin O is a bacterially encoded pore forming protein, and will lyse the membrane bound vacuole releasing the bacteria to the cytoplasm where it can replicate [123]. Also, similar to *Shigella*, this bacterium utilizes actin based motility to move internally within the cell [126].

Chapter 3

Antiviral Effect of *Tribulus Terrestris* and *Yucca Schidigera* Extracts

3.1 Introduction

Rotavirus is a leading cause of diarrheal disease in children under the age of five and responsible for over 300,000 each year. Rotavirus infections are common in both developed and developing countries and the virus likely infects every child in the world [84]. Adequate sanitation measures and improvements in safe drinking water do not seem to play a significant role in the transmission of rotavirus. The treatment of rotavirus induced diarrhea is nonspecific and mainly involves oral rehydration therapy (ORT) to replace lost fluids and often high mortality occurs in developing countries due to the lack of available ORT. Two live oral vaccines, Rotarix (GlaxoSmithKline) and RotaTeq (Medco) are effective in preventing severe diarrheal disease caused by the common rotavirus serotypes. However, global rotavirus vaccine administration has faced several challenges. A major hurdle is the current age restriction for initiation and completion of the vaccination regime. Children must be 6 weeks old for the administration of the first dose of Rotarix vaccine and the second dose must be given 4 weeks after the first dose and by 24 weeks of age. Additionally, the effectiveness of the vaccine in generating protective immunity tends to be generally low in developing countries or in some individuals in general. Rates of protection range from a low of 48.3% to as high as 98% against severe disease [129, 130]. Also, there exist safety issues associated with administrating the live attenuated rotavirus vaccine to immunocompromised children. These immunocompromised children are susceptible to the establishment of a chronic rotavirus infection and continued chronic shedding of the virus particles from these children provide a reservoir for spreading virus to new patients [131-133]. Therefore, the

need for an alternate rotavirus antiviral intervention exists and I hypothesize that saponins present in extracts from *Y. schidigera* and *T. terrestris* could be used to prevent rotavirus infections and reduce the need for vaccinations.

Reovirus, also known as respiratory enteric orphan virus is ubiquitous in the environment and commonly infects humans, causing asymptomatic infections. Even though reovirus infections result in asymptomatic infections there are several case reports linking reovirus infections with minor upper respiratory symptoms and gastrointestinal or central nervous system complications [134-136]. There are three reovirus serotypes known to infect humans: mammalian orthoreovirus 1 Lang (MRV-1 La), mammalian orthoreovirus 2 D5/Jones (MRV-2 Jo) and mammalian orthoreovirus 3 Dearing (MRV3 De). By using multiple viruses in our study we were able to demonstrate that antiviral activity is non-specific with regard to receptor utilization, as reovirus and rotavirus use different cellular receptors and mechanisms to cross the cellular membrane to initiate an infection of a cell. Additionally, a mouse cell line was used to support reovirus infections and a monkey cell line for rotavirus infections further demonstrating the wide range of saponin protection that is possible.

Previous work has demonstrated that *Q. saponaria* extracts, which contain a number of triterpenoid saponins, have antiviral activity *in vitro* on a broad range of viruses including reovirus and rotavirus. The current hypothesis on the mechanism of action of the *Q. saponaria* extract is through a disruption of cellular membrane protein receptors [8]. Viruses must utilize cell membrane receptors to initiate their entry into the cell. Saponins could offer a novel antiviral by preventing virus entry and therefore blocking infection of cells. My study explores the antiviral activity of *Y. schidigera* and *T. terrestris* saponin extracts which contain a mixture of steroidal saponins, against a major viral cause of childhood diarrhea: rotavirus and related reovirus.

3.2 Materials and Methods

3.2.1 Viruses Used in the Study

Two viruses were used in this study: rhesus rotavirus (RRV, ATCC VR-954) and human reovirus serotype 3 (ST3) strain Dearing (MRV-3DE- originally obtained from Dr. W.K. Joklik). Both viruses are from the same family of viruses, non-enveloped double stranded RNA segmented viruses. Rhesus rotavirus was chosen due to its similarity to human rotavirus and its ease of replication in the cell culture in comparison to human rotaviruses strains which replicate very poorly in cell cultures. Human reovirus strain MRV-3DE is one of the three known strains of reovirus known to infect humans.

3.2.2 Cell Lines Used in the Study

Two different cell lines were used in this study: MA104 (ATCC CRL-2378.1) is the Vervent monkey kidney epithelial cell line used for rhesus rotavirus propagation and L929 (ATCC CCL-1) is a mouse fibroblast cell line used for reovirus propagation. There are several reasons for selecting these cell lines. The MA104 cell line is highly susceptible to rotavirus infection. Rotavirus requires the presence of the proteolytic enzyme trypsin to successfully infect MA104 cells in culture. Due to trypsin cleavage of the spike protein or VP4 into two protein subunits VP5 and VP8, each subunit binds cell surface receptors that are present on the MA104 cells and virus uptake occurs by endocytosis. The L929 cell line is a preferred cell line for reovirus propagation due to the presence of required cell molecules for virus attachment and entry, as well as a cytopathic effect produced in this cell line upon infection that can be easily visualized and quantified, specifically lysis.

Both cell lines were maintained in 75 cm^2 tissue culture flasks at 37°C, 5% carbon dioxide (CO_2) in minimal essential media (MEM) with 1% antibiotic containing penicillin and streptomycin (P/S) along with 10% bovine calf serum or 5% fetal bovine serum (FBS). Every two to three days cells were checked for growth and confluency, and were passaged upon reaching 90-100% confluency aseptically in a biological laminar flow hood. The passaging of cells involves several steps. First, the current MEM was discarded, followed by rinsing with 1x Saline Sodium Citrate (1x SSC) solution. Next, cells were treated with 0.05% trypsin with 0.02% EDTA for 2-5 minutes to allow detachment of cells from the tissue culture flask. Once the cells detached, they were diluted 1:2 or 1:10 in fresh MEM and placed in a new flask.

3.2.3 Culturing Cells on Plates

Cells were cultured in 6, 12 or 24 well tissue culture plates. Following cell passaging, the cell suspension generated was pipetted up and down using a sterile pipette attached to the pipette gun to mix the cells evenly. To determine the number of cells per ml (cells/ml) in the cell suspension, I have used a hemocytometer to count the cells. Only one chamber of the hemocytometer was loaded with 10 µl of original cell suspension and the cells were counted in four corner squares as well as the center square. The following formula was used to determine the number of cells/ml.

Average number of cells counted per square x 10^4 = number of cells/ml

3.2.4 Saponin Extract Preparation

The *Y. schidigera* extract used in this study was obtained from Desert King International, San Diego, CA. One milliliter (ml) of the extract was lyophilized to remove the water leaving the dry material. The amount of dry material was determined by weighting. The *Y. schidigera* extract stock solution was determined to contain 40 mg per

ml (mg/ml) of solids, water-soluble saponins and minor contaminants. This was also confirmed by HPLC.

The *T. terrestris* was obtained from the general nutrition center (GNC). According to the manufacturer, the dried material was obtained from the aerial part of the *T. terrestris* plant. The dried material was used to make a *T. terrestris* extract stock solution used in the study. The extract preparation involved dissolving 1 gram of dried material in 10 ml of 1x SSC to yield a 100 mg/ml stock solution.

All saponin extract stock solutions were filtered sterilized with a 0.2 µm sterile syringe filter (Corning Inc.) and kept at 4°C for short term storage and at -20°C for long term storage. From the saponin stock, saponin extract was prepared by transferring the appropriate volume of extract stock solution to sterilized MEM with 5% FBS to yield the desired concentration of extract depending on the assay (Refer to sections: 3.2.7 and 3.2.8).

3.2.5 Virus Stock Preparation

Rhesus rotavirus and reovirus stocks were prepared in a similar manner with the exception of rotavirus requiring the addition of trypsin (0.5 µg/ml) to activate the virus particles are previously outlined. Virus stocks were obtained during three passages of virus in greater and greater cell numbers to yield a final high titer working stock. During the first passage, a cell culture flask (25 cm^2) was seeded with either MA104 or L929 cells and incubated at 37°C, 5% CO_2 until 90-100% confluency was reached. When the appropriate confluency was reached, the cells were infected with virus diluted in MEM with 1% P/S and 10% trypsin was included for MA104 infection with rotavirus. After virus addition the culture flask was incubated for one hour at 37°C and 5% CO_2 environment with rocking every 15 minutes to prevent cell drying and to ensure even virus distribution and attachment. After an hour of incubation, 5 ml 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 and monitored daily for cytopathic effect. When 50% of cells were lysed due to virus replication, the cells were sonicated, transferred to a 15 ml centrifuge tube and stored at --20°C.

First passage virus was used to infect confluent MA104 or L929 cell monolayer's in a 75 cm^2 cell culture flask and same infection protocol was carried out as explained for obtaining first virus passage. Following second passage, again confluent MA104 or L929 cell monolayer's, this time in 150 cm^2 cell culture flask were infected with second virus passage and the infection protocol was the same as for obtaining first virus passage. All three virus passage cell lysates were combined and stored at -20°C; they represent either rotavirus or reovirus stock which was used in further experiments.

3.2.6 Viral Titer Determination

A standard plaque assay was performed to determine the number of infectious virus particles per ml or plaque forming units per ml (PFU/ml) of rotavirus and reovirus stock. The cytopathic effect of both rotavirus and reovirus is cell lysis, which can be easily visualized as plaques on a cell monolayer.

Six well plates were seeded with the appropriate cell line (MA104 or L929) in MEM with 5% FBS and 1% P/S and incubated overnight at 37°C and 5% CO_2. After 24-48 hours cells that were 100% confluent were infected with their respective virus. The infection process involves making ten-fold serial dilutions (10^1-10^7) of rotavirus or reovirus stock in MEM with 1% P/S, trypsin was included for rotavirus. A volume of 250 µl of each virus dilution was then added to each well of a 6 well plate (three wells per virus dilution). Plates were incubated at 37°C and 5% CO_2 for 1 hour with gentle rocking every 15 minutes to allow even virus absorption. After 1 hour the virus inoculum was removed and replaced with 2 ml of 2% agar overlay. To make the agar overlay, 2x MEM with 10% FBS

and 2% P/S and melted 2% noble agar were placed in separate 125 ml glass bottles. Individual bottles were placed in the 42°C incubator for 40 minutes. After the agar and MEM have equilibrated to 42°C temperature, they were mixed together in 1:1 ratio. Two ml of agar overlay was added to each well slowly, and the agar was allowed to solidify before the plates were transferred to 37°C and 5% CO_2 environment for incubation. Every two days an additional 2 ml of agar overlay was added to each well with a total of three agar overlays. During the last agar overlay 4% neutral red was added to the overlay mixture and plaques were counted 24 hours later. The number of plaque forming units per ml (PFU/ml) was determined with the following formula:

PFU/ml = [# of plaques in a well / (dilution factor of virus stock in well * 4)]

3.2.7 Cell Cytotoxicity Assay

The trypan blue exclusion procedure was used to determine the highest concentration of saponin extracts tolerated by the cell lines used in this study. The trypan blue exclusion assay measures cell viability by assessing cell membrane integrity and stains nonviable cells blue. Indicated cells (MA104 or L929) were plated in a 24 well cell culture plate at 125,000 cells per well in 500 µl of MEM supplemented with 5% FBS and 1% P/S and incubated overnight at 37°C, 5% CO_2.

The saponin extract stock solution was ten-fold serially diluted in MEM with 5% FBS and 1% P/S for initial testing, with 10 mg/ml being the highest saponin extract concentration used for the experiment. Individual saponin dilutions (500 µl) were added to each well of a 24 well plate in triplicates and plate was incubated for 96 hours at 37°C and 5% CO_2 environment. Controls included treating the cells in parallel with MEM only with 5% FBS and 1% P/S. After 96 hours, media from the control and saponin treated wells were removed and 250 µl of 10% trypan blue in MEM with1% P/S was added to each well. Plates were incubated at room temperature for five minutes. Following

incubation, 500 cells per well were counted using an inverted microscope and the percentage of viable cells calculated as follows:

Viable cells (%) = Number of unstained cell / 500 (cells counted/well) *100%

After initial cytotoxicity testing, further testing was carried out by performing two-fold dilutions of saponin extract stock solution in MEM with 5% FBS and 1% P/S to determine the highest concentration of saponin extract that does not affect the growth and viability of cells.

3.2.8 Antiviral Assays

A plaque reduction assay was used to test the ability of each saponin extract to prevent virus infection in their designated cell lines. Various treatment conditions were used for the antiviral assay such as pre-treatment, co-treatment or post-treatment of cells with saponin extract to evaluate their effectiveness. The concentration of *Y. schidigera* extract used for the treatment of MA104 cells during antiviral assays was 0.005 mg/ml, and 0.0025 mg/ml for the L929 cells. The concentration of *T. terrestris* extract used for the antiviral assays was 0.8 mg/ml for both cell lines. A plaque assay was also carried out to examine any direct virucidal effect the saponin extracts might have on the viruses. Also, I explored the length of time that the cells show protection against virus infection when they were pre-treated with saponin extract for 1 hour.

3.2.8.1 Plaque Assay to Measure if Pre-treatment, Co-treatment and Post-treatment with *Y. schidigera* and *T. terrestris* Extract Can Block Infection

A plaque assay was used to determine if the presence of extract prior to, during or post-infection of cells in culture could prevent virus infection. A standard plaque assay was performed as outlined in 3.2.6 with slight modifications. First, cells were seeded into a 6 well tissue culture plate according to the protocol outlined in 3.2.3. After cells had reached complete confluence, the saponin extract stock solutions were diluted in medium

to a final concentration that produces no cell toxicity as previously established in the cytotoxicity assay for each cell line. This ensured that the impact of the saponin extract would be not against the cells themselves, but on the virus attachment and replication within the cells. For the pre-treatment assays, cells were pre-treated with saponin extract for 2, 4 or 6 hours prior to the addition of virus. For the co-treatment assays, saponin-containing medium was present during the addition of virus. Whereas, for the post-treatment assay, cells were exposed to virus and the saponin extracts were included post-infection. The Figure 3.1 shows conditions for the pre-treatment, co-treatment and post-treatment assays. At the end of each assay the number of plaques were counted and the viral titer determined. Each assay was carried out in triplicate and repeated to generate at least six measurements per time point.

3.2.8.2 Plaque Assay to Measure the Direct Virucidal Effect of *Y. schidigera* and *T. terrestris* Extracts

The ability of both *Y. schidigera* and *T. terrestris* saponin extracts to directly inactivate rotavirus or reovirus was examined using a standard plaque reduction assay. Briefly, 1 x 10^6 PFUs of each virus was mixed with saponin extract diluted in phosphate buffer saline (PBS) in the range of 0-50 mg/ml for *T. terrestris* or 0-40 mg/ml for *Y. schidigera*. The maximum concentrations tested were the highest concentrations of the extract stock solution available. Virus and saponin (test) or virus and PBS (control) suspension were incubated for up to 6 hours at 37°C and 5% CO_2. At 0, 1, 2, 4 and 6 hour time intervals, 0.1 ml of each virus suspension was used in a plaque assay. The visual yield obtained with saponin treated virus was compared to untreated virus to determine if the treatment had any direct virucidal effect on the virus. Assays were performed in triplicates and repeated to generate six measurements per time point.

3.2.8.3 Plaque Assay to Quantify the Lasting Effect of *Y. schidigera* and *T. terrestris* Extract Treatment on Cells

The potential for a lasting protective effect of the *Y. schidigera* and *T. terrestris* extracts was examined using a plaque assay as described previously (section 3.2.6). The modification to the plaque assay is that cells were treated with a concentration of saponin extract that demonstrated no cell toxicity for 1 hour. After treatment, the saponin-containing medium was removed, and 2 ml of fresh MEM was added to each well (Time 0). Immediately at time 0 or after 1, 2, 4, 8, 12, 16 and 24 hours, cells were infected with 1 x 10^6 PFUs of the indicated virus. From this point forward the standard plaque assay was performed. Figure 3.1 graphically depicts the parameters explored in this experiment.

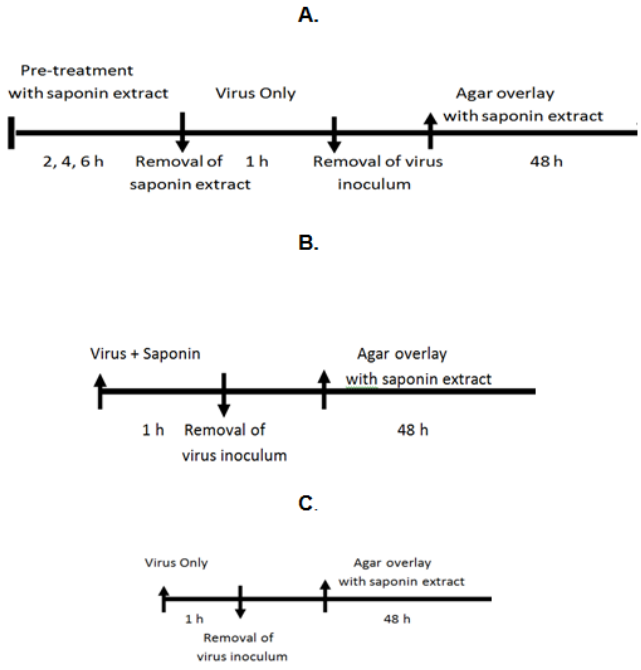


Figure 3.1. Conditions of Antiviral Assays A) Pre-treatment B) Co-treatment C) Post-treatment

3.3 Results

3.3.1 Titer of Rhesus Rotavirus and Reovirus Stock

A plaque assay was carried out to determine the titer of each virus stock as outlined in 3.2.6. Only wells of a 6 well plate with 30-300 plaques were counted. Dilution with appropriate number of plaques was within 10^{-5} dilutions (Table 3.1).

3.3.2 Cytotoxicity of *Y. schidigera* and *T. terrestris* on MA104 and L929 Cells

Cytotoxicity of *Y. schidigera* and *T. terrestris* saponin extracts on MA104 and L929 cells was obtained as outlined in 3.2.7. The highest concentration of *Y. schidigera* saponin extract that did not affect the growth or viability of the MA104 cell line was 0.005 mg/ml and for the L929 cell line, 0.0025 mg/ml (Figure 3.2). The growth and viability of the MA104 and L929 cells was not affected by 0.8 mg/ml for the *T. terrestris* saponin extract (Figure 3.2).

Table 3.1. Titer of rhesus rotavirus and reovirus stocks

	Plate 1	Plate 2	Plate 3	Avg +/- St. Deviation	Rotavirus Stock PFU/ml
Number of Rotavirus Plaques	42	36	33	37 +/-4.58	1.48×10^7
					Reovirus Stock PFU/ml
Number of Reovirus Plaques	130	80	110	106 +/-25.16	4.24×10^7

3.3.3 Antiviral Activity of *Y. schidigera* and *T. terrestris* on Rotavirus and Reovirus

To demonstrate the antiviral effect of *Y. schidigera* and *T. terrestris* saponin extracts, a plaque assay was performed under various treatment conditions as outlined in section 3.2.8.

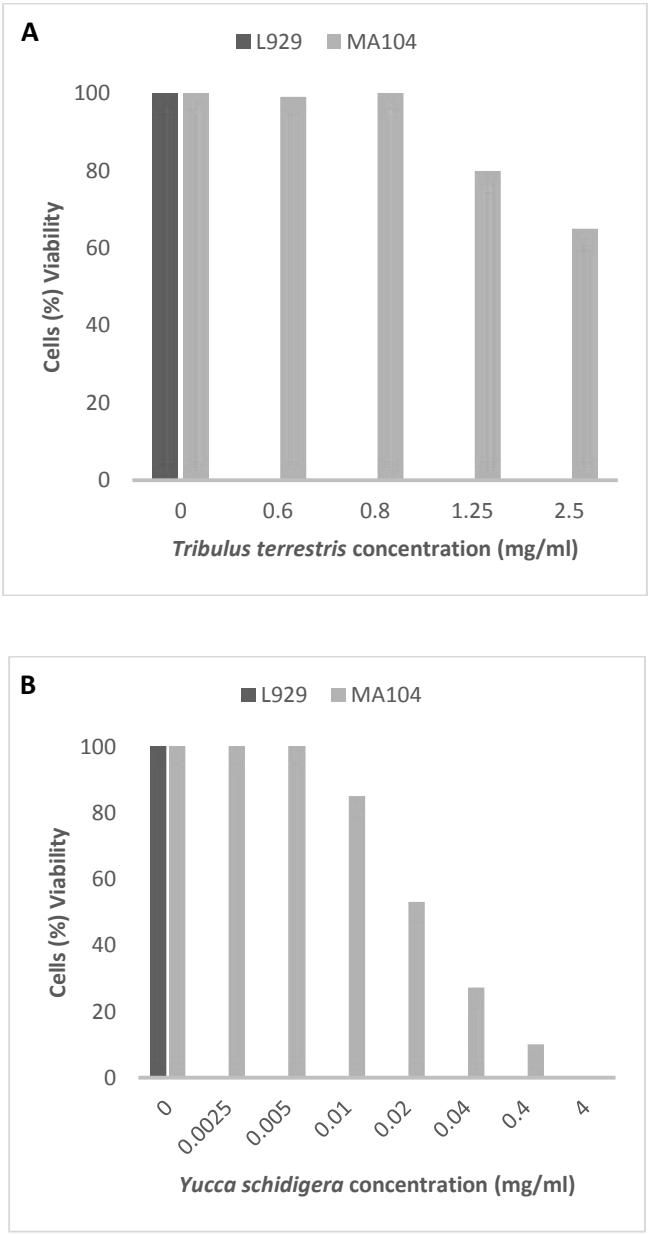


Figure 3.2. Cytotoxicity of A. *T. terrestris* and B. *Y. schidigera* extract on MA104 and L929 cells. Cytotoxicity was assessed by trypan blue exclusion procedure. Data are the means of three independent experiments in triplicates.

3.3.3.1 Does Pre-treatment with *Y. schidigera* and *T. terrestris* Extracts Protect Cells Against Infection?

To determine if the saponin extract pre-treated cells are no longer able to support virus infection, I treated cells with individual saponin extracts for two, four and six hours. The results of this assay are summarized in Table 3.2 and Figure 3.3-3.4. When the MA104 cells were pre-treated with *T. terrestris* saponin extract and then infected with rotavirus there is a 60% reduction in the number of plaques regardless of the amount of time cells were pre-treated (Table 3.2). However, when L929 cells were pre-treated with *T. terrestris* extract prior to reovirus infection there is a 37% decrease in viral replication after 2 hours pre-treatment and a steady decrease in virus production with each increase in the amount of time the cells were pre-treated with a maximum 74% reduction in virus production after 6 hours cell pre-treatment.

Pre-treatment of the MA104 cells with *Y. schidigera* saponin extract for two hours results in a 36% reduction in rotavirus production as compared to saponin untreated cells. An increase in the pre-treatment time of the MA104 cells with *Y. schidigera* extract resulted in a steady decrease in the level of protection against rotavirus infection. When the L929 cells were pre-treated with *Y. schidigera* saponin extract and then infected with reovirus there is a statistically significant reduction in viral yield following two, four and six hours of pre-treatment with up to a 72% reduction in reovirus production after 6 hours of pre-treatment.

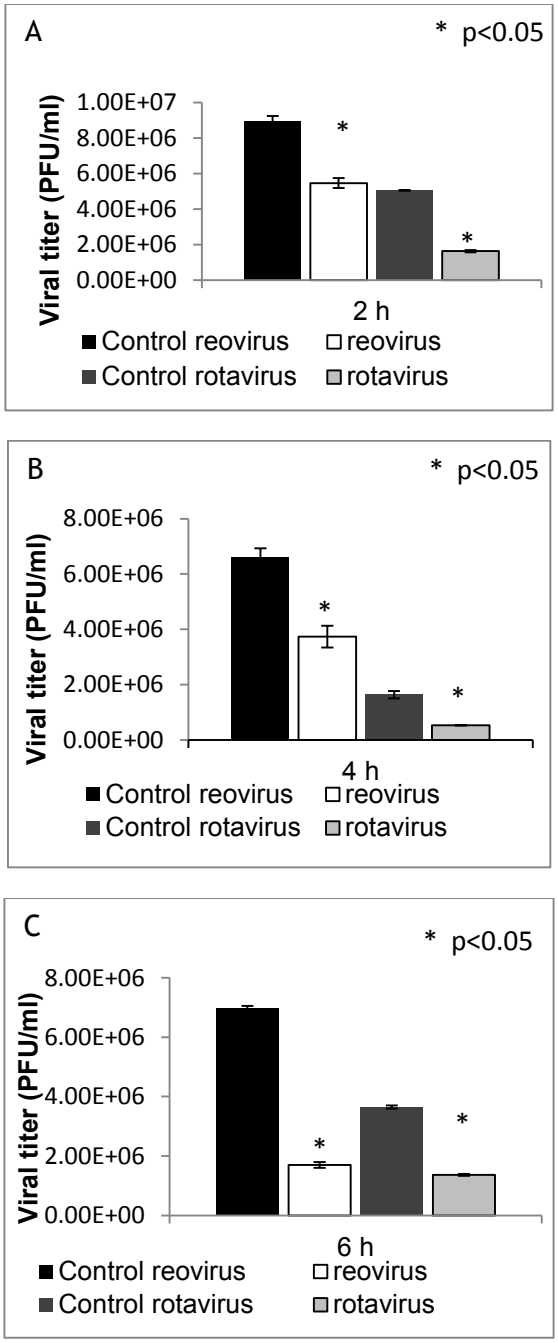


Figure 3.3. Antiviral activity of saponin extracts on cells pre-treated for 2, 4 and 6 hours. Antiviral activity is assessed as virus yield (PFU). Values are mean ± standard deviation of two independent experiments. The results are represented as (A) *T. terrestris* extract 2 h pre-treatment (B) 4 h (C) 6

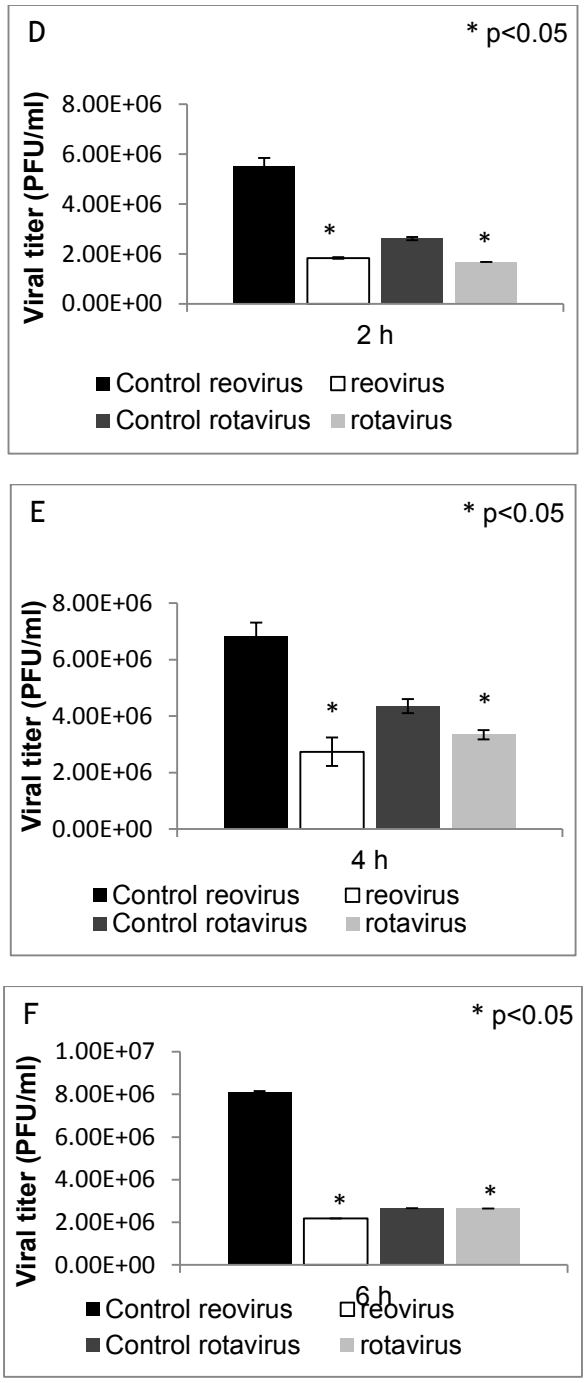


Figure 3.4. Antiviral activity of saponin extracts on cells pre-treated for 2, 4 and 6 hours. Antiviral activity is assessed as virus yield (PFU). Values are mean ± standard deviation of two independent experiments. The results are represented as (D) *Y. schidigera* extract 2 h pre-treatment (E) 4 h (F) 6 h

3.3.3.2 Does Co-treatment of Cells with *Y. schidigera* and *T. terrestris* Extracts Protect the Cells Against Infection?

To examine if the presence of individual saponin extracts during virus infection yields cells resistant to virus infection, MA104 cells were infected with rotavirus while L929 cells were infected with reovirus, in presence or absence of either *Y. schidigera* or *T. terrestris* extracts. As shown in Table 3.3 and Figure 3.5, co-treatment with either saponin containing extract was able to reduce both rotavirus and reovirus infectivity under conditions of co-treatment. The *T. terrestris* extract demonstrated a more robust activity, about 93% protection against reovirus and 90% protection against rotavirus.

3.3.3.3 Does Post-treatment of Cells with *Y. schidigera* and *T. terrestris* Extracts Protect the Cells Against Infection?

Another parameter of antiviral activity of the saponin extracts explored in this study was if the presence of saponin extract post-infection, that is only in agar overlay for the duration of the plaque assay, would be sufficient to protect the cells from rotavirus or reovirus infection. This explored the hypothesis that saponins could prevent virus spread from individually infected cells to surrounding cells, in contrast to the previous assays that examined blocking the initial infection and subsequent spread. As shown in Table 3.4 and Figure 3.6, the presence of *T. terrestris* saponin extract in the agar overlay post rotavirus or reovirus infection produced no reduction in virus production, and thus did not prevent new infections. In contrast, the presence of *Y. schidigera* extract post rotavirus infection results in a 1 fold reduction in rotavirus production, and a 2 fold reduction in reovirus production.

Table 3.2. Antiviral activity of *Y. schidigera* and *T. terrestris* extract in pre-treatment assay. Virus replication detected by plaques which were counted and expressed as plaque forming units per milliliter (PFU/ml). Values are mean ± standard deviation of two independent experiments in triplicates.

Virus	Time of exposure to extract prior to infection (hrs)	*Tribulus terrestris*			*Yucca schidigera*		
		Control	Extract treated	Percent reduction due to extract	Control	Extract treated	Percent reduction due to extract
Reovirus	2	$9.2(1.0)x10^6$	$5.80(7.2)x10^6$	36.96	$5.17(3.2)x10^6$	$1.80(1.6)x10^6$	65.18
	4	$6.93(2.3)x10^6$	$3.54(2.8)x10^6$	51.08	$7.31(1.7)x10^6$	$3.24(1.3)x10^6$	55.68
	6	$7.08(1.0)x10^6$	$1.83(0.8)x10^6$	74.15	$8.00(1.2)x10^6$	$2.18(1.1)x10^6$	72.75
Rhesus rotavirus	2	$5.06(6.4)x10^6$	$1.78(2.0)x10^6$	64.82	$2.64(0.3)x10^6$	$1.68(1.9)x10^6$	36.36
	4	$1.49(1.5)x10^6$	$5.20(1.3)x10^5$	65.10	$4.10(2.1)x10^6$	$3.17(0.4)x10^6$	22.68
	6	$3.72(3.9)x10^6$	$1.48(5.4)x10^6$	60.22	$2.66(5.1)x10^6$	$2.64(1.8)x10^6$	0.75

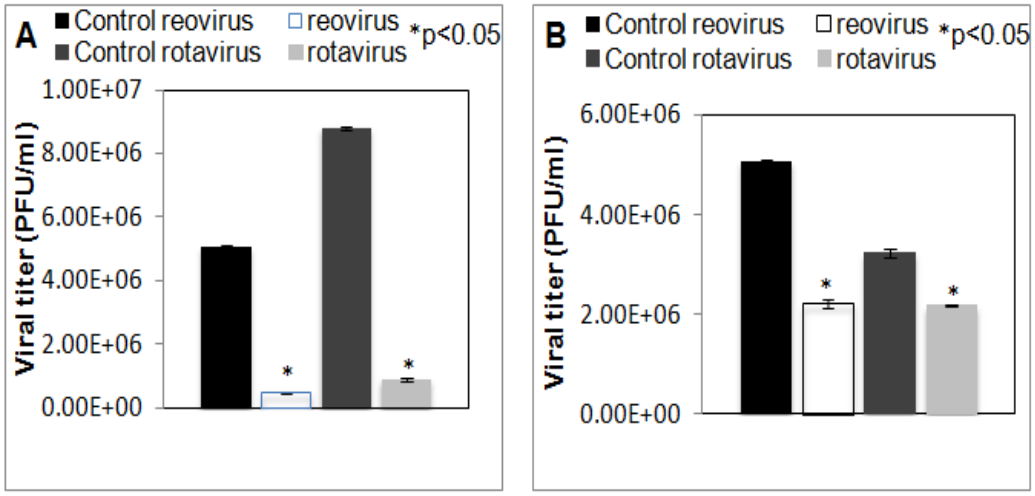


Figure 3.5. Antiviral activity of saponin extracts during co-treatment. Antiviral activity is assessed as virus yield (PFU). Values are mean ± standard deviation of two independent experiments. The results are represented as (A) *T. terrestris* extract (B) *Y. schidigera* extract

3.3.3.4 Direct Virucidal Effect of *Y. schidigera* and *T. terrestris* Extracts

The ability of the saponin extracts to directly inactivate both rotavirus and reovirus was tested. Each virus was treated with 0-50mg/ml of the *T. terrestris* extract and 0-40 mg/ml of the *Y. schidigera* extract. As demonstrated in Figure 3.7, extract treatment of each virus for 6 hours at 37°C did not reduce the virus infectivity. Concentrations as high as 50 mg/ml of *T. terrestris* is not effective at inactivating reovirus (Figure 3.7 B), while the concentration of 5 mg/ml of *Y. schidi*gera and around 15 mg/ml of *T. terrestris* are required to produce a 50% reduction in rotavirus infectivity (Figure 3.7, A). If *Y. schidigera* or *T. terrestris* saponin extract have a direct antiviral effect than it would occur at concentrations much higher than those tested and would prevent treatment of cells with such high concentrations as these concentrations are toxic to the cells themselves.

Table 3.3. Antiviral activity of *Y. schidigera* and *T. terrestris* extract in co-treatment assay.

Virus	Virus pre-incubated with extract	*Tribulus terrestris*			*Yucca schidigera*		
		Control	Extract treated	Percent reduction due to extract	Control	Extract treated	Percent reduction due to extract
Reovirus	1 hour	$6.20(0.6)x10^6$	$4.50(2.0)x10^5$	92.74	$5.03(4.3)x10^6$	$2.20(1.9)x10^6$	56.26
	None	$2.82(1.9)x10^6$	$1.72(3.1)x10^6$	39.00	$2.55(1.3)x10^6$	$1.20(2.3)x10^6$	52.94
Rhesus rotavirus	1 hour	$8.78(3.8)x10^6$	$9.00(6.0)x10^5$	89.75	$3.52(1.1)x10^7$	$1.08(1.1)x10^7$	99.69
	None	$1.58(0.2)x10^6$	$2.93(0.4)x10^6$	-85.44	$3.12(0.1)x10^6$	$2.28(1.4)x10^6$	26.92

Table 3.4. Antiviral activity of *Y. schidigera* and *T. terrestris* extract in post-treatment assay. Virus replication detected by plaques which were counted and expressed as plaque forming units per milliliter (PFU/ml). Values are mean ± of two independent experiments.

Virus	Virus titer (PFU/ml)[a]			
	Control	Post - treatment(mg/ml)	Control	
		Tribulus terrestris		Yucca schidigera
Reovirus	(2.82 +/- 1.9) x 10^6	(2.82 +/- 1.9) x 10^6	(2.55 +/- 1.3) x10^6	(1.20+/- 2.3) x 10^6
Rhesus rotavirus	(1.58 +/- 0.2) x10^6	(2.93+/- 0.4) x 10^6	(3.12 +/- 0.1) x10^6	(2.28 +/- 1.4) x10^6

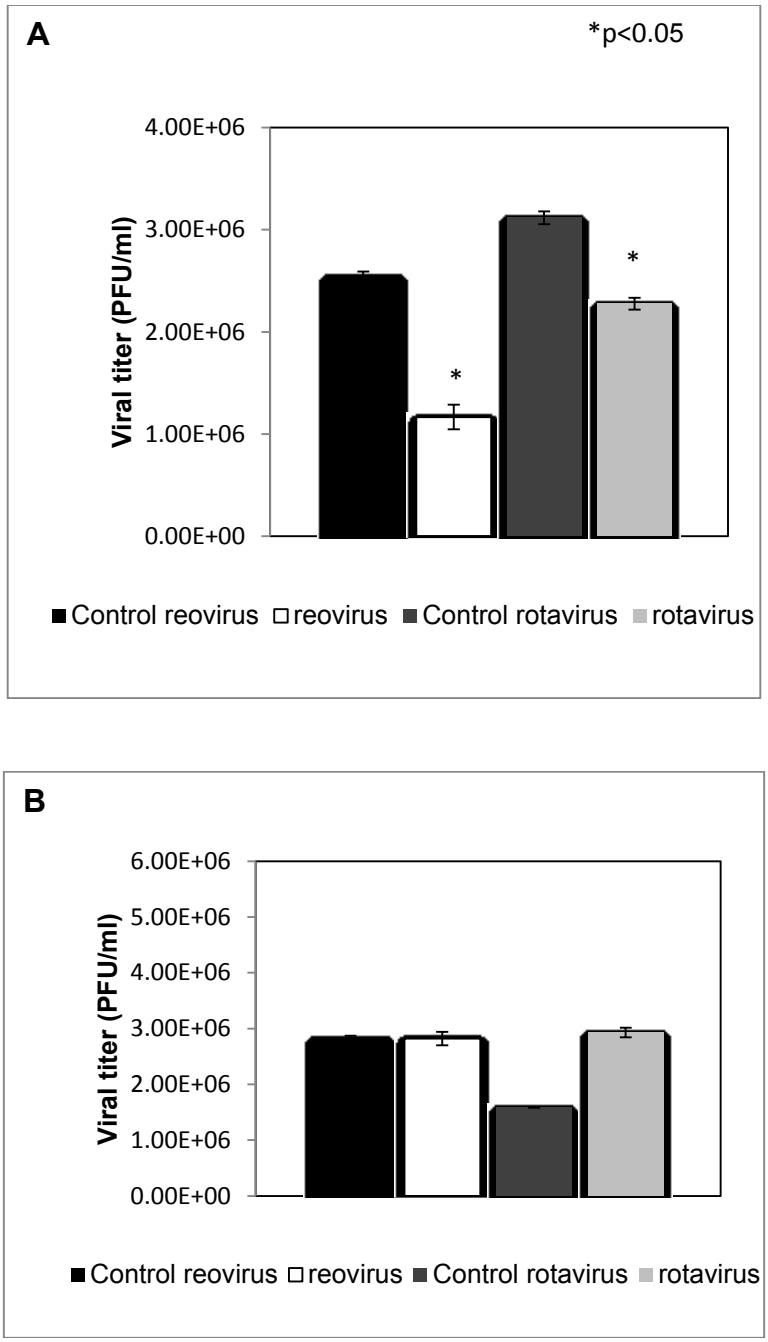


Figure 3.6. Antiviral activity of saponin extracts during post-treatment. Antiviral activity is assessed as virus yield (PFU). Values are mean ± standard deviation of two independent experiments. The results are represented as (A) *Y. schidigera* extract (B) *T. terrestris* extract

3.3.3.5 Lasting Effect of *T. terrestris* and *Y. schidi*gera Extract Treated Cells to Prevent Virus Infection

To measure the length of time cells would be protected from virus infection, cells were pre-treated with *T. terrestris* and *Y. schidi*gera saponin extract for 1 hour and then the extract removed. As indicated in Table 3.5, there was an approximately 20% reduction in reovirus replication when the L929 were infected immediately following *T. terrestris* treatment and steady decrease in protection was demonstrated over two and four hours. When the MA104 were infected with rotavirus immediately post *T. terrestris* treatment there was about 50% reduction in viral replication in comparison to untreated control. Protective effect of *Y. schidi*gera on either cell line was not significant. The two cell lines tested were completely susceptible to virus infection if they were infected at either 4 hours or 6 hours post-saponin treatment indicating little if any extended protection.

Table 3.5. Protection of cells against infection after 1 hour treatment with *Y. schidigera* and *T. terrestris* extract. Virus replication detected by plaques which were counted and expressed as plaque forming units per milliliter (PFU/ml). Values are mean ± standard deviation of two independent experiments.

Virus	Time after exposure to extract (hrs)	*Tribulus terrestris*			*Yucca schidigera*		
		Control	Extract treated	Percent reduction due to extract	Control	Extract treated	Percent reduction due to extract
Reovirus	0	$3.60(0.1)x10^6$	$2.80(1.8)x10^6$	22.22	$3.04(0.4)x10^6$	$2.88(0.7)x10^6$	5.26
	2	$2.80(2.7)x10^6$	$2.40(0.4)x10^6$	14.29	$3.04(0.4)x10^6$	$2.16(0.3)x10^6$	28.95
	4	$3.12(0.8)x10^6$	$2.88(2.1)x10^6$	7.69	ND	ND	ND
Rhesus rotavirus	0	$2.8(0.1)x10^6$	$1.28(0.6)x10^6$	54.29	$2.96(0.2)x10^6$	$3.12(0.2)x10^6$	-5.41
	2	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND

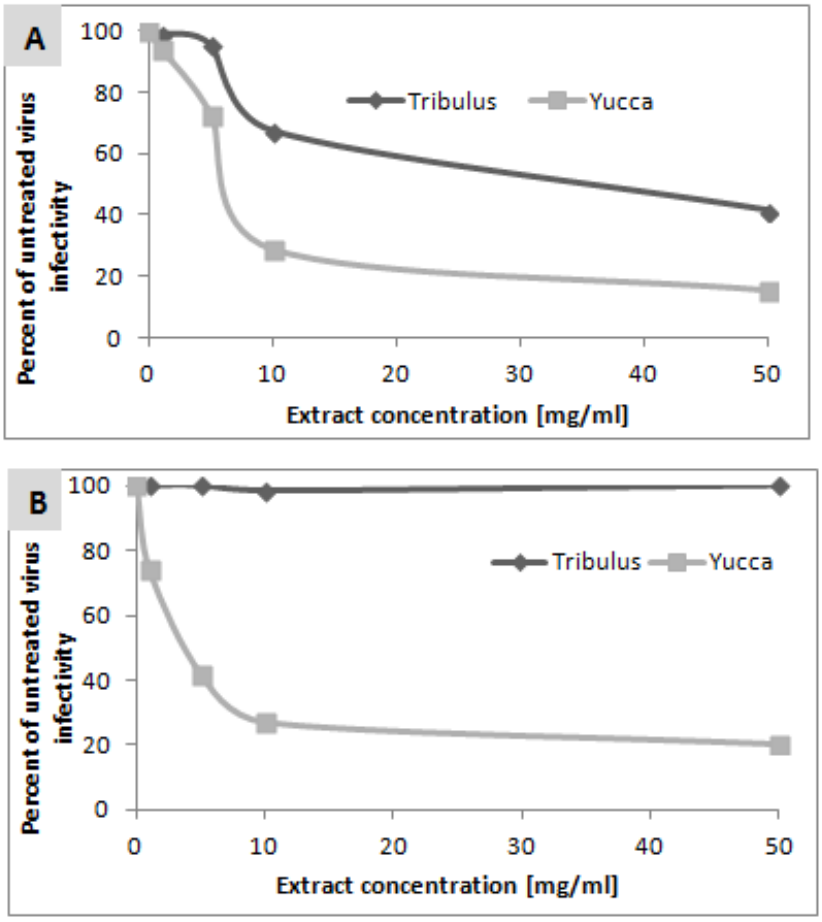


Figure 3.7. Percent inhibition of (A) rotavirus (B) reovirus infectivity due to *Y. schidigera* and *T. terrestris* saponin extracts. Data presented are means ± standard deviations of 2 separate experiments in triplicate.

3.4 Discussion

Rotavirus is the leading cause of severe diarrheal disease in children, accounting for 37% of all diarrheal deaths in children under the age of five. The high prevalence of rotavirus infections in young children is of concern. Rotavirus licensed vaccines have been shown to be safe and effective in both developed and developing countries [132]. Unfortunately, the high cost of the vaccines compromises access to the vaccine in

developing countries and prevents full vaccine coverage. Therefore, alternative measures are needed to reduce the number of deaths in young children and to relieve the great economic burden associated with rotavirus infections. In this study, I have evaluated the antiviral activity of two plant saponin-rich extracts: one from *Y. schidigera* and one from *T. terrestris* for their antiviral activity against rotavirus and reovirus. Both are aqueous extracts, and the *Y. schidigera* extract is approved by the FDA (under FDA 21 CFR 172.510.) for use in food and beverages. Both extracts contain a mixture of steroidal saponins with varying structures depending upon which sugars are attached to which carbons of the aglycone skeleton as well as the nature of the aglycone.

The results of my study demonstrate that *Y. schidigera* and *T. terrestris* extracts of ten-fold or higher concentrations than those toxic to MA104 and L929 cells, are required to cause a disruption in the virus particle rendering it non-infectious. Most likely, one of the reasons for this high concentration of extract required for a direct virucidal effect is the fact that rotavirus and reovirus are both non-enveloped viruses and are highly stable in the environment presenting few targets for disruption by detergent-like compounds. A number of studies exploring the mechanism of saponins action indicate that saponins exhibit their activity on membranes [41, 137-139], a structure found only with enveloped viruses. The concentrations of extract required to directly inactivate either rotavirus or reovirus are substantially greater than those used to demonstrate the antiviral activity of the extract. Therefore the antiviral activity of extracts from *Y. schidigera* and *T. terrestris* observed in this study are due to the protective effect of the extracts on the cells themselves and not due to inactivation of the viruses directly prior to infection. The results of this study demonstrate that both extracts are capable of up to a four-fold reduction in virus production when cells were pre-treated prior to infection. A stronger effect was observed with pre-treatment of cells with the *T. terrestris* extract. The pre-treatment of

cells with *T. terrestris* extract resulted in more than a 60% reduction in rotavirus production and a 70% reduction in reovirus production. The most robust antiviral effect was observed when saponin-containing extracts from *T. terrestris* were present during the virus infection, with a 93% inhibition of reovirus and a 90% inhibition of rotavirus production. When extracts were present post-infection, only *Y. schidigera* extracts demonstrated antiviral activity with a slight reduction of both rotavirus and reovirus production.

Also, in this study I was able to show that the protective effect of *T. terrestris* extract in protecting L929 cells against virus infection is not long lasting and cells are again susceptible to virus infection 4 hours after the initial treatment of cells. This indicates that no long term modification of the L929 cells due to *T. terrestris* extract treatment occurred.

Based on this study, I propose that it would be beneficial to examine if the antiviral effect can be enhanced by the presence of extract prior to and during the infection, also if any synergistic effect can be observed by combining the *Y. schidigera* and *T. terrestris* extracts during the virus infection of cells *in vitro*.

In conclusion, this data demonstrates for the first time that *Y. schidigera* and *T. terrestris* natural plant extracts have antiviral activity against rotavirus and reovirus. Overall, the *T. terrestris* extract had a higher protective effect *in vitro* against both viruses. The observed antiviral activity of *T. terrestris* extract *in vitro* can only be used as an indicator for the therapeutic benefit of the extract *in vivo*. Therefore, it would be valuable to examine if the antiviral effect can be translated *in vivo* as has been reported for extracts from *Q. saponaria.*

One unanswered question still remains and that is one surrounding the mechanism by which saponins reduce infection. Both saponin extracts used in this study

exhibit antiviral activity to differing degrees, suggesting the antiviral mechanism of action may be different. A large number of biological effects attributed to saponins are due to saponin interactions with cellular membranes. It is likely that the saponin interaction with the cell membrane may lead to modifications in the cell membrane and subsequently prevention of virus attachment and entry. Future studies are needed to pursue this further.

Chapter 4

Investigation of the Antibacterial Effect and the Mechanism of Saponin Extracts on Cell Membranes

4.1 Introduction

Infectious diarrheal disease is the second leading cause of morbidity and mortality globally. Bacteria have long been established as major food-borne and water-borne agents of diarrheal disease. Bacterial pathogens such as *V. cholerae*, *Salmonella*, pathogenic strains of E*. coli*, *Shigella*, *Yersinia* and *Listeria* cause much of the burden. [140].

Etiological agents causing diarrheal disease such as *V. cholerae* continue to be a worldwide health concern [141]. Due to poor sanitation, poverty and social strife, outbreaks continue to occur. The estimated numbers of cholera cases globally are 3-5 million per year with more than 100,000 deaths [94, 142]. Cholera is endemic in over 50 countries in Asia, parts of Africa and Latin America [143]. Two types of whole-cell killed vaccines are commercially available: Dukoral and Shanchol. Both vaccines contain whole killed *V.cholerae* O1 serotype along with recombinant cholera toxin B subunit [142]. These vaccines are available in countries where *V.cholerae* is endemic.

Salmonella enterica is another important water and food-borne pathogen with an estimated 93 million cases globally and 155,000 deaths due to *Salmonella* infections [144]. In the United States alone there are approximately 1.4 million salmonellosis cases annually resulting in over 1000 deaths [102]. It is likely that the actual number of salmonellosis cases is greater due to underreporting and asymptomatic cases.

Escherichia coli O157:H7 is an emerging foodborne pathogen that occurs globally. Since the first *E. coli* O157:H7 documented outbreak in 1982, it has continued to

cause outbreaks and sporadic cases in at least 30 countries on six continents [145]. However, a large number of cases of diarrheal disease due to this particular *E. coli* serotype commonly occur in the United States (US), Canada and the United Kingdom [146]. In the United States, *E. coli* O157:H7 is estimated to initiate 95,400 infections annually according to the CDC. This is a dangerous pathogen not only because of the diarrheal disease it causes but because of the potential for the development of hemolytic uremic syndrome (HUS) following infections, which is the leading cause of kidney failure in children [105, 147]. Treatment of diarrheal disease resulting from *E. coli* O157:H7 infection is often non-specific and includes ORT. Evidence does not exist that antibiotic treatment is helpful, and there is some evidence that sub-lethal concentrations of certain antibiotics may actually increase the risk of HUS [147].

Shigella also continues to be a global health burden since its discovery in the 1890s. Five to fifteen percent of all diarrheal disease cases worldwide are due to *Shigella* infections alone, resulting in 120 million cases annually, with 91 million infections in Asia and 8 million infections in Africa alone [113, 148]. The number of deaths due to *Shigella* infection is estimated to be 1.1 million and 60% of deaths are children under the age of five [149]. Currently, treatment strategies for shigellosis are inadequate and resistance of *Shigella* to most antibiotics is increasing. Vaccines could control and potentially eliminate *Shigella* infections but there are no licensed *Shigella* vaccines to date [114].

Yersinia enterocolitica is an important emerging water and food-borne human pathogen, frequently reported as a cause of gastroenteritis following *Campylobacter* and *Salmonella* [117]. *Y enterocolitica* infections occur in many countries worldwide, but the infections are much more common in colder climates such as northern Europe, Scandinavia, and Japan. The number of culture confirmed *Y. enterocolitica* cases in the

United States is about 5% of all bacterial enteric infections [150]. Treatment of patients with yersiniosis is primarily supportive, with hydration being the most important treatment.

In contrast to other enteric pathogens that cause gastrointestinal infections resulting in diarrheal disease, *L. monocytogenes* has a lower rate of occurrence but a higher rate of mortality, up to 30% [73]. Sporadic cases of listeriosis are more common, but large outbreaks can occur as well. According to CDC data, 1600 cases of *L. monocytogenes* occur annually with a mortality rate of 18%. Recently, an outbreak that originated in Colorado was the result of contamination in cantaloupes from Jensen Farms and spread to 28 additional states with 30% mortality and one woman experiencing a miscarriage. Globally the WHO estimates that rates of listeriosis not only vary between countries but also vary between consecutive years in the same country with typically 0.1-1.1 cases per 100,000 people are observed.

Treatment of diarrhea (caused by bacterial pathogens) with antibiotics in some cases can reduce morbidity associated with diarrheal disease. The problem exists that many bacterial enteric pathogens are becoming increasingly resistant to antibiotics as recorded by the National Antimicrobial Resistance Monitoring System. The extensive use of antibiotics in animal husbandry contributes to the burden of resistance, especially for food and water-borne pathogens such as *Salmonella* [140].

It is apparent that there is an urgent need to reduce the number of diarrheal disease associated cases. This study explored alternative measures in dealing with diarrheal disease. In this study I examined the antibacterial activity of three natural saponin-rich extracts: *Y. schidigera*, *Q. saponaria* and *T. terrestris*. These extracts are obtained from renewable resources at a low cost [43]. Infectious diarrhea caused by bacteria is initiated by adhesion to the host intestinal tissue and invasion of the host

tissue. The ability of these saponin-rich extracts was investigated to prevent these first steps in the infection process.

This study also examines two known effects of saponins as possible antimicrobial mechanisms of saponins in my antiviral and antibacterial studies. The two activities explored were (1) changes in the extracellular cholesterol and (2) changes in the intracellular Ca^{2+} levels both known to occur following exposure of mammalian cells to saponins.

4.2 Materials and Methods

4.2.1 Saponin Extract Preparation

The *Q. saponaria* extract used was obtained from Desert King International, San Diego, CA. This study used Vax Sap which is prepared by Desert King and is a purified medical grade version of their standard *Q. saponaria* extract. The saponin stock was prepared by dissolving the dried material into sterilized 1x SSC at a concentration of 0.2 gram of Vax Sap per 10 milliliter to yield a 20 mg/ml stock followed by filter sterilization. The final pH of the saponin extract solution was 7.2 to 7.4.

The *Y. schidigera* and *T. terrestris* extract stock solutions were used in the study and were prepared as outlined in 3.2.4.

4.2.2 Cell Lines and Culture Conditions

The HeLa cell line has been used extensively in the research literature for bacterial adhesion and invasion assays, primarily due to its pattern of growth in culture dishes allowing for easier access by the bacteria to the cells for subsequent adhesion and invasion by the bacteria (Figure 4.1). The MA104 and L929 cells were used for the cholesterol and calcium assays as they were the target cells for the antiviral assays. The

culture conditions for the cells are described in section 3.2.3. The cytotoxicity of each saponin extract was evaluated by measuring HeLa, L929 and MA194 cell viability as outlined in 3.2.7. Cytotoxicity of the extracts was reported: *Y. schidigera* and *T. terrestris* on MA104 and L929 cells was previously done (Figure 3.2).

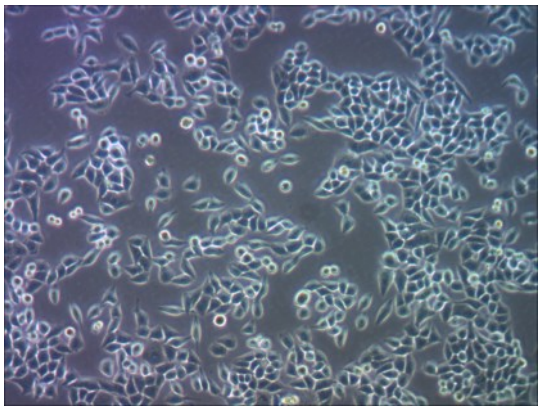


Figure 4.1. Sub-confluent HeLa cells in a well of a 6 well plate

4.2.3 Bacterial Strains and Growth Conditions

The bacterial strains used in the study and their American Type Culture Collection (ATCC) designation number are included in Table 4.1, along with growth medium used for cultivation of the strains and incubation times for the individual bacterial assays.

4.2.4 Preparation of Bacterial Working and Glycerol Stock

To prepare each bacterial working stock, bacteria were grown overnight in broth at 37°C statically or with agitation. The overnight culture was streaked onto a 100 mm x 15 mm petri dish, and incubated at 37°C for 24-48 h to obtain isolated colonies. The petri dish stock culture was kept at 4°C for a maximum of one month when a new stock was prepared. To prepare a long term storage glycerol stock, the same conditions of bacterial growth were applied as outlined above and 500 µl of each culture was added to 500 µl of 30% glycerol in a 2 ml cryo-vial tube, gently mixed and frozen at -80°C.

Table 4.1. List of bacteria used in the study. Growth medium is indicated and the incubation times for the adhesion and invasion assay

Strain	ATCC	Growth Medium	Adhesion assay incubation Time	Invasion assay incubation time
E. coli 0157:H7	43890	Luria Bertani (LB) Broth	6 hours	N/A
Yersinia enterocolitica	23715	Brain Heart Infusion (BHI) Broth	30 min	1 hr
Listeria monocytogenes	7646	Brain Heart Infusion (BHI) Broth	30 min	1 hr
Salmonella enterica serovar Typhimurium	6994	Luria Bertani (LB) Broth	30 min	1 hr
Shigella flexneri	12022	Nutrient (NA) Broth	2 hr	3 hr
Vibrio cholerae	51394	Luria Bertani (LB) Broth	30 min	N/A
Salmonella enterica serovar Typhimurium	14028	Luria Bertani (LB) Broth	30 min	1 hr

4.2.5 Measuring Cell Density Based on Optical Density (OD)

The bacteria in this study were cultured in their respective broth statically or with agitation at 37°C for the adhesion and invasion assays with the exception of *L. monocytogenes* which was cultured at 30°C for the invasion assay and 37°C for the adhesion assay. *Shigella flexneri* was cultured at room temperature for the adhesion and invasion assays while *Y. enterocolitica* was cultured at room temperature for the invasion

assay and at 37°C for the adhesion assay. To calculate colony forming units (CFU) per ml (CFU/ml), an OD_{600} of an overnight bacterial culture was prepared and the culture was serially diluted and plated onto agar plates in the absence of antibiotic. The counted colonies are used to calculate the CFU/ml, which was then set in relation to the measured OD_{600} value. The following formula was used for CFU/ml calculation: CFU/ml = Number of colonies on the plate/final plate dilution.

4.2.6 Growth Condition of Bacteria for Disk Diffusion Assay

To obtain a bacterial suspension for the disk diffusion assay, the colony suspension method was used. About two-three colonies were picked from the bacterial working stock plates and transferred to the 15 ml tube containing 10 ml of broth. The turbidity of each bacterial suspension was compared to McFarland standard (BioMérieux, Inc.). The bacterial suspension was adjusted to a McFarland standard number of 0.5 which is indicative of 1-2 x 10^8 CFU/ml. After the bacterial suspension was adjusted, it was used within 15 minutes to avoid any change in cell numbers due to growth or death within the culture.

4.2.7 Growth Condition of Bacteria for Broth Microdilution Assay

To obtain the bacterial inoculum for the broth microdilution assay, the growth method using overnight cultures was used. About two-three colonies were picked from the bacterial working stock and transferred to the 15 ml tube containing five-eight ml of indicated broth. Tubes were incubated overnight statically or with shaking at 37°C. Overnight bacterial suspension turbidity was adjusted using a McFarland standard, yielding a final inoculum size of approximately 5 x 10^5 CFU/ml and were used within 15 minutes to avoid changes in the cell number.

4.2.8 Growth Condition of Bacteria for Adhesion and Invasion Assay

The bacterial strains used in this study were cultured in their indicated broth statically or with shaking at 37°C overnight. After overnight incubation at 37°C the absorbance at OD_{600} was measured using a spectrophotometer. Based on the OD_{600} value each bacterial culture was diluted in MEM to either 2 x 10^6 CFU/ml for the co-treatment or 1 x 10^6 CFU/ml for the pre-treatment assay to yield a final total of 1 x 10^6 CFU/ml per well during the infection.

4.2.9 Disk Diffusion Assay

Prior to the disk diffusion assay, saponin extract saturated disks were prepared by the following procedure. Saponin extract stock solutions at a concentration of 1 mg/ml were two-fold serially diluted in phosphate buffer saline (PBS). About 10 blank sterile paper disks (Fisher Scientific Co Cat # B313390) were added to each 15 ml tube containing different saponin extract concentrations, 1 mg/ml being the highest saponin extract concentration tested. The disks were incubated for 24 hours at 4°C allowing the disks to be saturated with each extract preparation. Following the 24 hour incubation, individual disks were placed on the surface of the sterile Petri dish by picking each disk from the solution with sterile forceps. The disks were allowed to dry for 3 hours in a laminar flow hood.

Prior to the disk diffusion assay, bacterial suspensions for the assay were prepared as indicated in 4.2.6. This bacterial suspension was used to inoculate agar plates by dipping sterile cotton swabs (Fisher Cat # 18-366) into each bacterial suspension and spreading the bacterial suspension evenly across the agar plate. Previously dried saponin extract saturated disks were added to the inoculated agar plates with sterile forceps. Agar plates were incubated at 37°C for 24 hours. Antibacterial activity

of each saponin extract was determined by measurement of the resulting zone of inhibition in millimeters (mm) against each bacterial strain. Kanamycin 30 µg (BD Catalog # 230829) was used as a positive control and PBS served as negative control. The experiment was carried out in triplicates and repeated once.

4.2.10 Broth Microdilution Assay

The aim of this assay was to determine the lowest concentration of each saponin extract able to inhibit growth of each bacteria. The saponin extract stock solutions were two-fold serially diluted in the indicated broth into a 96 well plate by first adding 50 µl of broth to each well of a 96 well plate, followed by 50 µl of individual saponin extract. The highest concentration of *Y. schidigera* extract tested was 20 mg/ml, 50 mg/ml of *T. terrestris* and 10 mg/ml of *Q. saponaria* extract. Following saponin addition, 50 µl of bacterial inoculum was added per well of a 96 well plate with a multi-channel pippettor. Again, the bacterial suspension for this assay was prepared as indicated in section 4.2.7. Each 96 well plate was incubated for 20-24 hours at 37°C. The results of the assay were obtained by recording the absorbance at 600 nm using a plate reader (BioTek).The minimum inhibitory concentration was recorded as the lowest concentration of saponin extract that resulted in no growth of the bacteria. Background fluorescence (broth only or saponin extract diluted in broth) readings were subtracted from each test sample. Each experiment was performed in duplicate or triplicate and repeated at least 3 times.

4.2.11 Bacterial Adherence Assay

This assay was done in 6 or 12 well culture plates and was done in two ways to assess qualitative and quantitative adherence of bacteria to HeLa cells. For the qualitative adherence assay, HeLa cells were grown on glass coverslips. Prior to seeding

the HeLa cells onto tissue culture plates, sterile glass coverslips were placed onto each well of a culture plate using sterile forceps. Next, HeLa cells were seeded at about 1 x 10^6 cells per well in 2 ml of MEM with 5% FBS, 1% P/S and incubated overnight at 37°C, 5% CO_2 environment. After overnight incubation, cells that reached 70-80% confluence were washed two times with sterile PBS solution and infected with specific bacteria. HeLa cells were either pre-treated or co-treated with saponin extract at 0.0025 mg/ml of *Q.saponaria* and *Y. schidigera* or 0.8 mg/ml of *T. terrestris*.

For the pre-treatment assay, cells were treated with saponin extract for 24 hours. After treatment of the cells with each saponin extract, the saponin mixture was aspirated and cells were washed twice with sterile PBS solution. Cells were then infected with 1ml of 1 x 10^8 CFU/ml of individual bacteria diluted in MEM and incubated at 37°C, 5% CO_2 for the indicated times (Table 4.1).

During the co-treatment assay, 0.5 ml of MEM without saponin extract (control) or 0.5 ml MEM with saponin extract (treatment) and 0.5 ml of bacteria diluted in MEM that contained 4 x 10^8 CFU/ml were added simultaneously to HeLa cell monolayers. The plates were incubated at 37°C, 5% CO_2 for the indicated times (Table 4.1).

After each experimental condition, HeLa cell monolayers were washed with PBS four times to remove bacteria that did not adhere. Following the PBS wash, the glass cover slips were fixed with 100% methanol for 10 min and stained with 10% Giemsa stain for 20 min and examined microscopically under a 100x oil immersion lens. Each experiment was performed in duplicates and repeated once. The images of this qualitative adherence assay are included in Appendix C.

For the quantitative bacterial adherence assay certain modifications were made in comparison to the qualitative adherence assay. First, seeding of HeLa cells did not occur on the coverslips but directly onto the wells of a 6 well culture plate. Another

modification was that the bacterial inoculum added to the HeLa cells during infection was 1 x 10^6 CFU/ml. The final modification was that instead of cell fixation, the cells were lysed in 1 ml 0.1% Triton X-100 to release non adherent bacteria. The concentration of Triton X-100 used in this experiment ensures that bacterial lysis does not occur but the mammalian cells are completely lysed.

4.2.12 Bacterial Invasion Assay

The bacterial invasion assay is based on the gentamicin protection assay and measures the ability of invasive bacteria to enter the epithelial cells *in vitro* in the presence or absence of saponin extract. This assay was preformed similarly to the quantitative bacterial adherence assay (4.2.11) with the exception that cells post-infection were treated with 1 ml of 100 µg/ml gentamicin diluted in MEM for 2-3 hours to kill any extracellular bacteria.

4.2.13 Cholesterol Assay

Extracellular cholesterol was measured with a cholesterol-oxidase based fluorometric assay (The Amplex Red Cholesterol Assay Kit) from Invitrogen (Catalog no. A12216). The assay is based on an enzymatic reaction that can detect cholesterol at a concentration of 200 nM or lower. The basis of the enzymatic reaction is shown in Figure 4.2.

For the experiment, L929 or MA104 cells were seeded in a 24 well plate and incubated at 37°C, 5% CO_2 for 24 hours or until 100% confluency was reached. Next, the medium was removed and the cell monolayers were washed twice with 1x SSC solution. Following the SSC wash, cells were *Q. saponaria*, *T. terrestris* or *Y. schidigera* saponin extract treated. The saponin extracts were diluted in MEM with 1% P/S to the

concentrations not toxic to the cells (3.3.2) or MEM with 1% P/S and no saponin extract served as a control. The saponin extract containing medium (200 µl total volume) or medium alone was added to the individual wells of a 24 well plate in triplicate. The plates were incubated at 37°C, 5% CO_2 for 24 or 48 hours. Additionally, prior to cholesterol quantification, the cells were treated with 10 mM of methyl-β cyclodextrin (mβCD) which is known to bind cholesterol and pull it out of the membrane.

To quantify the extracellular cholesterol concentration, 50 µl of medium supernatant from saponin treated and untreated cells (control) was aspirated from each well and added to individual wells of a black with clear bottom 96 well culture plate. Once all of the samples were transferred from a 24 well plate to a 96 well plate, cholesterol standard was prepared in a 96 well plate. The remainder of the assay was preformed according to the Amplex cholesterol assay kit manufacturer instructions.

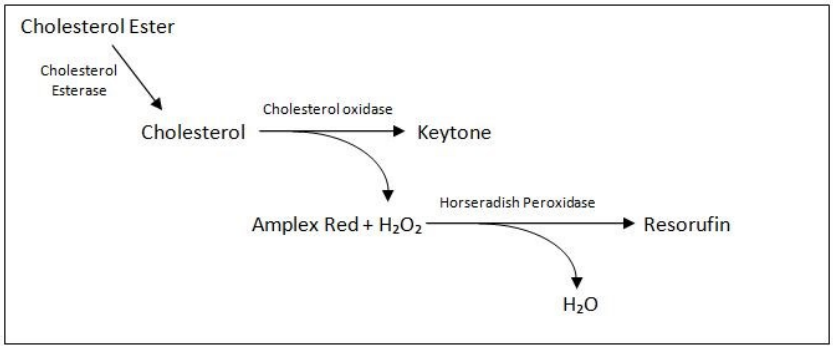


Figure 4.2. Enzymatic reaction for quantification of cholesterol**.**

4.2.14 Calcium Assay

Intracellular Ca^{2+} ion concentration was measured using a calcium ions-0-cresolphthalein chromogenic complex based assay (The Calcium Colorimetric Assay Kit) from BioVision Inc. (Catalog no. K3a80-250). The chromogenic complex can be detected by measuring absorbance at 575 nm (Figure 4.3). For the experiment MA104 and L929

cells were seeded in a 6 well tissue culture plate and incubated at 37°C, 5% CO_2 for 24 or 48 hours, until the cell density was approximately 1.2 x 10^6 cells per well. The cells were then treated with either *Q. saponaria* or *T. terrestris* saponin extract diluted in MEM with 1% P/S to the concentrations not cytotoxic (3.3.2) or MEM with 1% P/S and no saponin extract (control). The plates were incubated at 37°C, 5% CO_2 for 6 or 24 hours. Post-incubation, saponin-containing medium or medium only was aspirated and the cells were washed one time with 1x SSC solution, after which 60 µl of distilled water is added to each well of a 6 well plate. Cells were sonicated by inserting a sonicating probe to each well. Following sonication, 50 µl of cell lysate was transferred to individual wells of a 96 well plate and the calcium assay reagents added according to the manufacturer instructions. A calcium standard was prepared for each experiment. The 96 well plate was incubated at room temperature for 10 minutes in the dark. The absorbance at 575 nm was measured using a microplate reader. The background (well containing 0 µg of calcium) was subtracted from each sample reading. The assay was performed in duplicates and repeated once for the 6 hour assay but not for 24 hour assay.

Figure 4.3. Enzymatic reaction for quantification of intracellular Ca^{2+} ions. Chromogenic complex is formed when Ca^{2+} ion bind O-cresolphthalein whose absorbance can be detected at 575 nm wavelength

4.3 Results

4.3.1 Antibacterial Activity of Saponin Extracts

The results of disk diffusion assay (Appendix A) indicate that the highest concentration of saponin extract tested (1 mg/ml) had no inhibitory activity on bacterial

growth *in vitro*. The negative control (PBS) had no antibacterial activity while positive control (Kanamycin) had growth inhibitory effect with zones of inhibition ranging from 24-32 mm depending on the bacterial strain. Figure 4.4 shows the effect of saponin extract on the bacterial growth. The highest saponin extract concentration tested did not inhibit the growth of the gram-positive or gram-negative bacteria used in this study. It is worth mentioning that there is an increase in growth at low concentrations of saponin extract for most of the bacterial strains tested. Concentrations of *Y. schidigera*: 0.1 and 0.3 mg/ml had a growth promoting effect on most bacterial strains tested, while concentrations of *Q. saponaria*: 0.1, 0.3, 0.6 and 0.3 mg/ml of *T. terrestris* exhibited a similar growth enhancing effect on majority of bacterial strains tested.

4.3.2 Cytotoxicity of *Y. schidigera*, *T. terrestris* and *Q. saponaria* to HeLa Cells

The cytotoxicity of the saponin extracts on HeLa cells was performed as outlined in section 3.2.7. As indicated in Figure 4.5, the viability of HeLa cells was not affected with 0.0025 mg/ml of *Y. schidigera* and *Q. saponaria*, while 0.8 mg/ml of *T. terrestris* saponin extract does not affect the viability of HeLa cells under these treatment conditions.

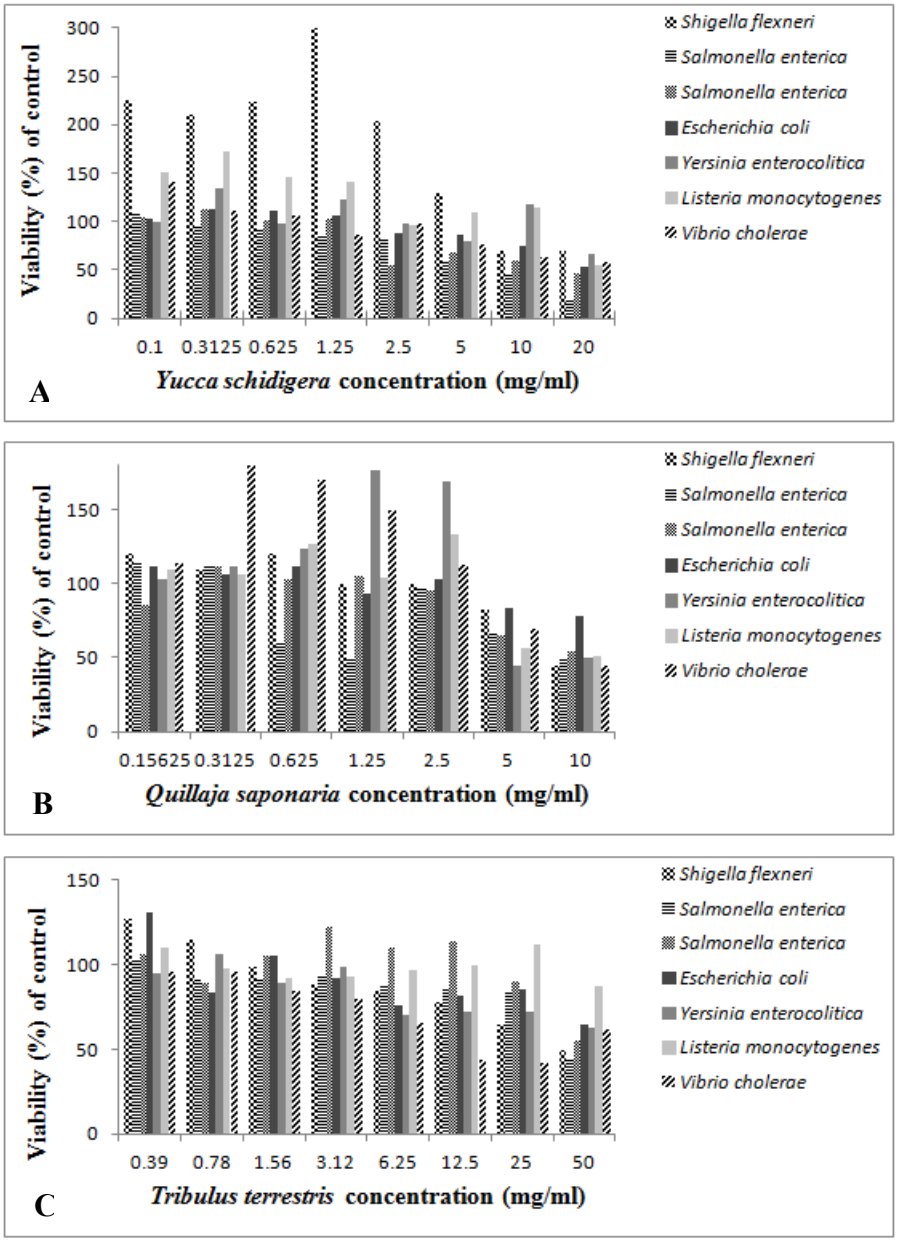


Figure 4.4. Growth of bacterial strains in the presence of (A*) Y. schidigera* extract, (B) *Q. saponaria* extract, (C) *T. terrestris* extract. The values represent mean ± standard deviation of 3 experiments in triplicates and are expressed as growth percentages relative to control which was assigned 100%.

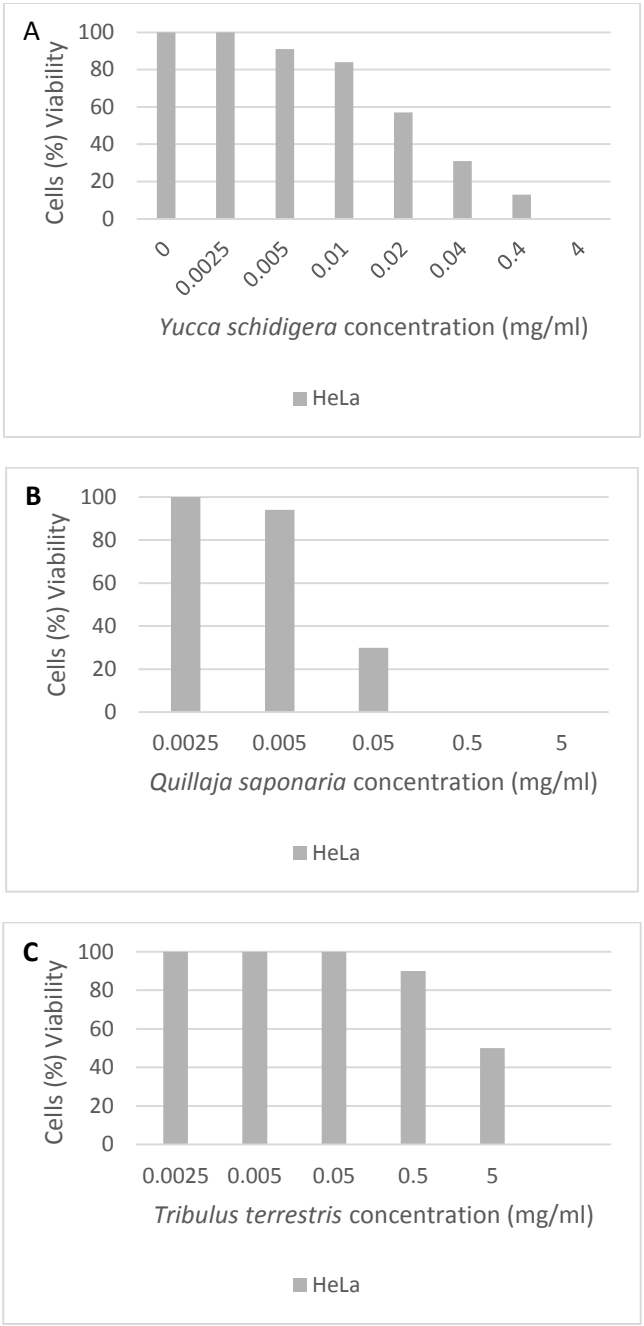


Figure 4.5. Cytotoxicity of (A) *Y. schidigera* (B) *Q. saponaria* and (C) *T. terrestris* extract on HeLa cells. Cytotoxicity was assessed by trypan blue exclusion procedure. Data are mean of three independent experiments in triplicates.

4.3.3 Adherence to HeLa cells Pre-treated with Saponin Extract

When HeLa cells were pre-treated for 24 hours with saponin extract-containing medium at non-cytotoxic concentrations (Figure 4.5) or medium alone (control) and then infected with individual bacteria, pre-treatment of cell with each of the saponin extracts failed to cause a significant reduction in the adhesion of bacteria with the exception of *E. coli* O157:H7 (Figure 4.7 E). Pre-treatment of cells with *Y. schidigera* extract resulted in significant (9.1×10^3 CFU/well) reduction in *E. coli* O157:H7 adherence in comparison to the untreated control (Figure 4.7 E). Adherence of *L. monocytogenes* was slightly reduced when compared to saponin extract pre-treated HeLa cells with a stronger effect observed when HeLa cells were pre-treated with *Y. schidigera* (220500 CFU/well). Adhesion of *S. enterica* (ATCC # 14028) to *Y. schidigera* pre-treated HeLa cells was also slightly reduced (3.1×10^4 CFU/well) (Figure 4.6 C). Also, there was slightly less CFU/well when *Y. enterocolitica* were added to *Y. schidigera* pre-treated HeLa cells, about 1.3×10^4 CFU/well difference from the control (Figure 4.7 G).

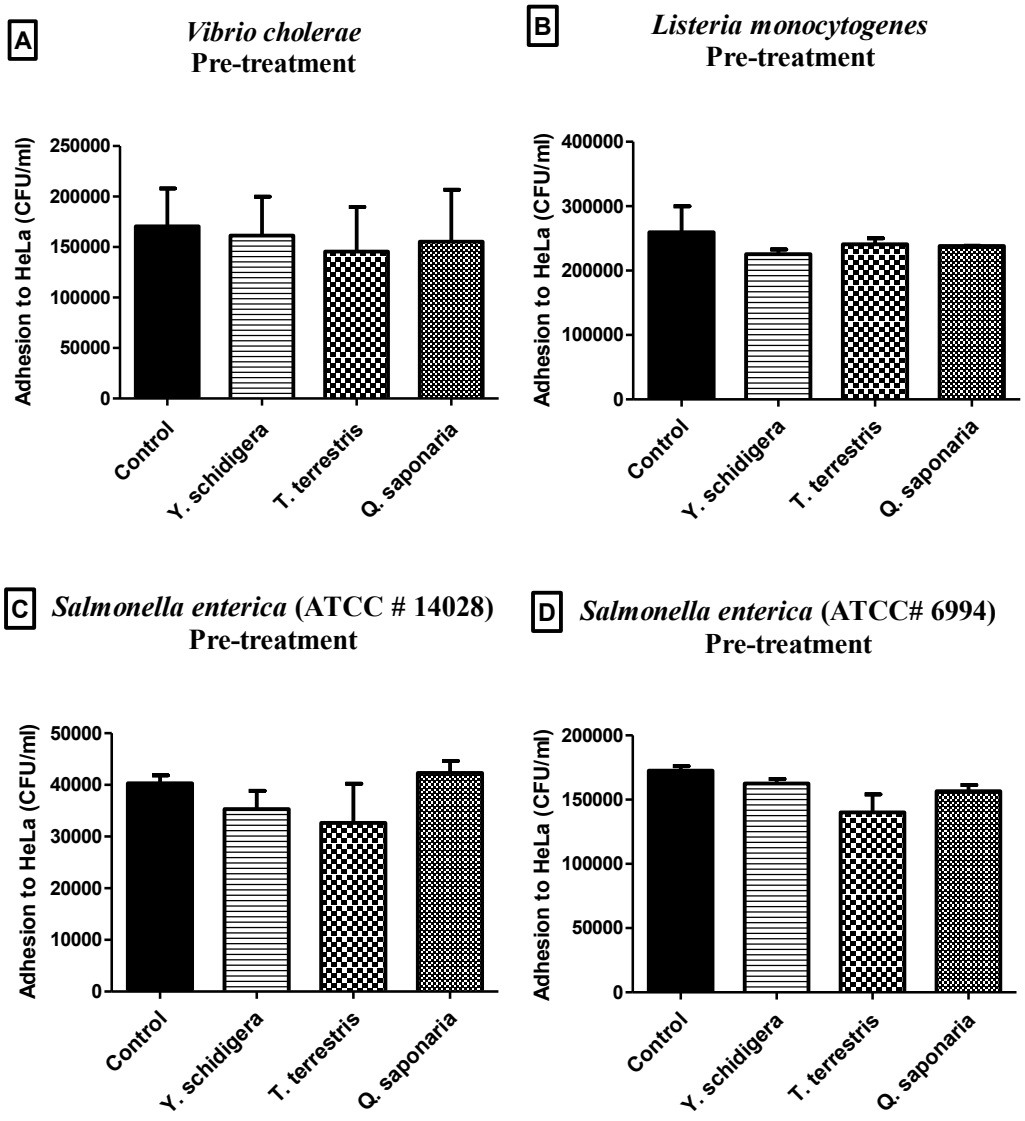


Figure 4.6. Quantification of *in vitro* adherence to epithelial cells of (A) *V. cholerae*, (B) *L. monocytogenes*, (C, D) *S. enterica*. Adherence to pre-treated HeLa cells (see material and methods, 4.2.11 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

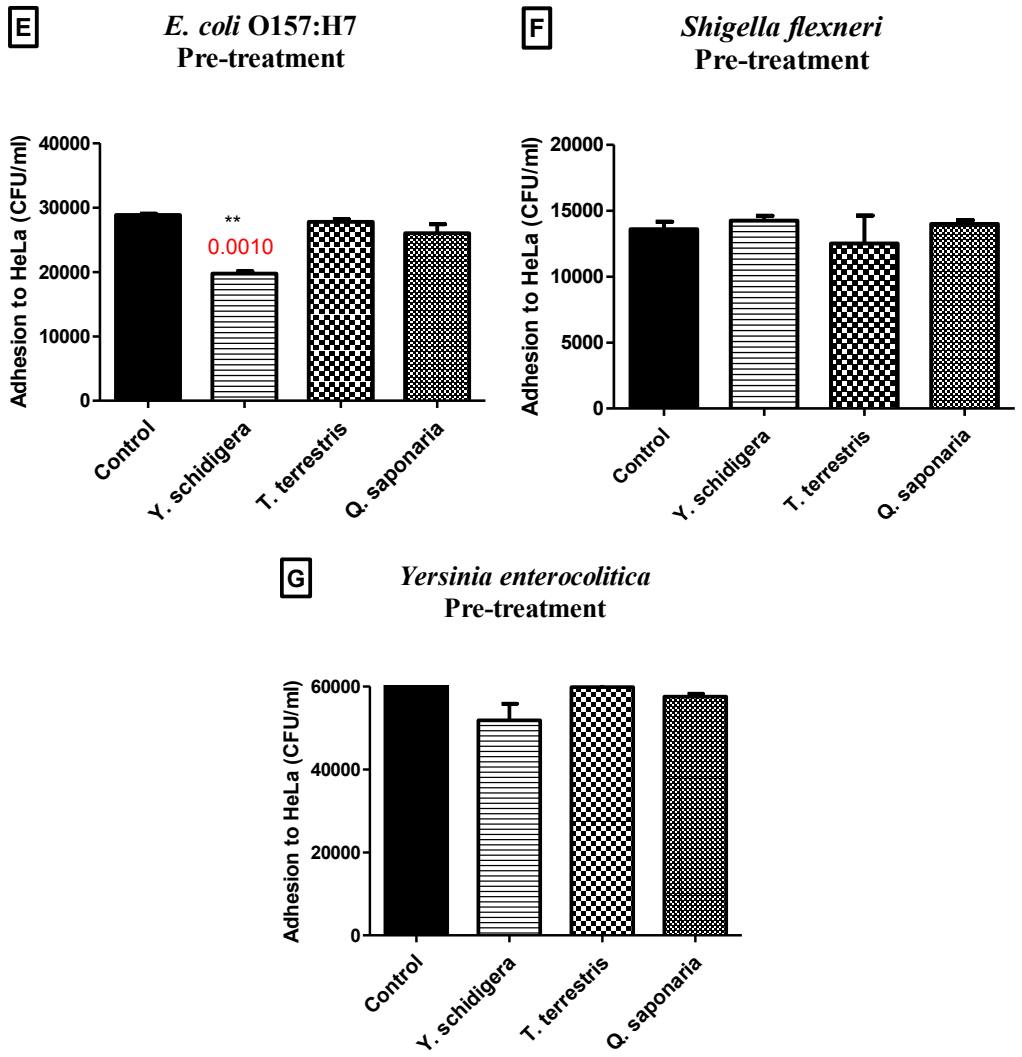


Figure 4.7. Quantification of *in vitro* adherence to epithelial cells of (E) *E. coli* O157:H7 (F*) S. flexneri* (G) *Y. enterocolitica.* Adherence to pre-treated HeLa cells (see material and methods, 4.2.11 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

4.3.4 Adherence to HeLa cells in Presence of Saponin Extract (Co-treatment)

The Co-treatment assay was used to evaluate if the presence of saponin extract during the infection period can prevent attachment of the bacteria to cultured HeLa cells. When individual bacteria and saponin extract were co-incubated simultaneously with the HeLa

cells, the number of bacteria attaching to cells was not significantly reduced in the presence of saponin extract for most bacterial strains tested (Figure 4.8-4.9). It is worth noting that simultaneous addition of bacteria and *Y. schidigera* extract had resulted in slight decrease in adhesion of *E. coli* O157:H7, *S. flexneri and Y. enterocolitica to* HeLa cells, by 5, 27 and 40% reduction in adhesion respectively (Figure 4.9 E, F and G).

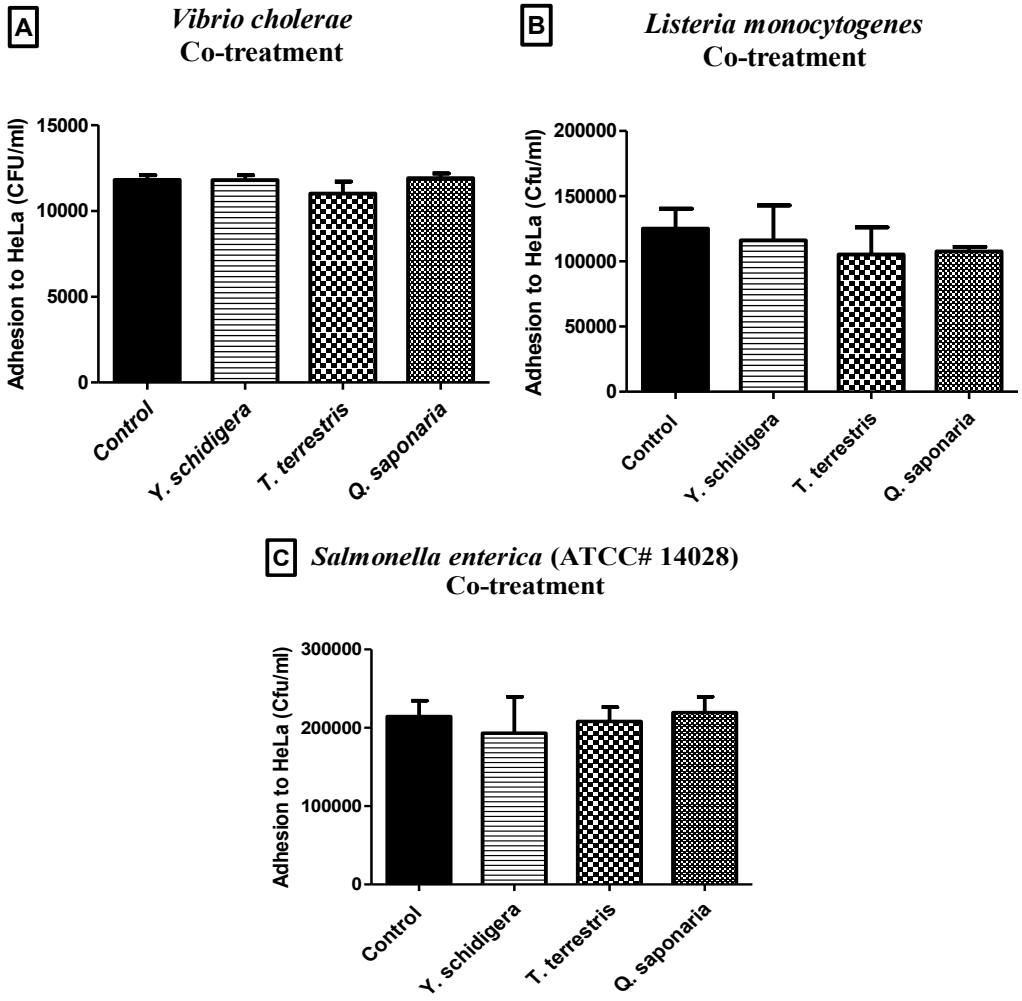


Figure 4.8. Quantification of *in vitro* adherence to epithelial cells of (A) *V. cholerae* (B) *L. monocytogene*s (C) *S. enterica*. Adherence to co-treated HeLa cells (see material and methods, 4.2.11 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

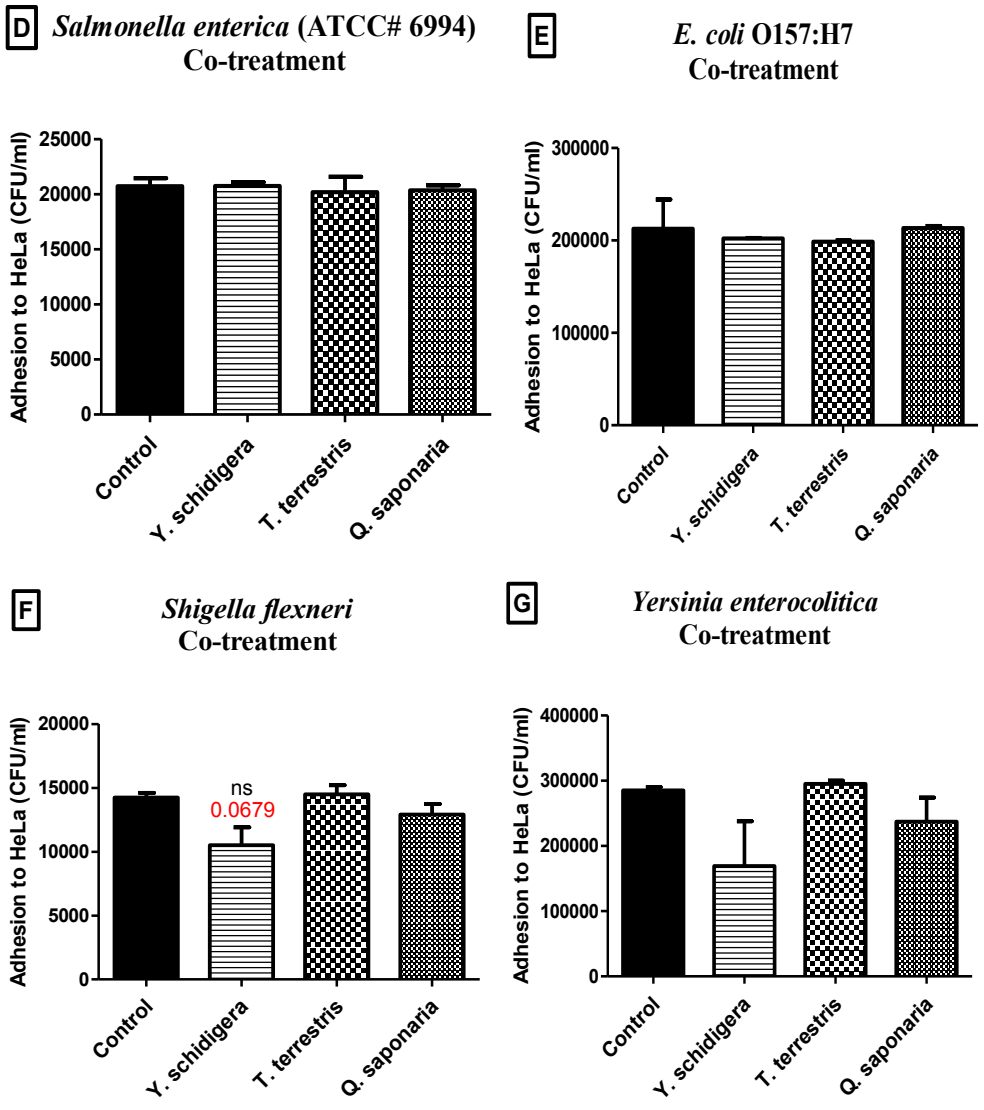


Figure 4.9. Quantification of *in vitro* adherence to epithelial cells of (D) *S. enterica* (E) *E. coli* O157:H7 (F) *S. flexneri* (G) *Y. enterocolitica.* Adherence to co-treated HeLa cells (see material and methods, 4.2.11 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

4.3.5 Invasion of HeLa cells Pre-treated with Saponin Extract

To examine the effect of saponin extracts on bacterial invasion efficiency, cells were pre-treated with individual saponin extracts for 24 hours prior to addition of bacteria. The results of this assay are presented in Figure 4.10 and 4.11. The *Y. schidigera* saponin extract treated cells were effective in reducing the number of invading bacteria. As demonstrated in Figure 4.10 A, the percentage of *L. monocytogenes* invading HeLa cells was modestly reduced (about 15%) but significantly (P= 0.02). Similarly, a statistically significant reduction of invasion was observed for *S. enterica* (P=0.02). About 17% reduction in invasion was observed for *S. flexneri* while *Y. enterocolitica* was reduced in invasion by 20% and significantly (P=0.01). Additionally, pre-treatment of HeLa cells with *T. terrestris* resulted in significant reduction (P=0.04) of *Y. enterocolitica* internalized by HeLa cells or close to a 15% reduction.

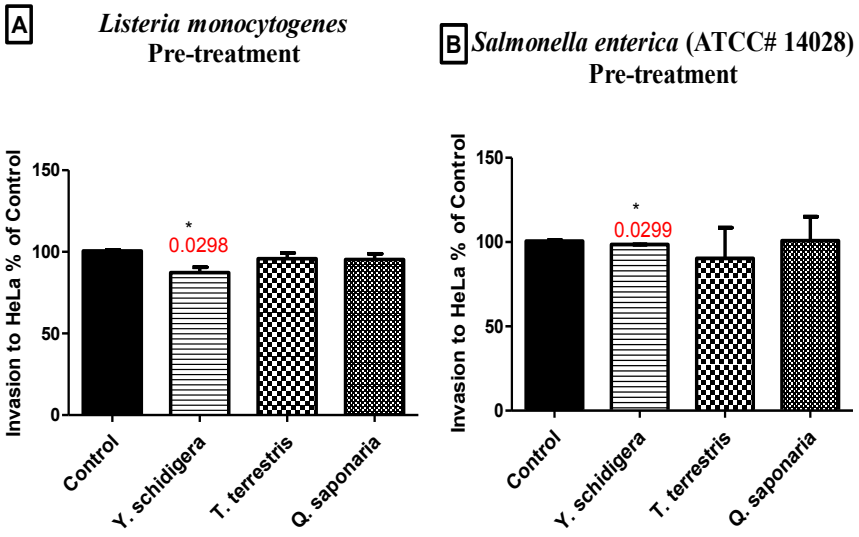


Figure 4.10. Quantification of *in vitro* invasion to epithelial cells of (A) *L.* monocytogenes (B) *S. enterica.* Invasion to pre-treated HeLa cells (see material and methods, 4.2.12 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates are expressed as percentages relative to control which was assigned 100% and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

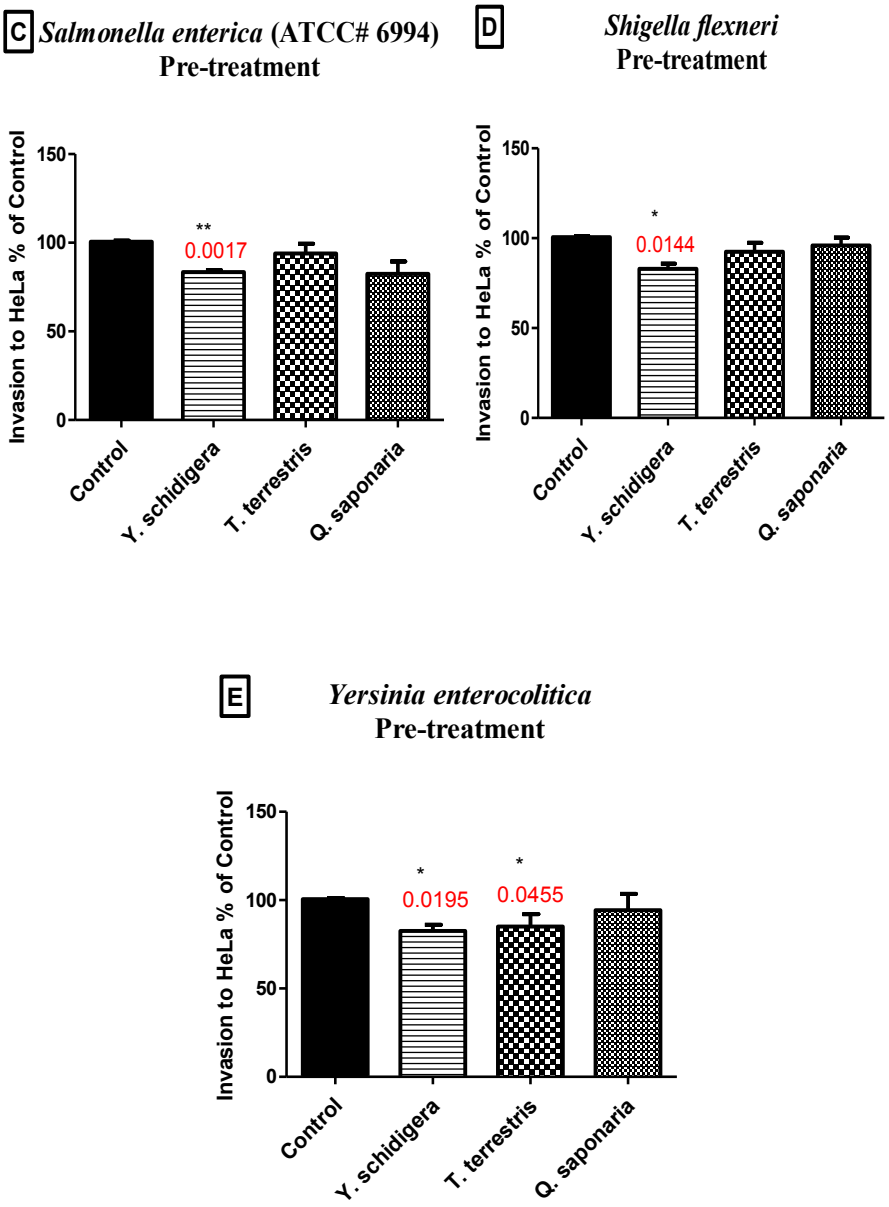


Figure 4.11. Quantification of *in vitro* invasion to epithelial cells of (C) S. enterica (D) *S. flexneri* (E) *Y. enterocolitica.* Invasion to pre-treated HeLa cells (see material and methods, 4.2.12 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates are expressed as percentages relative to control which was assigned 100% and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the Student t test.

4.3.6 Invasion of HeLa cells in Presence of Saponin Extract (Co-treatment)

The results of invasion efficiency of bacteria in the presence of saponin extract are shown in Figure 4.12 and 4.13. The presence of *Y. schidigera* saponin extract during infection resulted in a statically significant reduction in invasion of each of the bacterial strains tested. The invasiveness of *L. monocytogenes* was reduced by 46% (Figure 4.12 A), that of *S. enterica* by 35% (Figure 4.12 B), the reduction in invasion of a different strain of *S. enterica* was 64% (Figure 4.13 C), while S. *flexneri* invasion was reduced by 23% (Figure 4.13 D) and that of *Y. enterocolitica* by 45% (Figure 4.13 E). The presence of *T .terrestris* extract resulted in a moderate yet statistically significant reduction in *Y. enterocolitica* (32%), *L. monocytogenes* (9%) and both strains of *S. enterica* (17%, Figure 4.12 B and 30% Figure 4.13 C) internalized by the HeLa cells. The presence of *Q.saponaria* extract affected only the internalization of *S. flexneri* into HeLa cells and resulted in about 9% reduction in the *S. flexneri* invasion.

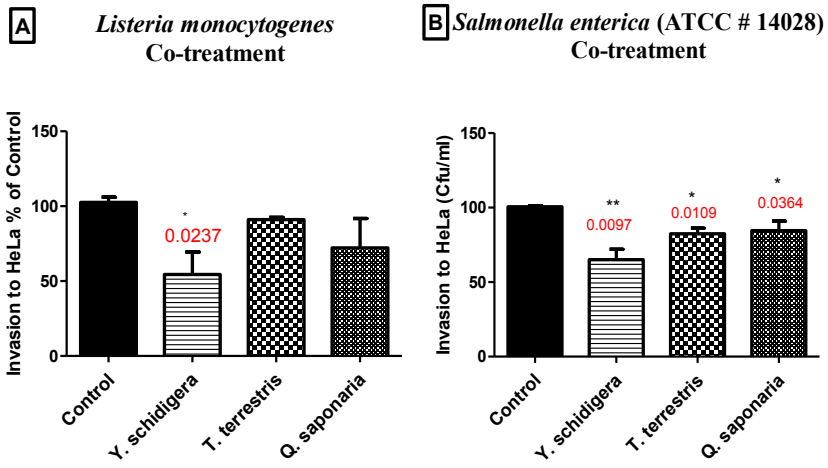


Figure 4.12. Quantification of *in vitro* invasion to epithelial cells of (A) *L*. monocytogenes (B) *S. enterica*. Adherence to co-treated HeLa cells (see material and methods, 4.2.12 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates are expressed as percentages relative to control which was assigned 100%.and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the t test.

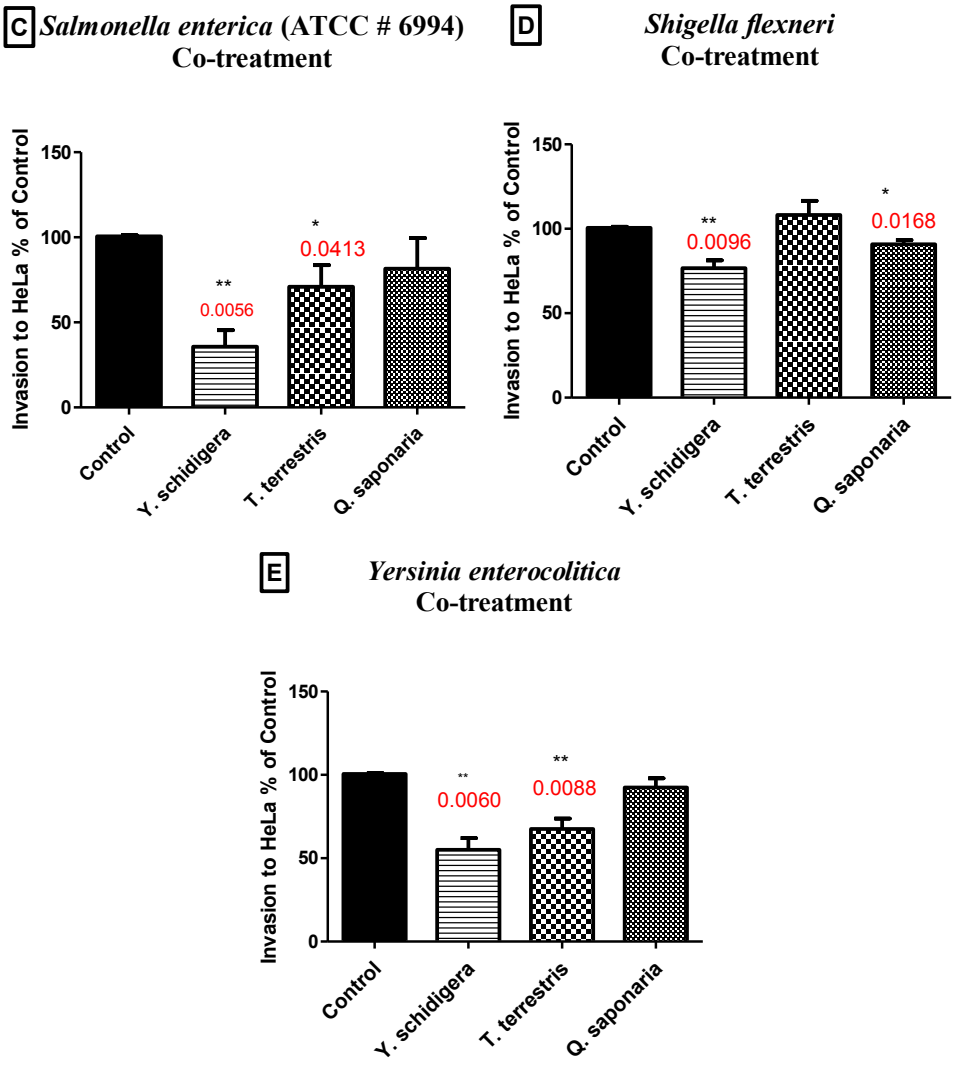


Figure 4.13. Quantification of *in vitro* invasion to epithelial cells of (C) *S. enterica* (D) *S. flexneri* (E) *Y. enterocolitica.* Adherence to co-treated HeLa cells (see material and methods, 4.2.12 for details) was quantified by determining CFU/ml. Results correspond to mean of two experiments preformed in triplicates are expressed as percentages relative to control which was assigned 100%.and bars represent standard deviation. Asterisks indicate p value (shown in red) generated by the t test.

4.3.7 Assessment of Extracellular Cholesterol Concentration in Cells Treated with Saponin Extract

The results of the cholesterol assay help address the question that if saponin extract treated cells undergo a change in the extracellular cholesterol concentration in comparison to untreated cells. As indicated in Figure 4.15 A-B, treatment of L929 and MA104 cells for 24 hours with the highest concentration of *Y. schidigera* saponin extract tolerated by these cells (0.0025 mg/ml) resulted in a reduction of extracellular cholesterol in those cells. The extracellular concentration of cholesterol in 24 hour treated L929 cells with *Y. schidigera* was decreased from 1.54 µg/well in control to a statistically significant 0.71 µg/well, while MA104 saponin treated cells saw a reduction in extracellular cholesterol from 1.10 µg/well in control to 0.83. In addition, 24 hour treatment of L929 cells with *Q.saponaria* extract yielded significant differences between the relative cholesterol content in treated L929 cells in comparison to untreated control (Figure 4.15 A). When MA104 and L929 cells were treated with *T. terrestris* (0.8 mg/ml) for 24 hours, there was a slight reduction in the cholesterol concentration from 1.10 µg/well to 1.0 µg/well for MA104 cells and 1.54 µg/well to 0.83 µg/well for L929 cells. Treatment of the MA104 or L929 cells for 48 hour with *T. terrestris, Y. schidigera* or *Q.saponaria* saponin extract did not result in significant change in the extracellular cholesterol concentration when compared to controls (Figure 4.16 C-D).

4.3.8 Evaluation of Ca^{2+} Intracellular Concentration in Cells Treated with Saponin Extract

As demonstrated in Table 4.2, the saponin extract treatment of L929 and MA104 cells for 6 hours or 24 hours did not significantly increase or reduce the concentration of intracellular Ca^{2+} ions in comparison to controls suggesting that the saponin extracts tested do not modulate levels of free intracellular Ca^{2+} under these test conditions when antiviral and antimicrobial activity is seen.

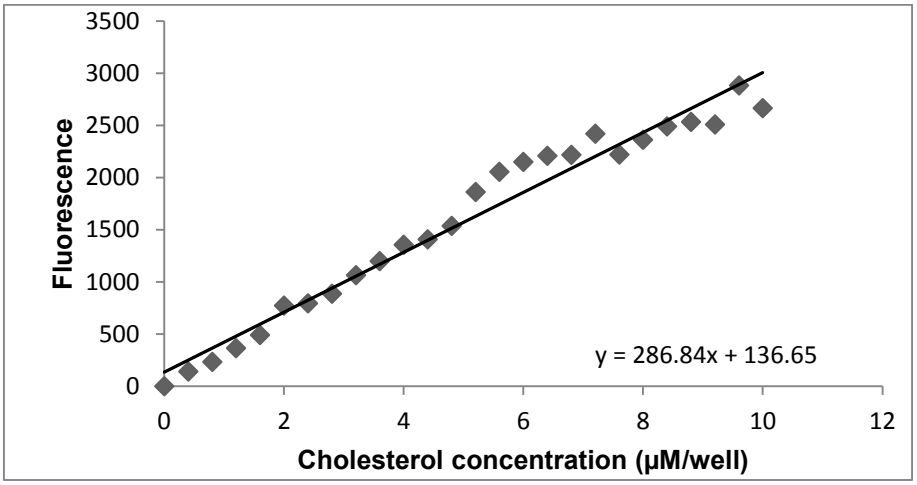


Figure 4.14. Detection of cholesterol using the Amplex Red cholesterol assay kit. Fluorescence of reaction samples with known cholesterol level was detected at excitation at 560 ± 10 nm and emission at 590 ± 10 nm with microplate reader and cholesterol standard curve generated.

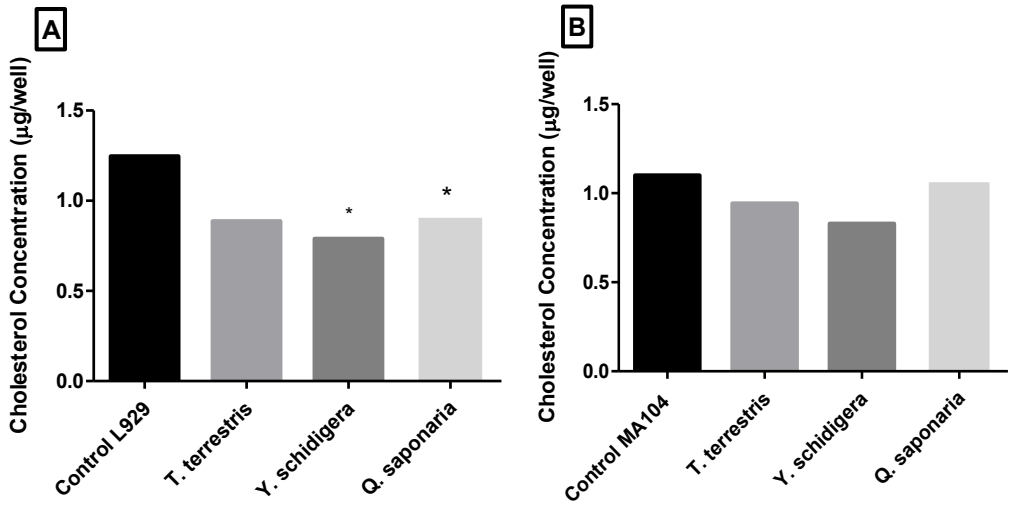


Figure 4.15. Fluorescence reading based on the known concentration of cholesterol in the experiment was used to calculate concentration of cholesterol in the test samples using TREND formula. Panel (A-B) L929 and MA104 cells treated or untreated (control) with saponin extract for 24 h. Results shown correspond to a single representative experiment preformed in triplicates. The ∗ indicates $p < 0.05$ (Student's t test).

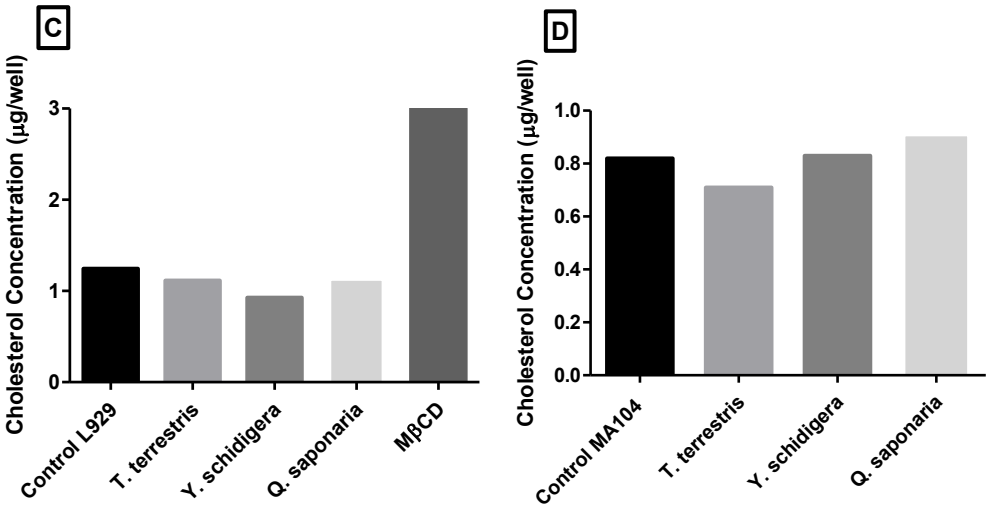


Figure 4.16. Fluorescence reading based on the known concentration of cholesterol in the experiment was used to calculate concentration of cholesterol in the test samples using TREND formula. Panel (C-D) L929 and MA104 cells treated or untreated (control) with saponin extract for 24 h. Results shown correspond to a single representative experiment preformed in triplicates. The ∗ indicates $p < 0.05$ (Student's t test). Panel C shows cholesterol levels in L929 cells treated with 10 mM mβCD.

Table 4.2. Calcium concentration (µg/well) after (A) 6 h (B) 24 h treatment with indicated saponin extracts. The results for 6 h are a mean of two experiments in duplicates while results for 24 h are a mean of single representative experiment in triplicates. Calcium standard curve generated was used to calculated calcium concentration in test samples.

A	**MA104 Control**	**MA104 *T. terrestris* 0.8 mg/ml**	**MA104 *Q. saponaria* 0.005 mg/ml**
Calcium Concentration (µg/well)	0.1	0.1	0.1
	L929 Control	**L929 *T. terrestris* 0.8 mg/ml**	**L929 *Q. saponaria* 0.0025 mg/ml**
Calcium Concentration (µg/well)	0.1	0.09	0.08

Table 4.2. *Continued*

B	**MA104 Control**	**MA104 *T. terrestris* 0.8 mg/ml**	**MA104 *Q. saponaria* 0.005 mg/ml**
Calcium Concentration (µg/well)	0.02	0.005	0.067
	L929 Control	**L929 *T. terrestris* 0.8 mg/ml**	**L929 *Q. saponaria* 0.0025 mg/ml**
Calcium Concentration (µg/well)	0.04	0.08	0.08

4.4 Discussion

Natural saponin extracts of *Y. schidigera*, *T. terrestris and Q. saponaria* were evaluated for their antibacterial effect. It was found that saponin extracts do not have a growth inhibiting effect on any of the bacteria used in the study as evidenced by disk diffusion and broth microdilution methods. In fact each of the saponin extracts had a growth promoting effect on both gram-positive and gram-negative bacteria up to 0.3 mg/ml concentration. A recent study, examining the effect of *Y. schidigera* plant powder and *Q. saponaria* saponins from various commercial sources also found that these extracts have growth promoting effects on *E. coli* K12 [37]. Several explanations for the observed growth promoting effect on bacteria exist. For example, saponins have been found to have a membrane permeabilizing activity, it is possible that increasing membrane permeability would increase the influx of nutrients into the cells and therefore result in increased growth. Saponins are natural detergents and as such they might increase flux of nutrients into the cells without destroying the cell membrane. Another scenario is that sugars that are linked via glycosidic bonds to the aglycone skeleton of

saponin molecule could be hydrolyzed by bacteria and utilized as a nutrient source. It is noteworthy that the concentration of 0.3 mg/ml of *Q. saponaria* to HeLa cells is highly toxic (data not shown), and this different effect of saponins on prokaryotic vs. eukaryotic cells is likely due to differences in their cell membrane architecture.

Next, I examined if saponins can prevent adhesion of bacteria to eukaryotic cells *in vitro*. Adhesion to host cells is an important step of bacterial pathogenesis; in fact adhesion and colonization are required for the subsequent development of diarrheal disease. The individual bacteria used in this study have different means of interacting with the cell surface to initiate adhesion. Most pathogens have genes encoding more than one adhesion factor and in addition use hydrophobic and other non-specific ways to interact with cells [151]. The prevention of an adhesion step in bacterial pathogenesis and subsequently infection is an attractive target for designing a microbiocide.

HeLa cells were used in this study to evaluate if the presence of saponin extract during bacterial infection or if pre-treating cells prior to infection would prevent bacterial adhesion. A quantitative adhesion assay demonstrated that the presence of saponin extracts during infection did not significantly reduce the bacterial adhesion to cultured HeLa cells. However, pre-treatment of cells with *Y. schidigera* led to a statistically significant decrease in adhesion of *E. coli* O157:H7 to HeLa cells with a moderate decrease in adhesion for other bacterial strains with the exception of *V. cholerae* and *S. flexneri.* The reason for this observed difference in reduction of adhesion of some but not all bacterial pathogens in the study is not known. It can be speculated that this differential effect observed is due to differences between bacterial adhesion factors utilized to interact with host cell membrane to facilitate their attachment. One explanation why pre-treatment of cells with *Y. schidigera* results in significant reduction of E*. coli* O157:H7 adherence is that this bacterium is the only pathogen in this study whose bacterially

encoded type III protein secretion system is used to insert particular protein (Tir) into host cellular membrane which is then used by the bacterial protein intimin to bind to cultured cells [152]. On the opposite spectrum, pre-treatment of cells with *Y. schidigera* extract had no effect on *V. cholerae* adhesion to the cells and speculatively can be contributed to the versatility of adhesion factors utilized by this pathogen. *Vibrio cholerae* is also an aquatic bacteria and has various adhesion factors to bind to a number of different surfaces in the aquatic environment such as a number of zooplankton [93].

The next question I wanted to answer involved examining the ability of saponin extracts to prevent invasion of bacteria into cultured cells. The majority of pathogenic bacteria causing diarrhea have invasive abilities as part of their pathogenesis making an inhibition of invasion an important target for disease prevention. This study shows that the presence of extract during infection results in a considerable level of inhibition of invasion. The presence of *T. terrestris* extract during infection can significantly reduce entry of both strains of *S. enterica* and *Y. enterocolitica* into HeLa cells. However, the presence of *Y. schidigera* extract during infection had a much more robust effect than the other extracts, preventing internalization of every bacteria used in the study. In addition, the presence of *Q. saponaria* extract during infection resulted in reduced invasion by only one of the strains of *S. enterica* and *S. flexneri.* Pre-treatment of cells prior to infection with *Y. schidigera* extract and not *T. terrestris* or *Q. saponaria* was also effective in reducing entry into the HeLa cells by all the bacteria tested. The pre-treatment of cells with *T. terrestris* reduced invasion by only *Y. enterocolitica*. Again, the reason for the observed differences in inhibition of invasion among bacteria can only be speculated, and it is possible that these differences exist due to different mechanisms used by bacteria to invade cells that have yet to be fully elucidated.

An additional question, for which an answer is not yet evident, is the mechanism by which saponins in these natural extracts might interact with cells resulting in an antimicrobial effect. It has been suggested that saponin interactions with cellular membranes is the likely mechanism by which saponins initiate a large number of the observed biological effects. The mechanism by which saponins interact with the cell membrane is not well understood and membrane components as well as the structure of saponins seem to play a role. The aglycone portion of saponins is known to interact with cholesterol in the cell membrane and could lead to its removal from the membrane [138]. Cholesterol is an important structural and functional part of lipid domains in the membrane and its removal subsequently would affect normal functioning of the cell membrane components [65, 68]. It is also possible that saponin molecules can distribute in the hydrophobic region of the cell membrane due to the lipid solubility of its aglycone skeleton. In either scenario, saponin molecules interacting with cellular membranes would lead to increase in membrane fluidity. An increase in membrane fluidly can affect various aspects of proper membrane functioning. Additionally, saponins have been found to either open or block ion channels causing a change in membrane polarization and therefore could use this pathway to alter membrane protein function [153].

Data generated in this study demonstrates that the treatment of L929 cells with *Y. schidigera* and *Q. saponaria* extracts for 24 hours but not 48 hours results in a statistically significant decrease in extracellular cholesterol concentration, indicating that saponin interaction with cell membrane cholesterol can lead to a reduction of free extracellular cholesterol in those cells. Saponin treatment of these cells did not result in a Ca^{2+} ion change indicating that this is not the likely mechanism by which saponins in the extract exhibit antimicrobial activity.

Chapter 5

Conclusion

Water is a basic human need. In developing and developed countries water safety is an ongoing issue. The problem is further elevated due to an increasing population density. One of the United Nations Millennium Goals is to increase the number of people that have access to safe drinking water. Some progress recently has been made in providing safe water. However, there is still 11% of the world population or 783 million people without access to safe drinking water. Even with this progress millions of people will die by 2020 of preventable waterborne disease [1].

Waterborne viral and bacterial pathogens: Rotavirus, *V. cholerae*, *S. enterica*, *S. flexneri*, *E. coli*, *Y. enterocolitica* will cause 1 in 9 deaths in children under the age of 5 globally, making diarrhea the leading cause of deaths among children according to the WHO. Therefore, diarrheal disease prevention is a highly relevant topic.

Saponins are a diverse group of glycosides that are widely distributed within the plant kingdom and have an equally broad range of biological properties. The natural saponin extracts used in the study contain a number of steroidal and triterpenoid saponins and most are approved by the Food and Drug Administration for inclusion in beverages or food. Evidence exists that the presence of natural saponin extracts of *Q. saponaria* can prevent rotavirus induced diarrhea in mice [7].

In this study, two natural saponin extracts of *Y. schidigera* and *T. terrestris* were evaluated against two dsRNA segmented viruses from the *Reoviridae* family: rotavirus and reovirus for their ability to prevent infection by these viruses in cultured cells. Rotavirus is the leading cause of severe diarrheal disease accounting for 37% of all diarrheal deaths in children. The results of this study demonstrate that co-treatment of saponin extracts during virus infection was most effective in reducing both rotavirus and

reovirus infectivity. The presence of *T. terrestris* during virus infection had a more robust activity resulting in a 90% reduction of rotavirus infection and over a 90% inhibition of reovirus infection in cultured cells. Adding *T. terrestris* extract post-infection did not result in a reduction in virus yield suggesting that saponins do not work on inhibiting virus replication steps post-entry. A procedure involving co-treatment was most effective in preventing virus infection confirming that the inhibition step is during virus entry.

Based on these results future research will explore the activity of extracts from *T. terrestris in vivo* against rotavirus. Specifically it would be beneficial to examine if the inclusion of *T. terrestris* to milk or water during exposure of animals to rotavirus would prevent rotavirus induced diarrhea in these animals. Additionally, our study suggests that longer pre-treatment (6 vs. 2 or 4 hours) of cells with *T terrestris* can translate to an increase in protection of those cells against infection. If the same effect can be translated *in vivo*, then it would be important to see if the presence of *T. terrestris* saponin extract prior to rotavirus exposure as well as during the exposure would have a higher protective effect against rotavirus induced diarrhea in the animals. This could be easily accomplished by simply adding saponin extracts to the fluids ingested by children prophylactically.

This study also explores if *Q. saponaria*, *Y. schidigera* and *T. terrestris* saponin extracts have the ability to inhibit cell association and invasion of HeLa cells by enteric pathogenic bacteria. Saponin extracts were not effective in preventing bacterial cell adhesion, with the exception of *Y. schidigera* inhibition of *E. coli* O157:H7 adhesion to HeLa cells, which can likely be contributed to the specific mechanism this pathogen exploits to associate with cells. Saponin extracts were effective in reducing invasion of pathogenic bacteria into HeLa cells. The *Y. schidigera* extract displayed high effectiveness in reducing invasion of each of the bacterial strains tested when the extract

was present during infection (co-treatment) as well as 24 hours prior to infection (pre-treatment). The presence of *Y. schidigera* extract during the bacterial infection generated a stronger antimicrobial effect again suggesting that the presence of extract in some way modifies the cell membrane function to prevent bacterial entry.

The mechanism by which saponins in natural extracts deliver their antimicrobial effect was also investigated. Interestingly, each pathogen examined has specific receptor(s) and specific mechanisms of interacting with cell membranes that lead to its subsequent uptake and infection of cells. Saponins in these extracts were able to interfere with entry of each pathogen in this study when extracts were present during infection suggesting that the mechanism of antimicrobial effect seems to involve an alteration of normal/proper cell membrane functioning thus blocking pathogen entry. The saponin interaction with cholesterol in the cell membrane accounts for many of its biological effects. The *Y. schidigera* extract is known to have antiprotozoal activity and it is effective in killing the intestinal pathogen: *Giardia lamblia* tropozoites to same extent as the metronidazole drug. Antiprotozoal activity of *Y. schidigera* is thought to occur due to saponin interactions with cell membrane cholesterol [45]. This study demonstrates that both *Y. schidigera* and *T. terrestris* saponin extracts generate a reduction in the amount of free cholesterol in cells treated with these extracts. A stronger reduction in the extracellular cholesterol was exhibited by the *Y. schidigera* saponin extract in both cell lines. The ability of saponin extracts to affect this parameter of the cell membrane may explain some of its *in vitro* antimicrobial activity. As mentioned earlier, cholesterol plays an important part in maintaining proper functioning of cell membrane proteins. Also, the cholesterol molecule provides rigidity and organization to the cell membrane and it is known from numerous studies that the cell membrane organization is critical for efficient orchestration of signaling during pathogen entry. The saponin extracts in this study

contain a mixture of saponins that can possibly interact with the cholesterol molecules in the cell membrane and consequently disturb the cell membrane organization and the ability of membrane receptors utilized by pathogens to function properly. It is reasonable to assume that this disorganization of the cell membrane will interrupt multivalent interaction required to occur between pathogen and cell membrane receptors. It is possible that hydrogen bonding and electrostatic interactions are responsible for the saponin-cholesterol interactions. The observed difference in antimicrobial effect among pathogens may be related to differences in the mechanism of action of not only saponins in the extract but also pathogenesis mechanisms implemented by each individual pathogen. As an example of differences in pathogenesis displayed by each pathogen, *S. enterica* effector protein known as Sip B binds cholesterol with high affinity prior to cellular invasion [154]. Whereas rotavirus entry is a multistep process and involves several interaction between virus and cell membrane receptors and lipid rafts [155].

Although bacterial pathogens employ different strategies that lead to bacterial uptake into the cell they still share some of the common targets within host cell. Pathogenic bacteria used in the study subvert host cytoskeleton machinery to induce their own uptake into the cell by modification of actin or GTP-binding proteins [156]. An extensive literature search on saponin mechanisms revealed that some saponin molecules can cause reorganization of the actin filament networks or interfere with tubulin dynamics which could then interfere with endocytosis in the cell [157-159]. Endocytosis is commonly employed by viruses during cell entry, while large size cargo such as bacteria are not thought to utilize this process during cell entry. However, recently it was found that *Listeria* can exploit endocytosis machinery to invade mammalian cells [160].

Additionally, to dissect further why *Y. schidigera* had more robust antibacterial activity while *T. terrestris* had more robust antiviral activity it would be beneficial to

examine if the saponin molecules present in the extract can interfere with cytoskeleton machinery thus disrupting cellular processes required for pathogen uptake. It remains to be explored further the mechanisms saponins in these natural extracts utilize to exert their antimicrobial effect. The interactions between the saponin molecule and cell membrane seem complex and due to structural diversity of saponin molecules in the extract it is likely that different mechanisms are involved.

In closing, I believe saponins could offer a novel microbiocide for the prevention of diarrheal disease caused by enteric viruses and bacteria.

APPENDIX
A

Antibacterial Activity of Saponin Extracts

Table A.1. The Mean Diameter (mm) of the Antibacterial Activity of Saponin extracts

Bacterial Strains	***Y. schidigera* (mg/ml)**	***Q. saponaria* (mg/ml)**	***T. terrestris* (mg/ml)**	**PBS**	**Antibiotic**
E. coli 0157:H7	>1	>1	>1	0	24
Yersinia enterocolitica	>1	>1	>1	0	31
Listeria monocytogenes	>1	>1	>1	0	28
Salmonella enterica (ATCC #6994)	>1	>1	>1	0	32
Shigella flexneri	>1	>1	>1	0	25
Vibrio cholerae	>1	>1	>1	0	31
Salmonella enterica (ATTC #14028)	>1	>1	>1	0	25
Enterococcus faecalis	>1	>1	>1	0	32
Campylobacter jejuni (ATCC# 33560)	>1	>1	>1	0	18
Campylobacter jejuni (ATCC# 700819)	>1	>1	>1	0	23

APPENDIX
B

Publications

1. Carraher Jr.,C.E.; Ayoub, M.; Roner, M.R.; **Moric, A**. and Trang, Nancy, T. 2013. Synthesis, Structural Characterization and Ability to Inhibit the Growth of Pancreatic Cancer by Organotin Polymers Containing Chelidonic Acid" JCAMS. Vol. 1, No. 1, 65–73.

2. Roner, M.R., Carraher, C.E., Jr., **Moric, A.,** Trang, N.T., Truong, N.T.C., Islam, Z. and Morrison, A. 2013. Comparison of Employing WI-38 and NIH/3T3 Cells for Evaluation of Chemotherapeutic Index and Growth Inhibition Values for Pancreatic Cancer Cell Lines for Group IVB Metallocene and Group VA Metal-Containing Polymers Derived from Glycyrrhetinic Acid, 3,5-Pyridine-dicarboxylic Acid, and Histamine. P*olymeric Materials: Science and Engineering. 108: 187-190.*

3. Roner, M.R., Carraher, C.E., Jr., **Moric, A.,** Trang, N.T., Islam, Z., Truong, N.T.C. and Williams-Sheffler, J. 2013. Comparison of WI-38 and NIH/3T3 Cells as Standards for the Evaluation of the Inhibition of Pancreatic Cancer Cell Lines by Organotin Polymers Containing Histamine and Phentolamine. P*olymeric Materials: Science and Engineering. 108: 195-197.*

4. Roner, M.R., Carraher, C.E., Jr., **Moric, A**., Trang, N.T., Islam, Z.and Morrison, A. 2012. Inhibition of Pancreatic Cancer Cell Lines by Group IVB Metallocene and Group VA Metal-Containing Polymers Derived from Glycyrrhetinic Acid, 3,5-Pyridine-dicarboxylic Acid, and Histamine. P*olymeric Materials: Science and Engineering. 107: 325-328.*

5. Roner, M.R., Carraher, C.E., Jr., **Moric, A.,** Trang, N.T., Ayoub, M. and Pham, N. 2012. Inhibition of Pancreatic Cancer Cell Lines by Organotin Polyesters Containing Thiodiglycolic Acid and Chelidonic Acid. P*olymeric Materials: Science and Engineering. 107: 329-331.*

6. Roner, M.R., Carraher, C.E., Jr., **Moric, A.,** Trang, A., Truong, N.T.C., Gupta, A., Islam, Z. and Williams-Sheffler, J. 2012. Inhibition of Pancreatic Cancer Cell Lines by Metal-Containing Polymers Derived from Glycyrrhetinic Acid, 6-Aminopenicillic Acid, Histamine, and Phentolamine (Regitine). P*olymeric Materials: Science and Engineering. 106: 492-497.*

7. Roner, M.R., Carraher, C.E., Jr., **Moric, A.,** Trang, A., Arnold, T., Lambert, R. and Morrison, A.. 2012. Inhibition of Pancreatic Cancer Cell Lines by Metal-Containing Polymers Derived from Thiamine, 3,5-Pyridinedicarboxylic Acid and Isomannide. P*olymeric Materials: Science and Engineering. 106: 498-502.*

8. Carraher Jr.,C.E.; Ayoub, M.; Roner, M.R.; **Moric, A.** and Trang, Nancy, T. **2013**. Synthesis, Structural Characterization and Ability to Inhibit the Growth of Pancreatic Cancer by Organotin Polymers Containing Chelidonic Acid" JCAMS. Vol. 1, No. 1, 65–73.

9. Carraher Jr.,C.E.; Morrison, A.; Roner, M.R.; **Moric, A.** and Trang, Nancy, T. 2013. Synthesis and Characterization of Organotin Polyesters Derived from 3,5-Pyridinedicarboxylic Acid. JIOMP. Accepted.

The publications listed above, features the work I did with examining a variety of polymers including metal-containing polymers as anticancer agents. I have tested over 300 different compounds to inhibit growth of various cancer cell lines (WI-38, 3T3, AsPC-1, PANC-1, 3465, 7259 and others) *in vitro.* The compounds that had growth inhibitory activity against cancer cell lines were further evaluated with apoptosis assay to determine the mechanism of cell death.
An additional publication to be published will feature my work with saponin extracts, described in the dissertation.

Presentations at Scientific Meetings

Poster presentation.
Annual Texas Branch American Society for Microbiology Meeting, New Braunfels, TX. 2013

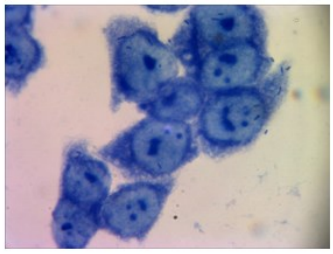
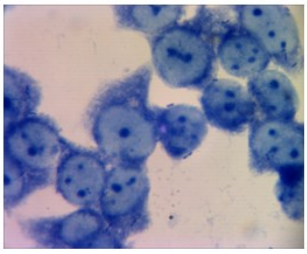
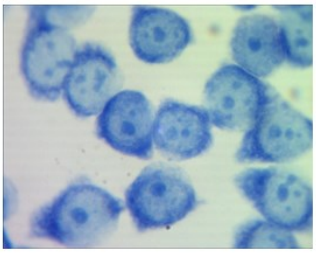
Oral presentation.
Annual Texas Branch American Society for Microbiology, University of Texas at Arlington.
S.E. Sulkin Award Recipient. 2011

Poster presentation.
29th Annual American Society for Virology meeting, Montana State University at Bozeman, Montana. 2010

APPENDIX
C

Qualitative Adhesion Assay Images

V. cholerae

Co-treatment	Pre-treatment	Post-treatment
Control	Control	Control
		
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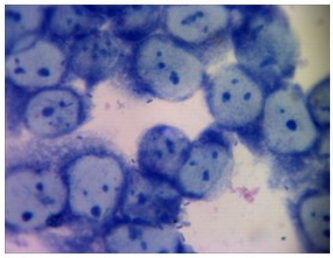	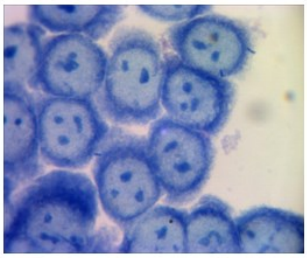	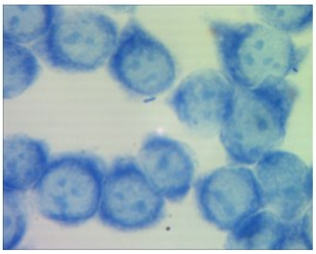
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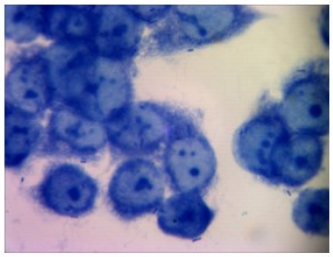	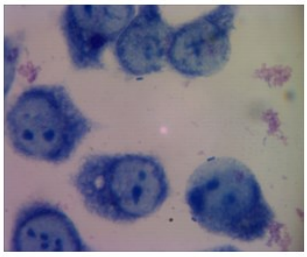	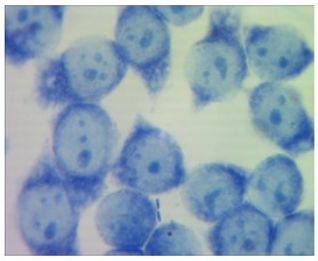
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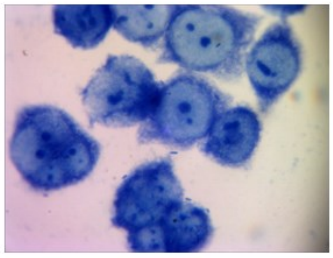
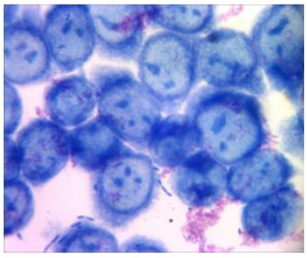
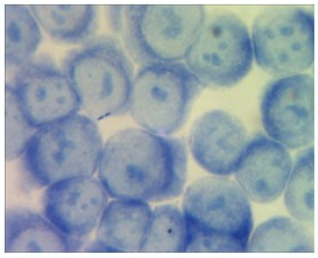
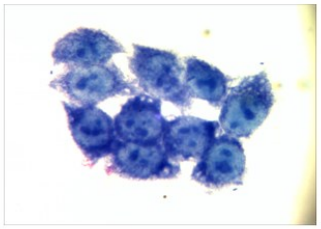
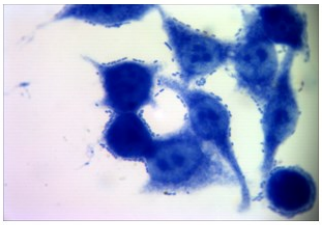
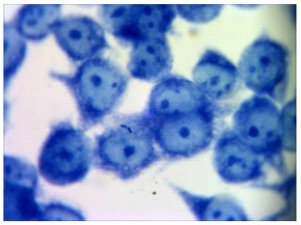
		

E. coli

Co-treatment	Pre-treatment	Post-treatment
Control	Control	Control
		
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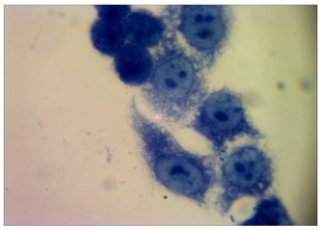	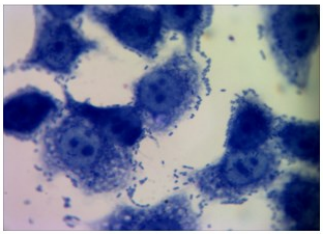	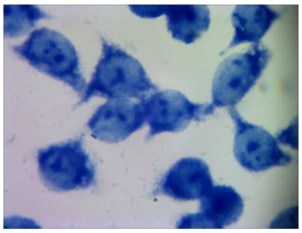
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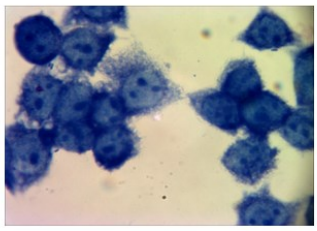	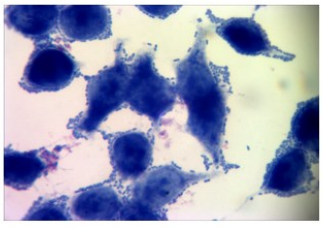	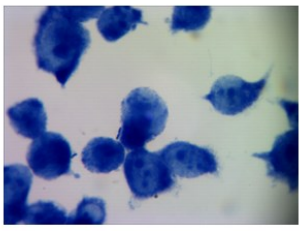
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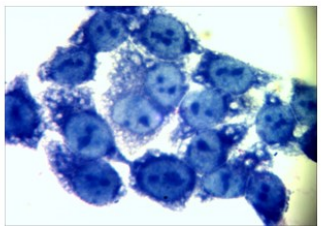
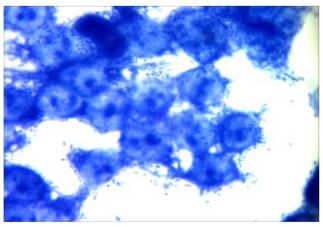
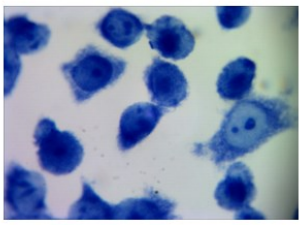
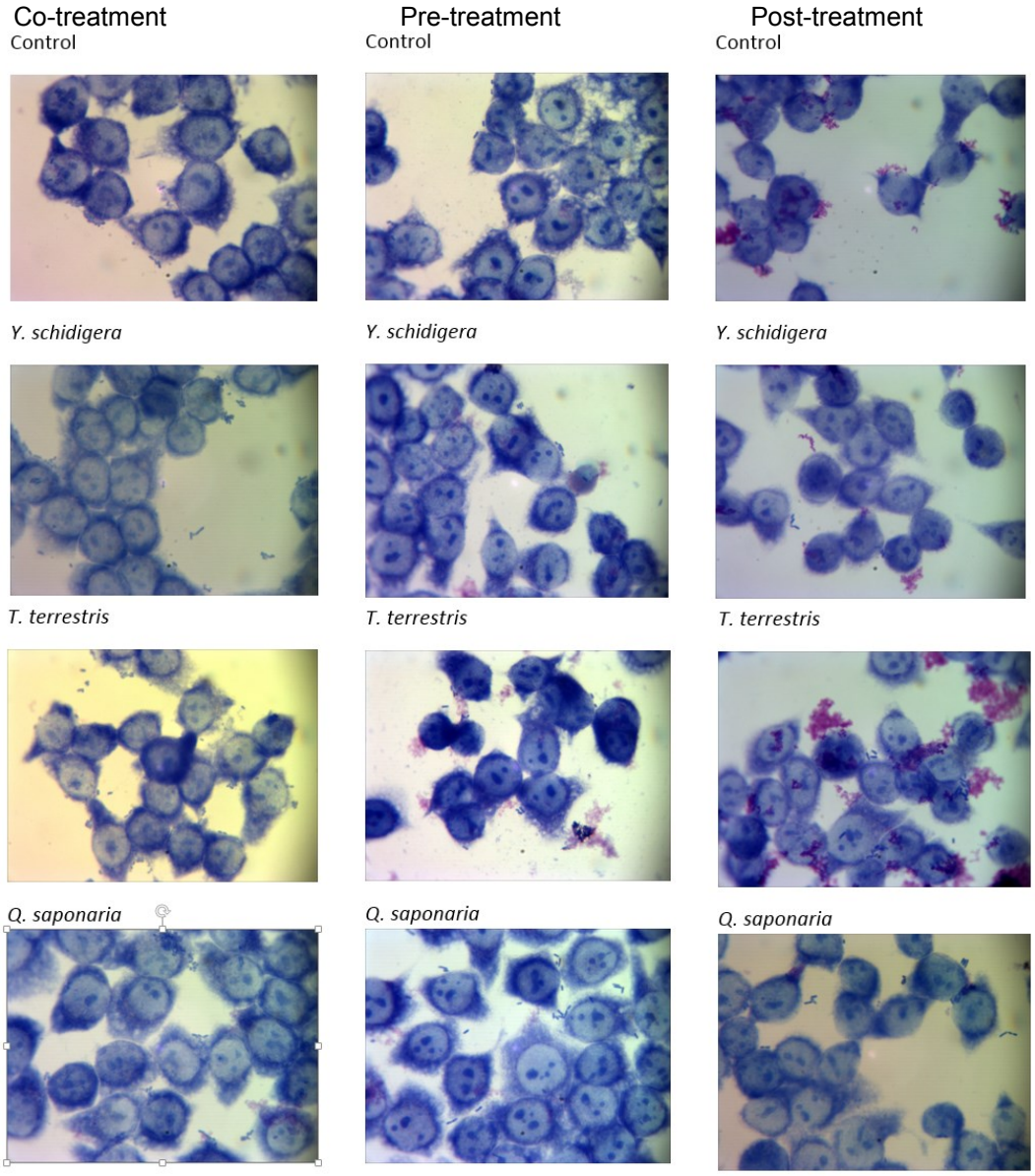
		

S. enterica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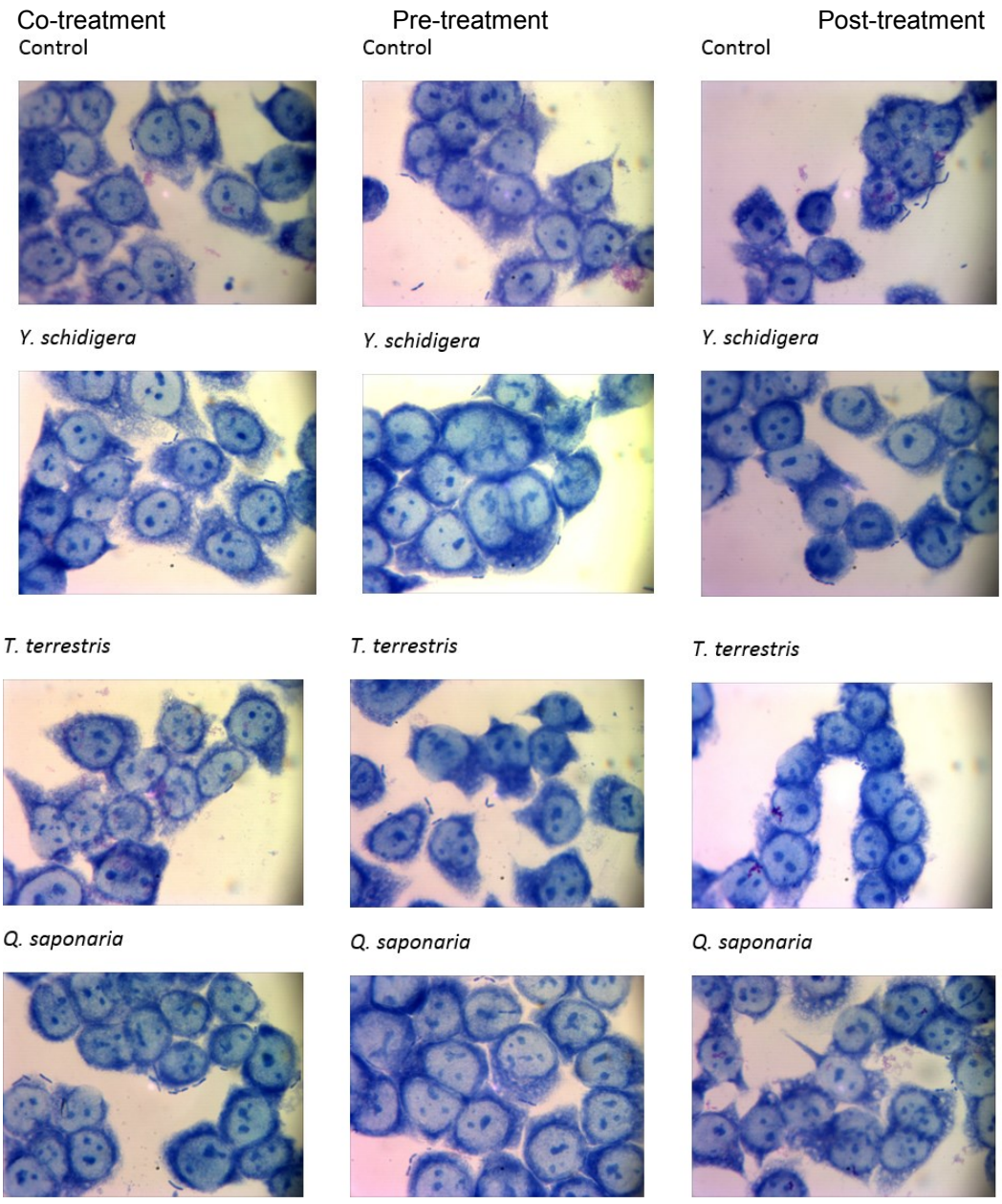

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Biographical Information

Alisa graduated from University of Texas at Arlington (UTA) in 2005 with a bachelor degree (BS) in Life Science. She started teaching High School in 2006 until 2009. After teaching at Fort Worth Independent School District for three years, Alisa entered BS-Ph.D. program in Quantitative Biology at UTA where she joined Dr. Michael Roner's Molecular Virology lab. Her dissertation work focused on evaluating ability of saponins from natural extracts to prevent viral and bacterial infections of cells *in vitro*. She has demonstrated that saponins from *Y. schidiger*a and *T. terrestris* plant are effective in blocking virus infection and subsequent replication, while saponins from *Y. schidigera* can reduce entry of bacteria to cultured cells. Alisa worked part-time at UTA as a graduate teaching assistant for the Introduction to Biology and Anatomy and Physiology I and II labs. In 2013, Alisa received her Ph.D. in Quantitative Biology. She would like to work in pharmaceutical industry. She wants to use knowledge in Microbiology to design microbicide for prevention or treatment of enteric infections.