



Anti-influenza activity of elderberry (*Sambucus nigra*)

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ABSTRACT

Elderberry extract is effective in treatment of flu. This study aimed to determine the mechanism of action of elderberry and its primary active compound, cyanidin 3-glucoside (cyn 3-glu), against influenza virus. The direct effect was studied via hemagglutination inhibition assay, plaque reduction assay, and flow cytometry analysis. In addition, to assess the indirect immunomodulatory effect, the modulation of pro-inflammatory cytokines was evaluated. Elderberry showed mild inhibitory effect at the early stages of the influenza virus cycle, with considerably stronger effect (therapeutic index of 12 ± 1.3) in the post-infection phase. Our data further support both direct effects of elderberry extract by blocking viral glycoproteins as well as indirect effects by increased expression of IL-6, IL-8, and TNF. Cyn 3-glu despite demonstrating a similar direct mechanism of action (IC50 of 0.069 mg/ml) compared to the elderberry juice, did not affect the expression of pro-inflammatory cytokines. In conclusion, elderberry exhibits multiple modes of therapeutic action against influenza infection.

1. Introduction

Influenza virus is one of the leading causes of morbidity and mortality worldwide. Seasonal outbreaks of the virus affect nearly 10% of the world population and may lead up to a million deaths annually (Layne, Monto, & Taubenberger, 2009). Although vaccines remain the primary mode of defense, the constant evolution of the virus renders them ineffective and requires the generation of new vaccines and annual revaccination (Ansaldi, Durando, & Icardi, 2011). Natural therapeutic foods have shown an increasing trend to complement pharmaceutical compounds for treating various illnesses, such as cold and flu (Makau, Watanabe, Mohammed, & Nishida, 2018; Watanabe, Rahmasari, Matsunaga, Haruyama, & Kobayashi, 2014). They may also serve an essential adjunctive role in disease management through modulation of immune response (Barak, Birkenfeld, Halperin, & Kalickman, 2002; Ho et al., 2015).

Influenza A viruses are divided into subtypes based on two glycoproteins on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). There are 18 different HA subtypes and 11 different NA subtypes, HA1 through HA18 and NA1 through NA11, respectively. The first three HAs, H1, H2, and H3, are found in human influenza viruses (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992).

Influenza virus infection is initiated by the binding of the host cell's α -sialic acid surface receptor to the viral glycoprotein HA (Tmprss, Bo,

Stein, Klenk, & Garten, 2011). After the entry of the virus into the host cell, Influenza A viral ribonucleoprotein (vRNPs) enter into the nucleus following transcription and replication of the viral genome, export of the vRNPs from the nucleus, and assembly of the viral membrane. Lastly, NA enzyme cleaves the terminal sialic acid residues to release virions from host cells (Webster et al., 1992).

Elderberry (*Sambucus nigra*) has been extensively used as a natural remedy against upper respiratory disorders. Based on *in vitro* and human clinical trials, elderberry extract is shown to be effective in mitigating the duration and severity of flu symptoms in several strains of influenza viruses (Mumcuoglu, Safirman, & Ferne, 2010; Zakay-Rones, Varsano, & Zlotnik, 1995). The anti-influenza activity of elderberry and its bioactive compounds, as well as their antiviral mechanisms of action, are still under investigation, and different theories have been proposed; such as (a) neutralizing the activity of the viral glycoproteins, HA (Roschek, Fink, McMichael, Li, & Alberte, 2009) and NA (Swaminathan, Dyason, Maggioni, von Itzstein, & Downard, 2013; Zakay-Rones et al., 1995), and (b) activating the healthy immune system and increasing production of inflammatory cytokines (Barak, Halperin, & Kalickman, 2001).

Antiviral mechanism of elderberry can be explained by blocking the function of the HA glycoprotein spikes found on the surface of influenza viruses. When these HA spikes are deactivated, the viruses can no longer attach to the cell wall or enter the cell and replicate. In a study

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using sheep red blood cell, incubation of influenza virus (8 HAU) with elderberry extract (2-fold diluted) for 1 h inhibited hemagglutination for several strains of influenza viruses (Zakay-Rones et al., 1995). In another study using mass spectrometry, two flavonoids from elderberry extract, 7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5 trihydroxycyclohexanecarboxylate and 5,7,3',4'-tetra-O-methylquercetin, inhibited influenza virus infection by interfering with host cell receptor recognition and/or blocking receptor binding (Roschek et al., 2009).

NA is another group of proteins found on the surface of influenza virus required for viral replication (Swaminathan et al., 2013). Since the cleavage of sialic groups is a crucial stage of influenza replication, blocking the function of NA with NA inhibitors is an effective way to treat influenza. Effective inhibitors have been designed based on the crystal structure of sialidase from influenza A virus (Bertozzi, Freeze, Varki, & Esko, 2009). The binding of the elderberry cyanidin 3-sambubioside to influenza NA has been demonstrated by mass spectrometry and molecular docking (Swaminathan & Downard, 2012). This natural anthocyanin has been shown to inhibit the activity of NA by blocking specific segments of the enzyme active site (NA residues 356–364 and 395–432), shielding proteases from releasing these peptide segments in the vicinity of the binding site (Swaminathan & Downard, 2012). This mode of binding has not been seen with other influenza NA inhibitors; therefore this compound and its derivatives offer a new potential to develop a new class of antivirals against influenza (Swaminathan et al., 2013).

The innate immune response is critical for a successful defense against viral infection. Cytokines, such as interferon, interleukin, and tumor necrosis factor, are a broad category of proteins produced by a wide range of cells, including immune cells such as macrophages, B lymphocytes, T lymphocytes and mast cells, as well as epithelial cells, endothelial cells, fibroblasts, and various stromal cells (Boyle, 2005). They serve to enhance the body's immune response by facilitating communication between cells and the external environment (Parkin & Cohen, 2001). For instance, IFN- α is an effective treatment for different viral diseases and is employed as a treatment procedure for hepatitis B virus (HBV) and hepatitis C virus (HCV) (Guterman, 1994).

Natural products from medicinal plants such as garlic, coneflower, Echinacea, thyme, and elderberry are potential candidates for the production of therapeutic drugs with immunomodulating activity (Cundell, Matrone, Ratajczak, & Pierce, 2003; Hwang, Dasgupta, & Actor, 2004; Manach et al., 1996; Rahimi, Teymouri Zadeh, Karimi Torshizi, Omidbaigi, & Rokni, 2011). Flavonoids such as flavonols and isoflavones, depending on their structure, can have profound effects on the function of immune and inflammatory cells (Middleton, Kandaswami, & Middleton, 1992). Evidence indicates that selected flavonoids such as quercetin may affect gene expression and release of cytokines and modulation of cytokine receptors (Middleton et al., 1992). Elderberry, a rich source of flavonoids, has been shown to exert protective effects against influenza virus by stimulating the immune system of the host through enhancing the production of cytokines (Kinoshita, Hayashi, Katayama, Hayashi, & Obata, 2012). Additionally, acid polysaccharides in elderberry such as pectins may contribute to boosting the immune function (Ho et al., 2015; Kinoshita et al., 2012), possibly through macrophage stimulation (Barsett, Aslaksen, Gildhjal, Michaelsen, & Paulsen, 2012).

In this study, we performed for the first time a comprehensive examination of the inhibitory effects of elderberry on influenza infection. Prior studies have examined isolated stages of the influenza cycle, such as viral entry or replication. However, the use of multiple assays to examine direct effects at different viral stages, as well as indirect effects on the immune system shed light on the anti-influenza mechanism of action of elderberry. Moreover, cyn 3-glu was specifically examined as a critical putative bioactive compound underlying the antiviral effects of elderberry.

2. Materials and methods

2.1. Materials

Madin-Darby canine kidney (MDCK Cells), well-characterized and recognized strain of influenza virus (A/Puerto Rico/8/1934 (H1N1)), Turkey Red Blood Cell (RBC), and specific antibodies against influenza virus (A/PR 8/34 (H1N1)) produced in ferrets, were gifts from the WHO Collaborating Centre for Influenza Reference and Research (VIDRL) (Melbourne, Australia). Receptor Destroying Enzyme (RDEII, Lyophilized) was supplied by Denka Seiken Co. Ltd (Tokyo, Japan). Fresh elderberries (*Sambucus nigra*) were provided from a commercial farm in Tasmania, Australia. Fetal bovine serum (FBS), Penicillin/streptomycin, HEPES, Bovine serum albumin (BSA), trypsin-EDTA, Trypsin TPCK treated from bovine pancreas, Glutamine, crystal violet, and LPS from *Escherichia coli* were purchased from Sigma Aldrich, Australia. Avicel RC-581 was supplied from FMC BioPolymer (Philadelphia, US). Viability dye (Zombie NIR fixable) was provided by Biologend (San Diego, US). Cytofix/Cytoperm™ Fixation/Permeabilization Kit and CBA Human Inflammatory Cytokines Kit were supplied from BD Biosciences. Anti-influenza A antibodies (fluorescently labelled (FITC) – IMAGEN influenza virus A and B test kit) were purchased from ThermoFisher. Ham's F-12 K (Kaighn's) Medium, Dulbecco's modified Eagle's medium (DMEM), and 2X DMEM were supplied from Gibco (Thermo-Fisher). A549 lung epithelial cells were supplied by American Tissue Type Culture Collection, (Water Soluble Tetrazolium Salt) WST-1 Reagent was purchased from Roche. Cyanidin 3-glycoside (Cyn 3-glu) was supplied by Polyphenols Laboratories, Norway.

2.2. Sample preparation

Fresh, commercially farmed Tasmanian elderberries (*Sambucus nigra*) were harvested and the fruits was separated from their stalks. Approximately 200 g of fruit was homogenized using Nutribullet blender for 2 min. A double layer cheesecloth sieve was used to separate the juice from the paste. The pH of the elderberry juice was 4.4 ± 0.1 , and its soluble solids content ($^{\circ}$ Brix) was 17.7%. The juice was pasteurized using high-pressure carbon dioxide treatment at 18 MPa and 45 °C for 90 min (Torabian et al., 2018) and diluted to the desired concentrations. Cyn 3-glu was serially diluted with the DMEM media to the concentrations of 5, 2.5, 1.25, 0.625, 0.315, 0.16, 0.08, and 0.04 mg/ml. Before analysis, all samples were filtered through a 0.22 μ m Whatman filter.

2.3. Hemagglutination inhibition assay

Immediately after receiving, turkey blood was centrifuged for 10 min at 200 g. The RBCs pellet was washed using 0.05% v/v BSA in PBS. The mixture was centrifuged twice for 5 min at 200 g, and the cells were diluted with PBS to the appropriate concentration (1% v/v).

Elderberry juice was treated with Receptor Destroying Enzyme (RDE) in a ratio of 1:4 to remove any nonspecific inhibitors that may exist in the elderberry. The mixture was incubated in a water bath at 37 °C overnight. The solution was further diluted by 1.6% w/v sodium citrate to a ratio of 1:5, which resulted in 30-fold final dilution of the juice. Subsequently, 25 μ l of PBS was distributed into each well of the first row of 96-well plate, followed by dispensing 25 μ l of elderberry RDE treated extract (1:30) in the first well and making two-fold serial dilution along the microplate. 25 μ l of 4 HAU influenza viruses were then added to the wells. After 2 h incubation at 4 °C, 50 μ l RBC were added to the wells to the final volume 100 μ l in each well.

As a negative control, 25 μ l of PBS, 25 μ l of 4 HAU influenza virus A/PR 8/34 (H1N1), and 50 μ l RBC were dispensed to each well. As a positive control, 25 μ l of PBS was distributed into each well, followed by dispensing 25 μ l of influenza virus A/PR 8/34 (H1N1) antiserum, in

the first well and making two-fold dilution across the microplate. Another 25 μ l of 4 HAU influenza virus was added to the wells. After 2 h incubation at 4 °C, 50 μ l RBC were added to the wells. The settling pattern of the RBCs suspension was observed by mixing 50 μ l of PBS with 50 μ l of RBCs and allowing the cells to settle. Results were read when the RBC control has settled. Hemagglutination appeared as a uniform salmon-pink film covering the entire bottom of the wells, while Hemagglutination inhibition occurred as a sharp red button on the bottom of the wells.

2.4. Cytotoxicity assay

The effect of elderberry juice on cell viability was assessed according to the manufacturer's specifications (Roche) using WST-1 (water-soluble tetrazolium salt) assay. Briefly, elderberry samples were serially 2-fold diluted in DMEM media supplemented with 1% v/v penicillin/streptomycin and added to a confluent monolayer of MDCK cells in a 96-well plate. The plates were incubated for 1.5 h following the addition of Cell Proliferation Reagent WST-1. The UV absorbance was measured at 450 nm against a background control as blank using Victor™ X3 Multilabel Plate Reader (PerkinElmer, USA). The reference wavelength was 620 nm, and each dilution was assayed in triplicate. MDCK and A549 cells, which were not treated with any compound served as the control and wells containing only the media were used as blanks. Cytotoxic concentration 50 (CC₅₀) which is the concentration of a compound at which 50% of the cells are viable, was calculated by regression analysis of the absorbance-concentration curves. This was further confirmed by examining the morphology of cells with a light microscope.

2.5. Plaque reduction assay

For assessment of the effect of elderberry juice on the late stages of viral infection, a confluent monolayer of MDCK cells, grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 6-well plate, was treated with 500 μ l solution containing 250 μ l of influenza virus (30 plaque-forming unit (PFU)/ml) in DMEM without serum and an equal volume of test samples. The solution was incubated for 40 min at room temperature before being added to the cells. After 1 h incubation at 35 °C in a humidified atmosphere of 5% CO₂, the mixture was aspirated and the cells were washed with PBS. The monolayer was overlaid with double-strength plaque assay medium containing 0.4% v/v BSA, 2% v/v penicillin/streptomycin, 0.4% v/v HEPES (25 mM), 2% v/v Glutamine and 2 μ g/ml TPCK-Trypsin plus 1.8% w/v Avicel RC-581 in a ratio of 1:1. To observe the effect of elderberry on the late stage of influenza cycle, elderberry sample was also added to the overlay. After 72 h incubation at 35 °C in a humidified atmosphere of 5% CO₂, the overlay was aspirated, and the cells were washed with PBS and fixed with methanol. Subsequently, the cells were stained with 1% w/v crystal violet. The number of plaques for each sample was counted manually by eye and compared to the control, which was infected MDCK cells that were not treated with elderberry. All experiments were performed three times in duplicate. The percent inhibition of plaque formation was calculated as follows:

$$\text{Percent inhibition} = \frac{(\text{Number of plaques in control} - \text{Number of plaques in sample})}{\text{Number of plaques in control}} \times 100$$

Inhibitory concentration 50 value (IC₅₀) which is elderberry concentration that results in 50% reduction in the number of plaques, was calculated by regression analysis of these curves.

2.6. Quantifying anti-influenza activity of elderberry by flow cytometry

Flow cytometric analysis was performed as another method for precise quantification of the anti-influenza activity of elderberry in the

early stages of viral infection (0–19 h) which is not possible to evaluate with the standard plaque reduction assay. Flow cytometry allows for the discrimination of different particles on the basis of size and colour. In this method by using a FITC conjugated antibody targeting the influenza A virus nucleoprotein, the fraction of the infected cells was enumerated.

A monolayer of MDCK cells (80–90% confluence) grown in a 24-well plate, was treated with 600 μ l solution containing 300 μ l of influenza virus (MOI 2) in DMEM without serum and an equal volume of the test sample. The MOI of the virus was optimized to induce maximal infection (90 \pm 10%). The solution was incubated for 40 min at room temperature before being added to the cells. After 1 h incubation at 35 °C in a humidified atmosphere of 5% CO₂, the mixture was aspirated, and the cells were washed with PBS. The monolayer was overlaid with medium containing 0.2% v/v BSA, 1% v/v penicillin/streptomycin, 0.2% v/v HEPES (12.5 mM), 1% v/v Glutamine and 1 μ g/ml TPCK-Trypsin. After 19 h incubation at 35 °C in a humidified atmosphere of 5% CO₂, the cells were detached using trypsin-EDTA. The cell suspension was transferred to FACS tubes, diluted by 3 ml PBS and centrifuged for 5 min at 200 g. The washing step was repeated to ensure residual FBS is removed. The cells were suspended in 100 μ l of diluted viability dye (Zombie NIR) (1:1000), followed by incubation at room temperature, in the dark, for 20 min. The dye was diluted by addition of 3 ml FACS buffer (1% v/v FBS in PBS) and centrifuged for 5 min at 200 g. After discarding the supernatant, the cells were fixed using Fix/Perm solution. Cells were subsequently washed three times with BD Perm/Wash reagent to deactivate the Fix/Perm solution and to maintain cells in a permeabilised state for intracellular staining. After spinning the tubes for 5 min at 500 g, cells were stained with 100-fold diluted fluorescently labelled (FITC) anti-influenza A antibodies. After incubation at 4 °C for 30 min in the dark, cells were washed three times with Perm/Wash buffer and re-suspended in 200 μ l staining buffer before flow cytometric analysis. The cell suspension was transferred into a round-bottom polystyrene tube with a cell strainer cap. The flow cytometry analysis was performed using FACS Canto II flow cytometer from Beckton-Dickinson, USA. Photomultiplier Tubes (PMT) voltage and compensation were set using single stained controls, including FITC and NIR fluorochromes. Fifty thousand cells were acquired for each reading. The gating strategy used for the analysis consisted of single cells gating, live cells gating, and gating of the infected cell in the histogram. The percent of infected and non-infected cells were enumerated in each sample, and the results expressed as percent inhibition.

2.7. Quantifying human inflammatory cytokines by flow cytometry

The immunomodulating activity of elderberry was evaluated by cytometric bead array (CBA) multiplexed assay according to the manufacturer's instruction (BD, Bioscience). The human alveolar carcinoma cell line A549 from the American Tissue Type Culture Collection was used as an *in vitro* model system for human epithelial lung cells. A monolayer of A549 cells (80–90% confluence) grown in a 6-well plate in Ham's F-12K (Kaighn's) Medium supplemented with 5% v/v FBS, 1% v/v penicillin/streptomycin and 1% v/v L-glutamine, was treated with different samples including elderberry sample (1:15), cyn 3-glu (2.5 mg/ml), and LPS (10 μ m/ml) in 600 μ l of medium without serum. The culture medium was changed to medium without serum 3 h before exposure of A549 cells to the samples, to gradually starve the cells and avoid undesirable stimulation by the serum components. After incubation at 37 °C in 5% CO₂ for 20 h, cells supernatant were analyzed using the CBA assay. The CBA human inflammatory cytokines kit quantitatively measured IL-8, IL-1 β , IL-6, IL-10, TNF, and IL-12p70 protein levels in a single sample. The level of cytokines expression was determined by measuring the geometric mean fluorescence intensity using a BD FACSCanto II flow cytometer (BD Bioscience). The constitutive expression of inflammatory cytokines was used to know the

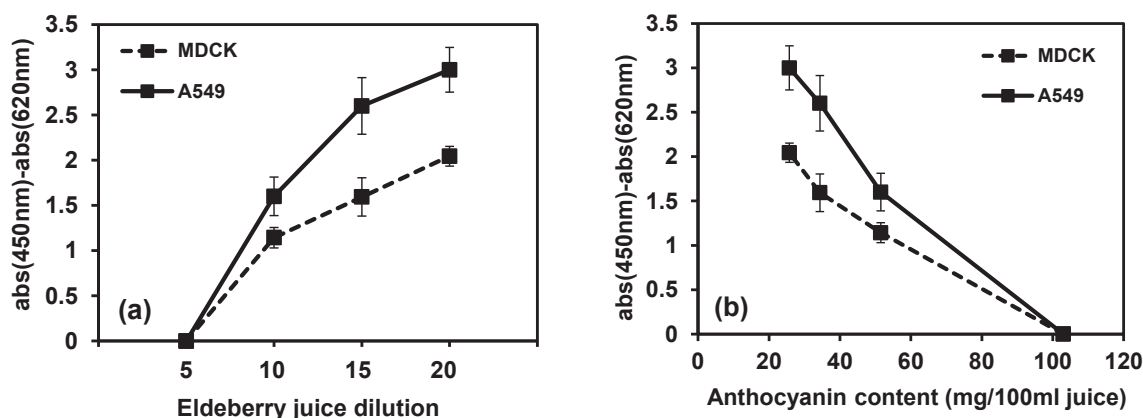


Fig. 1. The effect of elderberry juice on the viability of MDCK and A549 cells, (a) as a function of dilution and (b) as a function of the anthocyanin content, after treatment for 24 h, as analyzed by WST-1 assay.

baseline cytokine release by a control of only cells and media. The effect of the different elderberry samples on the induction of inflammatory/anti-inflammatory cytokine production (pg/ml) was expressed as Fold increase = $\frac{\text{Cytokine production induced by the sample}}{\text{Cytokine production by the cells only}}$

2.8. Statistical analysis

The results of the experiments were expressed as means \pm SD. Comparisons between groups were made using one-way ANOVA with a Tukey's multiple comparison tests. Statistical significance was defined at a p -value of < 0.05 . A labelling system of $^*p < 0.05$, $^{**}p < 0.01$ and, $^{***}p < 0.001$ was employed.

3. Results and discussion

3.1. Effect of elderberry on the viability of MDCK and A549 cells

A cytotoxicity assay was conducted to determine the CC50 of elderberry juice at different dilutions of 1:5, 1:10, 1:15, and 1:20 (with anthocyanin content of 103, 52, 34, 26 mg/100 ml juice) on MDCK and A549 cells after 24 h of exposure (Fig. 1). The effect of elderberry juice on cell viability was assessed using WST-1 assay and the results expressed as absorbance at 450 nm. The derived CC50 value is included in Table 1. On MDCK cells, elderberry juice had a CC50 of 77 ± 6 mg AC/100 ml juice (~ 9 -fold dilution). On A549 cells, the CC50 value was 81 ± 3 mg AC/100 ml juice (~ 7 -fold dilution). Therefore, A549 cells were less sensitive to elderberry juice than MDCK cells, which is consistent with prior published data that elderberry showed lower a level of toxicity on A549 cells (Shahsavandi, Majid, & Hasaninejad, 2017).

3.2. Direct mechanisms of anti-influenza activity of elderberry and cyn 3-glu

The direct inhibitory effect of elderberry and cyn 3-glu, in the early and late stages of influenza cycle, was studied by three different methods including (a) Hemagglutination inhibition assay, (b) plaque

Table 1

Therapeutic index of elderberry juice through plaque reduction assay on MDCK cells infected by A/PR 8/34 (H1N1). A: Treatment during and after the infection, B: Treatment only during infection.

Cytotoxicity (CC50, mg AC/100 ml juice)	Antiviral activity (IC50, mg AC/100 ml juice)		Therapeutic index (CC50/IC50)	
	A	B	A	B
77 ± 6	6 ± 0.8	17 ± 1.4	12 ± 1.3	4 ± 0.3

reduction assay, and (c) flow cytometry. Hemagglutination inhibition was used as a functional qualitative assay to confirm the antiviral activity of elderberry at the early stage of influenza cycle. Subsequently, the activity was analyzed through quantitative assays; plaque reduction and flow cytometry. Plaque reduction assay, a gold standard for analysing the antiviral activity, was developed to evaluate the antiviral activity of elderberry at the late stage of influenza cycle. However, the shortfalls of this method include subjective interpretation of the number of plaques, long duration of the assay, and technical complications such as cell detachment. Therefore, flow cytometry analysis enumerating individual dead and live infected cells was performed as another viral quantification method. This method offered a more selective, sensitive, reproducible, and faster method of analysis compared to plaque assays. Moreover, flow cytometric analysis was optimized to evaluate the inhibition of infection at the early stages of viral infection, which was not feasible by plaque reduction assay. However, considering the shorter duration of the flow cytometric assay, evaluating the effect of elderberry on the late stage of influenza cycle was not possible by this method. Nevertheless, by comparing the results of flow cytometry and plaque reduction assay, a comprehensive analysis was conducted on the inhibitory effect of elderberry on the entire cycle of influenza infection. The following sections provide an overview of the outcomes of each of the biological assays that were conducted in this study.

3.2.1. Anti-influenza activity of elderberry juice determined by hemagglutination inhibition assay

Hemagglutination inhibition assay is based on the ability of the HA protein of influenza viruses to bind to RBCs causing them to agglutinate. This assay detects the anti-influenza activity of test compounds related to inhibition of viral attachment and entry stage of the infection. Elderberry juice showed antiviral activity against influenza virus with a Hemagglutination inhibition titer of 60. The Hemagglutination inhibition titer is the highest dilution of the sample causing complete inhibition of hemagglutination. Partial inhibition occurred by 120-fold diluted RDE-treated elderberry juice (Fig. 2a). The results agree with the previous research on anti-influenza activity of elderberry extract that demonstrated the inhibition of hemagglutination by the extract (Zakay-Rones et al., 1995).

A sharp red button appeared in the RBCs control wells (Fig. 2b) illustrating that the RBCs were aggregated. In the negative control wells, where hemagglutination occurred by 4 hemagglutination unit (HAU) influenza virus A/PR 8/34 (H1N1), a uniform salmon-pink film covered the entire hemispherical bottom of the wells caused by dispersion of the cells (Fig. 2c). In the positive control wells, hemagglutination inhibition was caused by all dilutions of the antiserum against influenza virus A/PR 8/34 (H1N1) (Fig. 2d).

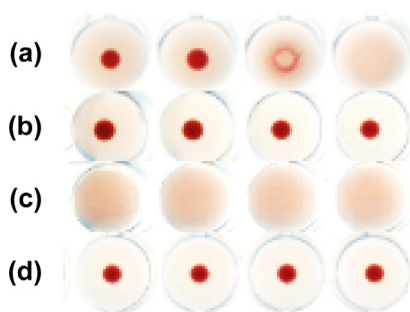


Fig. 2. (a) Hemagglutination inhibition by elderberry juice (30, 60, 120, and 240-fold diluted, from left to right) (b) RBC control, (c) Negative control: hemagglutination by 4 HAU influenza virus A/PR 8/34 (H1N1), (d) Positive control: hemagglutination inhibition by antiserum (2, 4, 8 and 16-fold diluted, from left to right).

3.2.2. Mechanisms of anti-viral activity of elderberry determined by plaque reduction assay

To evaluate the mechanism of anti-influenza activity of elderberry, we used classical plaque reduction assay that allows kinetic studies of the viral infection. We hypothesized that the elderberry has direct inhibitory effects against influenza infection by blocking the viral glycoproteins, HA and NA, in the early and late stage of influenza infection cycle respectively. The effects of pre-treatment and post-treatment of the virus or the cells with elderberry on inhibiting the influenza infection were assessed.

To determine the effect of pre-treatment, the antiviral activity of elderberry in three conditions were compared; (a) when viruses were pre-treated with elderberry juice before the infection, for 40 min at room temperature, (b) when virus and elderberry juice were added to the cells concurrently without any pre-treatment, and (c) when cells were pre-treated with elderberry juice (for 40 min at 37 °C) before the infection (Fig. 3a). Based on the results, a higher level of inhibition was achieved when viruses were pre-treated with elderberry juice before being exposed to the cells, which shows the ability of elderberry to block the active sites on the surface of the virus in the early stages of influenza cycle, including viral attachment and penetration into the host cell. However, no significant changes in viral inhibition were observed when cells were pre-treated with elderberry juice before the infection, which demonstrated that the extract inhibits HA of the viruses by binding to the virus itself and antiviral activity of elderberry was not attributed to any interaction with the cells.

The complete cycle of influenza virus replication, from entering into the cell to budding out from the cell, is between 24 and 48 h depending

on the viral strain. Although 30 min is sufficient for the virus to enter the cell, infection and accumulation of influenza nucleoproteins in cells usually occur after nearly 19 h. In typical plaque reduction assay, after 1–2 h antiviral agents are removed, cells are washed, and incubated for 72 h. Thus, the assays chiefly examine viral entry during the early stages of the influenza cycle. To observe the post-infection effect of elderberry, we modified the plaque reduction assay. After the aspiration of the virus/extract mixture, the elderberry extract was re-added to the cells to extend exposure through the 72 h post incubation period. With this extended exposure protocol, the antiviral activity of elderberry was significantly increased ($50 \pm 10\%$) (Fig. 3b). It was consequently speculated that elderberry might suppress the post-infection phase by blocking NA. Binding of natural anthocyanin inhibitor to influenza NA has been previously studied by mass spectrometry and molecular docking (Swaminathan et al., 2013).

3.2.3. Qualifying anti-influenza activity of elderberry juice by plaque reduction assay

Inhibition of influenza virus infection by treatment with various dilutions of elderberry juice (15–100-fold) during and after infection, was determined in dose-response curves (Fig. 4). Therapeutic index was calculated as a ratio of the CC_{50}/IC_{50} values. The therapeutic index was higher when the cells were treated during and after the infection compared to when treated only during the infection (Table 1). Elderberry juice held the antiviral activity when added to the cells during and after the infection, by obtaining the therapeutic index of 12 ± 1.3 . The therapeutic index > 10 defines elderberry as having antiviral activity (Kinoshita et al., 2012). The values for CC_{50} , IC_{50} , and CC_{50}/IC_{50} of elderberry juice are summarised in Table 1.

As shown in Fig. 4, treatment with 30-fold diluted elderberry juice yielded a significant reduction ($70 \pm 10\%$) in the number of the plaques compared to untreated virus-inoculated control. Moreover, elderberry reduced not only the number of plaques but also the size of some of the plaques was decreased, demonstrating inhibition of infection propagation. In a similar study, black currant extract (100 mg/ml) was capable of inhibiting the penetration and release of influenza type A and B viruses into/from the cells (Knox, Suzutani, Yosida, & Azuma, 2003).

3.2.4. Anti-influenza activity of the cyn 3-glu and its mechanism of action determined by plaque reduction assay

The anti-viral activity of elderberry has been attributed to particular flavonoid compounds present in the fruits of the plant (Roschek et al., 2009). Cyn 3-glu, which is the primary anthocyanin in elderberry, showed a strong anti-influenza effect with the IC_{50} of 0.069 mg/ml (Fig. 5a), and no toxic effect on MDCK and A549 cells at high

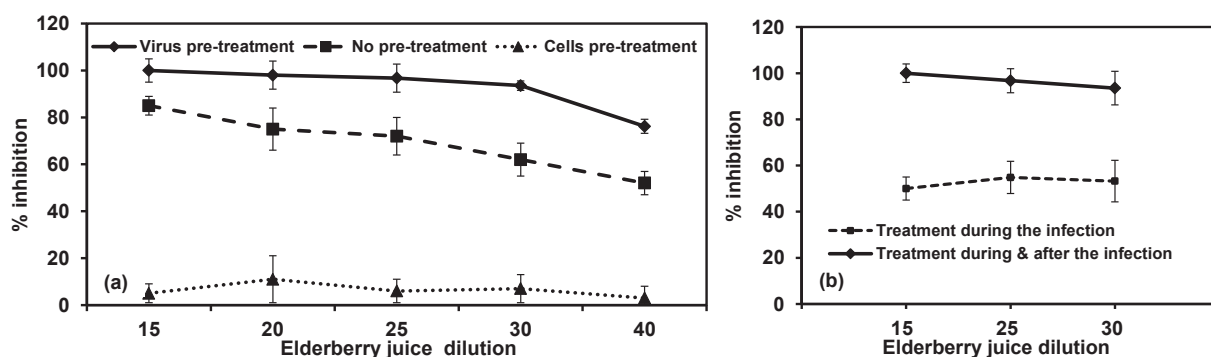


Fig. 3. Anti-influenza mechanisms of action of elderberry (a) Pre-treatment analysis. Plaque reduction assay on MDCK cells infected with influenza virus A/PR 8/34 (H1N1), Virus pre-treatment: when viruses were pre-treated with elderberry juice before the infection, for 40 min at room temperature, No pre-treatment: when virus and elderberry juice were added to the cells concurrently without any pre-treatment, Cells pre-treatment: when cells were pre-treated with elderberry juice before the infection for 40 min at 37 °C (the sample was exposed to the cells during and after the infection for the whole duration of the assay). (b) Post-treatment analysis. The anti-influenza effect of various concentration of elderberry juice on MDCK cells infected with influenza virus A/PR 8/34 (H1N1) when the sample was removed after the infection or when the sample was exposed to the cells during and after the infection for the whole duration of the assay (results expressed as percent inhibition).

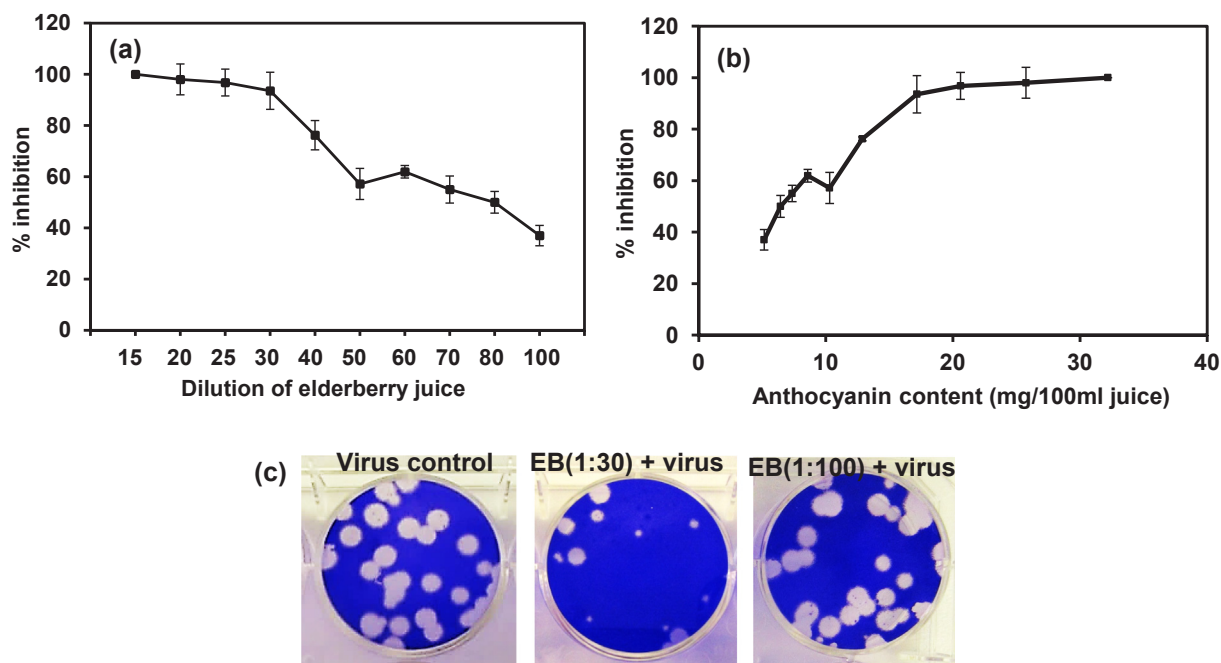


Fig. 4. Anti-influenza activity of elderberry juice. The inhibition of influenza infection through plaque reduction assay on MDCK cells infected by A/PR 8/34 (H1N1) when treated with elderberry juice during and after infection (a) as a function of dilution and (b) as a function of the anthocyanin content (results expressed as percent inhibition), (c) Plaque reduction assay on MDCK cells infected by A/PR 8/34 (H1N1) in virus control, infected cells treated with 30 and 100-fold diluted elderberry juice during and after infection.

concentrations (up to 10 mg/ml). The increasing inhibition with different treatment regimens (Fig. 5b) implies that cyn 3-glu has a capacity to both inhibit the virus in the early stages of the viral infection by blocking influenza HA, and to suppress the post-infection phase of the

influenza cycle.

As shown in Fig. 5b, for observing the inhibitory effect of cyn 3-glu on the early stage of infection, after 1 h infection the inoculum (virus/cyn 3-glu mixture) was removed, and the cells were washed. In

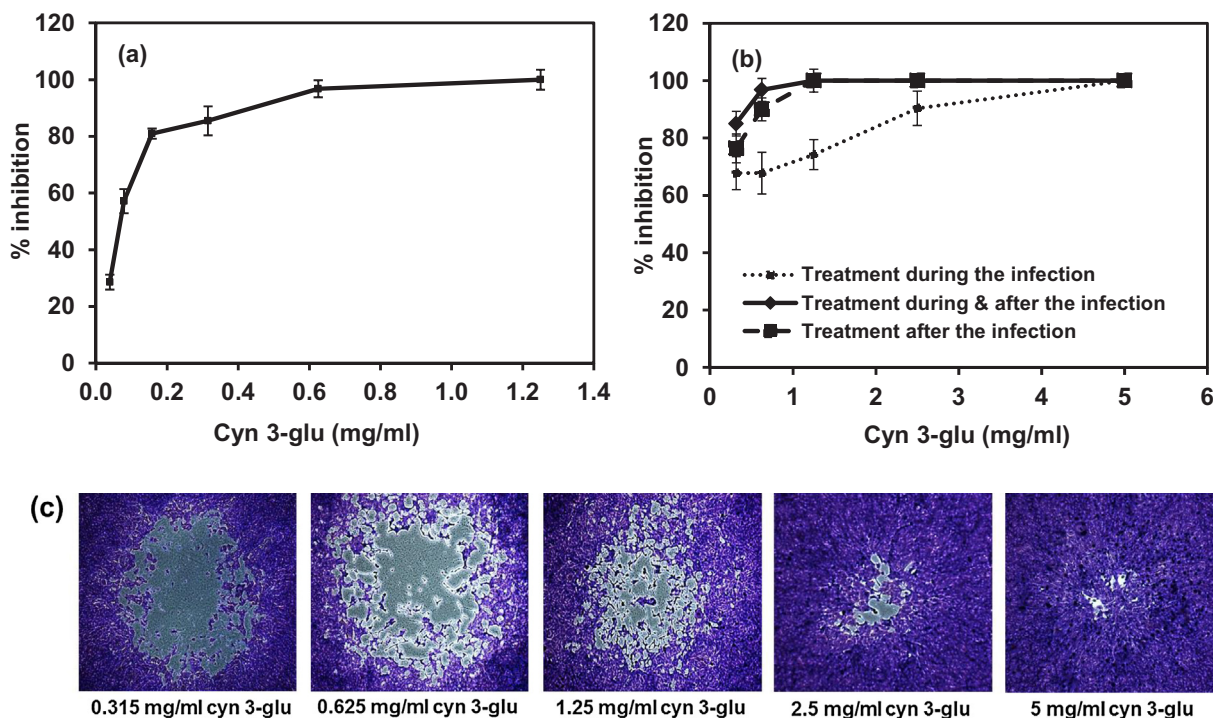


Fig. 5. Anti-influenza activity of cyn 3-glu (a) The inhibition of influenza infection through plaque reduction assay on MDCK cells infected by A/PR 8/34 (H1N1), when treated with various concentration of cyn 3-glu during and after infection for the whole duration of the assay. (b) The anti-influenza effect of various concentration of cyn 3-glu on MDCK cells infected with influenza virus A/PR 8/34 (H1N1) through plaque reduction assay, when the sample was removed after the infection, when the sample was exposed to the cells during and after the infection for the whole duration of the assay, or when the sample was added only after the infection (results expressed as percent inhibition). (c) Microscopic images (10X magnification) of representative plaque size at 0.315, 0.625, 1.25, 2.5, and 5 mg/ml cyn 3-glu respectively from left to right.

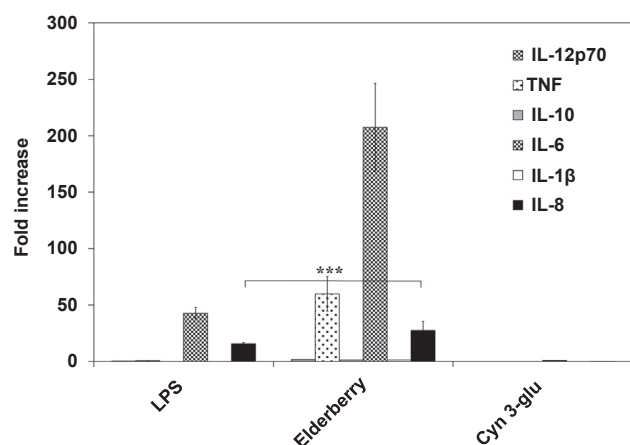


Fig. 6. Stimulation of human inflammatory cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF, and IL-12p70) on A549 cells after exposure to LPS (10 μ m/ml), elderberry sample (1:15), and cyn 3-glu (2.5 mg/ml) expressed as fold increase.

contrast, for assessing the activity of cyn 3-glu on the entire cycle of the influenza virus, after the infection, cyn 3-glu was re-added to the overlay/media. Lastly, for detecting post-infection inhibition, the inhibitor was added to the overlay/media only after the infection. Cyn 3-glu led to complete inhibition of influenza infection through the modified plaque reduction assay with the extended contact of cyn 3-glu (up to 1.25 mg/ml) with the infected cells during the assay; although less inhibition ($74 \pm 5\%$) was observed when the inoculum was removed after the infection. In addition to the significant effect of cyn 3-glu on reducing plaques number, plaques size was also remarkably decreased (Fig. 5c), which demonstrates effective inhibition of viral cell-to-cell transmission.

3.2.5. Anti-influenza activity of elderberry and cyn 3-glu determined by flow cytometry analysis

Different parameters of the flow cytometric assay, such as the multiplicity of infection (MOI) of the virus and duration of the incubation were optimized to study the effect of elderberry on the early stage of influenza cycle. MOI of 2 and period of 19 h were found to be optimal. Flow cytometry confirmed that the influence of elderberry on the early stage of influenza cycle is less substantial compared to its effect on the late stage of the viral infection cycle.

The viral inhibition by elderberry juice with the dilution of 1:20 and 1:30 were $10.3 \pm 5\%$ and $2.6 \pm 4\%$, respectively, which were significantly lower than nearly complete inhibition observed by the similar concentration of elderberry juice through the plaque reduction assay. Moreover, in flow cytometric analysis, only the highest concentration of cyn 3-glu (5 mg/ml) showed complete inhibition and the lower tested concentrations (2.5 mg/ml and 1.25 mg/ml) did not show any significant viral inactivation.

3.3. Indirect mechanisms of anti-influenza activity of elderberry and cyn 3-glu

The immunomodulatory property of elderberry juice on A549 cells was studied using a fluorescent microbead array probing for a panel of inflammatory cytokines. In this method, antibodies were conjugated to the surface of beads to capture analytes of interest. Six human inflammatory cytokines Interleukin-8 (IL-8), Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) were quantitatively measured using A549 cells exposed to elderberry juice (1:15) and cyn 3-glu (2.5 mg/ml). Lipopolysaccharide from *Escherichia coli* (LPS) (10 μ m/ml) was used as a control of cellular responses to cytokines (Fig. 6).

Elderberry juice significantly increased the production of IL-6

(207 ± 39 fold), IL-8 (28 ± 8 fold), and TNF (60 ± 15 fold). Similarly, it was previously demonstrated that goji berry extract modulated the immune response in adult mice by enhancing the secretion of IL-12 and TNF- α (Du et al., 2014).

Immune modulating activity was not attributed to cyn 3-glu, which is considered as a primary active compound in elderberry. These results are in accordance with previous research that showed weaker stimulation of immune response in mice treated with the low-molecular weight fraction of elderberry concentrate, which includes anthocyanins (Kinoshita et al., 2012).

Nevertheless, high-molecular-weight compounds including acidic polysaccharides derived from elderberry might be responsible for the stimulation of the immune function (Ho et al., 2015; Kinoshita et al., 2012). Polysaccharides from elderberries were reported to exhibit macrophage stimulating activity (Barsett et al., 2012). For instance, pectins have gained interest as health-promoting polysaccharides. These substances are a group of acidic heteropolysaccharides and one of the major components of the cell wall of many plants including elderberry (Paulsen & Barsett, 2005; Schols, Visser, & Voragen, 2009).

4. Conclusions

Elderberry showed potent antiviral activity with a therapeutic index of 12 ± 1.3 against influenza infection. Moreover, inhibition by elderberry was stronger against the late stage influenza cycle than the early stage. These findings are consistent with clinical data indicating that elderberry can mitigate the duration and severity of influenza symptoms in patients (Mumcuoglu et al., 2010). The action of elderberry is both direct – suppressing viral entry, affecting the post-infection phase, and viral transmission from cell to cell, and indirect – by modulating the release of cytokines such as IL-6, IL-8, and TNF. These data support the use of *Sambucus nigra* berries as nutraceutical ingredients for the management of influenza infection.

Ethics statement

This research did not include any human subjects and animal experiments.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2019.01.031>.

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