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Antiviral effects of blueberry proanthocyanidins against Aichi virus

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ABSTRACT

Blueberry polyphenols are known for their high antioxidant and antimicrobial potential. Aichi virus (AiV) is an emerging human enteric virus that causes gastroenteritis outbreaks worldwide. This study aimed to (1) determine the time- and dose-dependent effects of blueberry proanthocyanidins (B-PAC) against AiV over 24 h at 37 °C; (2) gain insights on their mode of action using pre- and post-treatment of host cells and Transmission Electron Microscopy; and (3) determine their anti-AiV effects in model foods and under simulated gastric conditions. AiV at ~5 log PFU/ml was incubated with equal volumes of commercial blueberry juice (BJ, pH 2.8), neutralized BJ (pH 7.0), B-PAC (2, 4, and 10 mg/ml) prepared either in 10% ethanol, apple juice (AJ), 2% milk, simulated gastric fluid (SGF, pH 1.5) or simulated intestinal fluid (SIF, pH 7.5), and controls (malic acid (pH 3.0), phosphate buffered saline (pH 7.2), apple juice (pH 3.6) and 2% milk) over 24 h at 37 °C, followed by standard plaque assays. Each experiment was replicated thrice and data were statistically analyzed. Differences in AiV titers with 1 mg/ml B-PAC were 2.13 ± 0.06 log PFU/ml lower after 24 h and ≥3 log PFU/ml (undetectable levels) lower with 2 and 5 mg/ml B-PAC compared to AiV titers in PBS after 24 h and 3 h, respectively. BJ at 37 °C resulted in titer differences (lower titers compared to PBS) of 0.17 ± 0.06, 1.27 ± 0.01, and 1.73 ± 0.23 log PFU/ml after 1, 3, and 6 h and ≥3 log PFU/ml after 24 h. Pre- and post-treatment of host cells with 0.5 mg/ml B-PAC caused titer decreases of 0.62 ± 0.33 and 0.30 ± 0.06 log PFU/ml, respectively suggesting a moderate effect on viral-host cell binding. B-PAC at 2 mg/ml in AJ caused titer differences of ≥3 log PFU/ml after 0.5 h, while differences of 0.84 ± 0.03 log PFU/ml with 5 mg/ml B-PAC in milk, and ≥3 log PFU/ml with B-PAC at 5 mg/ml in SIF after 30 min were obtained. This study shows the ability of BJ and B-PAC to decrease AiV titers to potentially prevent AiV-related illness and outbreaks.

1. Introduction

An estimated total of 48 million foodborne illnesses reportedly occur annually in the United States alone (CDC, 2016; Hall et al., 2016). Foodborne viruses have become increasingly widespread that cause non-bacterial acute gastroenteritis in humans (Blanton et al., 2006). During 2009–2010, a total of 29,444 cases of illness, 1184 hospitalizations, and 23 deaths were reported and among the 790 outbreaks, viruses were implicated in 336 (42%) of the cases (CDC, 2013). Common human foodborne viral diseases are typically associated with the recognized epidemiologically significant human noroviruses (HNoVs) and hepatitis A virus (HAV), though Aichi virus and other viruses are also associated with human gastroenteritis (Sair et al., 2002; CDC, 2017). Aichi virus (AiV) is small, 30 nM, non-enveloped, icosahedral in shape, containing 3 capsid proteins, with a single-stranded, positive-sense RNA genome that belongs to the genus *Kobuvirus* in the *Picornaviridae* family, the same family as foodborne HAV (Yamashita et al., 1991, 1998). The incubation period for AiV infection is 3–7 days with symptoms that include diarrhea, abdominal pain, nausea, vomiting and fever (Yamashita et al., 2001). AiV was first isolated in 1989 from stool samples of gastroenteritis patients who had reportedly consumed raw oysters (Yamashita et al., 1991). AiV is transmitted via the fecal-oral route and shed in the feces and hence filter-feeding shellfish from sewage-contaminated water can be a source of contamination (Reuter et al., 2009; Yamashita et al.,1991, 2001). AiV-related gastrointestinal cases have been reported worldwide, with prevalence in Asian, African, European and South American countries (Biscaro et al., 2018; Goyer et al., 2008; Jonsson et al., 2012; Kaikkonen et al., 2010; Lodder et al., 2013; Oh et al., 2006; Ribes et al., 2010; Sdiri-Loulizi et al., 2008; Sassi et al., 2018; Terio et al., 2018). AiV at high titers of up to 1.32×10^{12} RNA copies/g stool were reported in 10 of 499 samples in northern Germany, indicating the risk of spread through fecally polluted waters (Drexler et al., 2011). Thus, AiV is an emerging human pathogen with a global presence. Currently, there are a limited number

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of studies reported in literature regarding effective methods for their inactivation (Yamashita et al., 1998; Fino and Kniel, 2008; Cromeans et al., 2014). Improved methods for the control and prevention of AiV transmission are needed as commercial vaccines are currently unavailable against AiV.

The consumer demand and consumption of blueberries has increased due to increased awareness of healthy lifestyles and also their widely publicized broad spectrum of health benefits that aid in the prevention of various health disorders, taken together with their associated anti-inflammatory, anti-carcinogenic, antioxidative, cardio-protective, neuro-protective, and also antimicrobial properties (Zafra-Stone et al., 2007; Joshi et al., 2014, 2016; 2017). Some of these effects have been suggested to be associated with blueberry polyphenols including anthocyanins, flavonoids and B-type proanthocyanidins (B-PAC) (Howell et al., 2005; Huang et al., 2012). The antibacterial effects of blueberry components have been widely documented in literature (Ben Lagha et al., 2018; Lacombe et al., 2012; Joshi et al., 2014; Khalifa et al., 2015). However, there are currently only few reports on the antiviral effects of blueberry extracts or blueberry juice (BJ) stating activity against hepatitis C virus and herpes simplex virus (Fukuchi et al., 1989; Takeshita et al., 2009). The survival of HNoV surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) in blueberry juice (BJ) showed that FCV-F9 was undetectable after 24 h, whereas MNV-1 remained stable with a minimal decrease in titers even after storage for 21 days at 4 °C refrigeration (Horm et al., 2012). Our lab also showed that B-PAC and BJ, grape seed extract, pomegranate polyphenols and cranberry extracts possess antiviral effects against FCV-F9, MNV-1, and HAV at 37 °C and with published studies on some of the plant extracts in model foods and under simulated gastric conditions (Joshi et al., 2016, 2017; ; Su et al., 2010a, b, Su and D'Souza, 2011; Su and D'Souza, 2013). It should be noted that the main difference between cranberry proanthocyanidins (C-PAC) and blueberry proanthocyanidins (B-PAC) are their structure, where C-PAC primarily contains A-type linkages that are different from B-PAC that contains B-type linkages (Howell et al., 2005; Joshi et al., 2016). As reported earlier, these differences in structure and their monomeric and dimeric unit composition are known to affect their associated biological activities (Blumberg et al., 2013). Hence, the potential antiviral effects of BJ and B-PAC as well as their monomeric and dimeric units against AiV need to be investigated.

Given the reported antiviral properties of BJ and B-PAC against HNoV and HAV, the objectives of this study were to (1) determine the time- and dose-dependent effects of B-PAC and commercial BJ against AiV over 24 h at 37 °C; (2) gain insights on the mode of action of BJ and B-PAC against AiV using transmission electron microscopy (TEM), time of addition assays and the determination of anti-AiV activity of monomeric and dimeric catechins; and (3) determine the effects of B-PAC in model food systems and under simulated gastric conditions against AiV.

2. Materials and methods

2.1. Virus and host cell line

Aichi virus (AiV) kindly provided by Dr. Kalmia Kniel (University of Delaware) was used in this study. Previously reported procedures were used to maintain the host Vero cells using Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) with 2% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1x Anti-Anti (Antibiotic-Antimycotic, Life Technologies) at 37 °C in an atmosphere containing 5% CO_2 and AiV was propagated following procedures as described earlier (Fino and Kniel, 2008).

2.2. Antiviral effects of blueberry juice (BJ) and blueberry proanthocyanidins (B-PAC)

As reported earlier (Joshi et al., 2016; Howell et al., 2005), concentrated and purified B-PAC that was prepared and obtained from Dr. Amy Howell (Marucci Center for Blueberry and Cranberry Research, Rutgers University, Chatsworth, NJ; *see supplemental material* for preparation details) and commercial BJ that was purchased from local grocery stores were used in this study. Similar procedures as reported in earlier studies were used (Joshi et al., 2016; 2017) where stock solutions of 10 mg/ml B-PAC were prepared by first dissolving in 10% ethanol (10 ml 200 proof ethanol in 90 ml distilled deionized water) followed by filter-sterilization through 0.2-µm filters. Equal volumes of AiV at titers of ~5 log PFU/ml were individually mixed with commercial BJ (natural pH 2.8), BJ (neutralized pH 7.0), B-PAC (2, 4, and 10 mg/ml), malic acid (pH 3.0 as a low pH control), 10% ethanol or phosphate buffered saline (PBS; 7.2 as a neutral control) and incubated at 37 °C for 0, 5, 15, 30 min, and 1, 2, 6, or 24 h in a similar manner to that reported earlier for HNoVs and HAV (Joshi et al., 2016). After each incubation time, treatments were stopped using cell-culture media containing 10% heat-inactivated fetal bovine serum (FBS), followed by serial dilutions in cell-culture media containing 2% FBS, and 500 µl was used to determine infectivity of AiV. Standard plaque assays as described before were used with confluent Vero host cells in 6-well plates (Fino and Kniel, 2008). Each experiment was carried out thrice (each experiment on a different day) and assayed in duplicate.

2.3. BJ and B-PAC effects on AiV host-cell adsorption and AiV replication using time of addition experiments

To examine the effect of BJ and B-PAC treatment on the adsorption of AiV to the Vero host cells, these host cells were pretreated with B-PAC (0.5 mg/ml) or BJ for 20 min, followed by viral infection for 2 h at 37 °C as reported earlier for HNoVs and HAV (Joshi et al., 2016, 2017). Similar to earlier studies with HNoVs and HAV, in order to determine the effect of B-PAC and BJ on viral replication, host Vero cells were first infected with AiV for 2 h, followed by subsequent treatment with B-PAC (0.5 mg/ml) or BJ (50%) for 20 min at 37 °C. The Vero host cells were treated with DMEM–F12 containing 2% FBS as non-treatment controls either before-infection or post-infection with AiV. The media after the virus treatment times were first aspirated. The host cells were then overlaid with complete DMEM containing 0.75% agarose followed by incubation for 2 days at 37 °C under 5% CO_2. The cytopathic effect and plaque formation was visualized by staining with neutral red containing overlay media followed by enumeration of plaques as described earlier for HNoVs and HAV (Su et al., 2010a,b,c; Joshi et al., 2016, 2017)

2.4. Transmission electron microscopy (TEM) studies

AiV at ~7 log PFU/ml was mixed with equal volumes of B-PAC (2 mg/ml), or sterile distilled water (control) and incubated at 37 °C for 30 min (to allow for visualization and differentiation between intact and slightly damaged particles, rather than longer exposure or treatment with higher concentrations that would result in complete destruction and that could potentially prevent observation of any viral particle structures). Following previously described protocols, fresh glow discharged, formvar and carbon coated copper grids were coated with 10-µl of the control and treated AiV samples, stained with uranyl acetate for 1 min and then dried (Su et al., 2010c). The treated and control AiV samples were observed under a Hitachi H800 at 75 KeV (courtesy of Dr. Dunlap at the UT-Knoxville JIAM/Advanced Microscopy and Imaging Center) as described before (Su et al., 2010c).

Table 1
Effects of blueberry proanthocyanidins on Aichi virus over 24 h at 37 °C.

Time (Hours)	Recovered titer (Log PFU/ml)				
	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 ± 0.11B
1	4.62 ± 0.04^{A}	4.53 ± 0.06^{A}	3.12 ± 0.10^{C}	2.99 ± 0.26^{C}	2.86 ± 0.58C
3	4.53 ± 0.05^{A}	4.58 ± 0.08^{A}	2.69 ± 0.29^{C}	2.71 ± 0.12^{C}	Non-detectable levels*D
6	4.45 ± 0.11^{A}	4.59 ± 0.07^{A}	3.00 ± 0.36^{C}	Non-detectable levels*^{D}	Non-detectable levels*D
24	4.43 ± 0.06^{A}	4.52 ± 0.07^{A}	2.30 ± 0.00^{C}	Non-detectable levels*^{D}	Non-detectable levels*D

*- Detection limit of the assay is 2 log PFU/ml.
Non-detectable levels: ≤2 log PFU/ml.
-Different letters denote significant differences between treatments ($P < 0.05$).
–Initial AiV titer was ~5 log PFU/ml.
[a] PBS: phosphate buffered saline; B-PAC: blueberry proanthocyanidins.

2.5. Comparison of antiviral activity of monomeric and dimeric catechins against AiV at 37 °C over 24 h

Antiviral activity of catechin hydrate (Sigma Aldrich) and dimeric procyanidin B2 (Sigma Aldrich) against AiV was compared to the antiviral activity of B-PAC following previously established protocols (Su et al., 2010b; Joshi et al., 2017; Liu et al., 2018). Briefly, AiV at ~5 log PFU/ml was mixed with equal amounts of catechin hydrate (10 or 2 mg/ml), procyanidin B2 (1 or 2 mg/ml)(both were commercially obtained in powder form and were dissolved in 10% ethanol (1 ml of 200 proof ethanol with 9 ml sterile distilled de-ionzied water)), and treated for 0, 3, 6 and 24 h at 37 °C and compared to the results obtained with B-PAC. Reactions were stopped in DMEM containing 10% FBS and serially diluted ten-fold in DMEM-F12 containing 2% FBS. Viral infectivity was evaluated using standardized plaque assays, and data from three replicate treatments carried out on different days were statistically analyzed (Fino and Kniel, 2008; Su et al., 2010b; Joshi et al., 2016, 2017).

2.6. Determination of antiviral activity in model food systems

AiV was diluted to obtain a final titer of ~5 log PFU/ml after mixing with equal amounts of 2, 4 and 10 mg/ml B-PAC dissolved in either apple juice (pH 3.6), 2% milk, or malic acid (pH 3.0) along with controls that were juice (AJ; pH 3.6), 2% milk, and PBS alone (to determine differences between the effect of B-PAC in juice, or milk, or PBS) as reported earlier (Joshi et al., 2017). These treatments with virus were incubated at 37 °C for 0, 0.5, 1, 2, 3, 6 and 24 h and the treatment reactions were stopped by the addition to DMEM-F12 cell-culture media containing 10% FBS, followed by ten-fold serial dilutions in DMEM-F12 cell-culture media containing 2% FBS. The virus titers were determined using standard plaque assays as described above. Each experiment was replicated thrice (each replicate on a different day) and assayed in duplicate.

2.7. Antiviral effects of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) containing BJ and B-PAC against AiV

AiV at titers of ~6 log PFU/ml was treated in a 1:10 ratio (0.1 ml of virus with 0.9 ml of treatments) with phosphate buffered saline (PBS; 7.2 as control), malic acid control (pH 1.5), simulated intestinal fluid (SIF; pH 7.5, Fisher Scientific, USA), simulated gastric fluid (SGF; pH 1.5, Fisher Scientific, USA), or B-PAC at a final concentration of 5 mg/ml prepared in SIF or SGF and incubated at 37 °C for 0, 0.5, 1, and 3 h. The reaction mixture was then added to 10 times volume of 200 mM sodium carbonate (Na_2CO_3) for neutralization as described earlier and further serially diluted in DMEM with 2% FBS (Takagi et al., 2003). This was followed by standard plaque assays in duplicate, and data from triplicate experiments carried out on different days were then statistically analyzed.

2.8. Statistical analysis

Data obtained from three replicates of each treatment and control of AiV were statistically analyzed using ANOVA with SAS software (v 9.3, SAS Institute, Cary, NC, USA) and Tukey's test with a completely randomized design model as described in previous studies (Joshi et al., 2016; 2017; Su et al., 2010a, b).

3. Results

3.1. Antiviral effects of BJ and B-PAC on AiV over 24 h at 37 °C

B-PAC at 1 mg/ml caused differences in AiV titers of 0.48 ± 0.01, 1.50 ± 0.06, 1.84 ± 0.24, 1.45 ± 0.25 and 2.13 ± 0.06 log PFU/ml at 37 °C after 0.5, 1, 3, 6 and 24 h at 37 °C, respectively that were lower compared to the titers of AiV controls in PBS (Table 1). Increased concentration of B-PAC showed increased titer differences compared to control AiV in PBS where 2 mg/ml B-PAC caused AiV differences 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 AiV titers were undetectable (≥3 log differences, all treatment titers were lower than control) after 6 and 24 h. The highest tested final concentration of B-PAC at 5 mg/ml was found to be the most effective that caused AiV titer differences of 1.39 ± 0.04 and 1.76 ± 0.54 log PFU/ml after 0.5 and 1 h, respectively, and to undetectable levels (≤2 log detection) after 3 h (i.e. lower titers) compared to controls in PBS (Table 1). Treatment of AiV at ~5 log PFU/ml with BJ at 37 °C resulted in titer differences of 0.17 ± 0.06, 1.27 ± 0.01, and 1.73 ± 0.23 log PFU/ml after 1, 3 and 6 h, and AiV was undetectable (≥3 log differences, lower titers than control) 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 titer differences after 1, 3, 6 and 24 h compared to the control AiV in PBS. Malic acid (pH 3.0, acid control) was shown to have minimal effects on AiV infectivity over time though to a much lesser extent than BJ within 6 h, causing titer differences of 0.08 ± 0.0, 0.25 ± 0.06 and 0.84 ± 0.14 log PFU/ml after 1, 3 and 6 h (all lower titers than control) and AiV was undetectable (≤2 log detection) after 24 h, indicating that the low pH of malic acid also contributes to the antiviral effect of BJ over extended time (Table 2).

3.2. Effect of BJ and B-PAC on viral adsorption and replication of AiV

Pre-treatment of host cells with 0.5 mg/ml B-PAC before viral infection caused AiV titers to be lowered by 0.62 ± 0.34 log PFU/ml compared to the control. Average recovered titers for the pre-treatment control with DMEM-F12 were 4.71 ± 0.04 log PFU/ml, while for pre-treatments with 0.5 mg/ml B-PAC were 4.09 ± 0.38 log PFU/ml.

Table 2
Effects of blueberry juice on Aichi virus over 24 h at 37 °C.

Time (Hours)	PBS[a]	Recovered titer (Log PFU/ml)		
		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}	Non-detectable levels*^{D}	Non-detectable levels *^{D}	4.20 ± 0.17^{A}

*- Detection limit of the assay is 2 log PFU/ml; Non-detectable levels: ≤2 log PFU/ml.
-Different letters denote significant differences between treatments ($P < 0.05$).
–Initial AiV titer was ~5 log PFU/ml.
[a] –PBS: phosphate buffered saline.

Treatment with 0.5 mg/ml B-PAC post-viral infection caused AiV titer differences of 0.30 ± 0.07 log PFU/ml compared to the control. Average recovered titers for the post-treatment control with DMEM-F12 were 4.73 ± 0.03 log PFU/ml, while for post-treatments with 0.5 mg/ml B-PAC were 4.43 ± 0.10 log PFU/ml. Thus, treatment of host-cells directly with B-PAC before viral infections suggests that B-PAC could play a role (albeit minor considering the lower concentration used) in binding host cells (receptors) or directly binding virus particles and blocking attachment or binding to host cell and preventing host cell entry. However, from the results of this study, there is no conclusive evidence that this might be the case and one can only speculate that either the viral receptors or the host cell receptors are potentially blocked/damaged to prevent some amount of viral entry to the host. Alternately, direct binding could affect the capsid structure, which could be also determined by using TEM analysis as was performed and described below.

3.3. Transmission electron microscopy (TEM) observations

No significant structural changes or damage was observed for the B-PAC treated viral particles (except for slight reduction in size with the 2 mg/ml B-PAC treatment) when compared to the viral particles in the water control based on the quality of the TEM images obtained (data not shown).

3.4. Effect of monomeric catechin and dimeric procyanidin B2 on AiV titers at 37 °C over 24 h

Differences in AiV titers of 0.36 ± 0.20, 0.88 ± 0.00 and 0.95 ± 0.04 log PFU/ml were obtained with monomeric catechin at 5 mg/ml at 37 °C that were lower when compared to the PBS control, after 3, 6 and 24 h, respectively. With lower concentrations of catechin at 1 mg/ml, AiV titer differences between treatment and control of 0.77 ± 0.02, 0.82 ± 0.01, and 0.66 ± 0.19 log PFU/ml (that were lower than the control) were obtained after 3, 6 and 24 h, respectively. Dimeric procyanidin B2 had minimal effect on AiV titers, showing differences in titers between treatment and control of 0.41 ± 0.21, 0.49 ± 0.13 and 0.67 ± 0.19 log PFU/ml with 1 mg/ml and 0.23 ± 0.04, 0.12 ± 0.03 log PFU/ml differences with 0.5 mg/ml at 37 °C that were all lower in comparison to PBS control titers after 3, 6 and 24 h, respectively (Table 3).

B-PAC at 1 mg/ml caused AiV titer differences of 2.45 log PFU/ml after 24 h, however catechin and procyanidin B2 at 1 mg/ml led to differences of 0.66 and 0.67 log PFU/ml after 24 h, that were all lower in comparison to the PBS controls. These comparisons suggest that the individual subunits alone do not contribute to the antiviral activity but B-PAC in its entirety with its polymeric structure is necessary to cause higher differences in AiV titers compared to the controls.

3.5. Antiviral activity of BJ and B-PAC in model food systems

B-PAC made in AJ showed increased antiviral activity where AiV titer differences of ≥3 log PFU/ml (i.e. AiV was decreased to undetectable levels) was obtained after treatment with 2 and 5 mg/ml B-PAC in AJ after 30 min at 37 °C, compared to the PBS control. However, the AJ control by itself did not cause any significant change in titers after 3 h (Table 4). Reduced antiviral activity of B-PAC prepared in 2% reduced fat milk was observed, where AiV titer differences of 0.14 ± 0.01, 1.40 ± 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 which was lower in titer compared to the controls. AiV titer differences of 0.29 ± 0.01, 0.39 ± 0.19 and 0.52 ± 0.17 log PFU/ml were obtained with 2 mg/ml B-PAC in milk after 3, 6 and 24 h, that were lower titers compared to PBS controls. The milk control alone showed differences of 0.48 log PFU/ml after 24 h (lower titer) compared to PBS (Table 5).

3.6. Effect of BJ and B-PAC in SGF and SIF against AiV titers

As reported in earlier studies (Joshi et al., 2017), both SGF and SIF were chosen to study the effect of treatments under different pH conditions (pH of SIF is 7.5 and pH of SGF is 1.5) and gut enzymes such as pepsin (present in SGF) and pancreatin (present in SIF) similar to that encountered *in vivo*. AiV did not survive in SGF and further experiments with B-PAC were not carried out. AiV survived in the SIF control after 30 min to 3 h, but AiV titer differences were ≥3 log PFU/ml (undetectable) with 5 mg/ml B-PAC in SIF within 30 min (lower titers) compared to PBS controls (Table 6), indicating the retention of antiviral activity in SIF.

4. Discussion

Blueberry proanthocyanidins show promise in decreasing AiV titers. The antiviral activity was found to be both time and concentration dependent similar to the previously demonstrated study with HNoV surrogates and HAV (Joshi et al., 2016). It is well documented that blueberries possess antibacterial and antiviral properties (Zafra-Stone et al., 2007). Methanolic extracts from blueberry leaves were also shown to inhibit hepatitis C virus (HCV) by binding to nuclear ribonucleoprotein A2/B1 in turn affecting the subgenome expression in replicon cells (Takeshita et al., 2009). Blueberry leaf proanthocyanidin was reported to suppress the expression of NS-3 (non-structural gene-3) protein gene in HCV (Takeshita et al., 2009). While, herpes simplex virus was also shown to be inhibited by prevention of adsorption to African green kidney monkey and human adenocarcinoma host cells with 0.03 to 0.1mg/ml hydrolysable and galloylated tannins (Fukuchi et al., 1989). FCV-F9 titers were reported to be reduced to undetectable levels after 5 min with 1, 2 and 5 mg/ml B-PAC and after 60 min with 0.5 mg/ml B-PAC and MNV-1 titers were reduced to undetectable levels only after 3 h with 1, 2 and 5 mg/ml of B-PAC (Joshi et al., 2016). HAV titers were reduced to undetectable levels after 30 min with 2 and 5 mg/ml B-PAC (Joshi et al., 2016). When compared to these viruses, AiV was found to be sturdier, where AiV was non-detectable only after longer incubation of 3 h, requiring both higher concentration of 5 mg/ml B-PAC (compared to MNV-1) and longer time (compared to FCV-F9).

Commercially available blueberry juice (pH 2.8) showed AiV titers to be decreased to non-detectable levels after 24 h, similar to malic acid (pH 3). However, neutralized BJ (pH 7) did not show significant differences in AiV titers even after 24 h at 37 °C, compared to the PBS controls. From these results, it can be postulated that the lower pH of 2.8 of BJ played a role in the AiV titer differences compared to the PBS control, and/or that the activity of B-PAC (level/concentration not reported in the commercial product) in BJ was optimal at lower pH. However, the possibility also exists that the presence of polyphenols and other components of BJ could also play a role in these effects when combined with low pH as reported earlier (Joshi et al., 2017). More

Table 3
Effect of monomeric catechins and procyanidin B2 on Aichi virus at 37 °C over 24 h.

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 ± 0.02A
6	4.46 ± 0.03 A	3.58 ± 0.03 B	3.64 ± 0.02 B	3.97 ± 0.16 A	4.34 ± 0.06A
24	4.30 ± 0.22 A	3.35 ± 0.18 B	3.64 ± 0.03 B	3.63 ± 0.03 B	4.33 ± 0.05A

*- Detection limit of the assay is 2 log PFU/ml: Non-detectable levels: ≤2 log PFU/ml.
-Different letters denote significant differences between treatments ($P < 0.05$).
–Initial AiV titer was ~5 log PFU/ml.
– PBS: phosphate buffered saline.

Table 4
Effect of blueberry proanthocyanidins prepared in apple juice on Aichi virus over 24 h at 37 °C.

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

*- Detection limit of the assay is 2 log PFU/ml.
Non-detectable levels: ≤2 log PFU/ml.
B-PAC: blueberry proanthocyanidins; AJ: apple juice; NAJ: Neutralized apple juice.
-Different letters denote significant differences between treatments ($P < 0.05$).
–Initial AiV titer was ~5 log PFU/ml.
[a] –PBS: phosphate buffered saline.

Table 5
Effect of blueberry proanthocyanidins prepared in 2% milk on Aichi virus over 24 h at 37 °C.

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	4.60 ± 0.13A	4.35 ± 0.05A	4.31 ± 0.12A	4.46 ± 0.11A
6	4.58 ± 0.30A	4.13 ± 0.02A	4.19 ± 0.11A	3.18 ± 0.16C
24	4.43 ± 0.05A	3.95 ± 0.24B	3.91 ± 0.22B	3.59 ± 0.08B

*- Detection limit of the assay is 2 log PFU/ml.
B-PAC: blueberry proanthocyanidins.
–Different letters denote significant differences between treatments ($P < 0.05$).
—Initial AiV titer was ~5 log PFU/ml.
[a] –PBS: phosphate buffered saline.

Table 6
Effect of blueberry proanthocyanidins prepared in simulated intestinal fluid (SIF) on Aichi virus over 24 h at 37 °C.

Recovered titer (Log PFU/ml)			
Time (Hours)	PBS[a]	SIF (pH 7.5)	B-PAC in SIF (5 mg/ml)
0.5	4.79 ± 0.14A	4.41 ± 0.05A	Non-detectable levels*B
1	4.75 ± 0.07A	4.44 ± 0.21A	Non-detectable levels*B
3	4.53 ± 0.05A	4.41 ± 0.05A	Non-detectable levels*B

*- Detection limit of the assay is 2 log PFU/ml.
-Non-detectable levels: ≤2 log PFU/ml.
–Different letters denote significant differences between treatments ($P < 0.05$).
—Initial AiV titer was ~5 log PFU/ml.
[a] –PBS: phosphate buffered saline; SIF: simulated intestinal fluid; B-PAC: blueberry proanthocyanidins.

recently, AiV titers were reported to be decreased by 0.5 and 0.9 log PFU/ml with undiluted aqueous hibiscus extracts (200 mg/ml) after 2 and 6 h at 37 °C, respectively (D'Souza et al., 2016). However, increased reduction form initial AiV titers of 5 log PFU/ml to non-detectable levels was obtained after 24 h at 37 °C with 40, 100 and 200 mg/ml aqueous hibiscus extracts, similar to the results of this study with blueberry juice. Though, AiV treated with 100 mg/ml and 40 mg/ml diluted aqueous hibiscus extracts at 37 °C after 6 h was reported to cause only ~0.3 log PFU/ml decreased in viral titers. Thus, blueberry juice and its associated proanthocyanidins have antiviral effects capable of decreasing AiV infectivity.

Other studies have reported that AiV is resistant to some inactivation treatments. Essential oil extracts from *Origanum acutidens* (oregano) were shown to be ineffective in inhibiting AiV replication *in vivo* (Sokmen et al., 2004). With regards to physical treatments, AiV was reported to remain fully infectious after a 5-min treatment at 600 MPa high hydrostatic pressure in MEM (minimum essential medium) supplemented with 2% FBS as well as 10% FBS (Kingsley et al., 2004). While AiV reductions on lettuce, green onions, and strawberries after treatment with ultraviolet light at 240 mW s/cm^2 were 4.59, 2.49, and 1.87 log TCID50/ml, respectively (Fino and Kniel, 2008). This suggests that AiV is quite resilient to physical and some chemical inactivation treatments. Thus, the data on the antiviral effects of B-PAC against AiV shows promise for further research applications.

Other commonly used inactivation methods were recently tested against AiV (Cromeans et al., 2014). These researchers showed that heat treatment of 56 °C for 20 min decreased AiV titers by 4 log PFU, while AiV was shown to be resistant to alcohol treatments including 70% and 90% isopropanol and 70% and 90% ethanol, where less than 0.5 log PFU reductions in titers were noted by these researchers after 1 and 5 min using suspension tests. They also showed that 200 and 1000 ppm chlorine treatment for 5 min of AiV dried on stainless steel discs caused merely 0.5 log reduction in AiV RNA levels, and that AiV was highly resistant to high hydrostatic pressure treatment of 800 MPa without any significant change in infectivity, similar to the results reported by earlier studies of Kingsley et al., (2004). In addition, Cromeans et al. (2014) reported that AiV was quite stable after treatment with 100 mM citric acid buffer at pH 2, 3, 9 and 10 for 30 min at 37 °C in liquid suspension (Cromeans et al., 2014). These observations indicate the hardiness of this virus and that alternative methods of inactivation are necessary to prevent its spread. In this study, AiV titers remained undetectable after B-PAC treatments albeit after 3 or more hours with 5 mg/ml B-PAC at 37 °C.

To gain insights into the potential mode of action of B-PAC against AiV, the antiviral effects between monomeric, dimeric and polymeric catechins were compared. It was shown that B-PAC at 1 mg/ml could cause differences in AiV titers of 2.45 log PFU/ml that were lower in titers compared to the PBS control after 24 h at 37 °C. However, catechin and procyanidin B2 at 1 mg/ml caused only 0.66 and 0.67 log PFU/ml differences compared to the control after 24 h at 37 °C, respectively. Thus, PAC in its entirety with its intact polymeric structure appears to be necessary to decrease AiV titers and viral infectivity, and not just the individual monomeric units, somewhat similar to previous antiviral studies (Liu et al., 2018). Thus, the stability of B-PAC over time needs to be understood and during digestion to determine the optimal antiviral effects.

Furthermore, to determine the mode of action, pretreatment of Vero host cells with 0.5 mg/ml B-PAC before viral infection was carried out that resulted in 0.62 ± 0.34 log PFU/ml lower titer than the control. However, there is no concrete evidence on the potential to prevent infection by either destroying the viral receptors to prevent binding to the host cells or blocking the host cell receptors to prevent attachment of the virus. Further studies using TEM indicated that there was some alteration in the structure of the capsid, but these results are not conclusive as the quality of the TEM images was poor. In order to determine if B-PAC could be administrated after infection of host cells and prevent AiV replication in the host cell, 0.5 mg/ml B-PAC was added after AiV infection that resulted in a 0.30 ± 0.07 log PFU/ml lower titer compared to the control, indicating a moderate effect on inhibiting AiV replication.

When B-PAC was prepared in apple juice as a model food system, enhanced antiviral activity was observed where 2 mg/ml B-PAC caused AiV titers to be undetectable after 30 min, while there was a difference of 0.93 ± 0.10 log PFU/ml with 2 mg/ml B-PAC made in 10% ethanol after 1 h (lower titer) lower than that of the PBS control. The increased or synergistic effect could potentially be associated with the low pH of apple juice and natural polyphenols/components within the apple juice. Apple juice has been previously reported to contain hydroxycinnamic acids and chlorogenic acid as the main polyphenols that can have antiviral and antimicrobial effects as reported earlier (Kahle et al., 2005; Joshi et al., 2016, 2017). When B-PAC was prepared in 2% milk, a decline in the antiviral activity was observed. Compared to AiV titers that were undetectable with 5 mg/ml B-PAC prepared in 10% ethanol after 6 h, a lower titer difference of only 1.40 ± 0.14 log PFU/ml was obtained with 5 mg/ml B-PAC in 2% milk in comparison to PBS controls. It has also been suggested that the difference in activity could be due to the inhibition of antiviral activity caused by binding of B-PAC to proteins and lipids present in milk as reported earlier (Joshi et al., 2017). This is not surprising as previous research on the antiviral activity of grape seed extract (GSE) in the presence of bovine serum albumin (0.3 g/liter) showed decreased activity, suggesting that the decrease in the effectiveness of treatments could be due to or associated with the presence of high organic load (Li et al., 2012). Furthermore, GSE at 1, 2 and 4 mg/ml prepared in 2% milk, did not cause any significant reduction in FCV-F9 titers after 6 h at 37 °C (Joshi et al., 2015). GSE at 1, 2 and 4 mg/ml prepared in 2% milk caused 0.92 ± 0.01, 0.96 ± 0.02 and1.07 ± 0.01 log PFU/ml reduction of FCV-F9 titers after an increased incubation time of 24 h, respectively, without significant reduction in MNV-1 titers even after 24 h (Joshi et al., 2015). Similarly, 5 mg/ml B-PAC in 2% milk caused only 0.81 log PFU/ml reduction of MNV-1 and 1.09 log PFU/ml reduction of FCV-F9 after 24 h at 37 °C (Joshi et al., 2017). In addition, higher fat content of milk or food components was shown to inhibit the antimcrobial effects of test agents as shown in the study with cinnamon bark essential oil at 1000 ppm where a 1 log CFU/ml reduction of *L. monocytogenes* in whole milk was obtained, while a 3 log CFU/ml reduction in skimmed milk was reported (Cava et al., 2007).

In the present study, the 2% milk control was shown to cause 0.46 log PFU/ml difference (lower titer) in AiV titers after 6 h compared to the PBS controls. As reported earlier (Joshi et al., 2015, 2017), previous studies have shown that milk components like lactoferrin can inhibit adsorption of hepatitis C virus (HCV) (Ikeda et al., 2000), bind rotavirus particles and inhibit host cell adsorption (Superti et al., 1997), and inhibit poliovirus entry into host cells by receptor blocking (Marchetti et al., 1999). Thus, if B-PAC is prepared in or added to a food matrix that has components with antiviral activity, synergistic antimicrobial effects can be capitalized to make it a better antiviral and/or antimicrobial alternative as reported earlier (Joshi et al., 2017). Additionally, as suggested by several studies with antimicrobials, B-PAC can be encapsulated or formulated with other synergistic agents as an antiviral therapeutic to help retain antiviral activity against AiV and other enteric viruses even in presence of food matrices or perhaps in the presence of higher organic load (Joshi et al., 2015, 2016, 2017).

Additionally, as in previous studies (Joshi et al., 2015, 2017) experiments were carried out under simulated gastrointestinal conditions to understand its antiviral effect as a potential therapeutic after consumption. AiV was undetectable within 30 min with 5 mg/ml B-PAC in simulated intestinal fluid (SIF). Since AiV did not survive in the simulated gastric fluid (SGF), further experiments with AiV and B-PAC in SGF were not undertaken. This was similar to the results for the cultivable HNoV surrogates where B-PAC at 5 mg/ml in SIF was also reported to decrease titers of FCV-F9 and MNV-1 to undetectable levels within 30 min at 37 °C (Joshi et al., 2017).

In summary, this study showed AiV titer differences of 1.39 and 1.76 log PFU/ml after 0.5 and 1 h and AiV titer differences of ≥3 log PFU/ml (undetectable levels) after 3 h with 5 mg/ml B-PAC, with titers that were lower than PBS controls. Thus, overall B-PAC shows promise to decrease AiV titers with the potential to prevent AiV infections and/or alleviate the illness symptoms associated with AiV infections. However, further clinical trials have to be undertaken before any recommendations can be made as in the case for any new natural product as reported for other studies (Joshi et al., 2016, 2017).

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Appendix A. Supplementary data

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