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Blueberry polyphenols as natural antimicrobial agents against foodborne viruses: Towards understanding their mechanism and applications in food systems

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To the Graduate Council:

I am submitting herewith a dissertation written by Snehal Shrikant Joshi entitled "Blueberry polyphenols as natural antimicrobial agents against foodborne viruses: Towards understanding their mechanism and applications in food systems." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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Blueberry polyphenols as natural antimicrobial agents against foodborne viruses: Towards understanding their mechanism and applications in food systems

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To Aai and Baba (Jyotsna and Shrikant Joshi) for their immense love and support

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ABSTRACT

Foodborne viruses are recognized as public health concerns worldwide and therefore effective strategies to control their spread are being researched. Blueberries are known for their health benefits and antimicrobial properties. This study aimed to (1) determine the antiviral effects of blueberry juice (BJ) and blueberry proanthocyanidins (B-PAC) against the infectivity of hepatitis A virus (HAV), Aichi virus (AiV) and human norovirus surrogates (feline calicivirus (FCV-F9) and murine norovirus (MNV-1)) at 37ºC over 24-h using standard plaque assays; (2) evaluate antiviral effects in model foods (apple juice (AJ) and 2% milk) and simulated gastric conditions at 37^{o}C; and (3) determine the mechanism of action of B-PAC by comparing activity of monomeric catechins, procyanidin B2, B-type PAC from blueberries (B-PAC) and A-type PAC from cranberries (C-PAC), effects on viral structure using transmission electron microscopy, and on adsorption and replication. FCV-F9, MNV-1, HAV and AiV titers were reduced to undetectable levels after 5 min, 3-h, 30 min and 3-h with 1, 1, 2, and 5 mg/ml B-PAC, respectively. BJ reduced FCV-F9, MNV-1, AiV to undetectable levels and HAV by ~2 log PFU/ml after 3, 6, 24 and 24-h, respectively. All tested viruses were reduced to undetectable levels within 15 min with B-PAC (1, 2 and 5 mg/ml) in AJ (pH 3.6). B-PAC effects decreased in milk, where FCV-F9, MNV-1, HAV and AiV were reduced by ~ 1 log PFU/ml with 5 mg/ml after 24-h. B-PAC at 5 mg/ml in simulated intestinal fluid reduced all tested viruses to undetectable levels within 30 min. Monomeric catechins and procyanidin B2 were less effective than the polymeric B-PAC. Time of addition assays indicated that B-PAC had modest effects in preventing viral adsorption, with no significant effect on viral replication. TEM observations revealed moderate effects on virus structure with either damaged viral capsid or virus binding to

B-PAC, possibly preventing virus attachment to host cells. Overall, this study demonstrated the ability of BJ and B-PAC to reduce viral titers in model food systems and under simulated gastric conditions, suggesting their potential as preventive and therapeutic options against foodborne viral illnesses.

Keywords: blueberry polyphenols, human norovirus surrogates, hepatitis A virus, Aichi virus

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Introduction

Foodborne enteric viruses are commonly associated with non-bacterial acute gastroenteritis in humans and animals. The epidemiologically significant foodborne viruses include human noroviruses, hepatitis A virus, rotaviruses, Aichi virus, hepatitis E virus, adenoviruses, sapovirus, astrovirus, parvoviruses and other enteroviruses. These viruses are a growing cause of public health concern in the United States as well as worldwide with an increasing number of outbreaks reported every year. For instance, in the US among the 31 pathogens that are responsible 9.4-million foodborne illnesses, human noroviruses alone are reported to be responsible for an estimated 5.5 million foodborne illnesses. Foodborne viruses can be transmitted through the fecal-oral route by contaminated food and water, via person-to-person, contact with contaminated surfaces, and aerosolization. At-risk foods associated with viral outbreaks, include filter-feeding shellfish, fresh produce, juices, bakery items, deli meats, and other ready-to-eat (RTE) foods or undercooked foods. However, some of these viruses including human noroviruses and Aichi virus currently do not have vaccines available for their control. Give their widespread prevalence in the food chain, effective natural alternatives to control their spread are being researched. Natural plant extracts have been used for their valuable medicinal properties since centuries. Extracts from plant and fruit sources are known to contain abundant secondary metabolites mainly phenolic compounds including but not limited to anthocyanidins, proanthocyanidins, catechins, epicatechin gallate and tannins. Plant extracts are being studied

extensively as sustainable sources of potential therapeutic options and also being screened for their antiviral potential.

Blueberries and its polyphenols are known for their potential health benefits including anticarcinogenic, neuroprotective, cardioprotective, antibacterial, and antiviral properties. Blueberries contain structurally related polyphenols, including anthocyanins, flavonoids, and proanthocyanidins (PAC), sometimes referred to as condensed tannins (1). Human norovirus surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been studied for survival in commercial blueberry juice (BJ) at 4ºC, where FCV-F9 was undetectable after 24 h from initial 5 log PFU/ml, whereas MNV-1 showed minimal reduction after 21 days at 4ºC (2). Therefore, blueberries and blueberry phenols could potentially be used as part of a diet to prevent or treat human norovirus related gastroenteritis. This study aimed to explore the antiviral effects of blueberry polyphenols and blueberry juice against foodborne viruses (cultivable human norovirus surrogates and hepatitis A virus) *in vitro* at 37^{o}C, in model food systems, and under conditions mimicking digestion (simulated gastric conditions). In addition, this study also attempted to understand the mode of action of monomeric, dimeric and polymeric units of blueberry polyphenols against the tested viruses. Thus, insights on the benefits of blueberry polyphenols in improving gastrointestinal health by preventing or alleviating human norovirus and hepatitis A virus disease symptoms were obtained.

Chapter 1 Review of literature

Parts of this chapter have been submitted as chapters that have been accepted for publication.

1. Joshi, S.S. and D'Souza D.H. (2014). Health Benefits of Grape Seed Extract, "GRAPES": Production, Phenolic Composition, and Potential Biomedical Effects, Nova Science publishers Inc., New York.
2. D'Souza D.H. & Joshi S.S. (2015). Foodborne viruses of human health concern. Encyclopedia of Food and Health. Elsevier Publications.

Introduction to Foodborne Viruses

Annually, in the United States, among the 31 pathogens that cause an estimated 9.4-million foodborne illnesses, human noroviruses alone are reported to be responsible for an estimated 5.5 million foodborne illnesses (58%), 26% hospitalizations, and 11% deaths (3). As an overview about viruses, they are smaller in size (nM) and unique properties than bacterial pathogens, with lesser complexity. Viruses are obligate intracellular parasites that are dependent on a host cell to replicate and unlike bacteria cannot multiply in foods or the environment (4, 5). Foodborne viruses are generally environmentally stable, survive adverse conditions and are resistant to extreme pH conditions and enzymes of the gastrointestinal tract (4). They have low infectious doses, as few as 10-18 human noroviral particles can cause gastrointestinal illness (6).

Foodborne viruses can be transmitted through the fecal-oral route by contaminated food and water, via person-to-person, contact with contaminated surfaces, and aerosolization (7). Viral contamination of foods often occurs due to poor hygiene practices of infected foodhandlers, where public education and enforcement of hygienic practices can help reduce outbreak incidence. Fecally polluted waters that flow into farmlands, can contaminate crops and also shellfish can accumulate viruses in their gastrointestinal tract. Contaminated fresh produce and shellfish when eaten raw or undercooked can cause illness. At-risk foods associated with viral outbreaks, include filter-feeding shellfish, fresh produce, juices, bakery items, deli meats, and other ready-to-eat (RTE) foods or undercooked foods. Viruses that are associated with

foodborne and waterborne diseases include human noroviruses (HNoV), hepatitis A virus, rotaviruses, hepatitis E virus, adenoviruses, sapoviruses, astroviruses, aichi virus, parvoviruses, and other enteroviruses (8).

Human Noroviruses

Human noroviruses are considered as major causes of non-bacterial gastrointestinal outbreaks worldwide. Human noroviruses (HNoVs) belong to Caliciviridae family that consists of five major genera, Norovirus, Sapovirus, Vesivirus, Lagovirus, and the recent addition of Becovirus or Nebovirus (9). HNoVs were first reported to be discovered in an elementary school outbreak in 1968 in Norwalk, Ohio, where 50% of students and teachers developed gastrointestinal illness, from which the name "Norwalk-like" virus was derived by Albert Kapikian in 2000. HNoV transmission occurs all year round, however, outbreaks peak in winter (10). 'Winter vomiting disease' is the common seasonal syndrome caused by HNoV infection, characterized by mild gastroenteritis and symptoms like nausea, vomiting, abdominal cramps, fever and malaise, though emerging virulent strains are capable are causing death in the elderly and immunocompromised (11). The average incubation time of HNoV is 24-48 h and the self-limiting symptoms last from 18 to 48 h. Infected individuals can shed viruses for 72 h (and even longer, >week) after the first symptoms appear (12). This is an important consideration for food handlers/workers, who need to maintain good hygienic practices to prevent transmission upon return to work. In addition to outbreaks associated in food settings, HNoV outbreaks are reported to recur yearly on cruise-ships, in elderly care facilities, and in closed environments.

Noroviruses are non-enveloped, small about 27 to 32 nm in size, and possess a single-stranded positive-sense RNA enclosed in a capsid (outer shell protein), with a buoyant density of

1.33 to 1.41g/ml (13). HNoVs have icosahedral symmetry, with a capsid that comprises of 90 capsomeres protruding from the shell made of 90 dimers of capsid protein. The genome is about 7.3-7.7 kb long with three open reading frames (ORF's) (14). ORF1 encodes for a polyprotein that is cleaved to produce the N-terminal protein, the enzyme nucleoside triphosphatase, a 3A-like protein, the genome-linked viral protein (VpG), a 3C-like protease, and RNA-dependent RNA-polymerase (15). ORF2 encodes for the major capsid protein, viral protein, VP1; and ORF 3 encodes for a minor viral structural protein, VP2. Based on sequence analysis, the genus Norovirus is further divided into five genogroups, GI, GII, GIII, GIV and GV, and genogroups GI, GII and GIV, specifically infect humans (16, 17). Genogroup III includes bovine enteric calicivirus that infects cattle, and genogroup V infects mice. These genogroups are further subdivided into at least 32 genetic clusters (9, 18). Among the genogroups I and II, 8 genotypes are currently recognized in genogroup I and 19 genotypes are recognized in genogroup II. HNoV GII.4 genotype during the past decades has been the predominant cause of outbreaks in the United States, Europe, and Oceania that were responsible for 70%–80% of all HNoV outbreaks (particularly in health care settings) (19). More recently, canine norovirus infecting dogs was discovered with strains classified into genogroups IV and VI (20).

HNoV binds to the histoblood group antigens in human intestinal epithelial cells, where the site of replication is considered as the upper intestinal tract (21). Genetic determinants for these blood group antigens play a role in susceptibility of an individual to HNoV infection. Human volunteer studies have shown that H type I antigen likely serves as the receptor for HNoV, encoded by the fucosyltransferase (*fut2)* gene (22). Individuals that are recessive for this gene, lack the Type I antigen, and are inherently resistant to HNoV infections (22).

HNoV GI and HNoV GII can survive in the environment and were detected after 1-week storage on inoculated coupons at ambient temperature using RT-PCR (23). HNoVs are known to survive low pH of 2.7, can retain infectivity after 60°C for 30 min and also after exposure to 20% ether at 4°C for 18 h (13). Their resistance to inactivation is found to be higher in foods where steaming of mussels did not cause any significant reduction in HNoV titers (24). HNoVs were also found to be resistant to chlorine inactivation with a free residual chlorine of 0.5 to 1.0 mg/mL (25).

The inability to cultivate HNoVs in the laboratory has hindered the development of vaccines and effective control measures. There is no vaccine available to date for HNoV prevention. Cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been extensively used to understand antigenicity of HNoVs and evaluate methods of inactivation. Recently, Tulane Virus and porcine enteric Sapovirus have also been considered as alternate surrogates. FCV-F9 of the *Caliciviridae* family, is a small rounded, non-enveloped, single-stranded RNA virus with a 7.7 kb genome, has 3 ORFs, and is transmitted through air via fomites (26). However, since it is transmitted via a respiratory route, it may not completely mimic the survival of HNoV in the environment. In addition, unlike HNoVs, FCV-F9 is sensitive to low pH of 2.0 to 4.0 and pH 10.0 (27).

MNV-1 is currently the only known norovirus that replicates in the laboratory in murine macrophage RAW 264.7 cells (28). MNV-1 belongs to genogroup V norovirus, sharing sequence similarity to genogroup II that mainly infect humans (29, 30). MNV-1 is similar to HNoVs in immunological, biochemical, genetic and molecular properties, as MNV-1 is an icosahedral, non-enveloped, single-stranded RNA virus with 3 ORFs, 28-35 nm in diameter (28). Although it

causes a different disease in mice, it is transmitted via the fecal oral route similar to HNoVs (27). Unlike FCV-F9, it is less sensitive to pH within ranges of 2.0 to 10.0, however the thermal inactivation parameters processes like pasteurization at 63^{o}C were reported to be similar for both FCV-F9 and MNV-1 (27). D-values required for heat inactivation of FCV-F9at temperatures of 50, 65 and 72^{o}C were reported to be 19.95 ± 0.70, 0.72 ± 0.01 and 0.21 ± 0.01 min respectively. For MNV-1 the reported D-values at 50, 65 and 72^{o}C at were 36.28±3.21, 0.77±0.03 and 0.25±0.01 min, respectively (31).

Regarding application of surrogates for inactivation studies, both 200mg/ml sodium hypochlorite and 2% trisodium phosphate could reduce MNV-1 by 2-3 log PFU/ml and FCV-F9 to undetectable levels, respectively after 30 s at room temperature(32). Ozone at 6.25 ppm caused a~2-log Tissue culture infectious dose (TCID50/ml) reduction of FCV after 5 min and 2 log TCID50ml^{-1} reduction of MNV-1 after 3 min on lettuce and green onions (33). Hydrogen peroxide vapors (30% w/w) were found to cause 4 log reduction of FCV-F9 after 20 min on stainless steel, ceramic tiles, and PVC surfaces (34). MNV-1 at 7 log PFU was completely inactivated at a pressure of 450MPa after 5 min at 20^{o}C, while 250 MPa at 20^{o}C for 5 min reduced FCV titers by 5.1 log PFU/ml(35, 36).

Recently, Tulane virus (TV) representing a new genus *Recovirus* of the *Caliciviridae* family was isolated from the stool samples of a captive juvenile rhesus macaques (*Macacamulatta*) at the Tulane National Primate Research Center. TV is 36 nm in diameter, icosahedral in symmetry (37). TV has the shortest known genome of around 6.7 kb among the known caliciviruses, contains 3 ORFS and lacks the poly (A) tail. Pairwise homology and phylogenetic analysis of non-structural proteins of TV revealed highest identity with HNoVs.

TV can be readily grown *in vitro* in LLC MK2 (rhesus macaque kidney) cells. TV can bind to histoblood group antigens like HNoVs, suggesting that the antigenic determinants and structural properties might be comparable to HNoVs (30). Studies have shown no significant differences in heat inactivation parameters between MNV-1 and TV, though MNV-1 was found to be more pH stable and tolerant to chlorine, while significant reduction in TV titers were obtained at pH 2.0 and 9.0 and after 2 ppm chlorine treatments (30, 38). Therefore, the choice of appropriate cultivable surrogates for HNoVs is still debatable.

Porcine sapovirus (Cowden strain), an enteropathogen that causes gastroenteritis in gnotobiotic pigs is as another HNoV surrogate that can be cultivated in the swine kidney cell line, LLC-PK (39). Both porcine SaV and HNoV showed similar resistance to treatments at 56oC for 30 min and 2 h and with 2.5mg/Lor 10mg/L sodium hypochlorite for 1 min; similar to HNoV's, porcine SaV is stable at pH 3.0 to 8.0 (39). All these findings strongly support the use of porcine SaV as a surrogate to study HNoV.

Virus like particles (VLPs): Along with the use of above viruses as HNoV surrogates, virus like particles (VLPs) have also been used to understand and study HNoV replication in vivo or to develop vaccines (40). These virus-like particles (VLPs) are non-infectious (they lack the genome) of HNoVs and were first expressed in baculovirus-infected insect cells (41). The VLP's produced mimic the antigenic structure of the virus and also are acid and heat stable. These VLP's are also highly immunogenic and produce an immune response when delivered intranasal, making them an ideal candidate for use in developing vaccines (42). VLPs were also shown to bind to carbohydrate histo-blood group antigen (HBGA) displayed on mucosal cell surfaces similar to human norovirus (43).

Currently, there is no specific anti-viral treatment against HNoVs. Gastroenteritis and dehydration can be treated by oral or intravenous fluid therapy. To date, there is no vaccine available against HNoVs, the major barrier being the inability to culture them *in vitro*. Human volunteers re-infected with HNoV after exposure were found to be susceptible to the same strain as well as heterologous strains (44, 45). The spread and contamination can be prevented by proper sanitation and hygienic food handling practices or by heat, chemicals, or processing conditions described above (CDC, 2013).

Hepatitis A virus

Hepatitis A virus (HAV) belongs to the *Picornaviridae* family and is a positive-sense single-stranded non-enveloped RNA virus ranging in size from 27-32 nm, similar to HNoVs. The genome is 7.5 kb in length and unlike HNoVs consists of a single open reading frame. The P1 region of the genome encodes for three major viral capsid proteins VP1, VP2, and VP3. P2 and P3 regions encode for nonstructural proteins required for RNA replication and virion formation (46, 47). Only one serotype of HAV has been identified to date and a single exposure can render life-long immunity in an individual (48). However, genetic diversity in the strains worldwide led to their classification into seven genotypes designated I to VI. Genotypes I to IV are associated with human illnesses and genotypes V to VI include strains are obtained from affected simians (49).

The largest foodborne viral outbreak associated with 292,301 cases of HAV was attributed to eating raw clams in Shanghai, China in 1988 (50). The most recent HAV outbreak was linked to the frozen antioxidant mix that contained pomegranate seeds imported from Turkey (51). About

165 people were confirmed to be ill following the consumption of this mix and the genotype 1B was implicated where it was reportedly detected in 117 stool samples of the infected persons (51). Several other outbreaks have been linked to sundried tomatoes and green onions (52-54). Given the amount of imports from different regions of the world and increasing international travel there has been a rise in the number of HAV cases seen every year (55).

The average incubation period is long (unlike the short incubation period of HNoVs), approximately 28 days with severe symptoms that include jaundice, dark urine, vomiting, fever and weight loss that can last for 4 to 6 weeks. Among older children and adults, infection is usually symptomatic for 2 months, with jaundice occurring in 70% of patients. There are 4 phases of HAV infection: the asymptomatic incubation phase lasts for 10 to 50 days; prodromal or preicteric (56, 57) phase lasts for several days to week, characterized by fatigue, nausea, and loss of appetite, fever, dark urine, diarrhea and pale stools; the icteric phase where jaundice develops along with increase in liver enzymes (namely serum transaminase and alkaline phosphatase) and rise bilirubin levels in blood resulting in yellowing of urine and leading to jaundice, and in the 4^{th} phase (convalescent) symptoms start resolving with relapse that can occur in 3-20% of the cases (58).

There are few strains of HAV (HM-175, HAS-15, MBB 11/5) that are cell-culture adaptable and maintained using fetal rhesus monkey kidney (FRhK-4) and/or human fetal lung fibroblast (MRC-5) cells. These strains produce cytopathic effects causing cell death by inducing apoptosis (59, 60), and used in research for inactivation studies.

HAV is highly stable in the environment and retains infectivity in mineral water stored at room temperature even after 300 days (61). HAV remains infectious after a month at ambient

temperatures on environmental surfaces like stainless steel (62). HAV survives for >4 h on hands after experimental exposure (63) and during prolonged storage of spinach leaves at 5.4°C, with a D-value of 28.6 days (64). Sixty-two percent of inoculated HAV survived on romaine lettuce leaves that were stored for 12 days in a sealed-atmosphere plastic package at 4°C (65).

HAV survives in acid marinade at pH 3.75 over 4 weeks (66), and can retain its infectivity at pH 1.0 even after 5 h (67). HAV infectivity can be retained after 60 min at 60°C, being partially inactivated after 10-12 h at 56°C and can be inactivated in shellfish after heating at 85°C to 90°C for 1.5 min (67, 68), and reduced to undetectable levels from 7 log PFU/ml at 450MPa hydrostatic pressure for 5 min (69). These survival features play a significant role in the transmission of HAV. Once a person is infected with HAV, there are no specific treatment options available. However, symptoms of HAV infection may be alleviated by appropriate patient care that includes adequate hydration, nutrition, rest, and anti-emetics (70). For prevention, immunoglobulin (Ig) therapy is effective when administered within two weeks of viral exposure (through passive immunity). As preventive measures, inactivated and heat-killed vaccines against HAV are commercially available, that provide immunity against HAV for >20 years or lifetime (71). The U.S. Centers for Disease Control and Prevention (CDC) recommend routine vaccination against HAV for children aged between 12 to 23 months (55). When economically feasible, vaccination of food-handlers is recommended to prevent HAV transmission and to prevent outbreaks.

Aichi virus

Aichi virus (AiV) of the genus *Kobuvirus* in the *Picornaviridae* family was first isolated in 1989 from patient stool samples in a gastrointestinal outbreak involving oysters in Aichi, Japan (72). It has a positive-sense single-stranded RNA and a poly (A) tail, with a single ORF encoding for structural proteins VP0, VP1 and VP3, and non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C, 3D (73). AiV's are classified into three distinct genotypes A to C (74). AiV virions are icosahedral, 30 nm in diameter with three capsid proteins (73). AiV-related gastrointestinal cases have been reported from Asian, African, European and South American countries indicating its geographical spread and prevalence (75-80). AiV has been known to survive (without reduction) high pressure processing (HPP) treatment of 600 MPa for 5 min in minimum essential growth medium (MEM) with 10% fetal bovine serum (81). However, AiV was reduced by 1.87, 2.49 and 4.59 log $TCID_{50}$mlrespectively when treated by 240 mW s^{-2} of ultraviolet light treatment, that can be used to reduce AiV on fresh produce like strawberries, green onions and lettuce (82). AiV isolates were stable in 10% chloroform and MEM (pH 3.5) for 3h and survives heating at 50^{o}C and 60^{o}C for 30 min (72).

Hepatitis E virus

Hepatitis E virus (HEV) is a small non-enveloped RNA virus, 32-34 nm, primarily transmitted through the fecal-oral route usually through contaminated water (83, 84). This virus is mainly predominant in developing countries in Asia and Africa (85). HEV of the genus *Hepevirus* in the *Herpeviridae* family, comprises of a 7.2 kb single-stranded positive-sense RNA with three open reading frames (ORFs) (86). The average incubation period for HEV infection is between 2 to 10 weeks, with two distinct phases of illness. The pre-icteric phase is characterized by fever,

anorexia, vomiting and abdominal pain, while the icteric phase is characterized by jaundice and is self-limiting. Viral shedding begins 1-2 weeks before and 2-4 after onset of symptoms. A significant rise in serum alanine aminotransferase and aspartate aminotransferase occur, that is used as detection methods of liver injury (87). Due to its long incubation period, association of HEV with particular foods has not been established, however recent studies suggest HEV transmission is linked to consumption of uncooked pork meat (88). There is no specific treatment except patient care as described above for HAV symptom alleviation. Pegylated interferon administration has been tried in chronic infections with moderate success (89). Vaccine trials have been under study to determine preventive measures.

Rotavirus

Rotavirus (RV) of the *Reoviridae* family has a multilayered non-enveloped icosahedral capsid, ranging from 60-100 nm in size and is easily distinguishable from HNoVs or HAV by electron microscopy. RV has 11 segments of double-stranded RNA that code for structural proteins (VP1 to VP8) and non-structural proteins. Rotavirus (RV) is a common cause of acute gastroenteritis in children younger than 5 years (90). RV was first discovered in 1973 with electron micrographs of intestinal and stool biopsies of children suffering from gastroenteritis, with a typical wheel shape and hence the *Latin* name "Rota" meaning wheel (91). The incubation period for human RV is around 1 to 3 days. Symptoms occur 48 h after RV infection that include watery diarrhea, abdominal pain, vomiting, and dehydration, with severe diarrhea resulting in high mortality rates around the world (92). In 1980's, there were 870,000 deaths linked to RV infection globally, with current numbers around 450,000/year, and developing countries like the Democratic

Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan accounted for > half of total rotavirus related deaths, with India accounting for 22% (98,621) of deaths (93, 94). Many RV outbreaks are known to occur due to contaminated water and unhygienic and unsanitary conditions (95, 96).

Human RV can survive and retain antigenicity after 5 $mgml^{-1}$ chlorine treatment for 60 min (97), and is resistant to wipes containing 1000 ppm chlorine on stainless steel surfaces (98). High pressure processing at 350 MPa at $4^{o}C$ caused a 1.1 log PFU reduction in RV titers (99). RV subjected to wet ($100^{o}C$) and dry ($60^{o}C$) heat for varying periods of 1-20 min led to changes in virion morphology and loss of smooth capsid layer (100). Commercial cranberry and grape juices at native pH could reduce RV infectivity after 5 min at $23^{o}C$ (101). Other natural substances such as persimmon extract and wattle extract (0.5% solution) reduced human RV strain Wa titers by 5.3 log $PFUml^{-1}$ after 3 min at room temperature (102).

Animal model-systems have been developed to study and improve strategies for protection against infection from various RV serotypes/isolates. Two Simian RV strains SA11 and Rhesus Rotavirus (RRV) are being used as reference strains worldwide and some of the currently available reassortment vaccines are based on the RRV strain(103, 104). As a preventive measure against human RV infection, the World Health Organization (WHO) recommends vaccination that has significantly reduced rotavirus-related gastroenteritis and mortality (105).

Sapovirus

Sapovirus of the *Caliciviridiae* family is a positive-sense single-stranded 7.5 kb RNA virus, 41-46 nM in diameter, that infects humans and pigs (106). Sapporo virus (SaV), the human prototype was first identified in an infant home outbreak in Sapporo, Japan in 1977. It has 10

spikes on the outer surface. SaV is divided into five genogroups, GI to GV; Genogroups GI, GII, GIV and GV infect humans and GIII infects pigs. Genomes of GI, GIV, and GV contain three open reading frames (ORF's) and GII genomes contain two ORFs (107, 108). RNA-dependent RNA polymerase and a major capsid protein (VP1) are encoded by ORF1, ORF2 encodes a small protein similar to VP2 of NoVs and ORF3 encodes for a protein with unknown function (39). Outbreaks of SaV are less frequent than HNoV, however significant increase in SaV outbreaks has occurred in various settings such as elementary schools, high schools, hospitals and hotels (109). SaV is associated with treated and untreated sewage, and river water (110). The average incubation time is ~24-48h with symptoms like diarrhea, abdominal pain, nausea, vomiting, chills, malaise and fever (111). Human SaVs like HNoVs are uncultivable (112, 113). As mentioned above, porcine sapovirus that is cultivable in the lab is also studied as a surrogate for HNoVs (39).

Adenovirus

Adenoviruses (AdV) were first isolated from civilians and Army recruits who had respiratory disease (114, 115). AdVs of the genus *Mastadenovirus*, are 90-100 nm, non-enveloped, contain 26-45 kb double-stranded DNA enclosed in an icosahedral shell. Clinical manifestations of HAdVs range from upper and lower respiratory tract infections, pneumonia, to gastroenteritis and urinary tract infections (116, 117). HAdVs cause mild and self-limiting disease, and severe clinical presentations if any include cystits, enteritis, encephalitis and pneumonia (118). HAdV serotypes 40 and 41 are the second leading cause of viral gastroenteritis in children after rotavirus (119). Symptoms like diarrhea, dehydration, vomiting, and fever develop after an incubation period of 8-10 days and last for 7-8 day, with shedding for 7-14 days after infection

(120).

Other enteric viruses

Astroviruses are small, ~30 nm in size, star-shaped, non-enveloped RNA viruses, that were first identified in 1975 in children suffering from diarrhea in a maternity ward in England (121). Their name originates from the Greek word ‘astron’ meaning star, which represents the five star-like projections on the virus (122). Astroviruses belong to the family *Astroviridae* that is divided into two genera; *Mamastroviridae* (that infect mammals) and *Avastroviridae* (that infect avians) (123). Their genome is about 6.8 to 7.9 kb with a 5’ untranslated region and 3 ORFs, ORF1a, ORF1b and ORF2. ORF1 encodes a RNA dependent RNA polymerase and a protease while ORF2 encodes for the viral capsid (124). Astroviruses are not easily cultivable in vitro, hence the serological classification is mostly established on nucleotide and amino acid variability of ORF2 (124). Till date, there are eight known serotypes of human astroviruses (HAstV), with type 1 being the most prevalent (125-127). Along with the genetic diversity of ORF2, HAstV are further classified into genogroups A and B; genogroup A includes serotypes 1 to 5 and 8, genogroup B includes serotypes 6 and 7 (128). When culturable, the virus is grown using the human colon carcinoma (Caco-2) cell line (129).

HAstVs are reported to be a common cause of gastroenteritis in children (130). Symptoms related to HAstV develop 2-3 days after infection and include diarrhea, vomiting, abdominal pain, headache and dehydration. However, affected persons may not require hospitalization since the symptoms are self-resolving (120, 131). HAstV is known to infect the immunocompromised, elderly, and healthy adults, though children under the age of two are most

prone to infection (124, 132). Astrovirus is known to survive in water and surfaces for 60 days at 4^{o}C and in water at 20^{o}C and after 2 h in 1mg/L free chlorine (133).

Parvovirus of the *Parvoviridae* family is a small, 18-26 nM, non-enveloped single-stranded 5kb DNA virus, discovered in 1974 while testing for hepatitis B surface antigen (134, 135). It is icosahedral with a buoyant density of 1.39 to 1.42 g/ml. Parvovirinae are divided into three genera based on transcription maps, their terminal repeats and replication; *Parvovirus,* (autonomous), replication with helper virus *Dependovirus* and in erythrocytes *Erythrovirus*, and only *Dependovirus* and *Erythrovirus* are known to infect humans(136, 137). Parvovirus genome has 2 ORFs, transcribing viral proteins VP1 and VP2 and non-structural proteins, with VP2 the major structural protein comprising of 96% of total capsid protein (138).

Parvoviruses are resistant to pH between 3 and 9 and at 56^{o}C for 60 min, but can be inactivated by formalin and gamma-radiation (139). Parvoviruses have been linked to shellfish and the "cockle agent" parvovirus was associated with a large outbreak related with contaminated cockles in the UK involving around 800 people (137, 140).

With this information on the foodborne viruses and their transmission along with the lack of available vaccines in most cases, novel methods to control their spread are being researched. Ideally, methods should be cost-effective, easy-to-use, sustainable and natural with consumer appeal. Natural plant extracts and in particular plant polyphenols can serve as attractive alternate therapeutic or preventive options in the absence of vaccines and effective control measures.

Natural plant extracts

As indicated above, in the current absence of vaccines or effective treatment options for HNoV infections, natural alternatives are being evaluated (141). Plant derived polyphenols and their

associated health benefits as antimicrobials against bacteria are well established (142-145). In addition, there is an increasing demand from the consumers and the industry for natural alternates to synthetic chemical additives used in food systems. Extensive research on potential plant polyphenolic extracts, as natural antimicrobials are ongoing. Below are some of the widely used and studied natural plant extracts for their antimicrobial (and if known antiviral properties) and other health beneficial properties.

Blueberry polyphenols

Blueberries (genus *Vaccinium*) are perennial flowering plants native to North America. Blueberries are a rich source of polyphenols including anthocyanins, flavonoids and proanthocyanidins (PAC), also referred to as condensed tannins (1). Tannins (condensed and hydrolysable) differ from other phenolic compounds for they are able to precipitate proteins, known as astringency (146). Proanthocyanidins (PAC's) are dimers, oligomers, and polymers of catechins bound together by links between C4 and C8 (or C6) where the monomeric flavanols differ in the stereochemistry of C-3 and their hydroxylation pattern in ring A and B (147). Blueberries have been known to contain around 88-261 mg of proanthocyanidin/100 gram of edible portion as per the USDA, 2004 database for flavonoid content. Being a source of natural anthocyanin and proanthocyanidins, blueberries are known to have a broad spectrum of biomedical benefits such as treatment/prevention of cardiovascular disorders, advancing age-induced oxidative stress, inflammatory responses, and diverse degenerative diseases along with anticarcinogenic, neuroprotective, cardioprotective and antimicrobial properties (148).

Antibacterial properties: Berry ellagitannins are known to possess strong antimicrobial properties including prevention of adherence and colonization of bacteria (149). Blueberries contain polyphenols including anthocyanins and flavonoids and proanthocyanidins (PAC), also referred to as condensed tannins (1) are known to have antimicrobial activity against *Listeria monocytogenes*, *Helicobacter pylori*, *Salmonella* Typhimurium, *Escherichia coli* and *Candida albicans* (143, 150, 151). *Salmonella* Enteritidis *and Listeria monocytogenes*. *S.* Enteritidis was shown to be reduced by 5.85 log CFU/ml after 24 h and *L. monocytogenes* by 7.64 log CFU/ml after 24 h by 24 ppm of blueberry water extracts. Similar reduction of 5.50 log CFU/ml and 6.52 log CFU/ml was observed for *S.* Enteritidis and *L. monocytogenes* with blueberry ethanol extract after 24h (152). This data suggests that blueberry extracts can be used as antimicrobial agents in various food products as additives or consumed for treating infections arising from both *Listeria* and *Salmonella* species (152). Blueberry and cranberry contain two compounds (fructose and a high molecular weight polymeric compound) that inhibit adhesin, (required for the bacteria to adhere) thereby preventing attachment of pathogenic *Escherichia coli* to mucosal cells of the urinary bladder (151). Blueberry fractions that had concentrations of 8.9 g/L GAE for total blueberry extract, 8.0 g/L GAE for monomeric phenolics, 173.08 mg/L C3G for anthocyanins and proanthocyanidins, 139.8 mg/L C3G for anthocyanins, and 1.0 mM PA2 for proanthocyanidins was shown to cause 8 log CFU/ml reduction in growth of *L. monocytogenes* for all tested concentrations ranging from 0-4.45 g/L (gallic acid) and 0-86.54 g/L (cyaniding-3-glucoside) while *E. coli* O157:H7 demonstrated an 8-log CFU/ml reduction in growth for all fractions at their highest concentrations (143). *S.* Typhimurium demonstrated similar log reductions in growth to *E. coli* O157:H7, except for being more susceptible to monomeric

phenolics and anthocyanins and less susceptible to proanthocyanidins (143). Significant inhibition of about 50% of *Helicobacter pylori* (causative agent of ulcers) was demonstrated with elderberry (30%), bilberry (50%), blueberry (50.5%) and OptiBerry (62%) extracts. Addition of clarithromycin to the 0.25% berry concentrations, led to a significant increase (about 70%) in the bactericidal effects of the elderberry, bilberry, blueberry and OptiBerry extracts compared with the berry extracts alone. Addition of 1% clarithromycin led to more than 90% of inhibition of *H. pylori* for all berry extracts (150). Another study with blueberry extracts at 25-50% w/w in combination with an antibiotic such as trimethoprim at about 0.001% to 10% was found to enhance its antimicrobial activity against attachment and subsequent biofilm formation of urinary tract infections causing organisms *E. coli* and *C. albicans* (153). Mechanism of antibacterial action: The outer bacterial membrane of bacteria provides the fundamental barrier against any damage. Blueberry phenolics are known to have profound effects on membrane fluidity, inhibition of extracellular microbial enzymes, changing the fatty acid profile, and disrupting metabolism disruption (154). Proanthocyanidins from blueberry (0.15 g/L Gallic acid equivalent), were shown to increase membrane permeability in *E. coli* O157:H7 (155).

Antiviral properties: Only a few studies are reported in literature on the effects of blueberry extracts against viruses. The methanol extract fraction of blueberry leaves (proanthocyanidin more specifically epicatechin) was shown to inhibit hepatitis C virus (HCV) subgenome expression by binding to nuclear ribonucleoprotein A2/B1 that plays an indispensable role for HCV subgenome expression in replicon cells (156). The purified proanthocyanidin from blueberry leaf extracts was also found to suppress the expression of NS-3 (non-structural gene-3) protein gene in HCV (156). Similarly hydrolysable and galloylated tannins (0.03-0.1

microgram/ml) were shown to inhibit herpes simplex virus adsorption to its host, African green monkey kidney cells and human adenocarcinoma cells (157). Another study evaluated the influence of blueberry juice on the survival of human NoV surrogates (FCV-F9, MNV-1, and MS2) at refrigeration temperatures over 21 days, where FCV-F9 was shown to be completely reduced in BJ at 4°C after 1 day. MNV-1 showed minimal reduction on 14 days, and MS-2 complete reduction after 7 days (2).

Antigiardial properties: Trophozoites of *Giardia duodenalis*, a human intestinal parasite, were inhibited by blueberry polyphenols and other berry fruits like strawberry, blackberry, cloudberry at 166µg gallic acid equivalents. Incubation of *G. duodenalis* trophozoites in extracts of blueberries were also shown to cause their distortion, swelling, blebbing, flagellar truncation and increased killing, and increased the rate of spontaneous excystation of *C. parvum* oocysts (158).

Antioxidant properties: In blueberries, primarily anthocyanins along with hydroxycinnamates such as chlorogenic acid contribute to antioxidant properties (149). Studies have shown that blueberries help maintain healthy blood flow via several mechanisms including healthy low-density lipoprotein (LDL) oxidation, normal platelet aggregation, and maintenance of endothelial function (1, 159, 160). Fresh mature fruits of rabbiteye blueberry when assayed for their antioxidant properties were found to have the strongest antioxidant capacity, and the highest TPC (Total phenolic content; 9.44 mg gallic acid/g DW), TFC (Total flavonoid content; 36.08 mg rutin/g DW), and TAC (Total anthocyanin content; 24.38 mg catechin/g DW) values among other berries like blackberry and strawberry (1). These antioxidant properties are considered to be associated with phenolic acids, catechins (flavanols), and proanthocyanidins (condensed tannins) (1). In one study, a drink made form wild blue berries (containing approximately 375

mg of anthocyanins) was tested for its antioxidant potential in human volunteers for reducing oxidative stress and inflammation. After 6 weeks of consumption, significant reduction in levels of oxidized DNA was reported (161). Thus, blueberry juice consumption could be beneficial in relieving oxidative stress caused during chronic liver diseases.

Anti-diabetic: With increased incidences of diabetes worldwide, there is a need to search for alternatives to complement the currently available therapies. Blueberries with a wide range of health benefits and high amount of polyphenols are good candidates to screen for antidiabetic properties. Diabetes control approaches include reducing hyperglycemia through inhibition of carbohydrate hydrolyzing enzymes such as α -glucosidase. A study showed that phenolics from blueberry flowers had strong α -glucosidase inhibitory activity (162). Another study showed similar findings where anthocyanins and proanthocyanidins from fermented blueberry blackberry beverages were found to be inhibitors of carbohydrate-utilizing enzymes and inhibitors of inflammation (163). These studies show that consuming blueberries or their fermented beverages might be beneficial towards control of diabetes and inflammation. Blueberries have been shown to control diabetes by improving insulin sensitivity. When a cobiotic of inulin and sugar free blueberry pomace extract and oat was administered to a Type II diabetic patient for 8 weeks along with metformin (initial treatment for Type II diabetes), the blood sugar was shown to lower to 100mg/dL and weight loss of 5.5kg was reported. Such studies could lead into use of supplements from natural sources with anti diabetic properties to escalate the effect of commonly used drugs and reduce side effects (164).

Anticarcinogenic Effects: Cancer is a leading cause of death and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. Polyphenols have been reported to have effects of on cancer cells by affecting growth, differentiation and apoptosis (165). Some of the studies below have attempted to investigate the anticancer properties associated with blueberries and its consumption. When blueberry anthocyanin extracts were tested for anticancer potential against two breast cancer cell lines (MDA-MB-231 and MCF7) at 250 μg/ml was found to have a significant effect (65%) in reducing cell proliferation after 24 h of cell incubation (166). Anticarcinogenic effects of blueberry phenolics against breast cancer were recently demonstrated in a study (167), where rats on 5% blueberry diet (containing 38 mg/g of total phenolics and 21 mg/g of total anthocyanins) in chemopreventive and therapeutic modes were shown to have delayed tumor development by 28 and 35 days respectively to 17β-estradiol induced breast cancer. Additionally, 96% animals in a control group were shown to develop palpable mammary tumors compared to 60% and 55% animals in chemopreventive and therapeutic groups respectively (167). The study suggests anticancer and protective role of blueberries by regulating tumor development.

Cardioprotective effects: Cardio protective effects of blueberry extracts and its components are being extensively studied. Effects of blueberry supplementation in diets on metabolic syndrome, lipid peroxidation and inflammation were evaluated in obese men and women (168). After 8 weeks of dietary blueberry supplementation (50 g freeze-dried blueberries**;** 350 g fresh blueberries), a significant decrease of about 4.5% and 2.8% higher decrease in systolic and diastolic blood pressure were reported as compared to control groups. This study suggests that including blueberry as a dietary supplement might help in reducing the risk factors related to

cardiovascular disorders and reducing the intensity by a certain extent (168). Hypertensive rats on 2% w/w blueberry enriched diet for 6 to 12 weeks were reported to have lower blood pressure, improved glomerular filtration rate, and decreased renovascular resistance as compared to control groups. Long-term blueberry diet also showed significant decrease in total reactive oxygen species (ROS), peroxynitrite, and superoxide production rates kidney tissues (169). The results suggest that including blueberries in diet could prove beneficial for individuals suffering from hypertension.

Antiobesity: Obesity and its prevalence has increased significantly over the last few decades with the consumption of high fat diet and lack of exercise and sedentary lifestyle. Polyphenols present in green tea, grape seeds, orange, grapefruit and berries are known to have anti obesity effects (170). Antiobesity effects of blueberry juice were recently demonstrated in mice on a high fat diet (171). Four-week-old C57BL/6 mice were fed a high-fat diet (HFD) with or without blueberry juice for 12 weeks. Consumption of blueberry juice was shown to reduce the body weight of the mice by 7.3%. Liver lipids were reported to be ~ 10mg/ml lower in levels in blueberry juice fed rats as compared to HFD controls. Thus consumption of blueberry juice could help in tacking obesity issues (171). In a study dose-dependent effects of blueberry polyphenols were studied on mouse 3T3-F442A preadipocytes where blueberry polyphenols at concentrations of 150, 200, and 250 μg/mL led to a reduction of 27%, 63%, and 74% in intracellular lipid content respectively as compared to the control. All three concentrations of blueberry polyphenols were also reported to have significant inhibition of cell proliferation(170).

Bioavailability and delivery: Pastes, gel, powders can be potential delivery methods for targeting gastrointestinal conditions. Insuring bioavailability for any potential application is also an important consideration. Bioavailability of anthocyanins has been reported to be very low in human volunteers. A study carried out showed that when doses of 150 mg to 2 g total anthocyanins were given to the volunteers in the form of berries, berry extracts, or concentrates, levels of anthocyanins in plasma detected thereafter were reported to be as low as 10–50 nmol/L (172). Thus there is a need to develop effective delivery systems to ensure the delivery of bioactives. Antioxidant capabilities of blueberry and cranberry were investigated under simulated conditions of GI tract were demonstrated, where berry homogenates first exposed to digestive enzymes and then exposed to CaCo-2 cells did not show significant reduction in the antioxidant activity (173). Also, these treated homogenates were shown to annul the effects of ROS (reactive oxygen species) generated by hydrogen peroxide (173). Such critical evaluations are necessary to develop better delivery methods to the gut by way of encapsulation or controlled release so as to protect the beneficial properties from the gut enzymes and acids. More studies need to be carried out to validate its safety via feeding studies and clinical trials.

Grape seed extract

Grapes *(*genus *Vitis)* are native to southern Europe and Western Asia near the Caspian Sea, and were brought to North America and Europe. The medicinal and nutritional properties of grapes have been well known over centuries, where Egyptians and Greeks used the various forms of grapes (ripe, unripe, dried) in healing of various health conditions. Grape seed extract (GSE) is a natural by-product of the wine and grape juice processing industries (174). Grape seed and grape skin has been shown to contain bioactive components such as flavonoids, polyphenols,

anthocyanins, proanthocyanidins, procyanidins, and the stilbene derivative, resveratrol (175). Grape seeds are also known to have high amounts of monomeric phenolic compounds, such as epicatechin, epicatechin-3-O-gallate, catechins, and dimeric, trimeric and tetrameric procyanidins (176). Several pharmacological and therapeutic benefits such as antimicrobial, anti-inflammatory, antioxidative, cardioprotective, and neuroprotective effects have been reported to be associated with GSE (177). GSE has been approved as a generally recognized as safe (GRAS) by the U.S. Food and Drug Administration and hence is being studied extensively for its use as an antimicrobial agent in foods. ("GRAS" is Generally Recognized As Safe under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act) (178). GSE at doses of 50 to 100 mg/ml per day do not exhibit toxicity or adverse health effects in rats (176, 179, 180). This adds advantages to GSE that has great potential for use as a value-added product associated with its low cost, sustainability, and lack of evidence of being hazardous to the environment.

Antibacterial effects of GSE: GSE (both methanol and acetone extracts) were found to be more effective against Gram-positive bacteria when compared to Gram-negative bacteria, when tested against several foodborne bacterial pathogens (175).The minimum inhibitory concentration (MIC) with a methanol:water:acetic acid (90:9.5:0.5) extract was found to be 900 ppm for the gram-positive *Bacillus cereus, Bacillus subtilis* and *Bacillus coagulans* and 1000 ppm for *Staphylococcus aureus* (175).For the gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, the MIC was found to be 1250 and 1500 ppm, respectively. When the extract contained acetone (acetone: water: acetic acid (90:9.5:0.5)) instead of methanol, differences in MIC were observed for the gram-positive bacilli, resulting in MICs of 850 ppm for *B. cereus, B. subtilis* and *B. coagulans*, while the other organisms showed similar MICs to the methanol:

water: acetic acid (90:9.5:0.5) extract. These researchers determined that gallic acid was the bioactive component, with the three hydroxyl responsible for the antibacterial activity of GSE (175). Muscadine grape skin extracts were also found to be effective against *Helicobacter pylori* (causative agent of ulcers) with MIC ranging from 256 to 512 g/ml, and the MIC ranged between 256 to 1,024 g/ml for muscadine seeds (181). Resveratrol and ellagic acid were also found to inhibit this ulcer causing bacteria, with an MIC range of 6.25 to 50 g/ml, while myricetin was found to have no effect (181). GSE at a concentration of 5 mg/ml was also found to inhibit the dihydrofolatereductase activity and growth of *S. aureus* at 0.5 mg/ml after 6 h, however some injured cells were recovered after 12 h (182). For GSE at 1 mg/ml, these researchers reported 99% of inhibition after 24 h, with no observed cell recovery.

In another study, commercial grape juice and skin and grape seed extract were tested against the gram-positive foodborne pathogen, *Listeria monocytogenes*, of concern to immunocompromised individuals and pregnant women. Commercial grape juice decreased *L. monocytogenes* from 10^6 –10^7 CFU/ml to undetectable levels and *B. cereus* by 1-log within 10 min in the juice, without further reduction after 60 min (183). The seed and skin extracts (250–280 mg/L gallic acid) were reported to have similar anti-listerial activity to that of the commercial grape juice (183). Anti-bacterial activity of GSE was also tested against methicillin resistant *S. aureus* (MRSA), which is an on-going problem in nosocomial and other related infections, where health-care providers continue to have challenges in prescribing appropriate antibiotics against these infections (184). All of the 43 MRSA strains tested were found to be inactivated at 20.67 µg of GSE flavonoids (184). Another study showed that GSE at 1 and 5 mg/mL was found to be effective against MRSA strains resulting in a 2.9-4.0 log CFU/ml

reduction after 2 h at 37°C (145). These results suggest that natural products such as GSE can be used as effective antimicrobials for multidrug resistant strains as an alternative to regular antibiotics. Red GSE at 0.5 mg/ml was found to reduce plaque formation of *Streptococcus mutans in vitro* by 40% as compared to control (185), showing the potential of GSE to prevent dental caries.

Antiparasitic effects of GSE: GSE has been reported to have antiparasitic effects against *Histomonas meleagridis*, a protozoan parasite responsible for histomonosis with high mortality in turkey and chicken (186); *Blastocystis*, a prevalent unicellular parasite in human fecal specimens (187); *Trichomonas gallinae* that causes avian trichomonosis in various bird species, especially pigeons and doves (188)*;* and *Trichostrongylus colubriformis*a parasite of herbivorous mammals (189). GSE at 100 ppm for 6-12 h was shown to kill 50% of exposed *Lymneacailliaudi* snails (LC_{50}; lethal concentration 50) with higher molluscidal activity with increased exposure time to 12-24 h (190). However GSE showed weak effect against *Culex pipiens* larvae (responsible vectors for malaria, dengue and yellow fever) where their mortality did not increase over 50% even after increasing the GSE concentration to more than 200 ppm for 48h (190). GSE at 5 mg/ml was also found to completely inhibit (initial number of 10^3 histomonads) the growth of *Histomonas meleagridis, Trichomonas gallinarum* and *Blastocystis sp*. after 24 and 48 h (191). Another study tested a new product containing GSE against the avian parasite *H. meleagridis*, that showed inhibition of its growth at concentration of 2.5 or 5 µl/ml after 48 h (192). GSE at 10-20 mg/kg was reported to reduce mortality in chickens infected with *Eimeria tenella* and also alleviated the oxidative stress following infection and helped improve the weight

gain of chickens which is important for the poultry industry (193). Flavan-3-ols and galloyl derivatives of condensed tannins that are components of GSE were found to be completely inhibitory towards egg hatching of *Trichostrongylus colubriformis* at 1000 mg/ml, and epigallocatechin gallate at 100 mg/ml showed 100% inhibition of *T. colubriformis* larval development (194). Methanol extracts of grape seed at 300 and 600 mg were reported to be effective in killing the immature form of the leech *Limnatis nilotica* within 260 and 200 min, respectively, but was not effective towards the mature forms, suggesting that GSE should be used in combination with other treatments to boost their anti-leech effects (195).

Antiviral activity of GSE: In addition to bacteria, GSE is known to have effects against several viruses of human health concern. Human noroviruses are recognized as leading causes of non-bacterial gastroenteritis worldwide (3, 196). However, since these non-enveloped single-stranded RNA containing human noroviruses cannot be cultivated in the lab, cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) are used to determine inactivation by various chemical or processing technologies (197). Hepatitis A virus is another non-enveloped single-stranded RNA containing foodborne virus important because of the severity of the disease that affects the liver and lasts for more than a month. GSE at 0.25, 0.5, and 1.0 mg/ml for 2 h at 37°C was shown to reduce FCV-F9 titers by 3.64, 4.10, and 4.61 log PFU/ml; MNV-1 by 0.82, 1.35, and 1.73 log PFU/ml; and HAV by 1.81, 2.66, and 3.20 log PFU/ml (197). GSE was also shown to prevent replication of human norovirus surrogates using ELISA studies that were in agreement with the earlier studies of Su et al., 2011, where MNV-1 titers were shown to be reduced by 3 log PFU/ml when treated with 0.2 mg/ml (144). GSE was also evaluated for its anti-noroviral activity as a surface disinfectant by these researchers, where 0.2 mg GSE/ml,

showed a reduction of MNV-1 to undetectable levels without the presence of any protein load. In presence of 0.01% dried milk, reduction to undetectable levels was obtained only with 2 mg/ml GSE, while in tap water, GSE at 0.2 and 2 mg/ml was shown to cause 1 and 2 log PFU/ml reduction in MNV-1 titers (144). The effect of the symptoms of HIV-1 and AIDS is well publicized, emphasizing the need for improved control and preventive measures, where GSE may play an important role. GSE is also reported to interfere with the binding of the AIDS causing human-immunodeficiency virus (HIV-1) to its host cell receptor and thus prevents HIV-1 entry into the normal lymphocyte (198).

Mechanism of action of GSE against microbial pathogens: With regards to antibacterial properties of GSE, the bacterial cell membrane being semipermeable allows the entry of polyphenols that can interact with the cytoplasm or cellular proteins, with higher ability of GSE polyphenols (that are present in undissociated forms) to enter the cell and interact with cellular components (199). Thus, the benefits of the antimicrobial properties of GSE can be exploited for food or clinical applications. GSE was shown to have some effect on viral adsorption of FCV-F9, MNV-1, and HAV, with less effect on replication (197). These researchers showed that when the respective host cells were treated with GSE before viral infection, the titers of FCV-F9, MNV-1, and HAV were decreased by 0.8, 0.5, and 1.9 log PFU/ml, respectively. GSE treatment after the viral infection of the host cells showed a 0.1, 0.2, and 0.8 log PFU/ml reduction, respectively. The higher reduction obtained by GSE when used in pre-infection than post-infection, indicated that GSE either blocks virus binding to the host cell receptors or coats the virus to prevent attaching to the host-cell or causes clumping of viral particles. GSE has also been shown to inhibit the replication and expression of HIV-1, an enveloped virus with

glycoproteins gp 120 and gp41, by blocking binding of HIV-1 to the cell receptor and preventing HIV-1 entry into the normal lymphocyte (198). The study also showed that GSE at concentrations of 0.5 to 5 mg/ml over 24 h at 37°C was found to significantly down regulate expression of HIV-1 co-receptors, *CCR2b* and *CCR3* (B- chemokine receptors), in peripheral blood mononuclear cells (198).

Resveratrol (RV) is a non-flavonoid polyphenol found in grape seed and grape skin and is known to be synthesized in response to external stimuli and injury (200). RV has been known to affect several intracellular signaling pathways, and strongly inhibits the *in vitro* and *in vivo* replication of influenza virus (201). Virus replication was reportedly reduced by 20 μg/mL of RV and completely inhibited by 40 μg/mL of RV over 72 h of infection time. RV at 20 μM was also found to inhibit the replication of polyomavirus, with complete reduction of replication after 24 h of infection (202). Polyomaviruses are small, double stranded DNA viruses known to occur in immunocompromised individuals and may cause hemorrhagic cystitis in recipients of bone marrow transplantation and progressive multifocal leukoencephalopathy (203).

Studies were also carried out to gain insight about the mechanism of action of GSE and its components against bacterial pathogens, and it was proposed that the antibacterial activity of tannins could be due to the inhibition of extracellular microbial enzymes (146). Inhibitory effects of GSE could also be attributed to the constituent phenolic compounds that can form phenoxyl radicals upon entering the cell, or binding to the cellular membrane and causing cellular disruption (204). In addition, scanning electron microscopy studies carried out on MRSA cells treated with GSE showed the that the cell surface was rough and disrupted in comparison to the smooth and rounded surfaces of control cells (145). This suggests that GSE may be acting by

disrupting the bacterial cell membrane causing leakage of cellular material and thereby inactivation. In addition to the antimicrobial properties of GSE, research on other health benefits is also being carried out extensively.

Role of GSE in Anti-inflammation: Inflammation is theorized to be the underlying cause of most health issues and chronic inflammation has been linked to heart disease, hay fever, rheumatoid arthritis, obesity, and diabetes (205-208). When the anti-inflammatory and antioxidant properties of GSE were investigated in the case of septic shock caused by the intraperitoneal injection of lipopolysaccharide (LPS) derived from *Escherichia coli*, GSE at doses of 75 mg/kg/day had anti-inflammatory effects by decreasing the nitrate and nitrite (NOx) levels in the plasma, red blood cells, spleen, and liver (209). These researchers showed that high doses of 200 mg/kg/day could down regulate the genes *Il-6* (interleukin 6) and *iNos* (inducible nitric oxide synthase) associated with inflammatory responses against the LPS endotoxin (209). Murine studies showed that when GSE at 100 mg/kg was administered to acute asthmatic mice, there was a decrease in number of eosinophils, airway inflammation, hyper responsiveness, and oxidative stress (210).

GSE and its effects on Atherosclerosis and Heart Disease: GSE has been widely studied for their cardio-protective properties, and in reducing cholesterol and plasma lipoproteins. For example, GSE was shown to alleviate the side effects such as cardiac steatosis (adipose degeneration) and lipotoxicity in rats fed on a high fat diet for 45 days at levels of 500 mg/kg/bodyweight (211). GSE at doses of 50 and 100 mg GSE/kg body weight were found to reduce 49 and 63% foam cells, a biomarker of early stage atherosclerosis (212). Red grape seed extract at 200 mg/day for 8 weeks was found to reduce total cholesterol and oxidized low-density

lipoprotein particles by 10.68 mg/dL and 5.47 mg/dL, respectively (213). Thus, GSE consumption may also prove to be beneficial in reducing the risk of atherosclerosis (213). Additionally, dry GSE at a concentration of 100 mg was found to significantly decrease the amount of serum cholesterol levels in human volunteers (214). Taken together, these studies show the anti-atherosclerotic and cardio-protective effects of GSE.

GSE and its role in obesity and diabetes: GSE has also been explored for use in controlling obesity and preventing diabetes. Lipoprotein lipase (LPL) is an enzyme involved in hydrolysis of triacylglycerol by releasing free fatty acids for uptake into adipocytes, involved in obesity. GSE at 1 mg/ml was found to inhibit human pancreatic lipase activity by almost 80% within 5 min of incubation in adipocytes, suggesting the benefits of GSE against obesity (215). GSE was also found to up-regulate T_{regs} (regulatory T cells) and T helper 2 cytokine producing cells that play a role in suppressing excessive immune responses (216).

GSE was also shown to reduce postprandial plasma glucose in healthy individuals, where the plasma glucose concentrations at 15 min and 30 min after consumption of a high carbohydrate meal with 100 mg or 300 mg of GSE was significantly lower than that of a high carbohydrate meal without GSE (217). The study proposed the use of GSE as a preventive measure against diabetes in otherwise healthy populations. Mice on a high fat diet were shown to be protected from nerve fiber loss compared to controls when administered 100 mg/ml GSE for 12 weeks, suggesting that GSE may provide benefits towards the treatment of diabetic neuropathy (218).

Antioxidative effects of GSE: GSE is known to be an efficient antioxidant and free radical scavenger (175). GSE at 50 mg/l was shown to be an effective radical scavenger, being better than vitamins C and E (219). GSE components like procyanidin B4, catechins, and gallic acid at

concentrations of 10-25μmol/l were reported to be good preventive agents against cellular DNA oxidative damage, though, these compounds may induce cellular DNA damage at higher concentrations of 150 μmol/l (220). GSE at 2 mg/ml was found to inhibit the peroxidation of polyunsaturated fatty acids induced by UV–C (221).

Anticarcinogenic effects of GSE: When grape seed polyphenols were investigated for their anti-carcinogenic effects towards 12-O-tetradecanoylphorbol-13-acetate-(TPA)-induced tumor reduction as a topical application, tumor incidence was inhibited by 35 and 60%, tumor multiplicity was inhibited by 61 and 83% and tumor volume was inhibited by 67 and 87% at concentrations of 0.5 and 1.5 mg/ml/mouse/application on mouse skin (222). More recently in breast cancer studies, GSE at 100 μg/ml was found to increase apoptotic cell death significantly by up to 12% after 72 h incubation, when the highly metastatic MDA-MB 231 breast cancer line was used (223). Grape powder, resveratrol, and GSE when administered topically 30 min after 7,12-dimethylbenz [a] anthracene (DMBA, a skin carcinogen) exposure at concentrations of 1, 2, or 4 mg was shown to inhibit epidermal hyperplasia, proliferation, and dermal inflammation (224). GSE at 50 μg/mL was also reported to significantly induce apoptotic death in non-small-cell lung cancer cell lines by causing a release of cytochrome c in the cytosol and cleavage of caspases 3 and 9 that play a role in cell death (225).

Potential application of GSE for food industry as produce wash: Natural products that are cost-effective, environmentally friendly, and sustainable are of great interest to the food industry. GSE is one such natural product, which is being investigated as an antimicrobial surface disinfectant. Several studies have aimed to evaluate the potential of GSE for its use as a food additive or a pre-packaging wash or treatment. Kao et al., 2011 used pork slices as a “model”

food to evaluate its use in the food industry. Pork slices were first immersed in 20 mg/ml, 60 mg/ml, or 100 mg/ml of GSE solutions and then inoculated with *S. aureus* suspensions, and incubated at 37°C for 8 h and 24 h, after which >99% of inhibition was observed for pork slices treated with 100 mg/ml GSE (182). However, with such high concentrations of GSE used (due to its bitter taste), it is important to note that there may be issues with sensory acceptance of these products by consumers. In another study by Corrales et al., 2009, GSE was introduced into pea starch films at a concentration of 1% that showed growth inhibition of *Brochothrix thermosphacta* (spoilage organism) by 1.3 log CFU/mL in pork loins during the first 4 days of incubation (226). When GSE (commercially available Gravinol-S) was tested as an antimicrobial wash on fresh produce, MICs for *Listeria monocytogenes* and *L. innocua* ATCC 33090 were found to be 50 and 78 µg/ml (227). When tested against live cells using flow cytometry at sub-MICs for 2 min, GSE was found to cause rapid permeabilization and clumping of *L. innocua*. GSE treatment of 1250 µg/ ml was shown to yield the highest reduction of *L. monocytogenes* by 2 log CFU/ml after 2 min on spiked Roma tomatoes. These results suggest the potential application of GSE as a surface disinfectant for fresh produce (227). Conventional and electrostatic spraying of malic acid (MA), tartaric acid, gallic acid and GSE against *Salmonella* Typhimurium using spinach as a model system, showed that MA alone sprayed electrostatically caused reductions of 1.6 and 3.2 log CFU/g after 7 and 14 days, while GSE alone led to a reduction of 0 to 1.4 log CFU/g (228). These researchers reported that maximum reduction of 2.6 to 3.3 log CFU/g was observed for spinach leaves treated with 2% malic acid in combination with 3% GSE when sprayed electrostatically, while lower log reductions were observed (0 to 1.5 log CFU/g) when sprayed conventionally (228). These results showed that electrostatic

combination sprays containing MA and GSE were more effective in reducing the foodborne gram-negative *S.* Typhimurium that is linked to on-going produce outbreaks.

Consumer concerns regarding addition of nitrite in processed meats has led to finding natural alternatives such as GSE (142). GSE at 0.5% was found to reduce *L. monocytogenes* by 1.1 log CFU/g after 9 days at 10^{o}C in cured meats (142). When fresh pork slices that were first treated with GSE along with oregano, clove and pomegranate and then inoculated with low (10 CFU/m1) and high (10^5 CFU/mL) titers of *L. monocytogenes, S. aureus and S. enterica,* aerobically packed and stored at 20^{O}C, the treated samples showed reductions of 1.36, 0.96 and 1.75 log CFU/g for *L. monocytogenes, S. aureus,* and *S. enterica*, respectively after 9 days (179).

Irradiated meat samples when treated with a single 1% GSE treatment led to a reduction from of 6.5 to 4.5 log CFU/ml *L. monocytogenes* in 12 to 18 h but no subsequent further reduction were observed; while nisin (6400 IU/ml) treatment gave an initial reduction of 3 log CFU/ml in 12 h though the cell numbers rebounded after 18 to 24 h (180). However, these researchers showed that the combined treatment with 1% GSE and nisin (6400) IU led to reduction below detectable limits (180).

Application of GSE as an antiviral produce wash was also studied where 0.25 to 1 mg/ml GSE was found to reduce FCV-F9 and MNV-1 titers within 5 min on spiked lettuce and peppers (229). High titers (~7 log PFU/ml) of FCV-F9 on lettuce and peppers were found to be reduced by 2.71 log PFU and 3.05 with GSE at 1 mg/ml after 1 min, respectively, while MNV-1 was shown to be reduced by 0.2-0.3 log PFU on lettuce and 0.8 log PFU on peppers for low titers (~5 log PFU/ml) (229).

Pomegranate polyphenols

Pomegranate (*Punica granatum*) is a plant native to Iran, India, and the Mediterranean countries such as Turkey, Egypt, Tunisia, Spain, and Morocco (230). Pomegranate juice and its peel are known to be a rich source of polyphenols including flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid), which account for 92% of the antioxidant activity associated with the fruit (231). Among these, Ellagitannins and punicalagins were reported to be the main bioactive fractions in pomegranate (232). The anthocyanin content in pomegranate juice was shown to be around 135 mg/L; punicalagins and punicalins content were shown to be between 80 and 135 mg/L (233). In addition, it is also known to contain organic acids such as ascorbic acid, citric acid, fumaric acid, and malic acid (230).Methanolic extract of pomegranate was shown to inhibit the formation of biofilms by *S. aureus*, methicillin resistant *S. aureus*, *Escherichia coli*, and *Candida albicans* where the reported MIC was 1000µg/ml (234). The extract also disrupted pre-formed biofilms of *C. albicans.* Pomegranate extracts were also examined for their antimicrobial effects against *Clostridium difficile*, where the effective minimum inhibitory concentration ranged in between 12 to 25 mg/ml gallic acid equivalent (235). Aqueous pomegranate extracts were demonstrated to have activity against *E. coli* O157:H7 with an MIC of 0.19 mg/mL (236). Studies have proposed that the mode of action of pomegranate polyphenols is by disrupting the cellular membranes, inhibiting microbial enzymes and through interaction with proteins and co-aggregation of microorganisms (237, 238). Pomegranate extract was shown to have antifungal properties, where the crude extract at 5 mg/ml inhibited *Stemphylium botryosum* spore germination by 74% (232). Effect of purified pomegranate extract on influenza virus was studied

where punicalin (tannin) was shown to inhibit the virus replication and also inhibited agglutination of chicken red blood cells by the virus, and, when used in combination with the antiviral oseltamivir (237). Pomegranate polyphenol powder extract at 800 µg/ml was shown to reduce influenza virus titers by 3 log at room temperature within 5 min and it was suggested that this antiviral activity could be owing to the coating of the virus (239). In addition, pomegranate polyphenols were also shown to inhibit replication of Human immunodeficiency virus (HIV-1) by blocking the virus binding to CD4 host cell receptors (240).

Pomegranate juice was demonstrated to reduce titers of human norovirus surrogates; FCV-F9, MNV-1, and MS2 by 1.20, 0.06, and 0.63 log PFU/mL respectively at room temperature after 1h (241). When treated with polyphenols from pomegranate a reduction of 1.30, 2.11, 3.61 logPFU/mL for MNV-1 was obtained with 8, 16, and 32 mg/mL within 1h at room temperature. Titers of FCV-F9 were reduced to undetectable levels with 8 mg/ml within 1h at room temperature. The study suggested that this antiviral effect could be due to the possible binding of the polyphenols to the virus particle or by blocking host cell receptors (241).

Cranberry polyphenols

Cranberries (*Vaccinium macrocarpon*) are native to North American region and contains diverse group of phytochemicals, including flavonoids, hydroxycinnamic acid derivatives, organic acids, and isoprenoids including ursolic acid and lutein(242). The predominant bioactive compounds found in cranberries are the flavonols, the flavan-3-ols, the anthocyanins, the tannins (ellagitannins and proanthocyanidins), and the phenolic acid derivatives. The average flavonol content in cranberry is 410mg/100 g fresh fruit weight (243). Cranberries have a high amount of oligomeric and polymeric pigments, also referred to as condensed non-hydrolyzable tannins or

proanthocyanidins (PAC). 51-90% of the PAC's in cranberries are A-type PAC's. A-type PAC'shave anti-adhesion activity against the urinary tract infection causing P-fimbriated *E. coli* to uroepithelial cells and decrease in the expression of P-fimbrial gene (244). Commercial cranberry juice (CJ, pH 2.5) was found to reduce common gastrointestinal bacterial pathogens, *E. coli* O157:H7 and *L. monocytogenes* at 8 log CFU to undetectable levels after 15 min and 1 h of treatment at -23^{o}C (storage conditions) respectively. Cranberry proanthocyanidins (CPACs) at 25 mg/ml were found to inhibit infection and invasion of epithelial cells by enteropathogenic *Escherichia coli* (EPEC) and *Salmonella* Typhimurium by preventing the formation of actin pedestal required by EPEC for host infection (245). CJ was also found to inhibit biofilm formation of *E. coli, L. monocytogenes, Campylobacter jejuni, S.* Typhimurium and *S. aureus.* It was shown that CJ reduced the non-specific adhesion of bacterial cells to borosilicate glass (246). Oregano–cranberry (50:50) extracts at a concentration of 750 ppm in combination with 2% sodium lactate were shown to inhibit growth of *L. monocytogenes* by 1 log CFU/ ml in cooked ground beef samples. It was reported that in presence of 1mM proline, the effect of Oregano–cranberry with sodium lactate was significantly reduced indicating proline dehydrogenase to be the likely site of action for phenolic phytochemicals and lactate radical (247). In a similar study, cranberry juice was shown to reduce aerobic plate count for coliforms initial ~7 log CFU/ cm2 to 3.86 CFU/cm2 on chicken breasts (248). Studies on anti-adhesive effect of cranberry PAC on *E. coli* to uroepithelial cells showed that stationary phase strains was found to be about 52% and 75.5% inhibited in the presence of 100 μg/ml cranberry PAC A2 in pretreated uroepithelial cells (249). Cranberry proanthocyanidins (CPAC) at 1 mg/ml were also shown to reduce methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591 by 1.04,

1.68 and 1.84 log10 CFU/ ml after 1, 2 and 8 hours of treatment respectively. Complete reduction was shown after 24 hours at 37^0C, however MRSA ATCC 33593 was reported to reduce completely within 8 hours of treatment with 1mg/ml CPAC (145).

Cranberry juice (CJ) at concentrations ranging from 50%, 30%, and 10%, was shown to reduce bacteriophages T2 and T4 of *E. coli* strains C and B, and also simian rotavirus SA-11 after 30 min at 37^oC, 23^oC and 4^oC from 9 log PFU/ml to undetectable levels by plaque assays (101). However, concentrations lower than 10% did not show any significant reduction. Scanning Electron Microscopy (SEM) studies showed that CJ treated phage T4 was unable to attach to bacterial host cells as opposed to the control. Cranberry juice was also shown to inhibit hemagglutination of rotavirus, where 20% CJ led to a complete hemagglutination inhibition. It is proposed that CJ led to a change in glycoprotein moieties, which led to a failure in virus binding the receptor cells (101). Cranberry juice and cranberry PAC (proanthocyanidins) were also found to reduce human noroviral surrogates murine norovirus (MNV-1), feline calicivirus (FCV-F9), MS2 bacteriophage, and phiX-174. Cranberry juice at pH 2.6 and 7, and CPAC at 0.15 mg/ml and 0.3 mg/ml was shown to cause a complete reduction of FCV-F9 within 30mins. CJ at pH 2.6, 7.0 and CPAC at 0.15 mg/ml, 0.30 mg/ml PAC was found to cause log reductions of 1.90, 1.66, 2.24 and 2.94 log10 PFU/ml after 1h for MNV-1, respectively. For phi-X 174, reduction in titers of 1.66, 1.24, 2.15 and 2.82 log10 PFU/ml was achieved after 1 h with CJ at pH 2.6, CJ at pH 7.0, 0.15 mg/ml PAC and 0.30 mg/ml PAC (250).

Raspberries

Black raspberry (*Rubus coreanus*) is native to South Asian countries including China, Japan and Korea. They are known for their anti-inflammatory, antibacterial, and antiviral properties. The

fruit contains polyphenolic compounds including gallic acid and quercetin (251). When screened for their antibacterial activity, black raspberry was shown to effective against multi drug resistant *Staphylococcus aureus* (MRSA), *Bacillus anthracis* and *Acinetobacter baumannii* (252). MIC's of 250, 125 and 1000 µg/ml were reported for MRSA, *B. anthracis* and *A. baumannii*, respectively. At 1% concentration, raspberry extract caused 80% inhibition on the growth of Helicobacter pylori (150). Black raspberry juice (6%) was also shown to have antiviral effect on the HNoV surrogate, MNV-1 causing reduction in plaque titers by 99% after 1 h (253). Co-treatment (simultaneous addition of treatment and virus to the host cells) was demonstrated to exert maximal antiviral activity on bothFCV-F9 and MNV-1. The study suggested that the juice exerted its antiviral effect by inhibiting viral attachment to host cells, though gallic acid and quercitin did not have any significant effect (253).

Blackberries

Blackberry (*Rubus fruticosus*) fruits and plants have been used in herbal medicine for their antimicrobial, anticancer, antidiabetic, antidiarrheal, and antioxidant properties(254). Blackberries contain phenolic compounds including tannins, gallic acid, anthocyanins along with vitamin C, niacin, sugars, and some amounts of, citric acid and malic acid (255). Blackberries were shown to reduce the oral pathogen *Fusobacterium nucleatum* by 1.5 log CFU with 1400 µg/ml of blackberry extract after 24h at 37^{o}C (255). . Metabolic activity of *F. nucleatum* was reported to reduce by 40% with 700 µg/mL of blackberry extract, while a higher concentration of 1400µg/mL caused the metabolic activity to reduce by 84% (255).

Extract from blackberry leaves were reported to have minimum bactericidal concentrations (MBCs) against *H. pylori* strains G21 and 10K of 1200 µg/mL and 1500 µg/mL after 24h and 134 µg/mL and 270 µg/mL after 48 h, respectively(256). Blackberry extract at 56 µg/mL was also shown to inhibit herpes simplex virus (HSV-1) replication in oral epithelial cells by 99%(255). Higher concentration of 280 µg/mL was shown to affect the virus adsorption and entry into host cells.

Hibiscus extracts

Hibiscus sabdariffa commonly known as 'roselle' is an annual, tropical or subtropical shrub species native to countries including Sudan, Mexico, India, and Thailand. It belongs to family *Malvaceae* and calyces are often used in the preparation of beverages or for ornamental purposes (Morton, 1987). Hibiscus calyces contains bioactive compounds including anthocyanins, phenolic acids, organic acids, saponins, and alkaloids, gallic, chlorogenic, and protocatechuic acid and these flavonoid compounds are responsible for the deep red color of the calyces (257, 258). The total phenolic content is around 77.2 mg GAE (gallic acid equivalents)/g for an aqueous extract and 87.7 mg GAE/g for an ethanol extract (259). The calyces (aqueous extracts) are considered GRAS (generally recognized as safe) and are approved for use as a food additive by the U.S. by the Food and Drug Administration (21 CFR 172.510) in the flavoring of beverages. Delphinidin-3-sambubioside and cyanidin-3-sambubioside are two anthocyanins found in hibiscus extract that also contribute towards the antioxidant activity (260). Other compounds present are gossypetin, quercetin, pectin, saponins, cardiac glycosides, and alkaloids (261). These organic acids may also contribute to the antimicrobial activity, where the undissociated form of the organic acid can enter the cell wall of bacteria, thereby stressing the

cell by pH reduction cell and can lead to death (262). Organic acids including malic acid and tartaric acid are also identified in the calyces resulting in the low pH of approximately 2-2.5 (263). Calyces of *H. sabdariffa* are known to possess a wide range of health benefits including antioxidant, anti-diabetic, anticancer, cardio-protective and antimicrobial effects (264-266). Aqueous extracts were also shown to be effective against methicillin-resistant *S. aureus*, *Klebsialla pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* with the MIC's of 32, 48, 56 and 48 mg/l respectively (267). Crude extracts of *H. sabdariffa* calyces were reported to be effective against *Propionibacterium acnes* and *Staphylococcus epidermidis,* where the MIC was reported to be 2.5 mg/ml and 0.625 mg/ml, respectively and MBC (minimum bactericidal concentration) were reported to be to 5 mg/ml for both (268). Ethanol and aqueous extracts of *H. sabdariffa* were also shown to have inhibitory effects against foodborne bacteria such as *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes, Bacillus cereus*, and *S. aureus* when added at levels of 5 or 10 mg added to 100 g ground beef or 100 ml apple juice after 3 days of storage (269). Another study showed that the aqueous extracts at 100 % v/v when used as a produce wash could reduce 4 log CFU/g of *E. coli* O157:H7 and *Salmonella enterica* on lettuce after 24 h (270). In a recent study, *H. sabdariffa* extracts at concentrations of 0.5 to 2 mg/ml were also shown to inhibit biofilm formation in *Candida albicans* (271). Protocatechuic acid (PCA) is an essential component of *H. sabdariffa* and has been demonstrated to have several health beneficial properties (272). PCA has been shown to be the active component responsible for the antimicrobial activity of *H. sabdariffa* (267). Ferulic acid, a component of *Hibiscus mutabilis* has also been demonstrated to have antimicrobial properties against common food borne bacteria including *E. coli, S. aureus* and *L. monocytogenes* (273).

Ferulic acid was also demonstrated to possess antifilarial activity against *Setaria cervi* (274).

Conclusions

Overall, natural plant polyphenols appear to be promising alternates for use as preventive, therapeutic or control options against the spread of foodborne illness. It is recognized that effectiveness of antimicrobials can decrease in the presence complex food matrices where components of foods such as lipids, proteins or other acidic or alkaline conditions may interfere in the antiviral properties. Hence, it is necessary to determine their effects in model food systems, environment, and in simulated gastrointestinal fluids to better understand their effects and applications. To mimic the survival and behavior of viruses and antivirals in the gut, survival studies using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) are necessary. Survival studies in gastric environments have been reported previously with foodborne bacterial pathogens such as *E. coli, L. monocytogenes* and *V. vulnificus* (275-277). Apple juice and milk were chosen to study the effectiveness of tested treatments in this study. Apple juice is a food product mainly consisting of carbohydrates (sugars including glucose, fructose, sucrose and sorbitol), nonvolatile acids (malic, quinic, citric, shikimic, and fumaric), and phenolics (chlorogenic acid and hydroxymethylfurfural) (278). In a study, cinnamon bark essential oil at 1,000 was shown to have bactericidal against *L. monocytogenes* with 3 log CFU/ml reduction in skimmed milk and 1 log CFU/ml reduction in whole milk (279). The study suggested that intrinsic parameters like fat and protein contents of the milk interact with the antimicrobial substances that might reduce their activity. In addition to studying the effectiveness of these antivirals in foods systems and the gut environment, it is also interesting to look into their inactivation mechanisms. Several attempts have been made at understanding the mode of antiviral action against these noroviral surrogates using various techniques like electron

microscopy studies, binding studies, molecular assays and in vitro cell culture assays (144, 197, 253). Viruses are known to cross the host epithelial barrier through attachment to carbohydrate moieties of host cell glycoproteins, glycolipids such as sialic acid, and proteoglycans (280). Human noroviruses in addition to binding to histo-blood group antigens are also known to bind to porcine gastric mucin (PGM) (281). PGM has been used widely in studies to discriminate between infectious and non-infectious norovirus particles (282).

The objectives of this research were (1) to evaluate the antiviral properties of blueberry proanthocyanidins (B-PAC) and commercial blueberry juice (BJ) against human norovirus surrogates, namely feline calicivirus (FCV-F9) and murine norovirus (MNV-1); hepatitis A virus and Aichi virus; (2) to determine the antiviral activity of BJ and B-PAC against these test viruses in model food systems and under simulated gastric conditions and (3) to understand the mechanism of action of B-PAC and BJ against the tested viruses.

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Appendix A

Table 1.1 General properties of common foodborne viruses

	HNoV	**HAV**	**Aichi virus**	**Rotavirus**	**HEV**
Family Genus	*Caliciviridae; Norovirus*	*Picornaviridae Hepatovirus*	*Picornaviridae; Kobuvirus*	*Reoviridae; Enterovirus*	*Hepevirus; Hepeviridae*
Genome	(+) ssRNA, 7.5 kb	(+) ssRNA, 7.5 kb	(+) ssRNA, 8.2 kb	dsRNA, 7.5 kb	(+) ssRNA, 7.3 kb
Particle size (nm)	27-38	27-32	27-30	30	27-34
Envelope	No	No	No	No	No
Route of transmission	Fecal oral	Fecal oral	Fecal oral	Fecal oral	Fecal oral
Incubation period	24-48 h	4 weeks	24-48 h	2-4 days	40 days
Symptoms	Diarrhea, nausea, vomiting, abdominal pain	Malaise, dark urine, nausea, vomiting, jaundice	Diarrhea, abdominal pain, nausea, vomiting	Vomiting, diarrhea, dehydration, fever	Jaundice, anorexia, Acute liver failure
Vaccine	Not available	Available	Not available	Available	Available

*HNoV: human norovirus; HAV: hepatitis A virus; HEV: hepatitis E virus

Table 1.2 Human norovirus outbreaks

Year	Sample type	Contamination source/Location	Detection method	Reference
2014	Clinical	Cruise ship	Not known	(8)
2013	Not known	Unknown source (tourists), Yellow stone national park	Epidemiology	CDC, 2013
2012	Food	Imported frozen raspberries	Epidemiology	(283)
2011	Stool sample	Pasta, raw vegetables/ French military unit	RT PCR	(284)
2009	Clinical, food, environmental	Salad/German military base	RT PCR	(285)
2009	Stool	Water resort	RT-qPCR, Epidemiology	(286)
2009	Clinical	Cruise ship	RT-qPCR	(287)
2008	Water	Municipal drinking water/Sweden	Epidemiology	(288)
2008	Clinical	University dining hall	Epidemiology; RT PCR	(289)
2003	Food and clinical	Oysters	Epidemiology; RT PCR	(290)

Table 1.3 Hepatitis A virus outbreaks

Year	Sample type	Contamination source/Location	Detection method	Reference
2013	Food	Frozen berries in smoothies/ Nordic countries	Epidemiology	(291)
2011	Food	Sundried tomatoes	Epidemiology	(53)
2011	Serum	Sushi bar, Japan	RT PCR	(292)
2009	Serum	Sun dried tomatoes	RT PCR	(52)
2009	Serum	Well water	Epidemiology, detection of anti-IgM	(293)
2008	Serum/ stool	Thai navy base	Detection of anti-IgM, RT-PCR	(294)
2004	Clinical	Orange Juice/ Egyptian restaurant	RT PCR	(295)
2003	Serum	Green onions	Epidemiology, RT-PCR	(54)

Table 1.4 Rotavirus and Hepatitis E virus outbreaks

Virus Type	Year	Sample type	Contamination source/Location	Detection method	Reference
Rotavirus	2012	Clinical	French Reunion island	RT PCR	(296)
	2011	Clinical	Retirement communities	EIA, RT PCR	(297)
	2011	Clinical	Intensive care unit/Brazil	RT PCR	(298)
	2007	Clinical	Pediatric hospital/Mexico	ELISA	(299)
	1997	Stool, Serum	Pediatric unit/Toys	Epidemiology, EIA	(300)
Hepatitis E virus	2011	Serum	Italy	RT PCR, Detection of IgG and IgM by immunoassay	(301)
	2011	Food	Shellfish/England	RT PCR	(302)
	2007	Blood	Uganda	RT PCR	(303)

Table 1.5 Effect of grape seed extract (GSE) and its components against viruses

Type of bioactive	Concentration	Virus	Reduction	Reference
GSE	1 mg/ml	FCV-F9 MNV-1 HAV	4.61 log PFU/ml 1.73 log PFU/ml 3.2 log PFU/ml	(197)
	0.2 mg/ml	MNV-1	3 log PFU/ml	(144)
Resveratrol	40 μg/mL	Influenza virus	Complete inhibition	(201)
	20 uM	Polyomavirus	Complete inhibition	(202)
	10μM	Human immunodeficiency virus – 1	20-30% reduction in replication	(304)

*FCV-F9: feline calicivirus; MNV-1: murine norovirus; HAV: hepatitis A virus

Table 1.6 Antiviral activity of blueberry polyphenols

Type of bioactive	Concentration	Virus	Effect	Reference
Blueberry proanthocyanidin	5.47 µg/ml	Hepatitis C virus	Inhibition of subgenome expression	(156)
Tannins	0.03-0.1 µg/ml	Herpes simplex virus	Inhibition of adsorption to its host	(157)
Blueberry juice	4^0C after 1 day	FCV-F9	Reduction to undetectable levels	(2)

*FCV-F9: feline calicivirus

Table 1.7 Antiviral properties of additional fruit extracts

Type of bioactive	Concentration	Virus	Effect	Reference
Cranberry juice	30 min	FCV-F9	Reduction to undetectable levels	(250)
Cranberry juice	30%	Simian rotavirus SA-11	Reduction to undetectable levels	(101)
CPAC	0.15-0.3mg/ml	FCV-F9	Reduction to undetectable levels	(250)
Black raspberry juice	6%	MNV-1	99% reduction	(253)
Blackberries	56 μg/mL	HSV-1	99% reduction	(255)
Pomegranate polyphenols	800 μg/ml 5 min at room temperature	Human influenza viruses (H1N1, H3N2, and H5N1)	3 log PFU/ml reduction	(239)
Pomegranate juice	Room temperature 1h	FCV-F9 MNV-1 MS2	2.56 log PFU/ml 1.32 log PFU/ml 0.32 log PFU/ml	(241)

*FCV-F9: feline calicivirus; MNV-1: murine norovirus; HAV: hepatitis A virus

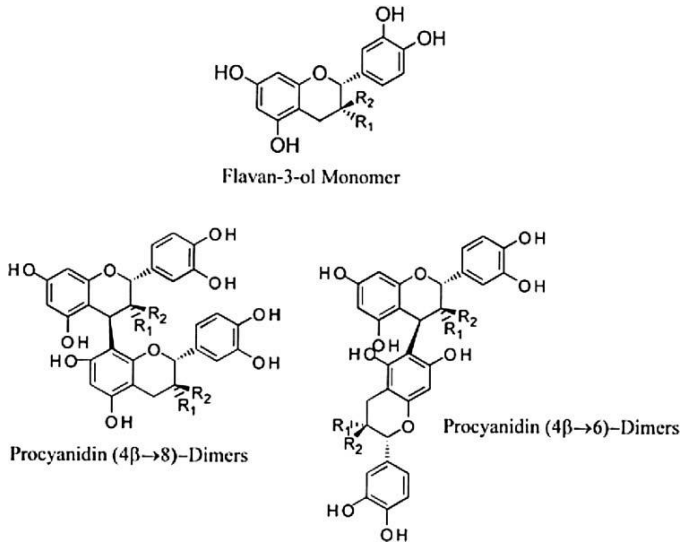

Figure 1.1 Representative structures of flavan-3-ol monomers and their dimers (B-type PACs). When R1= OH and R2= H, the monomer is (-)-epicatechin. When R1= H and R2= OH, then the monomer is (+)-catechin. (Adapted from Hammerstone et al., 2000).

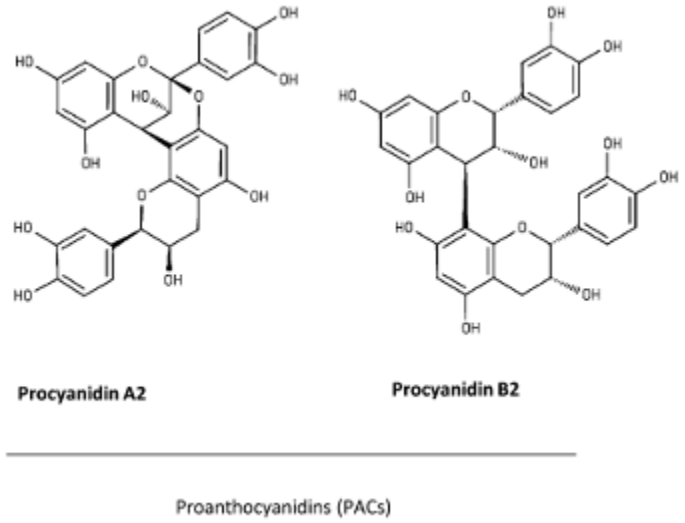

Figure 1.2. A-type and B-type PACs (Adapted from Blumberg et al., 2013)

Chapter 2 Reduction of enteric viruses by blueberry juice and blueberry proanthocyanidins

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Running Head: Enteric virus reduction by blueberry juice and proanthocyanidins

Key Words: enteric viruses, blueberry juice, blueberry proanthocyanidins, reduction

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Parts of this chapter have been submitted as a research article that is under review process.

Abstract

Blueberry and blueberry extracts are known for their health benefits and antimicrobial properties. Natural therapeutic or preventive options to decrease the incidences of foodborne viral illnesses are becoming popular and being researched. This study aimed to determine the antiviral effects of blueberry juice (BJ) and blueberry proanthocyanidins (B-PAC) against the infectivity of hepatitis A virus (HAV), Aichi virus (AiV) and human norovirus surrogates (feline calicivirus (FCV-F9) and murine norovirus (MNV-1)) at 37ºC over 24-h using standard plaque assays. Viruses at ~5 log PFU/ml were mixed with equal volumes of BJ (pH 2.8), neutralized BJ (pH 7.0), B-PAC (1, 2, 4, and 10 mg/ml), malic acid (pH 3.0), or phosphate buffered saline (pH 7.2) and incubated over 24-h at 37^{o}C. Each experiment was carried out in duplicate and replicated thrice. FCV-F9 titers were found to be reduced to undetectable levels with 1 and 2 mg/ml B-PAC after 5 min, after 1 h with 0.5 mg/ml B-PAC, and with BJ after 3-h. MNV-1 titers were reduced to undetectable levels after 3-h with 1, 2, and 5 mg/ml B-PAC and after 6-h with BJ. HAV titers were reduced to undetectable levels after 30 min with 2 and 5 mg/ml B-PAC, after 3-h with 1 mg/ml B-PAC, and by ~2 log PFU/ml with BJ after 24-h. B-PAC at 5 mg/ml reduced AiV titers to undetectable levels after 3 h, while BJ caused reductions to undetectable levels after 24 h. B-PAC shows potential to control enteric viruses in a dose and time-dependent manner, although further *in vitro* studies in model food systems and *in vivo* studies using animal models are warranted.

Introduction

Foodborne pathogens in the United States lead to an estimated 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths of which foodborne viruses such as human norovirus alone are responsible for 5.5 million illnesses (58%) per year (1). Recent reports indicate that this number has risen to 21 million illnesses per year (2). Human norovirus and hepatitis A virus have been linked to several foodborne and waterborne outbreaks in the US as well as worldwide (3). Aichi virus is an emerging pathogen is transmitted via the fecal-oral route and shed in the feces and hence seafood like shellfish from sewage-contaminated water can be a source of contamination (4, 5). AiV-related gastrointestinal cases have been reported all over the world including in Asian, African, European and South American countries (6-11). These foodborne viruses are now recognized worldwide as public health concerns and therefore effective natural alternatives to control their spread are being researched. This is important for the treatment or potential alleviation of illness symptoms and prevention of disease in the current absence of available vaccines for human noroviruses. Recently, the antiviral effects of grape seed extract (GSE) against human norovirus surrogates, FCV-F9 and MNV-1, and hepatitis A virus (HAV) at 37ºC for 2 h were reported (12). Cranberry juice and its proanthocyanidins were also reported to cause reduction of human norovirus surrogates (13).

Blueberries also contain structurally related polyphenols, including anthocyanins, flavonoids, and proanthocyanidins (PAC), sometimes referred to as condensed tannins (14). Blueberries and its polyphenols have been evaluated for their potential health benefits including their anticarcinogenic, neuroprotective, cardioprotective, antibacterial, and antiviral properties (15-19). Blueberry extracts at a concentration of 0.25% are known to have antimicrobial activity

and are reported to cause 50.5% inhibition of *Helicobacter pylori*, the bacterium associated with ulcers (20). Water and ethanol extracts of blueberries at 24 ppm were reported to cause a 5.90 log CFU/ml reduction of *Listeria monocytogenes* after 24 h at 37ºC *in vitro* (21). Recently, monomeric phenolics acids (0.4g/L gallic acid) from blueberries were shown to reduce *Escherichia coli* O157:H7 by 5 log CFU/ml along with cell-membrane damage after 24 h at37ºC *in vitro* (22). Additionally, using the HCV replicon cell system, the methanol extract fraction of blueberry leaves (0.112–2200 μg/ml) was shown to inhibit hepatitis C (HCV) virus subgenomic expression after 72h at 37ºC (23). Hydrolysable and galloylated tannin (0.03–0.1 μg/ml) was reported to inhibit the adsorption of herpes simplex virus to its hosts, African green monkey kidney cells and human adenocarcinoma cells at 37ºC (24). Human norovirus surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been studied for survival in commercial blueberry juice (BJ) at 4ºC, where FCV-F9 was undetectable after 24 h from initial 5 log PFU/ml, whereas MNV-1 showed minimal reduction after 21 days at 4ºC (25).

Blueberries and blueberry phenols could potentially be used as part of a diet to prevent or treat human norovirus related gastroenteritis. Therefore, the objective of this study was to determine the effect of blueberry juice (BJ) and blueberry proanthocyanidins (B-PAC) on the infectivity of hepatitis A virus (HAV), Aichi virus (AiV) and human norovirus surrogates (FCV-F9 and MNV-1) at 37ºC over a period of up to 24 h using standardized plaque assays.

Materials and Methods

Viruses and cell lines

Feline calicivirus (FCV-F9) and host Crandell Reese Feline Kidney (CRFK) cells were purchased from ATCC (ATCC identifying number VR-2057; Manassas, VA). Murine norovirus

(MNV-1) was kindly gifted by Dr. Skip Virgin (Washington Univ., St. Louis, MO) and RAW 264.7 cells were obtained from the University of Tennessee at Knoxville.Hepatitis A virus (HAV; strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were provided by our collaborator, Dr. Kalmia Kniel (University of Delaware). Aichi virus (AiV) was kindly provided by Dr. David Kingsley (USDA ARS, Delaware) and propagated using Vero host cells. Host cell lines were maintained using Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) containing 2 or 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1x Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) at 37ºC in an atmosphere containing 5% CO_2.

Propagation of viruses

To prepare stocks of FCV-F9, MNV-1, AiV and HAV, respective host cell lines at 90% confluency were inoculated with each virus and incubated for 2, 6, 3 and 8 days, respectively under 5% CO_2 at 37ºC. The viruses were harvested by 1 to 3 freeze thaw cycles followed by centrifugation at 5,000 x*g* for 10 min. The supernatant was filtered through a 0.2-μm filter, aliquoted, stored at -80ºC until further use and titers of the recovered viral stocks were determined using standard reported plaque assays (13, 26).

Propagation of viruses

To prepare stocks of FCV-F9, MNV-1, AiV and HAV respective host cell lines at 90% confluency were inoculated with each virus and incubated for 2, 6, 3 and 8 days, respectively under 5% CO_2 at 37ºC. The viruses were harvested by 1 to 3 freeze thaw cycles followed by centrifugation at 5,000 x*g* for 10 min. The supernatant was filtered through a 0.2-μm filter,

aliquoted, stored at -80ºC until further use and titers of the recovered viral stocks were determined using standard reported plaque assays (13, 26).

Isolation of PAC from blueberries

Proanthocyanidins were isolated from frozen highbush blueberry fruit (*Vaccinium corymbosum* L.) using solid-phase chromatography according to a well-established method for proanthocyanidin isolation (27). Briefly, blueberry fruit was homogenized with 70% aqueous acetone, filtered and the pulp discarded. The collected extract was concentrated under reduced pressure to remove acetone. The blueberry extract was suspended in water, applied to a preconditioned C-18 solid phase chromatography column and washed with water to remove sugars, followed by acidified aqueous methanol to remove acids. The fats and waxes retained on the C-18 sorbent were discarded. The polyphenolic fraction containing anthocyanins, flavonol glycosides and proanthocyanidins (confirmed using reverse phase HPLC with diode array detection) was eluted with 100% methanol and dried under reduced pressure. This fraction was suspended in 50% EtOH, applied to a pre-conditioned Sephadex LH-20 column which was washed with 50% EtOH to remove low molecular weight anthocyanins and flavonol glycosides. Proanthocyanidins adsorbed to the LH-20 were eluted from the column with 70% aqueous acetone, and monitored using diode array detection at 280 nm. The absence of absorption at 360 nm and 450 nm confirmed that anthocyanins and flavonol glycosides were removed. Acetone was removed under reduced pressure and the resulting purified proanthocyanidin extract freeze-dried. Electrospray mass spectrometry, ^{13}C NMR, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and acid catalyzed degradation with phloroglucinol have all

been utilized to confirm the purity of proanthocyanidins present in the extract obtained using this method (27).

Effect of BJ and B-PAC on host cells lines

Cytopathic effects of B-PAC, BJ (pH 2.8) and NBJ (neutralized blueberry juice; pH 7.0) treatments on the host cell lines were also determined. The maximum concentration of B-PAC used in the viral treatment and assays was 5 mg/ml. However, the host cell lines were exposed to only a 20-fold dilution of the treatment after neutralization/stopping of the treatment. Thus, each individual host cell lines on confluent 6-well plates were exposed to B-PAC at 0.5 mg/ml and BJ and NBJ for 2 h at 37^{o}C and observed under optical microscope (Fisher Scientific; 120 VAC) to determine visible cytopathic effects or changes.

Antiviral effect of BJ and B-PAC

Commercial blueberry juice (cocktail with grape and apple juice; preservatives listed include malic acid and sodium citrate) was purchased from local grocery stores and B-PAC was fractionated by Dr. Amy Howell (Marucci Center for Blueberry and Cranberry Research, Rutgers University, Chatsworth, NJ) and kindly gifted for this work. B-PAC was dissolved in 10% ethanol and filter-sterilized through 0.2-micron filters to obtain a stock of 10 mg/ml. Equal volumes of each virus at a titer of ~5 log PFU/ml was individually mixed with BJ (pH 2.8), B-PAC (1, 2, 4, and 10 mg/ml), malic acid (pH 3.0), neutralized BJ (pH 7 using 4 M sodium hydroxide, NaOH), 10% ethanol or phosphate buffered saline (PBS; 7.2 as control) and incubated at 37ºC for 5 (0.08 h), 15 (0.25h), 30 (0.5h), 60 (1h), 120 (2 h), 180 (3 h), 360 min (6 h) or 24h. Treatments were stopped using initial serial dilutions of the treated virus with cell-

culture media containing 10% heat-inactivated FBS, followed by dilutions in cell-culture media containing 2% FBS. The infectivity of viruses was evaluated using standard plaque assays as described before (13). Each experiment was replicated thrice and assayed in duplicate.

Statistical analysis

Statistical analysis was carried out using ANOVA with SAS software (version 9.3, SAS Institute, Cary, NC, USA) and Tukey's test with data obtained from the three replications for each individual virus as described in previous studies (12, 28).

Results

Effect of B-PAC and BJ on host cells lines

B-PAC at a concentration of 0.5 mg/ml was not found to be cytotoxic to the tested host cell lines. Undiluted BJ when added directly to the host cells had some minor effects after 2h. However, neutralized BJ (NBJ) did not show any morphological visual changes in the host cells and was used further in the time of addition experiments. In the experiment involving treatment of viruses with B-PAC, the cells are exposed to a maximum of 20-fold diluted B-PAC and hence the host cells do not show cytopathic effects.

Reduction of viral titers by B-PAC and BJ

All four tested viruses were found to be susceptible to the B-PAC treatment carried out at 37^{o}C. FCV-F9 from initial titers of ~5 log PFU/ml were reduced to undetectable levels (to $<10^{2}$PFU/ml per detection limit of the assay) after 5 min with 1, 2, and 5 mg/ml B-PAC, and to undetectable levels (> 3 log PFU/ml reduction) after 60 min with 0.5 mg/ml B-PAC. After 5 min, lower

concentration of 0.5 mg/ml B-PAC reduced FCV-F9 titers by only 1.33±0.03 log PFU/ml and after 30 min by 2.19±0.22 log PFU/ml (Table 2.1). MNV-1 was less sensitive to B-PAC treatments compared to FCV-F9, where MNV-1 at initial titers of~5 log PFU/ml were reduced to undetectable levels only after 3 h with 1, 2, and 5 mg/ml of B-PAC. Lower concentration of 0.5 mg/ml B-PAC caused MNV-1 reductions of 1.47±0.05 log PFU/ml after 3 h and caused slightly higher reductions of 1.83±0.10 log PFU/ml after 6 h. (Table 2.2). HAV titers were reduced to undetectable levels after 30 min with 2 and 5 mg/ml B-PAC, whereas 1 mg/ml B-PAC reduced HAV titers by 1.71±0.21 log PFU/ml after 30 min and to undetectable levels only after 3 h (Table 2.3). Lower concentration of 0.5 mg/ml B-PAC caused mere reductions of 0.55±0.04, 0.79±0.06 and 0.69±0.04 log PFU/ml in HAV titers after 1, 3 and 6 h at 37^{o}C. B-PAC (1 mg/ml) at 37^{o}C was shown to be effective in reducing AiV titers by 0.48±0.01, 1.50±0.02, 1.84±0.25, 1.45±0.35 and 2.13±0.06 PFU/ml after 0.5, 1, 3, 6 and 24 h, respectively. Increased concentration caused higher titer reduction where 2 mg/ml B-PAC caused reductions of 0.93±0.10, 1.63±0.22, and 1.82±0.07 log PFU/ml after 0.5, 1, and 3 h respectively and to undetectable levels after 6 and 24 h. The highest tested concentration of B-PAC at 5 mg/ml was found to be the most effective that caused reductions of 1.39 ± 0.04 and 1.76 ± 0.54 log PFU/ml after 0.5 and 1 h, respectively, and to undetectable levels after 3 h (Table 2.4).

Commercial BJ did not have a prominent effect on the viral titers after shorter contact times. Among the three tested viruses, FCV-F9 was the most sensitive, where BJ reduced FCV-F9 titers to undetectable levels after 3 h (Table 2.5). However, BJ did not have a significant effect on MNV-1 and HAV after 3 h. MNV-1 titers were reduced to undetectable levels only after 24 h with BJ while HAV was reduced by 1.86±0.05 log PFU/ml after 24 h with BJ (Table 2.6 and

2.7). Both, malic acid (pH 3.0, control) and neutralized BJ (pH 7.0) did not have any significant effect in reducing the titers of all FCV-F9, MNV-1 and HAV, compared to non-neutralized BJ (pH 2.8) that showed ~2-3 log PFU/ml reduction after 24 h. Thus, the reduction cannot be attributed solely to pH effects, indicating that BJ bioactives contributed to the reduction. Treatment of AiV at ~5 log PFU/ml with BJ at 37^{o}C resulted in titer reductions of 0.17±0.06, 1.27±0.01 and 1.73±0.23, log PFU/ml after 1, 3 and 6 h, and to undetectable levels after 24 h. Neutralized BJ (pH 7.0) showed 0.07±0.11, 0.0±0.0, 0.06±0.07 and 0.23±0.11 log PFU/ml after 1, 3, 6 and 24 h. Malic acid (pH 3.0, acid control) was shown to have minimal effects on virus infectivity over time though to a much lesser extent than BJ within 6 h, causing titer reductions of 0.08±0.0, 0.25±0.06 and 0.84±0.23 log PFU/ml after 1, 3 and 6 h and to undetectable levels after 24 h, indicating that low pH also contributes to the antiviral effect of BJ on AiV over extended time (Table 2.8).

Discussion

B-PAC and BJ were found to be effective in reducing the titers of all the three tested viruses. B-PAC showed dose and time-dependence effects against the four tested viruses where higher concentration and longer time had the greatest antiviral effects especially for MNV-1 and HAV. Between the two tested human norovirus surrogates, FCV-F9 was found to be the more sensitive surrogate to treatments than MNV-1. This is not surprising as similar patterns are reported when comparing FCV-F9 and MNV-1 using chemical treatments and plant-derived extracts such as trisodium phosphate (TSP) and GSE (12, 29). Previous studies have also shown that MNV-1 is the sturdier of the surrogates when exposed to extreme heat and pH (30). HAV was found to be moderately sensitive to B-PAC treatment where complete reduction was achieved with 2 and

5mg/ml B-PAC after 30 min. HAV is known to be highly stable in the environment and can retain infectivity for long periods of time, and can survive in mineral water stored at room temperature up to 300 days (31). It is also resistant to extreme acidic pH, where it was shown to survive in acidic marinade at pH 3.75 over 4 weeks and even at pH 1.0 even after 5 h (32, 33). Thus, the antiviral effect of B-PAC against HAV is quite noteworthy.

Bioactive compounds such as gallic acid, epicatechin, tannins, and proanthocyanidins found in berry fruits are known to be effective antimicrobial agents (34-36). Polyphenols from fruits such as pomegranate, cranberry, grapes (seed), and other plant extracts have been shown to possess antiviral properties including against human norovirus surrogates (13, 37, 38). FCV-F9 was found to be undetectable after 1 day in BJ as well as in orange and pomegranate juice blend at 4°C (25, 39). MNV-1 was reported to be completely reduced after 7 days in the orange and pomegranate juice blend, however only 1 log PFU/ml reduction of MNV-1 was observed in BJ at 4ºC after 14 days (25, 39). Black raspberry juice at 6% was found to reduce MNV-1 plaque formation by 99% after 1 h at 37ºC (38). However in the current study, BJ treatment needed longer contact time to achieve similar reduction, which could be attributed to the fact that black raspberry juice was freshly made by hand, and might have higher concentration of polyphenols, whereas commercially available BJ was used in our study. Grape seed extract (GSE), cranberry proanthocyanidins (CPAC) and pomegranate polyphenols (PP) have been reported for their antiviral properties against FCV-F9 and MNV-1 (12, 13, 37).FCV-F9 and MNV-1 were reduced to undetectable levels and by 2.95 log PFU/ml, respectively with 0.6 mg/ml CPAC after 1 h at RT (40).GSE at 0.5 and 1 mg/ml reducedFCV-F9 titers to undetectable levels within 15 min at 37^{o}C and RT, and MNV-1 titers were reduced by ~1 log

PFU/ml after 1 h at 37^{o}C and RT (12). FCV-F9 and MNV-1 were also sensitive to PP treatments at RT, where PP at 2 mg/ml decreased FCV-F9 titers to undetectable levels after 30 min and MNV-1 titers were reduced by 0.9 log PFU/ml after 60 min (37). In this study, B-PAC at 2 mg/ml reduced FCV-F9 titer to undetectable levels within 5 min and MNV-1 by 1.65 log PFU/ml after 60 min at 37^{o}C.When comparing these polyphenol extracts for their antiviral activity against FCV-F9 and MNV-1, their effect follows the order of CPAC>GSE>B-PAC>PP.

In comparison to human norovirus surrogates, fewer studies are reported in literature on the antiviral effects of natural extracts against HAV. In a previous study, GSE at 1mg/ml was shown to reduce HAV titers from initial 5 log PFU/ml by 2.89 log PFU/ml after 2h at 37^{o}C (12). HAV titers at initial 5.74 log PFU/ml were also shown to be decreased by 0.66 log PFU/ml when treated with 10 μg/mL Korean red ginseng, and decreased by 0.37 log PFU/ml when treated with 10 μg/mL purified ginsenoside extract after 24 h at 37^{o}C (41). B-PAC treatment, although at a higher concentration, was found to be effective in reducing HAV titers where 2mg/ml B-PAC caused reduction to undetectable levels within 30 min at 37^{o}C.

A few other studies have reported AiV to be resistant to inactivation treatments. Essential oil extracts from *Origanu macutidens* were shown to be ineffective in inhibiting AiV replication in vivo (42). AiV remained fully infectious after a 5-min treatment at 600 MPa in MEM (minimum essential medium) supplemented with 2 and 10% FBS, respectively (43). AiV reductions on lettuce, green onions, and strawberries at 240 mW s/cm2 were 4.59, 2.49, and 1.87 log TCID50 /ml, respectively (26). This suggests that AiV is quite resilient to physical inactivation treatments. In this study B-PAC treatments were more effective in comparison to most of the reported disinfectant treatments. AiV reductions of 1.39 and 1.76 log PFU/ml were

seen after 0.5 and 1 h with 5 mg/ml B-PAC and reduction to undetectable levels was obtained after 3 h with 5mg/ml B-PAC.

Neutralized BJ did not have any significant effect in reducing the viral titers. As PACs are known to be stable under acidic conditions, increasing the pH can change the structure of PAC, thereby potentially changing its bioactivity. Moreover, the malic acid (pH 3.0) pH control by itself alone also did not significantly contribute to the titer reduction. It could be speculated that both the bioactive components of BJ and the acidic pH together contributed towards the antiviral activity. Bioactive components of BJ were not individually analysed for antiviral activity in this study.

It is well recognized that effectiveness of antimicrobials can decrease in the presence complex food matrices where components of foods such as lipids, proteins or other acidic or alkaline conditions may interfere with the antiviral properties. Hence, along with studying the effect of these blueberry polyphenols on the viral infectivity *in vitro*, it is also crucial to further investigate their effectiveness in presence of food matrices and under simulated gastric conditions for use as potential antiviral therapeutics. Some studies have been conducted with plant extracts (hibiscus) in milk and apple juice against *E. coli* and *Staphylococcus aureus* where antimicrobial activity was shown to reduce in foods with higher lipid and protein load (44). A similar study was carried out using black tea and tea in milk against oral pathogen *Streptococcus mutans*, where reduced antimicrobial activity of tea made in milk was reported (45). This decrease in activity was attributed to complex formation of milk proteins and tea polyphenols and subsequent decrease in the bioavailability of polyphenols (45). Therefore, future studies are

aimed to determine the antiviral effects of B-PAC in food systems such as milk (high in lipids) and apple juice, and also simulated gastric conditions.

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Appendix B

Table 2.1 Effect of 0.5 to 5 mg/ml blueberry proanthocyanidins (B-PAC) on FCV-F9 at ~ 5 log PFU/ml at 37°C over 2 h.

Time (Min)	Recovered titer (Log PFU/ml)					
	PBS	Ethanol (10%)	B-PAC (0.5 mg/ml)	B-PAC (1 mg/ml)	B-PAC (2 mg/ml)	B-PAC (5 mg/ml)
5	4.87 ± 0.07^{A}	4.76 ± 0.05^{A}	3.54 ± 0.10^{B}	0^{D}	0^{D}	0^{D}
30	4.83 ± 0.05^{A}	4.77 ± 0.06^{A}	2.64 ± 0.27^{C}	0^{D}	0^{D}	0^{D}
60	5.03 ± 0.06^{A}	4.73 ± 0.06^{A}	0^{D}	0^{D}	0^{D}	0^{D}
120	5.03 ± 0.06^{A}	4.99 ± 0.06^{A}	0^{D}	0^{D}	0^{D}	0^{D}

$^{A, B, C, D}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 2.2 Effect of 0.5 to 5 mg/ml blueberry proanthocyanidins (B-PAC) on MNV-1 at ~ 5 log PFU/ml at 37°C over 6 h.

Time (Min)	Recovered titer (Log PFU/ml)					
	PBS	Ethanol (10%)	B-PAC (0.5 mg/ml)	B-PAC (1 mg/ml)	B-PAC (2 mg/ml)	B-PAC (5 mg/ml)
30	4.89 ± 0.08^{A}	4.56 ± 0.08^{A}	3.80 ± 0.06^{AB}	3.71 ± 0.05^{BC}	3.43 ± 0.09^{BC}	3.28 ± 0.22^{BC}
60	4.84 ± 0.10^{A}	4.82 ± 0.08^{A}	3.67 ± 0.13^{B}	3.49 ± 0.08^{BC}	3.19 ± 0.19^{C}	2.78 ± 0.28^{C}
120	4.76 ± 0.01^{A}	4.56 ± 0.08^{A}	3.70 ± 0.13^{B}	3.46 ± 0.06^{BC}	3.17 ± 0.12^{C}	2.76 ± 0.09^{C}
180	4.75 ± 0.07^{A}	4.75 ± 0.07^{A}	3.28 ± 0.12^{B}	0^{D}	0^{D}	0^{D}
360	4.63 ± 0.17^{A}	4.61 ± 0.07^{A}	2.80 ± 0.07^{C}	0^{D}	0^{D}	0^{D}

$^{A, B, C, D}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 2.3 Effect of 0.5 to 5 mg/ml blueberry proanthocyanidins (B-PAC) on HAV at ~ 5 log PFU/ml at 37°C over 3 h.

			Recovered titer (Log PFU/ml)			
Time (Min)	PBS	Ethanol (10%)	B-PAC (0.5 mg/ml)	B-PAC (1 mg/ml)	B-PAC (2 mg/ml)	B-PAC (5 mg/ml)
15	4.81 ± 0.05^{A}	4.80 ± 0.04^{A}	NP**	3.27 ± 0.08^{B}	2.79 ± 0.16^{C}	2.35 ± 0.12^{C}
30	4.78 ± 0.03^{A}	4.75 ± 0.03^{A}	NP**	3.07 ± 0.24^{C}	0^{D}	0^{D}
60	4.83 ± 0.07^{A}	4.71 ± 0.13^{A}	4.28 ± 0.11^{A}	2.58 ± 0.14^{C}	0^{D}	0^{D}
180	4.85 ± 0.12^{A}	4.77 ± 0.16^{A}	4.06 ± 0.18^{AB}	0^{D}	0^{D}	0^{D}
360	4.81 ± 0.13^{A}	4.61 ± 0.16^{A}	4.12 ± 0.09^{AB}	0^{D}	0^{D}	0^{D}

$^{A, B, C}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.
**NP= not performed

Table 2.4 Reduction of Aichi Virus (AiV) by blueberry proanthocyanidins (B-PAC) at 37°C over 24 h.

	Recovered titer (Log PFU/ml)				
Time (Hour)	PBS[a]	Ethanol (10%)	B-PAC (1 mg/ml)	B-PAC (2 mg/ml)	B-PAC (5 mg/ml)
0.5	4.75 ± 0.07^{A}	4.57 ± 0.14^{A}	4.27 ± 0.08^{AB}	3.82 ± 0.17^{B}	3.36 ± 0.11^{B}
1	4.62 ± 0.04^{A}	4.53 ± 0.06^{A}	3.12 ± 0.10^{C}	2.99 ± 0.26^{C}	2.86 ± 0.58^{C}
3	4.53 ± 0.05^{A}	4.58 ± 0.08^{A}	2.69 ± 0.29^{C}	2.71 ± 0.12^{C}	0^{D}
6	4.45 ± 0.11^{A}	4.59 ± 0.07^{A}	3.00 ± 0.36^{C}	0^{D}	0^{D}
24	4.43 ± 0.06^{A}	4.52 ± 0.07^{A}	2.30 ± 0.00^{C}	0^{D}	0^{D}

$^{A, B, C}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml

Table 2.5 Effect of blueberry juice (BJ) on FCV-F9 at ~ 5 log PFU/ml at 37°C over 6h.

Time (Min)	Recovered titer (Log PFU/ml)			
	PBS	Malic acid (pH 3.0)	Blueberry Juice (pH 2.8)	Neutralized Blueberry Juice (pH 7.0)
30	4.83 ± 0.05^{A}	4.85 ± 0.04^{A}	4.39 ± 0.08^{B}	4.87 ± 0.03^{A}
60	5.17 ± 0.10^{A}	4.85 ± 0.04^{A}	3.58 ± 0.09^{C}	4.84 ± 0.08^{A}
120	5.17 ± 0.10^{A}	4.80 ± 0.03^{A}	2.76 ± 0.30^{D}	4.74 ± 0.02^{A}
180	4.85 ± 0.05^{A}	4.39 ± 0.12^{B}	0^{E}	4.77 ± 0.04^{AB}
360	4.99 ± 0.07^{A}	4.39 ± 0.12^{B}	0^{E}	4.76 ± 0.03^{AB}

$^{A, B, C, D, E}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 2.6 Effect of blueberry juice (BJ) on MNV-1 at ~ 5 log PFU/ml at 37°C over 6 h.

Time (Hour)	Recovered titer (Log PFU/ml)			
	PBS	Malic acid (pH 3.0)	Blueberry Juice (pH 2.8)	Neutralized Blueberry Juice (pH 7.0)
1	4.57 ± 0.07^{A}	4.79 ± 0.04^{A}	4.33 ± 0.05^{A}	4.33 ± 0.05^{A}
3	4.75 ± 0.07^{A}	4.78 ± 0.02^{A}	4.26 ± 0.12^{B}	4.26 ± 0.12^{A}
6	4.50 ± 0.07^{A}	4.45 ± 0.05^{A}	4.02 ± 0.13^{AB}	4.02 ± 0.13^{AB}
24	4.04 ± 0.19^{C}	4.12 ± 0.09^{AB}	0^{D}	3.88 ± 0.18^{C}

$^{A, B, C}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 2.7 Effect of blueberry juice (BJ) on HAV at ~ 5 log PFU/ml at 37°C over 24 h.

Time (Hour)	Recovered titer (Log PFU/ml)			
	PBS	Malic acid (pH 3.0)	Blueberry Juice (pH 2.8)	Neutralized Blueberry Juice (pH 7.0)
1	4.78 ± 0.04^{A}	4.76 ± 0.05^{A}	3.63 ± 0.07^{A}	4.73 ± 0.05^{A}
2	4.77 ± 0.02^{A}	4.77 ± 0.07^{A}	3.85 ± 0.05^{B}	4.72 ± 0.02^{A}
3	4.90 ± 0.05^{A}	4.76 ± 0.05^{A}	3.63 ± 0.32^{BC}	4.79 ± 0.03^{A}
6	4.80 ± 0.05^{A}	4.47 ± 0.12^{A}	3.45 ± 0.05^{C}	4.52 ± 0.22^{A}
24	4.63 ± 0.07^{A}	4.32 ± 0.04^{A}	2.77 ± 0.12^{D}	4.52 ± 0.22^{A}

$^{A, B, C, D}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 2.8 Effect of blueberry juice (BJ) on Aichi virus (AiV) at ~ 5 log PFU/ml at 37°C over 24 h.

Time (Hours)	Recovered titer (Log PFU/ml)			
	PBS[a]	Malic acid (pH 3.0)	Blueberry Juice (pH 2.8)	Blueberry Juice (pH 7.0)
1	4.62 ± 0.04^{A}	4.54 ± 0.04^{A}	4.45 ± 0.10^{A}	4.55 ± 0.15^{A}
3	4.53 ± 0.05^{A}	4.28 ± 0.11^{A}	3.26 ± 0.04^{B}	4.53 ± 0.05^{A}
6	4.45 ± 0.11^{A}	3.61 ± 0.25^{B}	2.72 ± 0.34^{C}	4.39 ± 0.18^{A}
24	4.43 ± 0.06^{A}	0^{D}	0^{D}	4.20 ± 0.17^{A}

$^{A, B, C, D}$ Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml

Chapter 3 Blueberry proanthocyanidins against human enteric viruses in model foods and simulated gastric conditions

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Key words: feline calicivirus, murine norovirus, hepatitis A virus, Aichi virus , blueberry polyphenols, model foods, simulated gastric conditions

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Abstract

Blueberry proanthocyanidins (B-PAC) are known to decrease foodborne virus titers. The application of B-PAC as therapeutic or preventive options against foodborne viral illness needs to be determined using model foods and simulated gastric conditions in vitro. The objective of this study was to evaluate the antiviral effect of B-PAC in model foods (apple juice; AJ and 2% reduced fat milk) and simulated gastrointestinal fluids against human norovirus surrogates (feline calicivirus; FCV-F9 and murine norovirus; MNV-1), hepatitis A virus (HAV) and Aichi virus (AiV) over 24 h at 37^{o}C. Equal amounts of each virus (5 log PFU/ml) were individually mixed with either B-PAC (1, 2 and 5 mg/ml) made in AJ or 2% milk or simulated intestinal fluid controls or controls including phosphate buffered saline or malic acid (pH 7.2) or apple juice or 2% milk over 24h at 37^{o}C. All tested viruses were reduced to undetectable levels within 15 min with B-PAC (1, 2 and 5 mg/ml) in AJ (pH 3.6). B-PAC activity was reduced in milk, where significant reduction in titers were obtained only after 24 h. FCV-F9 was reduced by 0.4 and 1.09 log PFU/ml with 2 and 5 mg/ml, respectively and MNV-1 titers were reduced by 0.81 log PFU/ml with 5 mg/ml after 24 h in milk. For HAV, reductions of 0.54 and 0.83 log PFU/ml with 2 and 5 mg/ml, respectively after 24 h in milk were obtained. With AiV, reductions of 0.52 and 0.84 log PFU/ml were obtained with 2 and 5 mg/ml after 24h. B-PAC at 5 mg/ml in simulated intestinal fluid reduced titers of all four tested viruses to undetectable levels within 30 min. Overall, these results show potential for B-PAC as preventive and therapeutic options for foodborne viral illnesses.

Introduction

Foodborne enteric viruses are commonly associated with non-bacterial acute gastroenteritis in humans (1). Epidemiologically significant foodborne diseases include human noroviruses, hepatitis A virus, rotaviruses, Aichi virus, hepatitis E virus, adenoviruses, sapovirus, astrovirus, parvoviruses and other enteroviruses (2). However, some of these viruses such as human norovirus and Aichi virus do not have vaccines available for their control. Natural plant extracts have been used for their valuable medicinal properties since centuries (3). Extracts from plant and fruit sources are known to contain abundant secondary metabolites mainly phenolic compounds including but not limited to anthocyanidins, proanthocyanidins, catechins, epicatechin gallate and tannins (3). Plant extracts are being studied extensively as sustainable sources of potential therapeutic options, due to their low cost and consumer appeal (4). Among the array of potential health benefits, these extracts are being screened for their antiviral potential as well (3, 4).

In absence of specific treatment strategies or control measures for many of these viruses, natural extracts provide novel alternatives to control of their spread and prevent their transmission. Blueberries have gained increasing popularity for consumption due to their associated broad spectrum of health benefits in the prevention of cardiovascular disorders and age-induced oxidative stress, combined with antioxidative, anti-inflammatory, anti-carcinogenic, neuro-protective, cardio-protective, antibacterial, and antiviral properties (5). Blueberries contain polyphenols including anthocyanins, flavonoids and proanthocyanidins (PAC) (6). Our lab has recently reported the antiviral effects of blueberry juice and blueberry juice proanthocyanidins (B-PAC) against human norovirus surrogates and hepatitis A virus at 37^{o}C in vitro. However, for

B-PAC to be used as a therapeutic antiviral agent, its effectiveness in complex food matrices and gastrointestinal fluids that mimic digestion needs to be evaluated. It is well known that the effectiveness of antimicrobials decrease in the presence complex food matrices. Food components such as carbohydrates, lipids, proteins, salts, or other acidic or alkaline conditions may affect the antimicrobial properties of a given compound (7-14). Apple juice and milk have been used extensively as model food systems in various studies that evaluated efficiency of antimicrobials in food environments (7, 14, 15). With this information, the objective of this study was to assess the antiviral effects of B-PAC in model food systems (apple juice and 2% reduced fat milk) and in simulated gastrointestinal fluids against human noroviruses (using cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1)), hepatitis A virus (HAV) and Aichi virus (AiV) over 24h at 37^{o}C.

Materials and methods

Viruses and host cell lines

Feline calicivirus (FCV-F9) and host Crandell Reese Feline Kidney (CRFK) cells were purchased from ATCC (Manassas, VA). Murine norovirus (MNV-1) was kindly gifted by Dr. Skip Virgin (Washington Univ., St. Louis, MO) and RAW 264.7 cells were obtained from the University of Tennessee at Knoxville. Hepatitis A virus (HAV; strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were provided by our collaborator, Dr. Kalmia Kniel (University of Delaware). Aichi virus (AiV) was kindly provided by Dr. David Kingsley (USDA ARS, Delaware) and propagated using host Vero cells. Host cell lines were maintained using Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan,

UT) containing 2 or 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1x Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) at 37ºC in an atmosphere containing 5% CO_2 as described earlier (16).

Assessment of antiviral activity in model food systems

Each virus was individually mixed with equal amounts of treatments that included B-PAC (2, 4 and 10 mg/ml dissolved in either apple juice (pH 3.6) or 2% milk to obtain a final titer of ~5 log PFU/ml. Controls included each individual virus mixed in apple juice (AJ; pH 3.6) or 2% milk or malic acid (pH 3.0) to obtain a final titer of ~5 log PFU/ml. Treatments were incubated at 37ºC over 5, 15, 30, 60, 120, 180, 360 min or 24h and stopped/neutralized by adding to cell-culture media containing 10% heat-inactivated fetal bovine serum (FBS), followed by serial dilutions in cell-culture media containing 2% FBS. The infectivity of the viruses was evaluated using standard plaque assays as described before (17). Each experiment was replicated thrice and assayed in duplicate.

Antiviral activity under simulated gastrointestinal conditions

Equal amounts of viruses at (~6 log PFU/ml) were mixed in a ratio of 1:10 of with phosphate buffered saline (PBS; 7.2 as control) or malic acid control (pH 1.5) or simulated intestinal fluid (SIF; pH 7.5, obtained from Fisher Scientific, USA, Waltham, MA, USA), simulated gastric fluid (SGF; pH 1.5, obtained from Fisher Scientific, USA, Waltham, MA, USA), or B-PAC (5 mg/ml) made in SIF or SGF and incubated at 37ºC for 1, 3, 6 and 24 h. Sodium carbonate (Na_2CO_3; 200mM) was used to neutralize the reaction mixture as described earlier and followed by serial dilution in cell-culture media containing 2% FBS (18). Virus infectivity was evaluated using standard plaque assays that were carried out in duplicates and replicated thrice.

Statistical analysis

Data obtained from the three replications for each individual virus were statistically analysed using ANOVA with SAS software (version 9.3, SAS Institute, Cary, NC, USA) and Tukey's test using a completely randomized design model as described in previous studies to determine statistically significant differences between the viral titers recovered from treatments and controls.

Results

Antiviral activity in model food systems

Antiviral activity of B-PAC was shown to be the highest when prepared in apple juice (pH 3.6). FCV-F9 titers were reduced to undetectable levels (< 2 log PFU/ml; limit of detection of the assay) within 15 min at 37^{o}C with 1, 2 and 5 mg/ml of B-PAC (Table 1a). However, the antiviral activity was impeded to a large extent when B-PAC was made in 2% reduced fat milk. Titer reductions of 0.40±0.03 and 1.12±0.01 log PFU/ml were obtained after 24h with 1, 2 and 5mg/ml respectively. After 6h of treatment, reductions of 0.33±0.05, 0.44±0.16 log PFU/ml were obtained with 2 and 5mg/ml respectively (Table 3.1). The milk control caused a reduction of 0.96±0.23 log PFU/ml after 24 h (Table 3.2).

MNV-1 showed a similar trend, where reduction to undetectable levels was obtained when B-PAC was prepared in AJ with all three concentrations (1, 2 and 5 mg/ml) within 15 min (Table 3.3). However, when treated with B-PAC in 2% milk, minor reductions of 0.18±0.01 and 0.05±0.02, PFU/ml were obtained after 6h with 2 and 5 mg/ml, respectively and 0.81±0.18 log PFU/ml after 24h with 5 mg/ml (Table 3.4).

B-PAC prepared in AJ caused HAV titers to reduce to undetectable levels within 15 min with 1, 2 and 5 mg/ml (Table 3.5). When B-PAC was prepared in 2% milk, reductions of 0.54±0.15 and

0.83±0.04 log PFU/ml were obtained after 24h treatment with 2 and 5 mg/ml concentration of B-PAC. After 6h of treatment, reductions of 0.15±0.01 and 0.13±0.02 log PFU/ml were obtained for 2 and 5 mg/ml B-PAC (Table 3.6).

B-PAC made in AJ had increased antiviral activity where reduction to undetectable levels (< 2 log PFU/ml) was obtained after treatment with 2 and 5 mg/ml B-PAC in AJ after 30 min at 37^{o}C. However, the AJ control by itself did not cause any significant change in titers after 3 h of treatment (Table 3.7). Reduced antiviral activity of B-PAC was seen when prepared in 2% reduced fat milk, where titer reductions of 0.14±0.01, 1.4±0.14, 0.84±0.03 log PFU/ml were obtained with 5 mg/ml B-PAC in milk after 3, 6 and 24 h. Reductions of 0.29±0.01, 0.39±0.19 and 0.52±0.17 were obtained with 2 mg/ml B-PAC in milk after 3, 6 and 24 h. The milk control caused a reduction of 0.48±0.19 log PFU/ml after 24 h (Table 3.8). No significant reduction with B-PAC in milk at any concentration was obtained with 1 or 3h treatment for FCV-F9, MNV-1, HAV or AiV. Moreover the AJ control did not change titers of the four tested viruses over 3h.

Antiviral activity under simulated gastrointestinal conditions

Simulated gastric fluid (SGF; pH 1.5) experiments were not conducted with FCV-F9 given its sensitivity to low pH (19). MNV-1, HAV and AiV did not survive in the SGF control; hence the treatments with B-PAC under gastric conditions were inconclusive. With SIF (pH 7.5), B-PAC at 5 mg/ml reduced titers of all four tested viruses within 30 min (Table 3.9).

Discussion

B-PAC and BJ have been shown to be highly effective in reducing the titers of all the four tested viruses when made in 10% ethanol where the antiviral activity was found to be both time and concentration dependent. However, for potential application of B-PAC as an antiviral

therapeutic, evaluating its activity in model food systems and gastrointestinal conditions is necessary. Amid the two food systems, the effectiveness of treatments was seen to be higher in AJ compared to the effects of B-PAC in 10% ethanol, where higher titer reductions in lesser time were obtained. For instance, MNV-1 titers were reduced to undetectable levels after 3h with 5 mg/ml B-PAC, however when 5 mg/ml B-PAC was made in AJ, similar reduction was obtained within 15 min. When tested as a control, AJ at pH 3.6 alone did not cause any significant reduction in titers over 3h for all four viruses. These observations suggest that the enhanced antiviral activity of B-PAC in AJ could be a synergistic effect of PAC together with the low pH of AJ as well as the presence of polyphenols like hydroxycinnamic acid and chlorogenic acid in AJ (20).

Previous research carried out to test effectiveness of antimicrobials in food systems points to similar observations. A study examined the antimicrobial effect of seaweed *Himanthalia elongata* in carbohydrate (glucose) and protein (bovine serum albumin) model food systems against *Salmonella abony* and *Listeria monocytogenes* (21). The extract at concentration of 8 mg/ml was shown to cause 100% inhibition of both *Salmonella abony* and *L. monocytogenes* after 24h at 37^{o}C. The efficacy was reported to be dose dependent with lower concentration of 2 mg/ml causing only 48.4% inhibition and no inhibition at 1 mg/ml. In protein system, 100% inhibition for both organisms was reported at 8 mg/ml extract and lower inhibition with lower concentrations. An interesting observation reported by the study was that the antimicrobial efficacy of the extract increased with increasing concentration of glucose, which was attributed to a possible synergistic effect where glucose can lower water activity restricting microbial growth.

When B-PAC was prepared in 2% milk, a sharp decline in effectiveness was seen where significant titer reductions were obtained only after 24h of treatment for all tested viruses. Similar results were demonstrated in a previous study where grape seed extract in the presence of bovine serum albumin (0.3 g/liter) was reported to have reduced antiviral activity against MNV-1, implying that organic load such as protein interferes with the effectiveness of treatments (22). In this study too, reduced activity with increasing organic load was observed in model food systems. This could possibly be due to the inhibition from the carbohydrates, lipids and proteins present in the milk matrix. Peppermint oil and eugenol were tested in a study for their antimicrobial activity against *Bacillus cereus, Staphylococcus aureus,* and *Escherichia coli* in model food systems. In cabbage and barley model systems, significant reduction was reported for all four organisms with 0.4 % and 0.4% concentration after 28 days of storage at 37^{o}C (23). In another study, cinnamon bark essential oil at 1,000 was shown cause a 1 log CFU/ml reduction of *L. monocytogenes* in whole milk as compared to 3 log CFU/ml reduction in skimmed milk indicating that higher fat content can hamper the antimicrobial effect (14). In the present study, 2% milk control was seen to cause a reduction of 0.96 and 0.46 log PFU/ml in FCV-F9 and AiV titers after 24 h. Previous studies have shown that milk components like lactoferrin can inhibit adsorption of hepatitis C virus (HCV) to host cells by binding to the envelope proteins E1 and E2 (24, 25). Similarly, lactoferrin was reported to inhibit adsorption of rotavirus to its host cells by binding to the virus particle (26). Lactoferrin was also reported to inhibit entry of poliovirus into host cells by blocking of receptors (27). However, lactoferrin might lose some of its activity after pasteurization. Thus if B-PAC is prepared in a food matrix that has inherent antiviral activity, synergistic effects can be further exploited to make it a better

antiviral alternative. B-PAC can be encapsulated or nano-emulsions can be developed using conjugates such as sodium caesinate for better dispersion and solubility. In a study, sodium caesinate encapsulated thymol was shown to have better dispersion properties and significantly higher antimicrobial activity against *L. monocytogenes* in milk as compared to the free thymol (28). The conjugate was shown to be stable at neutral pH, however at pH of 4.6, soybean polysaccharide was used for preventing aggregation. Similar approach could be used to develop B-PAC as an antiviral therapeutic thus helping it retain its antiviral activity even in presence of food matrices.

To be able to exploit antiviral properties of B-PAC, it is particularly important to study its effect under conditions that it will possibly encounter during consumption. Furthermore, it is also important that the antiviral effects of the said therapeutic be maintained in the low pH and conditions of the gastrointestinal tract. Both SGF and SIF were chosen to study effect of treatment under different pH conditions (pH of SIF is 1.5 and pH of SGF is 7.5) and gut enzymes like pepsin and pancreatin, as they would be subject in vivo. However, the tested viruses did not survive in the simulated gastric fluid (pH 1.5). This could possibly be due to the fact that MNV-1 as a human norovirus surrogate was used and it could not mimic the survival of human norovirus at such low pH. The same could be true with HAV, since the strain HM175 used in the study is a lab-adapted strain that did not respond to the low pH in a manner similar to the wild type strain. Another possible reason for their survival *in vivo* could also be due to their binding to mucus or protection from food during the stomach transit, so they are protected from the enzymatic environment. Antimicrobial activity of *T. camphoratus* crude methanolic and water extract (minimum inhibitory concentration of 2 mg/ml) at 37^{o}C after 24h was reduced after exposure to

simulated gastric fluid (29). The study suggested that exposure to gastric fluid caused chemical alteration or degradation of the active compounds leading to decreased antimicrobial activity. In this study, since the viruses did not survive in the gastric fluid, it was difficult to conclude the fate of B-PAC upon exposure to gastric environment.

This study will help provide a better understanding on the effective optimum concentration levels and time required to inactivate the target viruses for use as antiviral therapy. The reported results might be able to provide useful data for designing where time-release or dosage for these antiviral compounds. Additionally, animal feeding studies with blueberries and B-PAC using pre- and post-challenge should be carried out to determine antiviral effects. In addition, "omics" approaches (metagenomic analysis and next generation sequencing) can be used to determine changes in the gut microbiota that could enhance growth of beneficial microflora to prevent gastrointestinal disease. However, before any recommendations for consumption as therapeutic options can be suggested or health claims made, clinical trials and regulatory approvals will be needed and hence *in vitro* data must be interpreted with caution as indicated in earlier reports (30).

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Appendix C

Table 3.1 Effect of blueberry proanthocyanidins (B-PAC) in apple juice (AJ) on FCV-F9 titers over 3h at 37^0C

Time (min)	Recovered titer (Log PFU/ml)				
	PBS[a] (pH 7.2)	AJ (pH 3.6)	B-PAC in AJ (1 mg/ml)	B-PAC in AJ (2 mg/ml)	B-PAC in AJ (5 mg/ml)
15	4.79 ± 0.14^{A}	4.70 ± 0.10^{A}	0^{*B}	0^{*B}	0^{*B}
30	4.83 ± 0.05^{A}	4.88 ± 0.05^{A}	0^{*B}	0^{*B}	0^{*B}
60	5.03 ± 0.06^{A}	4.86 ± 0.06^{A}	0^{*B}	0^{*B}	0^{*B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.2 Effect of blueberry proanthocyanidins (B-PAC) in 2% milk on FCV-F9 titers over 24h at 37^0C

Time (Hours)	Recovered titer (Log PFU/ml)			
	PBS[a]	2% Milk	B-PAC in 2% Milk (2 mg/ml)	B-PAC in 2% Milk (5 mg/ml)
3	4.85 ± 0.05^{A}	4.10 ± 0.02^{AB}	4.76 ± 0.02^{A}	4.67 ± 0.11^{A}
6	4.99 ± 0.07^{A}	4.10 ± 0.05^{AB}	4.66 ± 0.12^{A}	4.55 ± 0.23^{A}
24	4.75 ± 0.07^{A}	3.79 ± 0.30^{B}	4.35 ± 0.10^{A}	3.66 ± 0.06^{B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.3 Effect of blueberry proanthocyanidins (B-PAC) in apple juice (AJ) on MNV-1 titers over 3h at 37^0C

Time (min)	Recovered titer (Log PFU/ml)				
	PBS[a] (pH 7.2)	AJ (pH 3.6)	B-PAC in AJ (1 mg/ml)	B-PAC in AJ (2 mg/ml)	B-PAC in AJ (5 mg/ml)
15	4.74 ± 0.24 ^{A}	4.76 ± 0.19 ^{A}	0 ^{*B}	0 ^{*B}	0 ^{*B}
30	4.89 ± 0.08^{A}	4.76 ± 0.19 ^{A}	0 ^{*B}	0 ^{*B}	0 ^{*B}
60	4.84 ± 0.10^{A}	4.76 ± 0.19 ^{A}	0 ^{*B}	0 ^{*B}	0 ^{*B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.4 Effect blueberry proanthocyanidins (B-PAC) in 2% milk on MNV-1 titers over 24h at 37^0C

Time (Hours)	Recovered titer (Log PFU/ml)			
	PBS[a]	2% Milk	B-PAC in 2% milk (2 mg/ml)	B-PAC in 2% milk (5 mg/ml)
3	4.90 ± 0.10 ^{A}	4.92 ± 0.02 ^{A}	4.51 ± 0.07 ^{A}	4.53 ± 0.09 ^{A}
6	4.75 ± 0.07^{A}	4.94 ± 0.02 ^{A}	4.57 ± 0.06 ^{A}	4.80 ± 0.05 ^{A}
24	4.50 ± 0.07^{A}	4.91 ± 0.01 ^{A}	4.66 ± 0.04 ^{A}	3.69 ± 0.25 ^{B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.5 Effect of blueberry proanthocyanidins (B-PAC) made in apple juice (AJ) on HAV titers over 3h at 37^0C

Time (min)	Recovered titer (Log PFU/ml)				
	PBS (pH 7.2)	AJ (pH 3.6)	B-PAC in AJ (1 mg/ml)	B-PAC in AJ (2 mg/ml)	B-PAC in AJ (5 mg/ml)
15	5.09 ± 0.04^{A}	4.87 ± 0.05^{A}	0^{*B}	0^{*B}	0^{*B}
30	4.81 ± 0.05^{A}	4.86 ± 0.06^{A}	0^{*B}	0^{*B}	0^{*B}
60	4.78 ± 0.03^{A}	4.70 ± 0.05^{A}	0^{*B}	0^{*B}	0^{*B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.6 Effect of blueberry proanthocyanidins (B-PAC) made in 2% milk on HAV titers over 24h at 37^0C

Time (hours)	Recovered titer (Log PFU/ml)			
	PBS[a]	2% Milk	B-PAC in 2% milk (2mg/ml)	B-PAC in 2% milk (5mg/ml)
3	5.09 ± 0.04^{A}	4.87 ± 0.05^{A}	4.87 ± 0.03^{A}	4.80 ± 0.08^{A}
6	4.90 ± 0.05^{A}	4.76 ± 0.07^{A}	4.75 ± 0.04^{A}	4.77 ± 0.07^{A}
24	4.98 ± 0.08^{A}	4.67 ± 0.14^{A}	4.44 ± 0.23^{A}	4.15 ± 0.12^{B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Table 3.7 Effect of blueberry proanthocyanidins (B-PAC) prepared in apple juice (AJ) on Aichi virus (AiV) at ~ 5 log PFU/ml over 24h at 37°C

Time (Hours)	Recovered titer (Log PFU/ml)				
	PBS[a] (pH 7.2)	AJ (pH 3.6)	NAJ (pH 7.0)	Blueberry PAC in AJ (1 mg/ml)	Blueberry PAC in AJ (2 mg/ml)
0.5	4.79 ± 0.14^{A}	4.70 ± 0.10^{A}	4.49 ± 0.09^{A}	0^{*B}	0^{*B}
1	4.75 ± 0.07^{A}	4.80 ± 0.10^{A}	4.42 ± 0.04^{A}	0^{*B}	0^{*B}
3	4.53 ± 0.05^{A}	4.56 ± 0.22^{A}	4.42 ± 0.15^{A}	0^{*B}	0^{*B}

Table 3.8 Effect of blueberry proanthocyanidins (B-PAC) prepared in 2% milk on Aichi virus (AiV) over 24h at 37°C

Time (Hours)	Recovered titer (Log PFU/ml)			
	PBS[a]	2% Milk	Blueberry PAC in 2% Milk (2 mg/ml)	Blueberry PAC in 2% Milk (5 mg/ml)
3	4.60 ± 0.13^{A}	4.35 ± 0.05^{A}	4.31 ± 0.12^{A}	4.46 ± 0.11^{A}
6	4.58 ± 0.30^{A}	4.13 ± 0.02^{A}	4.19 ± 0.11^{A}	3.18 ± 0.16^{C}
24	4.43 ± 0.05^{A}	3.95 ± 0.24^{B}	3.91 ± 0.22^{B}	3.59 ± 0.08^{B}

Table 3.9 Effect of B-PAC (5 mg/ml) prepared in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) against FCV-F9, MNV-1, HAV and AiV (~ 5 log PFU/ml) over 1 h at 37ºC

Virus	Treatment	Time in hours	
		0.5	1
		Recovered titer (Log PFU/ml)	
FCV-F9	PBS[a] (pH 7.2)	4.76 ± 0.06 ^{A}	4.80 ± 0.03 ^{A}
	SIF (pH 7.5)	4.79 ± 0.25 ^{A}	4.79 ± 0.25 ^{A}
	B-PAC in SIF	0 ^{*B}	0 ^{*B}
MNV-1	PBS[a] (pH 7.2)	4.95 ± 0.07 ^{A}	4.95 ± 0.07 ^{A}
	SIF (pH 7.5)	4.84 ± 0.09 ^{A}	4.77 ± 0.09 ^{A}
	SGF (pH 1.5)	0 ^{*B}	0 ^{*B}
	B-PAC in SIF	0 ^{*B}	0 ^{*B}
HAV	PBS[a] (pH 7.2)	4.77 ± 0.01 ^{A}	4.78 ± 0.04 ^{7A}
	SIF (pH 7.5)	4.61 ± 0.01 ^{A}	4.50 ± 0.03 ^{A}
	SGF (pH 1.5)	0 ^{*B}	0 ^{*B}
	B-PAC in SIF	0 ^{*B}	0 ^{*B}
AiV	PBS[a] (pH 7.2)	4.79 ± 0.14 ^{A}	4.75 ± 0.07 ^{A}
	SIF (pH 7.5)	4.41 ± 0.05 ^{A}	4.44 ± 0.21 ^{A}
	SGF (pH 1.5)	0 ^{*B}	0 ^{*B}
	B-PAC in SIF	0 ^{*B}	0 ^{*B}

a - phosphate buffered saline; * - Detection limit of the assay is 2 log PFU/ml. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

Chapter 4 Understanding the mechanism of antiviral activity of blueberry juice and blueberry proanthocyanidins

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Keywords: Blueberry polyphenols, transmission electron microscopy

Abstract

Blueberry polyphenols have demonstrated effective antiviral properties against cultivable human norovirus surrogates, Aichi virus, and hepatitis A virus. The objectives of this study were to (a) compare the antiviral activity of the monomeric catechin monohydrate, procyanidin B2, B-type PAC from blueberries (B-PAC) and A-type PAC from cranberries (C-PAC) against hepatitis A virus (HAV), Aichi virus (AiV) and the cultivable human norovirus surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) at 37ºC by plaque assays; (b) determine the effect of blueberry proanthocyanidins (B-PAC) on viral adsorption and viral replication of HAV, AiV, FCV-F9 and MNV-1 at 37ºC using plaque assays; (c) determine effects of B-PAC on virus structure using Transmission Electron Microscopy (TEM) and enzyme linked immunosorbent assays (ELISA). Experiments were carried out in duplicate, replicated thrice, and data were analyzed statistically analyzed. Monomeric catechins at 1 mg/ml reduced FCV-F9 titers to undetectable levels within 1 h and MNV-1 after 24h, AiV by 0.66 log PFU/ml after 24h, without any significant effect on HAV. Procyanidin B2 at 1 mg/ml reduced FCV-F9 to undetectable levels after 3h, with 1.23, 0.04 and 0.67 log PFU/ml reduction for MNV-1, HAV and AiV after 24h. Pre-treatment of host cells with 0.5 mg/ml B-PAC showed that FCV-F9, MNV-1, HAV and AiV titers were reduced by 0.63, 0.53, 0.34 and 0.62 log PFU/ml indicating that B-PAC had modest effects on preventing viral adsorption. However, lower reduction after post-infection treatment was obtained. TEM observations revealed moderate effect on the structure of the viral particles with either damaged viral capsid or binding by PAC, possibly preventing their attachment to host cells. These results provide insights for use of blueberry polyphenols in developing preventive antiviral therapies.

Introduction

With the rise in number of foodborne diseases caused by enteric viruses, there is a need to develop effective mitigation strategies for their control. In the current absence of vaccination or effective treatment options for human norovirus infections, natural alternatives are being evaluated (1). Plant derived polyphenols and their associated health benefits as antimicrobials against bacteria are well established (2-5). Likewise, blueberries are known to have a broad spectrum of health benefits such as prevention of cardiovascular disorders, age-induced oxidative stress, inflammatory responses, and diverse degenerative diseases along with anti-carcinogenic, neuro-protective, cardio-protective, antibacterial, and antiviral properties (6). Blueberries contain polyphenols including anthocyanins, flavonoids and proanthocyanidins (PAC), also referred to as condensed tannins (7). Tannins (condensed and hydrolysable) differ from other phenolic compounds for they are able to precipitate proteins, known as astringency (8). PAC's are dimers, oligomers, and polymers of catechins bound together by linkages between C4 and C8 (or C6) where the monomeric flavanols differ in the stereochemistry of C-3 and their hydroxylation pattern in ring A and B (9). Many plant foods, including blueberries, apples and grapes contain high amounts of PACs, but only a few contain A-type PACs (10). The polymerized PACs in blueberries contain A-type linkages, with different profiles and amounts as compared to cranberries (11). Cranberries contain PAC's with A-type linkage accounting for 51– 91% of total PACs (10). B-type proanthocyanidins can be converted to A-type proanthocyanidins by radical oxidation (12) Distinction between A- and B-type PAC structures is of importance because the difference can influence their biological properties. The A-type PACs exhibit significantly greater inhibition of adhesion of P-fimbriated *Escherichia coli* to uroepithelial cells *in vitro* than

the B-type PACs, which is the initial step in the development of urinary tract infection (UTI) (13). Blueberries have been known to contain around 88-261 mg of proanthocyanidin/100 gram of edible portion as per the USDA database for flavonoid content (USDA Database for the proanthocyanidin Content of Selected Foods, August 2004). The recommended dose for blueberries in daily diet can be 1 to 2 cup serving of fresh or frozen blueberries per day, and consumption of more than 100 gram fruit per day may be required for health benefits. The blueberry PAC concentration range used in our study is from 1 to 5 mg/ml that compares to eating about ¼ of the daily serving size.

Several attempts have been made at understanding the mode of antiviral action of natural plant polyphenols against human noroviral (HNoV) surrogates, feline calicivirus (FCV-F9), murine norovirus (MNV-1) using various techniques such as electron microscopy studies, binding studies, molecular assays and *in vitro* cell culture assays (4, 14, 15). Viruses are known to cross the host epithelial barrier through attachment to carbohydrate moieties of host cell glycoproteins, glycolipids such as sialic acid, and proteoglycans (16). Human noroviruses in addition to binding to histo-blood group antigens are also known to bind to porcine gastric mucin (PGM) (17). PGM has been used widely in studies to discriminate between infectious and non-infectious norovirus particles (18). ELISA (enzyme-linked immune sorbent assay) involving differential binding of viral particles with intact and non-intact capsid to PGM has been used to determine viral infectivity (Hirneisen and Kniel, 2012). Briefly, only those viruses that have an intact capsid after inactivation treatments will bind to the PGM in the assay. The study assessed attachment of MNV-1 after heat, high-pressure, ozone and UV inactivation treatments, and found that only heat treatments at 80 and 100^{o}C for 5 min caused a significant change in the

binding ability whereas other treatment did not have a significant effect on the capsid (19). Saliva-binding ELISA showed that grape seed extract (GSE) treatments of 0.2 and 2 mg/ml at 37°C for 1 h caused a significant difference in the binding ability of HNoV's, indicating that loss of infectivity caused by GSE treatments was due to capsid damage and thus loss in binding ability (4). Similar approaches could be used for determining the mechanism of action of blueberry juice (BJ) and B-PAC on foodborne viruses. Such assays would be useful in understanding the effect of treatments on capsid integrity/structure and thereby their decrease binding abilities. Correlation between binding studies, infectious plaque assays and TEM analysis could help elucidate the exact mechanism of action of these polyphenols against these viruses.

The objectives of this study were to (a) compare the antiviral activity of the monomeric unit (Catechins) and dimeric procyanidin B2 (B- type PAC present in blueberries) to PAC in blueberries (B-PAC, previously published data) on infectivity of hepatitis A virus (HAV), Aichi virus (AiV) and human norovirus surrogates, FCV-F9 and MNV-1 at 37ºC; (b) determine the effect of blueberry juice (BJ) and blueberry proanthocyanidins (B-PAC) on viral adsorption and viral replication of HAV, AiV, FCV-F9 and MNV-1 at 37ºC and (c) determine effects of B-PAC on virus structure using Transmission Electron Microscopy and ELISA.

Materials and Methods

Viruses and cell lines

Feline calicivirus (FCV-F9) and host Crandell Reese Feline Kidney (CRFK) cells were purchased from ATCC (Manassas, VA). Murine norovirus (MNV-1) was kindly gifted by Dr.

Skip Virgin (Washington Univ., St. Louis, MO) and RAW 264.7 cells were obtained from the University of Tennessee at Knoxville. Hepatitis A virus (HAV; strain HM175) and fetal rhesus monkey kidney (FRhK4) cells were provided by our collaborator, Dr. Kalmia Kniel (University of Delaware). All three host cell lines were maintained using Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) containing 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1x Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) at 37ºC in an atmosphere containing 5% CO_2. Aichi virus (AiV) was kindly provided by Dr. David Kingsley (USDA ARS, Delaware). Host Vero cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) with 2% FBS heat-inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1x Anti-Anti (Antibiotic-Antimycotic, Invitrogen, Grand Island, NY) at 37ºC in an atmosphere containing 5% CO_2 as described earlier (20).

Comparison of antiviral activity of components and types of PAC's

Antiviral activity of catechin hydrate (Sigma Aldrich) and procyanidin B2 (Sigma Aldrich) against the four viruses; FCV-F9, MNV-1, HAV and AiV was compared to the antiviral activity of PAC's isolated from blueberries (B-type PAC; kindly provided as a gift by Dr. Amy Howell, Rutgers University) and cranberries (A-type PAC; previously published data;(21)). Equal amounts of each virus at 5 log PFU/ml were mixed with equal amounts of catechin hydrate (10 or 2 mg/ml), procyanidin B2 (1 or 0.5 mg/ml), or B-PAC (all three were in powder form and were dissolved in 10% ethanol) and treated for 3, 6 and 24 h at 37^{o}C. Reactions were stopped in DMEM containing 10% FBS and serially diluted in DMEM with 2% FBS. Viral infectivity was evaluated using standardized plaque assays (20, 21).

Effect of B-PAC and BJ on host cells lines

Cytopathic effects of B-PAC, BJ (pH 2.8) and NBJ (neutralized blueberry juice; pH 7.0) treatments on the host cell lines were also determined. The maximum concentration of B-PAC used in the viral treatment and assays was 5 mg/ml, however, the host cell lines were only exposed to a maximum 20-fold dilution of the treatment after neutralization/stopping of treatment. Thus, each individual cell lines were exposed to B-PAC at 0.5 mg/ml and BJ and NBJ for 2h at 37^{o}C and observed under optical microscope to determine visible changes.

Effect of B-PAC and BJ on adsorption and replication

To determine the effect of B-PAC and NBJ on viral replication, host CRFK, RAW 264.7, FRhK4, and Vero cells in 6-well plates were first infected with FCV-F9, MNV-1, HAV, and AiV respectively for 2 h, followed by treatment with B-PAC (0.5mg/ml) or NBJ for 20 min at 37°C. To determine effects on viral adsorption/binding, the host cells were pretreated with B-PAC (0.5 mg/ml) or NBJ for 20 min, followed by viral infection for 2 h at 37°C. Cells were then overlaid with complete DMEM containing 0.75% or 1% agarose. After incubation for 2 to 8 days at 37°C under 5% CO_2, a second overlay containing neutral red was added followed by incubation to allow the visualization of plaques and plaques were counted and recorded as plaque forming units (PFU/ml) (21).

Transmission electron microscopy (TEM) analysis

FCV-F9, MNV-1, HAV or AiV at ~6.5 log PFU/ml were mixed with equal volumes of B-PAC (2 mg/ml), BJ, or sterile distilled water (control) and the individual viruses were incubated at 37^{o}C for 15 min, 1 h, 30 min and 1 h respectively (based on reduction of viral titers obtained with treatments). Fresh glow discharged, formvar and carbon coated copper grids were coated with

10-µl of the viral samples, stained with uranyl acetate for 1 min and allowed to dry. The samples were then observed under a Hitachi H800 at 75KeV (courtesy of Dr. Dunlap at the UT-Knoxville Advanced Microscopy and Imaging Center).

ELISA PGM binding assay

The protocol for PGM binding ELISA was adapted from (19). ELISA plates pre-coated with poly-D-lysine (ThermoFisher Scientific) were coated with 100 µl of porcine gastric mucin (Sigma) at 10 µm/mL in Tris Buffered Saline (TBS; pH 7.6) and were incubated for 24h at 4^{o}C. Plates were washed with deionized double distilled (dDI) water thrice to remove unbound material. Following this, the plates were blocked with Animal-Free Blocking Buffer (Vector Labs, Burlingame, CA) for 2 h at room temperature. The wells were washed twice with dDI water to remove excess blocking buffer. MNV-1 samples (treatment or control) diluted in TBS (upto 5 serial dilutions) were added to the wells and were allowed to incubate at 37^{o}C for 1 h. Wells were washed thrice with dDI water followed by addition of 1:1000 dilution of monoclonal anti-MNV IgA (gifted by Dr. Christiane Wobus, University of Michigan) diluted in TBS and allowed to incubate at 37^{o}C for 1 h. Unbound anti-MNV IgA was removed by washing the wells with TBS with 0.05% Tween- 20 (TTBS). Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary IgA (diluted 1:5000 in TTBS) (KPL) was used for detection of bound primary antibody followed by incubated at 37^{o}C for 1 h. Unbound secondary antibody was removed by washing thrice with TTBS followed by addition of 80 µL of 3,3', 5, 5' tetramethylbenzidine (TMB) liquid substrate (Thermo Fisher) and incubation at room temperature for 10 min. The reaction was stopped by the addition of 2M H_2SO_4 and absorbance was read at 450 nm.

Statistical analysis

Statistical analysis was carried out using ANOVA with SAS software (version 9.3, SAS Institute, Cary, NC, USA) and Tukey's test on a completely randomized design with data obtained from the three replications for each individual virus as described in previous studies (14, 22).

Results

Comparison of antiviral activity of monomeric and polymeric PAC's

Polymeric B-PAC at 1 mg/ml was shown to reduce FCV-F9, MNV-1 and HAV titers to undetectable levels within 3 h of treatment, while longer incubation time of 6h was needed to reduce AiV titers to undetectable levels at 37^{o}C. Monomeric catechins at 1 and 5 mg/ml reduced FCV-F9 to undetectable levels after 6 and 3h, respectively. B-type PAC, procyanidin B2 at both 0.5 and 1 mg/ml reduced FCV-F9 to undetectable levels after 3h (Table 4.1). MNV-1, the sturdier of the surrogates showed lower reduction with both monomeric catechin and procyanidin B2. Reductions of 0.42±0.02 and 1.76±0.13 log PFU/ml were obtained for MNV-1 with 1 and 5 mg/ml monomeric catechin after 3h. MNV-1 titers reduced to undetectable levels after 6h with 5 mg/ml monomeric catechin and after 24h with 1 mg/ml monomeric catechin. No significant reduction in MNV-1 titers was obtained with procyanidin B2, except with a reduction of 1.23±0.11 log PFU/ml after 24h with 1 mg/ml (Table 4.2). Similarly for HAV, no significant reductions were obtained either with monomeric catechin or procyanidin B2 even after 24h (Table 4.3). AiV titer reductions of 0.36±0.20, 0.88±0.0 and 0.95±0.04 log PFU/ml were obtained with monomeric catechin at 5 mg/ml after 3, 6 and 24h, respectively. Monomeric catechin at 1mg/ml reductions of 0.77±0.02, 0.82±0.01 and 0.66±0.19 log PFU/ml after 3, 6 and 24h respectively were obtained. Procyanidin B2 at 1 mg/ml had minimal effect on AiV titers,

with reductions of 0.41±0.21, 0.49±0.13 and 0.67±0.19 log PFU/ml and 0.23±0.04, 0.12±0.03 log PFU/ml with 5 mg/ml after 3, 6 and 24h respectively were obtained (Table 4.4).

Effect of B-PAC and BJ on host cells lines

B-PAC at a concentration of 0.5 mg/ml was not found to be cytotoxic to the four tested host cell lines. Undiluted BJ when added directly to the host cells had some minor effects after 2h. However, neutralized BJ (NBJ) did not show any morphological visual changes in the four host cells and was used further in the time of addition experiments. In the experiment involving treatment of viruses with B-PAC, the cells are exposed to a maximum of 20-fold diluted B-PAC and hence the host cells do not show cytopathic/cytotoxic effects.

Effect of B-PAC and BJ on viral adsorption and viral replication

Treatment of host cells with 0.5 mg/ml B-PAC before viral infection reduced the titers of FCV-F9, MNV-1, HAV and AiV by 0.63±0.01, 0.53±0.03, 0.34±0.07 and 0.62±0.34 log PFU/ml, respectively (Table 4.5). When the cells were treated with 0.5 mg/ml B-PAC post viral infection, reductions in viral titers of 0.37±0.01, 0.23±0.06, 0.03±0.01 and 0.30±0.07 log PFU/ml were obtained for FCV-F9, MNV-1, HAV and AiV respectively (Table 4.6). These results suggest that B-PAC at 0.5 mg/ml plays a modest effect in the prevention of virus attachment to the host and adsorption with a lesser effect on inhibiting replication. NBJ did not have any significant effect on viral adsorption or viral replication of all the four tested viruses.

Transmission electron microscopy studies using B-PAC

TEM studies were carried out with B-PAC (0.5 mg/ml) to elucidate the mechanism of action of B-PAC on the virus structure. B-PAC was shown to encapsulate FCV-F9 viral particles while some treated viruses also had structural damage with capsid disruption by TEM as shown (Fig 4

– 1a and b). Similar results were observed with MNV-1 where the particles were coated or adhered with the treatment material (Fig 4 – 2a and b), and similarly treated HAV samples appeared to be coated/encapsulated by B-PAC compared to the water control (Fig 4- 3a and b). However, TEM observations for AiV did not show any significant change in the viral particle or damage to the capsid after B-PAC treatment (Fig 4, 4a and b).

ELISA binding study

The PGM coating on the plate led to a very high background, without any significant difference between the controls and test wells. Further investigation revealed that the primary antibody (IgA) bound unspecifically to the PGM resulting in detection even in control wells. Hence, no conclusive results were obtained at this time while the screening for additional specific antibodies continues to be researched.

Discussion

As there are an increasing number of studies that examine antiviral properties of natural polyphenols, it is equally important to assess them in terms of their antiviral potential, compare their effect when these polymeric PAC's are isolated from different sources and when used as individual monomeric units. One of the objectives of this study aimed to compare antiviral activities of the monomeric and polymeric units. Overall, the polymeric PAC's showed a higher antiviral effect as compered to monomeric catechins. PAC's isolated from both blueberries and cranberries were observed to have higher effect than commercial procyanidin B2. HAV was found to be the most resistant to monomeric treatments amongst the four tested viruses, followed by AiV and MNV-1. This provides insights about the structural bioactive units that can be beneficial in the design of appropriate preventive anti-viral therapies from natural sources.

Extensive research is being carried out to understand the interaction of natural polyphenols with the viruses and their host cells. Researchers have proposed several theories as to the effects of plant polyphenols *in vitro*. Berry PACs were reported to have an effect on penetration of herpes simplex virus-2 (HSV-2) to the host cells, without much effect on viral attachment (23). In another study with HSV-2, PAC from an African resurrection plant was found to inhibit both virus attachment and penetration (24). Black raspberry juice was shown to be effective in reducing plaque formation of MNV-1 with pre-treatment of host cells that prevented binding of the viruses and thus subsequent infection of host cells by treated viruses, however post treatment of cells after infection was not shown to have any significant effect suggesting that antiviral activity occurred at the point of attachment or entry and not after infection or on replication (15).

In this study, B-PAC was found to prevent viral adsorption to the host cells to a certain extent, with lesser effects on viral replication, similar to the results reported by Oh et al., 2012 with black raspberry juice. These findings are also in agreement with the study conducted with permisson extract where no significant effect pre or post infection was reported and that the maximal antiviral activity was obtained by direct treatment of the virus particles (25). Similar findings were reported for ginsenoside extract (5 μg/mL) treatment against HAV, where a very low reduction in titers was reported when the host cells were pre treated with the extract (26).

In previous studies, similar results with GSE on viral adsorption of FCV-F9, MNV-1, and HAV was reported, with less effect on replication (14). However, PAC from blueberry leaves was shown to have a pronounced effect on replication of hepatitis C (HCV) (which is an enveloped virus) virus by inhibiting expression of NS-3 gene (non-structural gene-3), along with

blocking of nuclear ribonucleoprotein essential for subgenomic replication (27). Thus, the mechanism of action seems to differ depending on the virus (enveloped versus non-enveloped) and the source of PAC.

Antiviral activity of monomeric and polymeric units for PAC was compared in this study in order to understand if a particular subunit had a higher antiviral activity and that which component contributes more towards the antiviral effect of B-PAC. FCV-F9 titers were reduced to undetectable levels with 1 mg/ml B-PAC after 5 min, whereas with catechin hydrate and procyanidin B2, a treatment time of 3 h was needed to exert the same effect. In case of MNV-1, 1 mg/ml B-PAC reduced titers to undetectable levels within 3 h, however procyanidin B2 at 1 mg/ml reduced MNV-1 titers by 1.23 log PFU/ml and catechin hydrate to undetectable levels only after 24h. Similar observations were reported for HAV with reduction to undetectable levels within 3h with 1 mg/ml B-PAC, however no significant change in titers was observed with either catechin or procyanidin B2 after 24h. B-PAC at 1 mg/ml reduced AiV titers by 2.45 log PFU/ml after 24h, however catechin and procyanidin B2 at 1mg/ml led to a reduction of 0.66 and 0.67 log PFU/ml after 24h. These comparisons indicate that the individual subunits alone do not contribute to the antiviral activity but the PAC in its entirety with its polymeric structure is necessary to cause viral reduction.

With regards to effects on viral structure, in the current study, TEM observations showed that the B-PAC treatment had a pronounced effect on the structure of the viral particles where visible damage to the outer capsid was seen with disruption of the capsid and release of internal contents for FCV-F9, MNV-1 and HAV. The treatment material was also seen to bind to viral particles in some fields of observations. Binding of the treatment material to viral particle could

block the binding of the virus capsid to the host cell receptors, and prevent host attachment. This finding is also consistent with the reduction in the titers observed in the host binding/attachment assays. Thus, the B-PAC treatment seems to exert its antiviral activity by destroying the viral capsid and blocking the binding receptors, both leading to prevention of host attachment. However, no significant damage to the viral capsid by the treatment was observed for Aichi virus.

With regards to understanding the effect of treatments on viral capsid through ELISA, no clear mechanism could be established given the high background, and the non-specific binding of the primary antibody to PGM. Thus, further research to screen for specific primary antibodies for use and optimization of the ELISA-PGM binding assay is on-going. There are several other methods proposed in the literature that can be carried out to distinguish between intact or damaged capsid. Intercalating dyes such as propidiummonoazide along with RT-PCRhave been used to distinguish between infectious and non-infectious enteric virus (Coxsackievirus, poliovirus, echovirus, and Norwalk virus) particles and could distinguish noninfectious or inactivated viruses treated at 72°C and 37°C, however, the method could not detect enteroviruses that were rendered noninfectious by treatment at 19°C (28). The principle of PGM binding to distinguish intact and non-intact capsid could also be tested using a different method of detection such as RT-PCR, which could overcome the problem of high background in the control (29).

Nonetheless, this data presented in this research provided insights on the structural units of polyphenols that have higher antiviral activity, their effects on viral attachment to host and replication, and on viral structure by TEM. Overall, gaining insights on the effect of individual subunits of the tested polyphenols and their mode of action on the target viruses is

essential towards designing future animal feeding studies, clinical trials and potential antiviral therapies.

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Appendix D

Table 4.1 Effect of monomeric catechins and procyanidin B2 on FCV-F9 titers at 37°C over 24h

Time (hour)	Recovered titer (Log PFU/ml)				
	PBS (pH 7.2)	Catechin hydrate (5 mg/ml)	Catechin hydrate (1 mg/ml)	Procyanidin B2 (1 mg/ml)	Procyanidin B2 (0.5 mg/ml)
1	4.71 ± 0.18^{A}	0^{C}	4.73 ± 0.37^{A}	4.04 ± 0.24^{AB}	4.45 ± 0.05^{A}
3	4.71 ± 0.15^{A}	0^{C}	3.97 ± 0.16^{B}	0^{C}	0^{C}
6	4.77 ± 0.01^{A}	0^{C}	0^{C}	0^{C}	0^{C}
24	4.73 ± 0.04^{A}	0^{C}	0^{C}	0^{C}	0^{C}

Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 4.2 Effect of monomeric catechins and procyanidin B2 on MNV-1 titers at 37°C over 24h

Time (hour)	Recovered titer (Log PFU/ml)				
	PBS (pH 7.2)	Catechin hydrate (5 mg/ml)	Catechin hydrate (1 mg/ml)	Procyanidin B2 (1 mg/ml)	Procyanidin B2 (0.5 mg/ml)
3	4.80 ± 0.07^{A}	3.04 ± 0.20^{B}	4.38 ± 0.09^{A}	4.55 ± 0.16^{A}	4.65 ± 0.05^{A}
6	4.41 ± 0.04^{A}	0^{C}	4.63 ± 0.03^{A}	4.67 ± 0.08^{A}	4.49 ± 0.08^{A}
24	4.64 ± 0.04^{A}	0^{C}	0^{C}	3.41 ± 0.15^{B}	4.43 ± 0.07^{A}

Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 4.3 Effect of monomeric catechins and procyanidin B2 on HAV titers at 37°C over 24h

Time (hour)	Recovered titer (Log PFU/ml)				
	PBS (pH 7.2)	Catechin hydrate (5 mg/ml)	Catechin hydrate (1 mg/ml)	Procyanidin B2 (1 mg/ml)	Procyanidin B2 (0.5 mg/ml)
3	4.68 ± 0.08 ^{A}	4.64 ± 0.03 ^{A}	4.56 ± 0.16 ^{A}	4.64 ± 0.04 ^{A}	4.77±0.04 ^{A}
6	4.64 ± 0.10 ^{A}	4.64 ± 0.05 ^{A}	4.72 ± 0.05 ^{A}	4.68 ± 0.04 ^{A}	4.70±0.03 ^{A}
24	4.40 ± 0.04 ^{A}	4.64 ± 0.03 ^{A}	4.74 ± 0.04 ^{A}	4.44 ± 0.05 ^{A}	4.30±0.03 ^{A}

Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 4.4 Effect of monomeric catechins and procyanidin B2 on AiV titers at 37°C over 24h

Time (hour)	Recovered titer (Log PFU/ml)				
	PBS (pH 7.2)	Catechin hydrate (5 mg/ml)	Catechin hydrate (1 mg/ml)	Procyanidin B2 (1 mg/ml)	Procyanidin B2 (0.5 mg/ml)
3	4.40 ± 0.06 ^{A}	4.04 ± 0.26 ^{A}	3.63 ± 0.04 ^{B}	3.99±0.27 ^{A}	4.17±0.02 ^{A}
6	4.46 ± 0.03 ^{A}	3.58 ± 0.03 ^{B}	3.64 ± 0.02 ^{B}	3.97±0.16 ^{A}	4.34±0.06 ^{A}
24	4.30 ± 0.22 ^{A}	3.35 ± 0.18 ^{B}	3.64 ± 0.03 ^{B}	3.63±0.03 ^{B}	4.33±0.05 ^{A}

Different letters denote significant differences when compared between each column ($p<0.05$). Detection limit for the assay is 2 log PFU/ml.

Table 4.5 Effect of blueberry proanthocyanidins (B-PAC at 0.5mg/ml) on binding of FCV-F9, MNV-1, HAV and AiV at 37°C

Virus	Recovered titer/ Reduction (Log PFU/ml)			
	Control (DMEM)		Blueberry PAC (0.5 mg/ml)	
FCV-F9	6.17 ±0.02^{A}	0	5.54±0.10^{A}	0.63
MNV-1	5.76±0.07^{A}	0	5.22±0.10^{A}	0.54
HAV	5.83±0.02^{A}	0	5.49±0.09^{A}	0.34
AiV	4.71±0.04^{A}	0	4.09±0.38^{A}	0.62

Table 4.6 Effect of blueberry proanthocyanidins (B-PAC at 0.5mg/ml) on replication of FCV-F9, MNV-1, HAV and AiV at 37°C

Virus	Recovered titer/Reduction (Log PFU/ml)			
	Control (DMEM)		Blueberry PAC (0.5 mg/ml)	
FCV-F9	6.33±0.02^{A}	0	5.95±0.03^{A}	0.38
MNV-1	5.72±0.03^{A}	0	5.49±0.09^{A}	0.23
HAV	5.73±0.03^{A}	0	5.7±0.04^{A}	0.03
AiV	4.73±0.03^{A}	0	4.43±0.10^{A}	0.30

Figure 4.1 TEM images of FCV-F9 (1a), MNV-1 (2a), HAV (3a) and AiV (4a) controls in sterile distilled deionized water and FCV-F9 (1b), MNV-1 (2b), HAV (3b) and AiV (4b) treated with B-PAC at 0.5 mg/ml for 15min, 1 h, 30 min and 1 h respectively; controls in sterile distilled deionized water; and B-PAC background control (5).

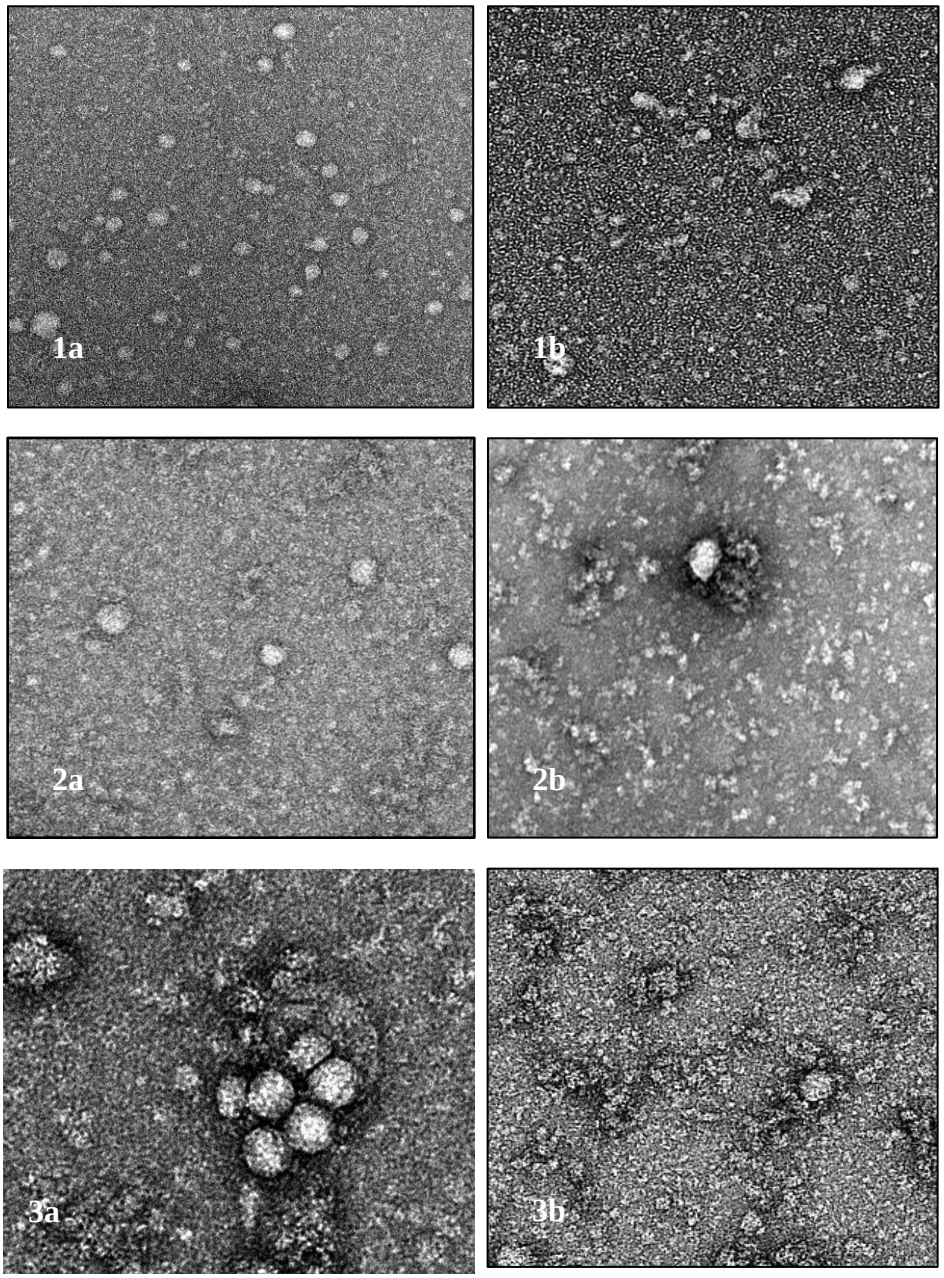
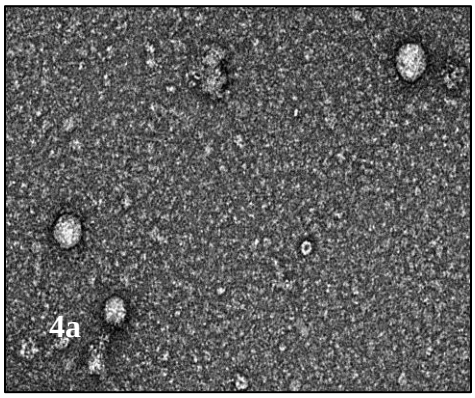
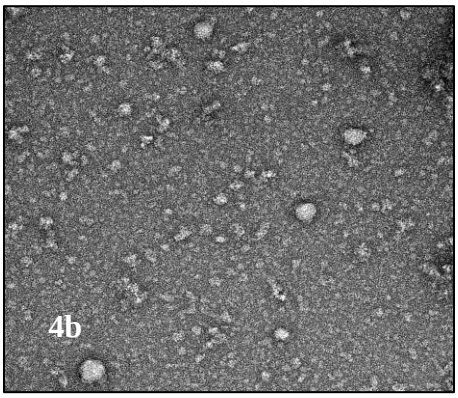


Figure 4.1 Continued





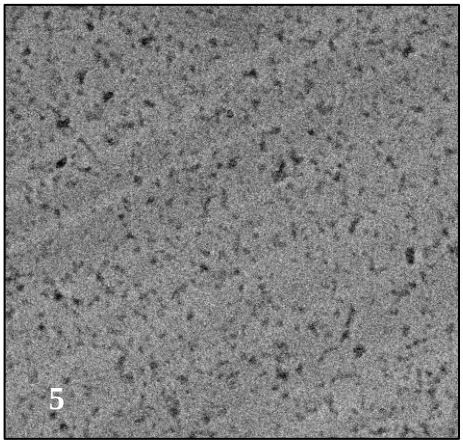


Figure 4.1 Continued

Chapter 5 Conclusion

With the rise in the number of foodborne diseases caused by enteric viruses, there is a need to develop effective mitigation strategies to control their spread. In the current absence of vaccination or effective treatment options for human norovirus infections, natural alternatives are being evaluated (141). Plant derived polyphenols and their associated health benefits as antimicrobials against bacteria are well established (142-145). Blueberries are known to have a broad spectrum of health benefits such as prevention of cardiovascular disorders, age-induced oxidative stress, inflammatory responses, and diverse degenerative diseases along with anti-carcinogenic, neuro-protective, cardio-protective, antibacterial, and antiviral properties (148).

This study determined that blueberry proanthocyanidins (B-PAC) and commercial blueberry juice (BJ) have a significant effect in reducing the titers of human norovirus surrogates (FCV-F9 and MNV-1), HAV, and AiV in a dose (for B-PAC) and time-dependent manner. For B-PAC to be used as a therapeutic antiviral agent, evaluation of its effectiveness in model food systems is important. B-PAC was examined for it antiviral activity in two model food systems, namely apple juice and 2% milk. These systems were chosen for their high carbohydrate and sugar content (apple juice) and for their high protein and lipid content (milk). Antiviral activity of the treatments was enhanced in the presence of apple juice, whereas the activity though retained, was at a reduced level in the presence of milk.

The study of the effects of B-PAC as an antiviral also includes the determination of its potential effects after consumption. Effectiveness of B-PAC in the gastrointestinal environment when consumed orally as potential treatment options against the above foodborne viruses was studied using simulated gastrointestinal fluids (in the absence of available animal models). The results showed that B-PAC had a significant effect on viral titers under simulated intestinal

conditions. This study also aimed at understanding the possible mode of action of B-PAC and its effect on the infectivity and integrity of the tested viral particles through time of addition assays and transmission electron microscopy. Results showed moderate effect on the viral particles with either damaged viral capsid or binding by PAC, possibly preventing their attachment to host cells.

The overall results from this study provided an understanding of the effective optimum concentration levels of B-PAC and time required to reduce the titers of the target viruses, in model food systems and under simulated gastrointestinal conditions. Additionally, insights on the mechanism of action were obtained. In future, studies on developing strategies for sustained release or encapsulation may be needed. Furthermore, feeding studies using animal models (using surrogate viruses for human noroviruses in the current absence of suitable animal models or human feeding studies) as well as toxicity studies are necessary. However, recommendations for consumption as therapeutic options or health claims should not be made without supporting clinical trial data and appropriate regulatory approvals.

VITA

Snehal S. Joshi was born in Thane, Maharashtra, India on March 1, 1988. She grew up in Kalyan and received her Bachelor's degree in Biotechnology from Mumbai University and her Master's degree in Biotechnology from Mumbai University. She later earned an PhD degree in Food Science and Technology with a concentration in Food Microbiology from the University of Tennessee, Knoxville. Her research interests are in the area of food and molecular microbiology and functional genomics to help combat foodborne outbreaks and enhance food safety. Snehal will pursue a career in the area of Food Safety and Microbiology.