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Elderberry flavonoids bind to and prevent H1N1 infection *in vitro*

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ABSTRACT

A ionization technique in mass spectrometry called Direct Analysis in Real Time Mass Spectrometry (DART TOF-MS) coupled with a Direct Binding Assay was used to identify and characterize anti-viral components of an elderberry fruit (*Sambucus nigra* L.) extract without either derivatization or separation by standard chromatographic techniques. The elderberry extract inhibited Human Influenza A (H1N1) infection *in vitro* with an IC_{50} value of 252 ± 34 μg/mL. The Direct Binding Assay established that flavonoids from the elderberry extract bind to H1N1 virions and, when bound, block the ability of the viruses to infect host cells. Two compounds were identified, 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) and 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate (**2**), as H1N1-bound chemical species. Compound **1** and dihydromyricetin (**3**), the corresponding 3-hydroxyflavonone of **2**, were synthesized and shown to inhibit H1N1 infection *in vitro* by binding to H1N1 virions, blocking host cell entry and/or recognition. Compound **1** gave an IC_{50} of 0.13 μg/mL (0.36 μM) for H1N1 infection inhibition, while dihydromyricetin (**3**) achieved an IC_{50} of 2.8 μg/mL (8.7 μM). The H1N1 inhibition activities of the elderberry flavonoids compare favorably to the known anti-influenza activities of Oseltamivir (Tamiflu®; 0.32 μM) and Amantadine (27 μM).

1. Introduction

The chemical complexity of botanical extracts has made mass spectrometric characterization of whole extracts difficult due to the lack of reliable extraction methodologies that yield optimized extracts with dose-to-dose reliable chemical compositions (Schmidt et al., 2007). A relatively new ionization source in mass spectrometry, termed DART (Direct Analysis in Real Time) (Cody et al., 2005), is coupled to a time-of-flight mass spectrometer, making it possible to rapidly and accurately identify the chemical components in botanicals and extracts at atmospheric pressure, typically with no sample preparation or processing requirements.

The DART ion source utilizes electronic excited-state species, such as metastable helium and nitrogen atoms, as plasmas. These excited atoms ionize samples directly for mass spectrometric analysis. The most common ions produced during DART analysis are the $[M+H]^+$ cations and the $[M+NH_4]^+$ adducts (observed if ammonium hydroxide is present near the DART source); however metal, cation adducts are never observed (Cody et al., 2005). DART is capable of analyzing surface materials without direct exposure of the samples to elevated temperatures and/or electrical potentials as occurs during atmospheric pressure chemical ionization (Sciex, 1992) and electrospray ionization (Pramanik et al., 2002) mass spectrometric techniques. Fragmentation of the samples during DART ionization can be induced by adjusting the mass spectrometer voltages, allowing for more detailed structural information (Cody et al., 2005). Recently, DART TOF-MS was used to determine the molecular formulae and structures of toxoid compounds in cell cultures of *Taxus wallichiana* (Banerjee et al., 2008), and alkaloids expressed in the hairy roots of *Rauvolfia serpentine* (Madhusudanan et al., 2008).

The combination of enhanced super critical CO_2 extraction technologies and affinity chromatography has enabled the production of optimized and dose-reliable botanical extracts from variable feedstocks that possess a defined bioactive profile (Alberte et al., 2007). These extraction technologies were employed herein to generate reproducible extracts of elderberry (*Sambucus nigra* L.) fruits for both chemical characterization and assessment of biological activity. Elderberries are known to be rich in phenolic compounds, including phenolic acids, flavonoids, catechins, and proanthocyanidins (de Pascual-Teresa et al., 2000; Hakkinen et al., 1999), as well as possessing a variety of anti-oxidant properties (Abuja et al., 1998; Rice-Evans et al., 1996; Seeram and Nair, 2002; Wang et al., 1997), and enhancing the immune response (Barak et al., 2001; Zakay-Rones et al., 1995). In addition, elderberry extracts have shown anti-influenza activity in human clinical trials (Zakay-Rones et al., 2004).

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We utilized an optimized elderberry extract as well as a newly developed Direct Binding Assay to identify key bioactive flavonoids in elderberry fruits that contribute to the reported anti-influenza activities. The identified flavonoids bind to Human Influenza A (H1N1) viruses and block viral infection *in vitro*.

2. Results and discussion

2.1. Anti-viral activity of elderberry fruit extracts

A viral focus reduction assay was used to characterize the *in vitro* anti-influenza activity of the elderberry extract. Human influenza A (H1N1) virus particles were used to infect Madin-Darby canine kidney NBL-2 (MDCK) cells. The elderberry extract showed clear dose-dependent inhibition of H1N1 virus infection (Fig. 1). The 50% inhibition concentration (IC_{50}) of the extract for H1N1 was 252 (±34) μg/mL, while 100% inhibition of H1N1 infection was achieved at 1000 μg/mL, the highest concentration tested.

To address possible extract-induced cellular toxicity, and to confirm that the viral inhibition effects of the elderberry extract were not due to non-specific effects of the extract on target cells, the elderberry extracts were evaluated in a MTT cell viability assay using the target MDCK cells. No non-specific effects on cell viability or cellular toxicity were observed up to 2000 μg/mL of the extract (data not shown), well above the IC_{100} concentration for viral infection.

2.2. Identification of elderberry extract compounds that bind to H1N1 virions

Fig. 2 shows the DART TOF-MS fingerprint of the compounds that are bound to H1N1 virions (Fig. 2A) and those compounds that do not bind to the virus particles (Fig. 2B). This was accomplished by incubating the H1N1 virus in the elderberry extract and removing the unbound components by washing through a membrane filter. One of the extremely abundant compounds (*m/z* $[M+H]^+$ = 369.353) was identified as cholestadiene. This compound was present in the washing medium and, from our control experiments, did not possess any anti-influenza activity.

Because the AccuTOF mass spectrometer accurately determines isotopic abundances (JEOL, 2007) and yields high resolution mass measurements from the time-of-flight mass spectrometer, it was possible to determine the candidate molecular formulae for the compounds at *m/z* = 359.325 and 479.232 amu found to bind to the H1N1 particles. It was also determined, based upon the molecular formulae for each of these compounds, that many of the abundant compounds bound to H1N1 were DART-generated fragments of the parent ions. The structures of compounds **1** and **2** were determined by: (1) measuring the accurate isotopic abundance ratios; (2) determining the precise molecular formula based on these isotopic ratios; (3) monitoring the DART-generated fragmentation of these compounds bound to H1N1 virions; and (4) molecular modeling of various proposed structures based on the determined molecular formulae.

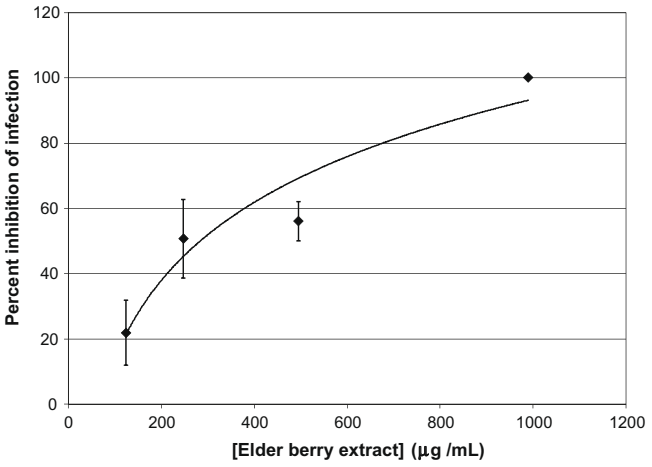

Fig. 1. The dose-dependent inhibition curve of influenza A (H1N1) virus infection of MDCK cells incubated with an elderberry fruit extract. The IC_{50} and IC_{100} values were determined using the line-of-best-fit (R^2 = 0.92; n = 22).

The first flavonoid (**1**) at *m/z* $[M+H]^+$ = 359.325 amu (with corresponding DART-generated fragments at *m/z* $[M+H]^+$ = 341.310, 331.289, 313.275, and 285.205 amu, representing the loss of water and/or CO from the parent ion) (Cuyckens and Claeys, 2004) was identified as 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) (Fig. 3). The second flavonoid identified (**2**) at *m/z* $[M+H]^+$ = 479.232 amu was esterified with 3,4,5-trihydroxy-cyclohexanecarboxylic acid (Fig. 3) on the 3-OH of the 3-hydroxyflavonone C-ring. Compound **2** contains multiple stereogenic centers, and as such, we have presented one of several possible diastereomers. However, free-energy minimizations of compound **2** indicate that the ester functionality of compound **2** is not likely a requirement for the anti-influenza activity observed here (see below) and therefore, racemic dihydromyricetin (**3**), the free 3-hydroxyflavonone of **2**, was pursued for synthesis.

To validate the entry inhibition mechanism-of-action of the elderberry extract, the H1N1 virus particles were subjected to the Direct Binding Assay. After incubation of the H1N1 viruses in the elderberry extract, unbound chemicals were removed, and the H1N1 virus particles with bound compounds were allowed to infect MDCK cells. The identified flavonoids were found to bind to H1N1 virions in a ratio (2.9:1; **1**:**2**) different from the ratio of these compounds in the elderberry extract (1.5:1) indicating that binding of these flavonoids to H1N1 is not non-specific.

When H1N1 virions were incubated at the IC_{50} and IC_{100} concentrations determined for the elderberry extract (252 and 1108 μg/mL, respectively), 58% and 95% inhibition of H1N1 infection was achieved. This level of inhibition indicates that the active chemicals in the elderberry extract bind stoichiometrically to H1N1 virions, and when bound, block viral infection *in vitro*.

To verify the proposed mode-of-action established with the elderberry extract, and to confirm the proposed structures as well as the *in vitro* anti-influenza activity of these compounds, 5,7,3′,4′-tetra-*O*-methyl quercetin (**1**) and dihydromyricetin (**3**) were synthesized. Compound **1** was synthesized in two steps from Rutin (**4**; Fig. 4), while racemic dihydromyricetin (**3**) was synthesized in five steps by coupling acetophenone (**6**) and benzaldehyde (**7**; Fig. 5). The anti-influenza mode-of-action of compounds **1** and **3** was confirmed by utilizing the Direct Binding Assay. Fig. 6 shows the DART TOF-MS fingerprints of the compounds bound to the H1N1 virions. The binding of dihydromyricetin (**3**) and compound **1** to H1N1 viruses (Fig. 4A and B, respectively) confirms the entry inhibition mode-of-action of these flavonoids.

The synthesized flavonoids were also subjected to focus-forming inhibition assays against the H1N1 virus. Dihydromyricetin (**3**) achieved 50% inhibition of H1N1 infection at a concentration of 2.8 μg/mL (8.7 μM) (Table 1), which is approximately 100 times lower than the IC_{50} of the elderberry extract (252 μg/mL). Based on the results of the Direct Binding Assay for the elderberry extract, we expected compound **1** to have an IC_{50} 7–10× lower (more active) than dihydromyricetin (**3**). In fact, compound **1** achieved an IC_{50} of 0.13 μg/mL (0.36 μM) (Table 1), which is 20× lower than the IC_{50} determined for (±)-dihydromyricetin, and 3-orders-of-magnitude (1000×) lower than the elderberry extract. Of particular interest, 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) achieved an IC_{50} against H1N1 similar to that of Oseltamivir (Tamiflu®; 0.32 μM),

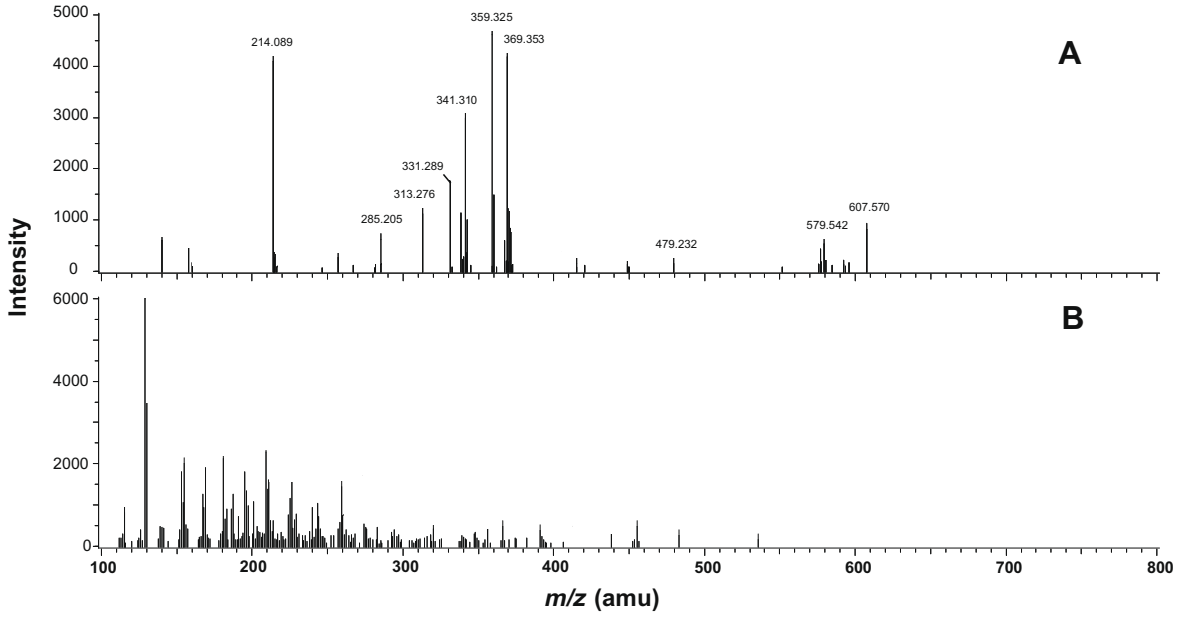

Fig. 2. DART TOF-MS fingerprints of the compounds present in the elder berry extract that are bound (A) and not bound (B) to H1N1 virus particles after 1-h incubations.

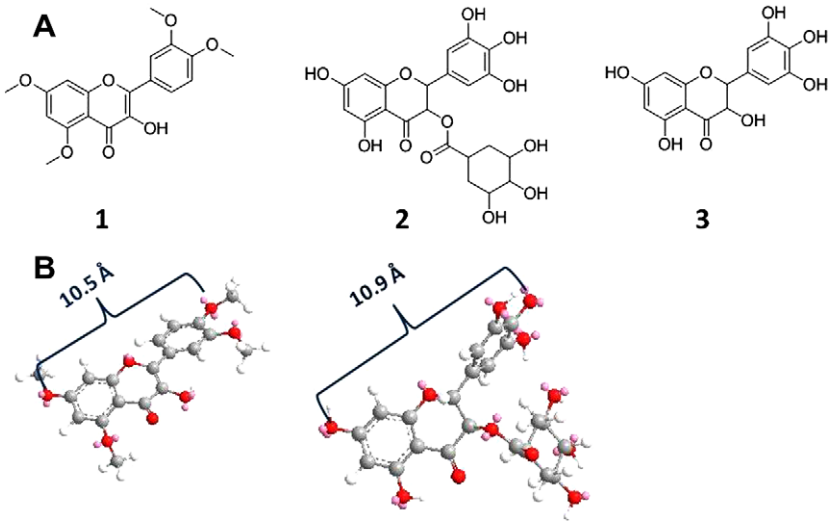

Fig. 3. (A) The structures of 5,7,3′,4′-tetra-*O*-methylquercetin (**1**), 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate (**2**), and (±)-dihydromyricetin (**3**). (B) The region most likely to bind to the hemagglutinin proteins of Influenza A is bracketed in the free-energy minimized three-dimensional structure of **1** and **2**.

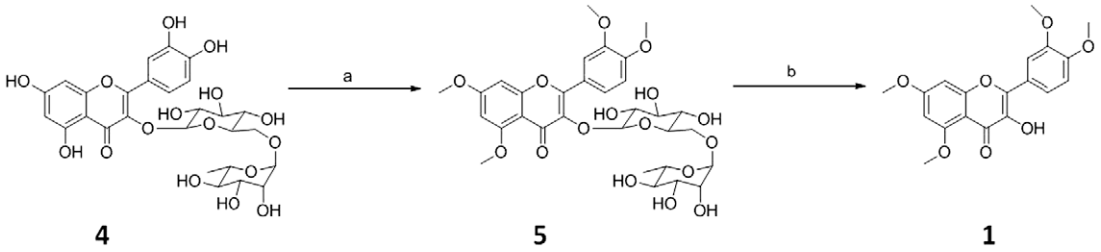

Fig. 4. The synthesis of 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) from Rutin (**4**). (a) DMS, K_2CO_3, acetone, reflux, 70 h; (b) 20% HCl, reflux 2 h.

a known influenza neuraminidase (N1) inhibitor that prevents the release of progeny from infected cells, and 27× lower than that of the known influenza M2 proton channel inhibitor Amantadine (27 μM) (Pinto and Lamb, 2006). Dihydromyricetin (**3**) had an IC_{50} value 3× higher (less active) than Oseltamivir and ca. 3× lower than Amantadine (Table 1).

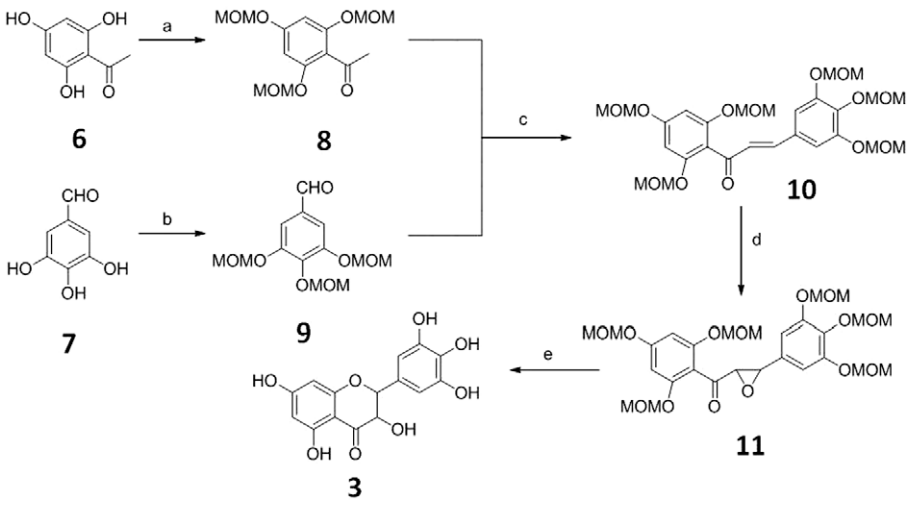

Fig. 5. The synthesis of (±)-dihydromyricetin (**3**) from **6** and **7**. (a) NaH, MOM-Cl, DMF, 0 °C; (b) K_2CO_3, MOM-Cl, acetone, 10 °C; (c) 40% KOH, EtOH, 20 °C; (d) H_2O_2, 2 N NaOH, MeOH, 20 °C; and (e) 20% HCl, MeOH, 45 °C.

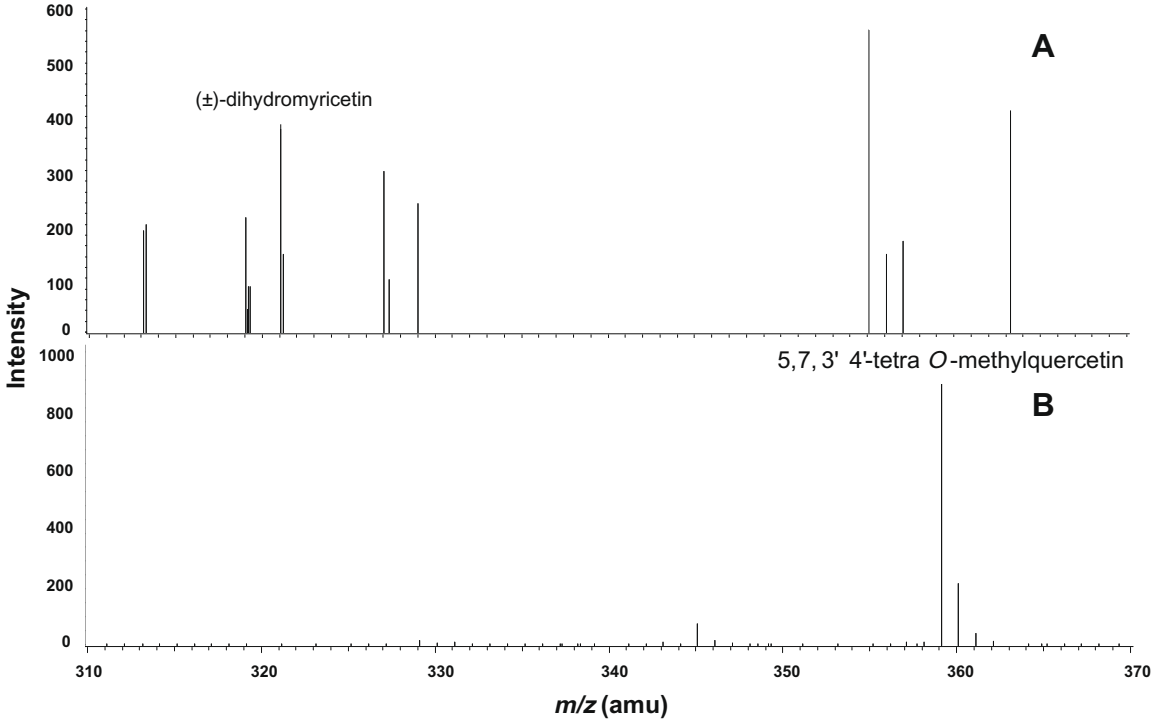

Fig. 6. DART TOF-MS fingerprints of (A) (±)-dihydromyricetin (**3**) (m/z = 321.0674), and (B) 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) (m/z = 359.2825) bound to H1N1 virions.

Minimum free-energy modeling analysis revealed that the A and B rings of compounds **1** and **2** form an axis with inter-phenolic ring distances of 10.5 Å and 10.9 Å, respectively (Fig. 3). This distance is well within the size constraints of the hemagglutinin (HA) binding domain pocket (14–15 Å) of influenza viruses, which is responsible for host cell receptor binding and viral entry (Stevens et al., 2004). The phenolic regions of dihydromyricetin (**3**), as well as compound **1**, most likely bind to the viral mannose-rich HA binding domains and, as such, the proposed bound orientation of dihydromyricetin (**3**) would leave the esterified functionality of **2** free to interact with the immune system, potentially increasing an immune response to the viral particles *in vivo* (Vigerust and Shepherd, 2007).

Proposed mechanisms for anti-H1N1 activity *in vitro* by commonly studied polyphenols (e.g. catechin, quercetin, cyanidin) include the prevention of endosome acidification (Imanishi et al., 2002), the inhibition of membrane fusion (Nagai et al., 1995), the inhibition of progeny virion release (Knox et al., 2001), the inhibition of neuraminidase activity (Knox et al., 2001; Macdonald et al., 2004; Song et al., 2005), and the inhibition of intercellular replication (Serkedjieva et al., 1990). These previously described mechanisms are different from the proposed molecular mode-of-action of compounds **1** and **2**. We show that the synthesized compounds, as well as the elderberry extract, inhibit H1N1 infection by binding to the viral envelope, most likely the HA domains involved is host cell binding and recognition. This binding mechanism subse-

Table 1
The IC_{50} values determined for 5,7,3′,4′-tetra-*O*-methylquercetin (**1**), the (±)-dihydromyricetin (**3**), Oseltamivir, and Amantadine.

Compound	IC_{50} (μg/mL)	IC_{50} (μM)
5,7,3′,4′-Tetra-*O*-methyl quercetin (**1**)	0.15	0.36
(±)-Dihydromyricetin (**3**)	2.8	8.7
Oseltamivir	0.1	0.32
Amantadine	4.1	27

quently blocks the ability of the H1N1 virions to bind to, and consequently enter, host cells.

3. Conclusions

Through the use of the Direct Binding Assay and DART TOF-MS analysis, it was possible to identify and characterize the molecular mode-of-action of two anti-influenza flavonoids in an optimized elderberry fruit extract. The identified compounds were 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate (**2**) and 5,7,3′,4′-tetra-*O*-methylquercetin (**1**). These flavonoids are the major contributors to the anti-influenza activity of the elderberry extract. The molecular mode-of-action of these flavonoids was determined by demonstrating their direct binding to H1N1 virus particles resulting in the inability of the H1N1 viruses to enter host cells, effectively preventing H1N1 infection *in vitro*. This mode-of-action was further verified using synthesized 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) and racemic dihydromyricetin (**3**) which bind to H1N1 virions and, when bound, blocked H1N1 infection *in vitro*.

DART TOF-MS analysis of botanical extracts is a highly accurate and efficient method for identifying compounds in complex mixtures. DART TOF-MS coupled with traditional analytical techniques will dramatically broaden and greatly enhance the understanding of the chemical complexity of botanical extracts.

4. Experimental

All solvents were purchased from Thermo Fisher Scientific (Fairlawn, NJ) unless specified below.

Elderberry extract preparation: Wild crafted elder berries (*Sambucus* nigra L., Caprifoliaceae) from Hungary were purchased from Blessed Herbs, Inc. (Oakham, MA; Product No. 724, Lot No. L10379w). The polymer adsorbent extract was obtained by extracting 20 g of ground elderberries using supercritical CO_2 at 60 °C and 300 bar for 2 h, followed by two extractions using EtOH:H_2O (100 mL, 4:1, v/v) EtOH for 2 h each. The combined extracted slurry was filtered through Fisherbrand P4 filter paper and centrifuged at 537×*g* for 20 min. The supernatant was vacuum distilled to remove EtOH, and the final solution concentration was ~35 mg/mL for polymer adsorbent loading.

Adsorption experiments were carried out at room temperature in an open batch system. The ADS5 polymer adsorbent (Nankai University, China) was washed with EtOH to remove monomers and impurities and soaked in distilled H_2O overnight before packing. The column was loaded with 60 mL of the above prepared solution and washed with two column volumes of distilled H_2O. The column was eluted using EtOH/H_2O (40 mL, 4:1, v/v). The collected fraction was dried at 50 °C overnight to yield a dark purple crystalline powder. This procedure was repeated multiple times to ensure reproducibility of the extract.

HPLC analysis of elderberry extracts: The extracts were characterized by HPLC–UV on a Shimadzu LC-10AVP system (Shimadzu, Singapore) with a LC10ADVP pump and a SPD-M 10AVP diode array detector at 280 and 350 nm by injecting 10 μL of a diluted extract solution in EtOH onto a reversed phase Jupiter C_{18} column (250 × 4.6 mm I.D., 5 μ, 300 Å; Phenomenex, Torrance, CA) with a flow rate of 1 mL/min. The column temperature was held at 25 °C. The mobile phase consisted of 5% (v/v) HCO_2H (solvent A) and MeOH (solvent B). The following linear gradient was used: 0–2 min, 5% B; 2–10 min, 5–24% B (hold 5 min); 15–30 min, 24–35% B (hold 5 min); 35–50 min, 45% B (hold 5 min); 55–65 min, 5% B (hold 3 min).

DART TOF-MS analysis of elderberry extracts, synthetic flavonoids and viral-bound compounds: The JEOL DART™ AccuTOF mass spectrometer (JMS-T100LC; Jeol USA, Peabody, MA) was used for chemical analysis of the elderberry fruit extracts and was executed in positive ion mode $[M+H]^+$. The needle voltage was set to 3500 V, heating element to 300 °C, electrode 1–150 V, electrode 2–250 V, and He gas flow to 3.98 L/min. For the mass spectrometer, the following settings were loaded: orifice 1 set to 20 V, ring lens voltage set to 5 V, and orifice 2 set to 5 V. The peak voltage was set to 1000 V in order to give peak resolution beginning at 100 *m/z*. The microchannel plate detector (MCP) voltage was set to 2550 V. Calibrations were performed internally with each sample using a 10% (w/v) solution of PEG 600 (Ultra Chemical, North Kingston, RI) that provided mass markers throughout the required mass range of 100–1000 amu. Calibration tolerances were held to 5 mmu. Samples (as dry powders for the extracts and synthetic flavonoids, and fixed virions for the Direct Binding Assays) were introduced into the DART He plasma using the closed end of a borosilicate glass melting point capillary tube until a signal was achieved in the total-ion chromatogram (TIC). The next sample was introduced when the TIC returned to baseline levels.

Candidate molecular formulae were identified using elemental composition and isotope matching programs in the Jeol MassCenterMain Suite software (JEOL USA, Peabody, MA). The candidate molecular formulae were assigned with a confidence level greater than 90%. The candidate molecular formulae were then used to determine plausible chemical structures (JEOL, 2007).

MassCenterMain was also used to determine candidate molecular formulae for the compounds that bind to the H1N1 virus. The DART-generated fragments were confirmed using the determined molecular formulae for each of the masses identified as bound to the H1N1 virions.

Three-dimensional free-energy minimizations: Chem3D Ultra (Cambridgesoft, Cambridge, MA) molecular modeling package was employed for the free-energy minimizations of the identified compounds using the molecular mechanics two level of theory.

Viral focus reduction infection assays: The viral focus reduction infection assays were modified from the procedure described by Okuno et al. (1990). The specific procedure used is described below.

The virus for this study was Influenza A (H1N1) virus strain A/PR/8/34 (ATCC, Manassas, VA; ATCC No. VR-1469). The elderberry extract and synthesized compounds were dissolved in a minimal volume of EtOH (USP grade) prior to dilution in DMEM (pH 7.4). Approximately 100 focus-forming units (FFU) of influenza virus were incubated with dilutions of the elder berry extract solution in DMEM for 1 h at room temperature and then allowed to infect confluent MDCK cells for 1 h at room temperature. After infection, cells were fixed with Formalde-fresh then permeabilized with EtOH (USP). The FFU's were visualized using goat anti-influenza A virus IgG polyclonal antibody, rabbit Anti-Goat IgG (H&L) horseradish peroxidase conjugated affinity purified antibody (Chemicon, Temecula, CA) and AEC chromogen substrate (Dako, Carpinteria, CA). These same methods were employed for the re-infection assays with viral-bound compounds.

Extract non-specific and cytotoxicity assessments: The possible non-specific effects of the elderberry extract on viral infection (e.g. positive control) as well as the potential toxicity of extracts was measured by monitoring mitochondrial reductase activity in

MDCK cells using the TACS™ MTT cell proliferation assay (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Direct Binding Assay: A Direct Binding Assay was developed to determine which compounds in the elderberry extract bind to H1N1 virions. The H1N1 virus particles were incubated in the elderberry extract or the synthesized flavonoids, and were then washed 3 times on an Amicon 100 kDa filter (Ultracel PL-100; Millipore Corp., Billerica, MA) with PBS to remove unbound compounds. The virus particles were then collected and a portion was fixed in 100% (USP) EtOH for DART TOF-MS analysis. In addition, the washed fractions containing the unbound chemicals were collected and analyzed directly by DART TOF-MS for comparison. The closed end of a borosilicate glass capillary tube was immersed in the virion solution (in EtOH) and passed through the DART He plasma to obtain mass spectra of virion surface-bound compounds as described in Section 4.2.

Compound synthesis: The synthesis of the 5,7,3′,4′-tetra-*O*-methylquercetin (**1**) and racemic dihydromyricetin (**3**) have been adapted from previous reports (Koeppen et al., 1962; Li et al., 1990; Rao and Weisner, 1981). Specific methodologies are described below.

4.1. Synthesis of 5,7,3′,4′-tetra-O-methylquercetin (*1*)

Dimethyl sulphate (138 g, 109 mmol) was added slowly to a mixture of Rutin monohydrate (50 g, 82 mmol) and powdered K_2CO_3 (210 g, 152 mmol) in acetone (1 L) at RT over a period of 30 min. The reaction was heated to reflux and maintained for 80 h. The reaction was cooled to RT, filtered through Celite and washed with acetone (250 mL). The combined acetone layer was concentrated under vacuum to give a pale yellow gummy solid (**4**, 48 g). The gummy solid was dissolved in 20% (v/v) HCl in H_2O (500 mL), heated to 100 °C and maintained for 3 h. The reaction mixture was cooled and extracted with CH_2Cl_2 (4 × 500 mL). The combined organic layer was washed with H_2O (1 L), brine (1 L) and dried (Na_2SO_4). The organic layer was filtered and concentrated under vacuum to give a dark solid (21 g) which was purified by silica gel column chromatography and eluted with EtOAc:hexanes (1:1) followed by CH_2Cl_2:MeOH (4:1). The CH_2Cl_2:MeOH fractions were concentrated under vacuum and yielded a brown residue which was triturated with neat *iso*-PrOH (100 mL) and stirred for 1 h. The resulting pale green solid was filtered, washed with cold *iso*-PrOH (25 mL) and dried at 60 °C under vacuum for 12 h resulting in an off-white powder (9.0 g, 31% overall yield). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 7.82 (1H, *s*, H-2′), 7.41 (1H, *s*, H-3′), 6.99 (1H, *s*, H-6′), 6.55 (1H, *s*, H-8), 6.35 (1H, *s*, H-6), 3.99 (6H, *s*), 3.96 (3H, *s*), 3.92 (3H, *s*). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 172.0 (C-4), 164.5 (C-7), 160.7 (C-5), 159.0 (C-9), 150.5 (C-3′ and C-4′), 142.2 (C-2), 137.7 (C-3), 124.0 (C-1′), 120.8 (C-6′), 111.3 (C-2′), 110.9 (C-5′), 106.3 (C-10), 95.8 (C-6), 92.6 (C-8), 56.2, 55.9. ESI-MS (positive): $[M]^+$ = 358.2; $[M+H]^+$ = 359.3; $[M+H-CH_3]^+$ = 344.4; $[M+H-2\times CH_3]^+$ = 329.3.

4.2. 2,4,6-Tris(methoxymethoxy)acetophenone (*8*)

A mixture of 2,4,6-trihydroxyacetophenone (**6**, 1 g, 5.4 mmol) in dry DMF (20 mL) was added to a slurry of NaH (60% in mineral oil, 0.9 g, 20 mmol) in dry DMF (10 mL) at 0–5 °C over a period of 30 min under N_2 and stirred for 1 h at RT. The reaction mixture was cooled to 0–5 °C; a solution of chloromethyl methylether (1.75 g, 22 mmol) in dry DMF was added slowly over a period of 15 min. The reaction mixture was stirred at RT for 4 h, poured in to ice-cold H_2O (100 mL), and extracted with EtOAc (2 × 50 mL). The combined organic layer was washed with distilled H_2O (50 mL), brine (50 mL) and dried (Na_2SO_4). The filtered organic layer was concentrated under vacuum and the resultant oily residue was purified by silica gel column chromatography by eluting with hexanes:EtOAc (9:1) followed by hexanes:EtOAc (8.5:15) to give compound **8** (0.78 g, 48%). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 6.51 (2H, *s*, H-3 and H-5), 5.14 (6H, –C*H*$_2$–, *s*), 3.47 (3H, –OC*H*$_3$, *s*), 3.45 (6H, –OC*H*$_3$, s), 2.50 (3H, C(O)C*H*$_3$, *s*). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 204.1 (*C*=O), 163.0 (C-4), 160.2 (C-2 and C-6), 116.1 (C-1), 95.1 (3× –*C*H$_2$–), 93.8 (C-3, C-5), 55.7 (3× –OC*H*$_3$), 33.0 (C(O)*C*H$_3$). HRMS (positive ion) = 301.1281 (calcd. for $C_{14}H_{21}O_7$ = 301.1287).

4.3. 3,4,5-Tris(methoxymethoxy)benzaldehyde (*9*)

A mixture of 3,4,5-trihydroxy benzaldehyde (**7**, 0.5 g, 2.9 mmol), K_2CO_3 (4 g, 29 mmol), and dry acetone (100 mL) were placed in a 2-necked RB flask under N_2 and the mixture was cooled to 10–15 °C. Chloromethyl methyl ether (1.44 g, 18 mmol) was added slowly over a period of 30 min at 10–15 °C, and the reaction mass was allowed to reflux slowly over a period of 6 h. After refluxing, the reaction mixture was filtered, washed with acetone (50 mL), concentrated under vacuum and extracted with EtOAc (2 × 25 mL). The combined organic layer was washed with distilled H_2O (25 mL), brine (25 mL) and dried (Na_2SO_4). The filtered organic layer was concentrated and the resultant oily residue was purified by silica gel column chromatography by eluting with hexanes:EtOAc (4:1) to give compound **9** (0.6 g, 72%). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 9.87 (1H, *s*, C*H*(O)), 7.40 (2H, *s*, H-2 and H-6), 5.29 (6H, *s*, –C*H*$_2$–), 3.66 (3H, *s*, –OC*H*$_3$), 3.54 (6H, *s*, –OC*H*$_3$). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 191.9 (*C*=O), 151.2 (C-3 and C-5), 140.3 (C-4), 132.2 (C-1), 106.2 (C-2 and C-6), 98.0 (–*C*H$_2$–), 94.9 (2× –*C*H$_2$–), 55.9 (3× –OC*H*$_3$). HRMS (positive ion) = 287.1138 (calcd. for $C_{13}H_{19}O_7$ = 287.1131).

4.4. 2′,4′,6′,3,4,5,-Hexakis(methoxymethoxy)chalcone (*10*)

A solution of 40% KOH in EtOH (20 mL) was added to a mixture of **8** (1 g, 3.33 mmol) in EtOH (5 mL) cooled to less than 20 °C. After stirring for 15 min, a solution of **9** (1 g, 3.5 mmol) in EtOH (5 mL) was added slowly over a period of 10 min and allowed to stir overnight at RT. The reaction was quenched with distilled H_2O (50 mL) and extracted with EtOAc (2 × 50 mL). The combined organic layer was washed with distilled H_2O (50 mL), brine (50 mL) and dried (Na_2SO_4). The organic layer was concentrated under vacuum to give compound **10** as a pale yellow solid (1.5 g, 78%). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 7.22 (1H, *d*, *J* = 12 Hz, H-α), 7.04 (2H, *s*, H-3′ and H-5′), 6.87 (1H, *d*, *J* = 12 Hz, H-β), 6.57 (2H, *s*, H-2 and H-6), 5.20 (6H, *s*), 5.17 (2H, *s*), 5.11 (4H, *s*), 3.61 (3H, *s*), 3.51 (3H, *s*), 3.49 (6H, *s*), 3.39 (6H, *s*). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 192.9 (*C*=O), 164.2 (C-4′), 163.6 (C-2′, C-6′), 151.0 (C-3, C-5), 145.3 (C-β), 133.1 (C-4), 127.2 (C-α), 126.5 (C-1), 112.9 (C-1′), 103.6 (C-2, C-6), 95.3 (3× –*C*H$_2$–), 94.7 (3× –*C*H$_2$–), 55.9 (6× –OC*H*$_3$). HRMS (positive ion) = 569.2251 (calcd. for $C_{27}H_{37}O_{13}$ = 569.2234).

4.5. 2′,4′,6′-Tris(methoxymethoxy)phenyl(3-(3,4,5-tris(methoxymethoxy)phenyl)oxiran-2-yl)methanone (*11*)

H_2O_2 (50% [v/v], 1 mL, 17.35 mmol) was added to a mixture of chalcone **10** (1 g, 1.8 mmol) and 2 N NaOH (3 mL), and stirred for overnight at RT in MeOH (30 mL). The MeOH was concentrated under vacuum and the resultant residue was extracted with EtOAc (2× 50 mL). The combined organic layer was washed with distilled H_2O (50 mL), brine (50 mL) and dried (Na_2SO_4). The organic layer was concentrated under vacuum to give compound **11** as a thick pale yellow oil (0.72 g, 70%). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 6.77 (2H, *s*, H-2 and H-6), 6.49 (2H, *s*, H-3′ and H-5′), 5.13 (12H, *s*, –C*H*$_2$–), 3.91 (1H, *d*, *J* = 2 Hz, H-α), 3.83 (1H, *d*, *J* = 2 Hz, H-β), 3.58

(3H, *s*), 3.44 (9H, *s*), 3.37 (6H, *s*). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 196.5 (C=O), 163.5 (C-4′), 162.2 (C-2′ and C-6′), 150.9 (C-3 and C-5), 134.1 (C-4), 131.6 (C-1), 103.4 (C-1′), 99.7 (C-2 and C-6), 95.2 (3× $-CH_2-$), 94.9 (3× $-CH_2-$), 69.1 (C-α), 58.2 (C-β), 55.5 (6× $-OCH_3$). HRMS (positive ion) = 585.2193 (calcd. for $C_{27}H_{37}O_{14}$ = 585.2183).

*(±)-Dihydromyricetin (**3**):* A mixture of **11** (0.2 g) and HCl in MeOH (1.25 M, 3.0 mL, 3.75 mmol) was stirred at 45 °C for 30 min. The MeOH was concentrated under vacuum and the resultant dark residue was purified by silica gel column chromatography by eluting with EtOAc:hexanes (1:1) followed by CH_2Cl_2:MeOH (9:1) to give compound **3** as an off-white powder (0.70 g, 66%). ^{1}H NMR ($CDCl_3$; 400 MHz) δ 6.62 (2H, *s*, H-2′ and H-6′), 5.98 (1H, *s*, H-8), 5.94 (1H, *s*, H-6), 4.96 (1H, *d*, *J* = 12 Hz, H-2), 4.57 (1H, *d*, *J* = 12 Hz, H-3). ^{13}C NMR ($CDCl_3$; 400 MHz) δ 197.9 (C-4), 167.6 (C-7), 164.8 (C-9), 164.0 (C-5), 146.1 (C-3′), 134.0 (C-1′), 128.9 (C-4′), 107.9 (C-2′ and C-6′), 101.4 (C-10), 96.8 (C-8), 95.7 (C-6), 84.4 (C-3), 72.9 (C-2). HRMS (positive ion) = 320.0541 (calcd. for $C_{15}H_{12}O_8$ = 320.0532).

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