Antiviral Effect of Extract from Fagopyrum buckwheats Against Two Nonenveloped Viruses

Xueling Shao¹, Lu Wang², Sydney W. Gu¹, Tinchun Chu³ and Stephen Hsu¹,4,*

¹Department of Oral Biology and Diagnostic Sciences, Dental College of Georgia, Augusta University, Augusta, GA.
²DST Pharma, Inc., Duluth, GA.
³Seton Hall University, South Orange, NJ.
⁴Camellix Research Laboratory, Augusta GA.

Correspondence: Stephen Hsu, Department of Oral Biology and Diagnostic Sciences, Dental College of Georgia, Augusta University, Augusta, GA.

Received: 29 April 2020; Accepted: 18 May 2020


ABSTRACT
Nutraceuticals have been a rich source of prophylactic and therapeutic benefits to humans against various diseases. Golden buckwheat (Fagopyrum dibotrys [F. dibotrys or F. dibityo]) has been used in Chinese traditional medicine and herbal medicine to treat inflammatory conditions. Extracts from the leaves and roots of the plant contain unique combinations of compounds that may have potential for future medications due to their anti-cancer, anti-oxidant, anti-inflammatory, anti-aging, and hepatoprotective properties. However, the potential antiviral activity of the plant extract has not been explored. The purpose of the current study is to investigate if Golden buckwheat or 5,7,3’,4’-tetrahydroxyflavan-3-ol C4-C8 dimer, the major compound of Golden buckwheat, possess antiviral activity against alcohol-resistant nonenveloped viruses such as norovirus and hepatitis A viruses. Methods used in the study include cytotoxicity assay (MTT assay), viral infection assays, and TCID50 assay. The results demonstrate that a single dose of both the extract of Golden buckwheat and 5,7,3’,4’-tetrahydroxyflavan-3-ol C4-C8 dimer (双聚原矢车菊甘元 in Chinese) are able to inhibit feline calicivirus (a surrogate for human norovirus) and human hepatitis A virus, when added either before viral infections or after viral infections without cytotoxicity. To the best of our knowledge, this is the first discovery that Golden buckwheat and its major component 5,7,3’,4’-tetrahydroxyflavan-3-ol C4-C8 dimer exhibit strong antiviral activities against nonenveloped viruses causing humans acute symptoms. As of today, there is no therapeutic method to treat norovirus or hepatitis A virus infection, these nutraceuticals may provide solutions for future prophylactic and therapeutic methods, pending future research and development.

Keywords
Golden buckwheat, Fagopyrum, Nutraceuticals, 5,7,3’,4’-tetrahydroxyflavan-3-ol C₄-C₈ dimer, Norovirus, Hepatitis A virus.

Introduction
Nutraceuticals, a word invented by Dr. Stephen L. DeFelice, has been defined as naturally occurring dietary substances that could provide medicinal or health benefits to prevent and treat diseases [1]. Nutraceuticals may include food and foodstuffs, dietary regimen, nutrition supplements, herbal products, functional foods, fortified foods, and dietary supplements [2]. Many plant extracts belong to this category of naturally occurring compounds that possess medicinal properties. The genus Fagopyrum belongs to the flowering plant family Polygonaceae, which comprises 15 species mainly found in the northern hemisphere. There are 10 buckwheat species present in China, with three important species: Fagopyrum esculentum (F. esculentum) Moench. (common buckwheat), Fagopyrum tataricum (F. tataricum) (L.) Gaertn. (tartary buckwheat), and Fagopyrum dibotrys (F. dibotrys or F. dibityo) (D. Don) Hara. (perennial buckwheat or Golden buckwheat) [3]. These Fagopyrum buckwheats contain flavonoids, phenolics, fagopyritols, triterpenoids, steroids and fatty acids, and have been used in traditional Chinese medicine for multiple ailments and conditions [3]. Previous studies have demonstrated that Fagopyrum buckwheats and their extracts possess many bioactive properties including anti-tumor, anti-oxidant, anti-inflammatory, anti-aging, hepatoprotective, hypoglycemic, anti-allergic, and anti-fatigue activities [4-11]. In 1974, Fagopyrum buckwheats (F. dibotrys or F. dibityo, Golden buckwheats) was reported to
treat acute inflammation in a clinical trial, and the major active ingredient was identified as 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer [12], and referred to as a new flavonoid in 1987 [13]. Other major compounds found in golden buckwheat include rutin, quercetin, and hecogenin [3, 14].

However, the potential anti-viral effects of Golden buckwheat in either extract form or purified compound form of the major component were not reported. The purpose of the current study is to investigate if Golden buckwheat extract (referred to as GBE hereafter) or 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer exhibit antiviral activity that may provide potential therapeutic methods for viral infections. The goal of the current study is to evaluate the potential antiviral activities of GBE and 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer against feline calicivirus (FCV) and human hepatitis A virus (HAV). The rationale for testing FCV and HAV is that FCV is a well-recognized test surrogate for human norovirus. Both norovirus and HAV are nonenveloped viruses, and they both cause acute illness in humans. Currently, there is no vaccine or therapeutic for treatment of norovirus infection. If GBE or 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer demonstrates antiviral activity against norovirus, testing another nonenveloped virus causing human acute disease could unveil the potential broad spectrum antiviral activities for the nutraceuticals. If GBE or 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer possess antiviral activity against HAV, which infection also lacks treatment method, they could have a broad-spectrum of antiviral activities against hepatitis viruses and other viruses. Therefore, the class of plants and their phytochemicals warrant further studies to discover the potential antiviral properties for the use of treatment and prevention of viral infection associated with human illnesses.

Material and Methods

Cells and viruses

Human hepatitis A virus (VR-1402), feline calicivirus (VR-782), FRhk-4 feline rhesus monkey kidney cells (CRL-1668) and CRFK cat kidney cells (CCL-94), DMEM medium (30-2002), and EMEM medium (30-2003) were purchased from ATCC. Cell culture, viral propagation and harvesting were performed according to supplier’s protocols, which received approval from Augusta University Institutional Biosafety Committee. GBE was purchased from Xi’an Orient Biotechnology, Co., Ltd., China. Purified 5,7,3’,4’-tetrahydroxyflavan-3-ol C7-C8 dimer (referred to “dimer” here after) were provided from Changxing Sanju Biotechnology Ltd, China.

MTT assay

Cell viability assay (MTT assay) was performed according to the method previously described [24]. Briefly, cells were cultured in a 96-well plate until confluent. Cell culture medium containing specific agents were incubated with the monolayer of the cells for 1 h before the medium was changed. After overnight incubation, the plate was removed from the cell culture incubator and an MTT assay was performed as described [24].

To determine if GBE is associated with cytotoxicity, GBE was dissolved in either EMEM or DMEM for CRFK or FRhK cells respectively. Cells were grown in 96 well plate until 90% confluent. GBE in EMEM or DMEM was added to the wells in quadruplicates at 0, 0.1, 1, and 2%, followed by incubation overnight. MTT assay was performed on the cells, and cell viability was calculated as previously described [24].

Viral infectivity assays

TCID50 assay (50% tissue culture infectious dose assay) was used to determine viral titers and the inhibitory effects of the agents. The infectivity of HAV and FCV was measured with or without treatment of the plant agents to determine three aspects of the antiviral capabilities: pre-infection, simultaneous infection, and post-infection. Pre-infection experiments test the viral infectivity after cells are pre-treated with the plant agents for 1 h, followed by viral infection at different dilutions for 1 h, before TCID50 assay. Simultaneous infection experiments test the effect of the plant agent in direct contact with the virus, when they were mixed prior to infecting the cells for 1 h, in different dilutions, followed by TCID50 assay. Post-infection experiments test the viral infectivity with the addition of the plant agents after 1 h of viral infection at different dilutions, followed by TCID50 assay. Controls of the assay were uninfected (negative) and infected but untreated cells (positive). All assays were repeated three times independently.

Pretreatment of CRFK cells with different concentrations of GBE for 1 h before TCID50 assay

CRFK cells were plated in 96-well tissue culture plates in EMEM culture medium with 10% fetal bovine serum (FBS) and antibiotics at 37°C, 5% CO₂. When cells covered the surface of each well to >90%, GBE dissolved in EMEM culture medium with 10% FBS was added at 0, 0.1, 0.2 and 1% in triplicates of 100 µl/well, followed by incubation for 1 h. GBE medium then was removed and FCV was added at different dilutions by Hanks balanced salt solution (HBSS) from 10⁻⁵ to 10⁻⁷. After 1 h of absorption, virus was removed and EMEM culture medium with 0.2% FBS was added to each well. Cytopathic effect (CPE) was recorded and result was calculated after 5 days.

GBE and FCV added to cells simultaneously

GBE was dissolved in EMEM containing 10% FBS. To 0.45 ml of the medium containing GBE at 0, 0.1, 0.2 or 1%, 50 µl FCV was added and incubated for 1 h at room temperature. 100 µl of the mix was added to 0.9 ml EMEM containing 10% FBS. This is a 10⁻² viral/GBE mix. A series of dilutions of this mix at 10⁻² to 10⁻⁴ was made, and 100 µl from each dilution in quadruplicates was added to the wells and incubated for 1 h. The mix was replaced with EMEM containing 0.2% FBS, and the plate was incubated for at least 5 days in a tissue culture incubator with 5% CO₂ at 37°C, until CPE was observed for TCID50 assay.

GBE added after FCV viral infection of CRFK cells

CRFK cell monolayer in a 96-well plate was infected with FCV in a series dilution from 10⁻⁴ to 10⁻⁶ in quadruplets in HBSS. After 1 h incubation, the virus/HBSS was replaced with GBE-containing
EMEM with 10% FBS, and incubated for 1 h prior to medium change of EMEM containing 0.2% FBS. CPE was observed from day 5 under a microscope and TCID50 values were calculated.

HAV infectivity assay was conducted in 48-well plates. FRhK-4 cells in complete DMEM medium containing 10% FBS were plated in each well to allow the cells to form a monolayer. To measure the viral titer, 50 µl HAV virus was added to 450 µl HBSS. This is 10^{-1} dilution of viral mix. A series of dilutions by adding 100 µl of the mix to 900 µl of HBSS up to 10^{-6}. To a 48-well plate, 250 µl from each dilution (10^{-3} to 10^{-6}) was loaded to the designated three repeating wells per dilution. After 1 h absorption, the virus/HBSS mix was removed and DMEM containing 2% FBS was added to each well. The plate was incubated for at least 8 days in a tissue culture incubator with 5% CO₂ at 35°C until CPE was observed for TCID50 assay calculation.

**Pre-infection viral infectivity assay**

GBE dissolved in DMEM containing 2% FBS was added to FRhK cell monolayer in a 48 well plate at 0, 0.1, 0.2 and 1% and incubated for 1 h. The GBE/medium was then replaced with a series dilution of HAV from 10^{-3} to 10^{-6} in HBSS in triplicates per each dilution, and the virus was allowed to be absorbed for 1 h before the virus was removed, and DMEM containing 2% FBS was added to each well. The plates were incubated at 35°C with 5% CO₂. On day 5, the medium was changed. CPE was observed from day 8 under a microscope and TCID50 result was calculated.

**GBE and HAV virus were added to cells at the same time**

GBE was dissolved in DMEM containing 2% FBS. To 0.45 ml of the medium containing 0, 0.1, 0.2, and 1% GBE, 50 µl HAV was added, and incubated for 1 h at room temperature. 100 µl of the mix was added to 0.9 ml DMEM containing 2% FBS. This is 10^{-2} viral/GBE mix. A series of dilutions of this mix were made at 10^{-3} to 10^{-6}. 250 µl of each dilution was loaded to each well in triplicates and incubated for 1 h. The mix was replaced with DMEM containing 2% FBS, and the plate was incubated for at least 8 days in a tissue culture incubator with 5% CO₂ at 35°C until CPE was observed for TCID50 assay.

**Post-infection assays**

FRhK cell monolayer in a 48-well plate was infected with HAV in a series dilution from 10^{-3} to 10^{-6} in triplicates in HBSS. After 1 h incubation, the virus/HBSS was replaced with GBE-containing DMEM and incubate for 1 h prior to medium change of DMEM containing 2% FBS. CPE was observed from day 8 under a microscope and TCID50 was calculated.

**Infectivity assays of 5,7,3’,4’-tetrahydroxyflavon-3-ol C₄-C₈ dimer against FCV and HAV**

Procedures are identical to the infectivity assays using GBE except the concentration used was 0.1%.

**Statistical analysis**

All assays were performed three times. The paired t tests were used to analyze the data between treatment and control at the p < 0.05 level of significance. One-way analysis of variance (ANOVA) was carried out to analyze the samples treated with different concentrations before, simultaneously, or after viral infection, respectively.

**Results**

**Cell viability test results after incubation with GBE**

To determine if GBE is associated with cytotoxicity, GBE was dissolved in either EMEM or DMEM for FCV F9 or HAV viral infections in CRFK or FRhK cells, respectively. Figure 1 demonstrates that GBE did not reduce cell viability in CRFK cells even at 2%. Statistical analysis was performed using one-way ANOVA and t-test. The results demonstrate that there is a significant difference among all samples tested ANOVA p=0.015). Result from t test indicates that the only differences among samples are between 2% and control (0%), and 2% and 0.5% (p<0.05). That is, GBE at 2% significantly increased cell viability in CRFK cells.

**GBE and HAV virus were added to cells at the same time**

GBE was dissolved in DMEM containing 2% FBS. To 0.45 ml of the medium containing 0, 0.1, 0.2, and 1% GBE, 50 µl HAV was added, and incubated for 1 h at room temperature. 100 µl of the mix was added to 0.9 ml DMEM containing 2% FBS. This is 10^{-2} viral/GBE mix. A series of dilutions of this mix were made at 10^{-3} to 10^{-6}. 250 µl of each dilution was loaded to each well in triplicates and incubated for 1 h. The mix was replaced with DMEM containing 2% FBS, and the plate was incubated for at least 8 days in a tissue culture incubator with 5% CO₂ at 35°C until CPE was observed for TCID50 assay.

**Post-infection assays**

FRhK cell monolayer in a 48-well plate was infected with HAV in a series dilution from 10^{-3} to 10^{-6} in triplicates in HBSS. After 1 h incubation, the virus/HBSS was replaced with GBE-containing DMEM and incubate for 1 h prior to medium change of DMEM containing 2% FBS. CPE was observed from day 8 under a microscope and TCID50 was calculated.
Results of inhibitory effects of GBE on feline calicivirus (FCV) F9

Pretreatment of CRFK cells with different concentrations of GBE for 1 h before TCID50 assay. Figure 3 demonstrates that results from three independent experiments indicate a significant inhibition of viral infection at all concentrations of GBE, even though there was no direct contact of GBE and virus. Specifically, GBE at all concentrations reduced FCV F9 infection by more than 50% if GBE was incubated with CRFK cells for 1 h before FCV F9 infection (n=3, p<0.01). There is no statistical difference among all concentrations (ANOVA, p=0.62).

GBE and FCV were added to cells at simultaneously

Figure 4 shows that GBE at all concentrations significantly inhibited FCV viral infection in CRFK cells. Data was obtained from 3 independent experiments. The interesting observation is that lower concentration of GBE has significant higher efficacy than higher concentrations. Specifically, GBE at 0.1% showed significantly higher efficacy (17.79% ± 0 infectivity) than other concentrations (48.00% ± 14.16 and 52.58% ± 14.16 infectivity). ANOVA indicate the differences are statistically significant (p=0.0048). There is no statistical difference between 0.2% and 1%.

In summary, FCV F9, a surrogate of human norovirus that is resistant to alcohol, can be effectively inhibited by GBE with different incubation methods.

Results of Effects of GBE on human hepatitis A virus (HAV)

Pretreatment of FRhK cells with different concentrations of GBE for 1 h before TCID50 assay.

Figure 5 shows the results from three independent experiments that without direct contact with the virus, GBE significantly lowered FCV F9 infection. Specifically, GBE at all concentrations significantly reduced FCV F9 infection (n=3, p<0.001). There is no statistical difference among the concentrations.

Results of inhibitory effects of GBE on human hepatitis A virus (HAV)

Pretreatment of FRhK cells with different concentrations of GBE for 1 h before TCID50 assay. Figure 6 demonstrates the results from three independent experiments. GBE at all concentrations led to a significant inhibition of HAV viral infection, even though there was no direct contact of GBE and virus. Statistical analysis shows that GBE at 0.2% and 1% significantly reduced HAV infection rate in FRhK cells (n=3, p<0.05). At 1%, pre-incubation of GBE with FRhK cells for 1 h reduced HAV infection rate to 26.59%.
FRhK cells after the cells were pre-treated with different concentrations of GBE for 1 h. Reduction of viral infectivity values are: 0.1% GBE, 59.09% ± 23.15, 0.2% GBE, 34.94% ± 18.57, and 1% GBE, 26.59% ± 20.64.

On the other hand, there is no statistical difference among the concentrations on the effect of HAV (ANOVA, p=0.221). The result indicates that GBE is effective in reducing HAV infection of FRhK cells if GBE is incubated with FRhK cells prior to HAV infection, and the apparent dose response is statistically insignificant.

GBE and HAV virus were added to cells simultaneously.

Figure 7 shows that GBE at all concentrations significantly inhibited FCV F9 viral infection in CRFK cells. Data was obtained from three independent experiments. The interesting observation is that lower concentration of GBE has a higher efficacy than higher concentrations. Statistical analysis using t-test shows that GBE at all concentrations significantly reduced HAV infection rate in FRhK cells (n=3, p<0.05). There is no statistical difference among the concentrations on the effect of HAV (ANOVA, p=0.426). The result indicates that GBE is effective in reducing HAV infection of FRhK cells when GBE was incubated with HAV and FRhK cells during HAV infection.

Figure 7: Result of the inhibitory effect of GBE on FRhK cells after the cells were infected by HAV in the present of different concentrations of GBE. At 0.1%, the infectivity was reduced to 21.37% ± 0. At 0.2%, the viral infectivity was reduced to 33.13% ± 11.56, while 1% GBE reduced the viral infectivity to 29.47% ± 14.02.

GBE added after HAV infection of FRhK cells

Figure 8 shows the results from three independent experiments. Without direct contact with the virus, GBE at all concentrations significantly lowered HAV infection. Statistical analysis shows that GBE at all concentrations significantly reduced HAV infection rate in FRhK cells (n=3, p<0.05). Result from one way ANOVA shows there is no difference among GBE concentrations (p=0.183). The result indicates that GBE is effective in reducing HAV infection of FRhK cells after HAV infection.

In summary, HAV can be effectively inhibited by a single dose of GBE using different incubation methods. HAV is one of the most difficult to inactivate virus due to its size and nonenveloped structure similar to poliovirus and feline calicivirus. Thus, GBE is a strong inhibitor of HAV.

Results of the Effect of C. 5,7,3',4'-tetrarydroxyflavon-3-ol C$_4$-C$_8$ dimer on FCV and HAV

We used purified 5,7,3’,4’-tetrarydroxyflavon-3-ol C4-C8 dimer to test on the inhibitory effect of human hepatitis A virus (HAV) to see if they are able to inhibit this non-enveloped virus. The method used for viral inhibition was identical to the method described above, except the concentration used for the purified compounds was 0.1%.

Figure 9 demonstrates that 5,7,3’,4’-tetrarydroxyflavon-3-ol C4-C8 dimer is effective against FCV infection comparable to quercetin, better than rutin. ANOVA indicates there is no statistical difference between the control and pre-treatment (p=0.071). But both post-treatment and simultaneous treatment significantly inhibited viral infectivity (p<0.002).

Figure 9: Results of the inhibitory effects of 5,7,3’,4’-tetrarydroxyflavon-3-ol C$_4$-C$_8$ dimer on FCV F9 using three different treatment methods. Before, CRFK cells were pre-treated with 5,7,3’,4’-tetrarydroxyflavon-3-ol C$_4$-C$_8$ dimer for 1 h prior to FCV F9 infection with reduction of 33.87% ± 45.76*. Sametime, the dimer was mixed with the virus prior to dilution and infection, infectivity reduced by 38.11% ± 14.54. After, the dimer was added after viral infection, 28.44% ± 15.82. *only two data points available with large variation.
Noroviruses are a group of single-stranded, positive sense RNA nonenveloped viruses constituting the Norovirus genus in the family Caliciviridae [16]. Noroviruses have been recognized as the most important cause of viral epidemic acute gastroenteritis affecting people of all ages. In the United States, norovirus cases cause 19 to 21 million cases of acute gastroenteritis each year, leading to 1.7 to 1.9 outpatient visits and 400,000 emergency department visits each year, and contribute to about 56,000 to 71,000 hospitalizations and 570 to 800 deaths, mostly among young children and the elderly (US CDC, U.S. Trends and Outbreaks). Norovirus is the leading cause of foodborne illness in the United States. On a worldwide basis, noroviruses lead to 218,000 deaths in developing countries and 1.1 million episodes of pediatric gastroenteritis annually in developed countries [17]. Thus, norovirus associated diseases have been a heavy burden to public healthcare. Noroviruses are difficult to control owing to their widespread nature and the lack of effective vaccines and antiviral drugs.

Transmission of these highly infectious plus-stranded RNA viruses occurs primarily through contaminated food or water, but also through person-to-person contact and exposure to objects that have been contacted with the virus. Symptoms of norovirus include fever, cramps, head and body aches, along with profound gastroenteritis, diarrhea, and vomiting. Symptoms can arise gradually or abruptly and usually resolve within 48 to 72 h. Currently there is no treatment for norovirus [18]. During an active norovirus infection, it is important for the infected person to intake a sufficient amount of fluids to avoid dehydration. Intravenous fluid delivery is necessary if the infected person is not able to drink sufficient fluids. Loss of fluid due to vomiting and diarrhea can lead to severe dehydration, and if untreated, it may lead to more severe complications and even death [19].

Hepatitis is an inflammation of the liver caused by hepatitis viruses and other infections, toxic substances like alcohol and drugs, and autoimmune diseases. The five main hepatitis viruses are: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). HAV and HEV cause acute hepatitis, while HBV, HCV, and HDV are the cause of chronic viral hepatitis. Chronic infection with hepatitis can lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma if left untreated [20]. HBV and HCV are responsible for 96% of mortality from viral hepatitis. According to the World Health Organization (WHO), in 2015, viral hepatitis was estimated to have caused 1.34 million deaths worldwide, which is a 22% increase since 2000. HAV infection causes acute hepatitis as one of the most common infectious diseases worldwide. According to WHO, HAV infection resulted in 13.7 million illnesses and 28,000 deaths in 2010 [21,22]. Unlike HBV and HCV, HAV is a positive single-stranded, nonenveloped ribonucleic acid (RNA) virus [23].

To the best of our knowledge, we report for the first time that GBE and its major component 5,7,3’,4’-tetrahydroxyflavan3-ol C4-C8 dimer possess significant antiviral activities against FCV, a surrogate for human norovirus, and HAV, which causes acute hepatitis in humans. In addition, GBE is not cytotoxic to mammalian cells at or under 2% (Figures 1 and 2). At the concentration range of 0.1% to 1%, a single dose of GBE significantly reduced FCV infection regardless if administered before, simultaneously, or after FCV infection in CRFK cells (Figures 3, 4, 5). FCV F9, as well as human norovirus, are among the most difficult to inactivate viruses due to their sizes and nonenveloped structure similar to poliovirus. Thus, GBE can be categorized as a strong inhibitor of FCV/norovirus. Treatment of CRFK cells for 1 h without direct contact to the virus led to >50% reduction in infected cells by FCV (Figures 3). When GBE was mixed with FCV and infected the cells, a protective effect was observed, with the low dose (0.1%) showing a higher effect than the higher doses (Figures 4). This result suggests that contact inhibition may not be dose-dependent. In fact, dose ranges of 0.1, 0.2 and 1% did not show a
dose-dependent pattern for FCV viral infection. A single dose of GBE added after FCV infection inhibited >50% viral infection of the cells, without significance among the doses (Figure 5). These results suggest that GBE, a documented Chinese herbal remedy, could be used as an herbal medicine to treat norovirus infection, pending additional research and development.

Similarly, GBE significantly inhibited HAV infection of FRhK cells regardless if administered before or after HAV infection or added as a mixture with the virus (Figures, 6, 7, 8). Again, dose-dependent effect was not apparent, and lower concentration (0.1%) showed a higher activity when GBE was mixed with HAV to infect the cells (Figure 7). This observation also suggests that lower concentration leads to a greater contact inhibition as shown in FCV test result (Figure 4). The underlying mechanism of this observation needs further investigation. It is interesting to know that Golden buckwheat used in Chinese herbal medicine was prepared in boiling water for an extended period of time to extract the contents from parts of the plant prior to oral administration. The dose of GBE in water (approximately 250 ml) going through the digestive system and absorbed into the blood stream is diluted into low concentration ranges. The above results indicate that Golden buckwheat and its chemical contents could be used to effectively prevent or treat norovirus and HAV infections.

In summary, GBE is able to inhibit FCV and HAV without cytotoxicity. We also determined the antiviral activities of the major compound present in GBE: 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer. The compound can be extracted from GBE by water extraction. This compound also has strong activity against FCV and HAV at 0.1% concentration (Figures 9 and 10). When cells were treated with 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer either during or after viral infection, cells infected by FCV were reduced to <40% (Figure 9). The inhibition effects of the compound at 0.1% is comparable to that of GBE, except for direct contact inhibition (same time added to cells). This result suggests that the contact inhibition of FCV demonstrated by GBE may rely on a combination of the compounds present in GBE.

Surprisingly, 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer demonstrated very strong inhibitory effects against HAV, especially in the post-infection of the cells, with >98% cells protected (Figure 10). In addition, the contact inhibition was also about ten-fold higher than that of GBE, with only 4.61% cells infected vs. 55.16% (Figure 10 vs. Figure 8). These results suggest that 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer may be suitable for therapeutic use against HAV infection due to the potency shown here. In comparison to norovirus, HAV is a relatively slowly growing virus, similar to HBV and HCV (incubation time 15-45 days, 30-180 days, and 15-150 days, respectively) [24]. These results suggest that the efficacy of 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer can serve as a base to explore therapeutic drug development. As of today, there is no effective drug for HAV infection. Drugs for HBV, based on chemical or biologicals, i.e. nucleotide analogues or interferon, are not associated with high efficacy, especially at late stages of the diseases. On the other hand, HCV infection can be cured with newly developed drugs [24].

In conclusion, we discovered that GBE and its major component 5,7,3′,4′-tetrarydroxyflavon-3-ol C₄-C₈ dimer, possess strong antiviral properties against norovirus (FCV as human norovirus surrogate) and HAV, which are two nonenveloped viruses causing acute symptoms without treatment. These results suggest that compounds derived from buckwheat family (Fagopyrum, part of the flowering plant family Polygonaceae) could be used, either as a crude extract, or as purified phytochemicals to prevent and/or treat norovirus or hepatitis virus infections. The antiviral mechanisms, as well as whether they have a broad-spectrum antiviral activity require additional studies. Since these nutraceuticals have been widely used in human populations, this discovery may lead to new drug development against viral hepatitis and norovirus infection pending further work such as animal model safety and efficacy tests, and eventually leading to clinical trials.

Acknowledgement
This work was supported by a grant from DST Pharma to SH, and in part by the Dental College of Georgia, Augusta University. The authors thank Larry Wang for proof-reading and edits.

References