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Antiviral Research 88 (2010) 1–9

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Influenza virus variation in susceptibility to inactivation by pomegranate polyphenols is determined by envelope glycoproteins

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ARTICLE INFO

Article history:
Received 27 August 2009
Received in revised form 5 June 2010
Accepted 14 June 2010

Keywords:
Influenza A virus
Pomegranate
Polyphenols
Plant extract
Antiviral

ABSTRACT

Pomegranates have high levels of polyphenols (PPs) and may be a rich source of compounds with antiviral activity. We evaluated the direct anti-influenza activity of three commercially available pomegranate extracts: pomegranate juice (PJ), a concentrated liquid extract (POMxl), and a 93% PP powder extract (POMxp). The acidity of PJ and POMxl solutions contributed to rapid anti-influenza activity, but this was not a factor with POMxp. Studies using POMxp showed that 5 min treatment at room temperature with 800 μg/ml PPs resulted in at least a 3 log reduction in the titers of influenza viruses PR8 (H1N1), X31 (H3N2), and a reassortant H5N1 virus derived from a human isolate. However, the antiviral activity was less against a coronavirus and reassortant H5N1 influenza viruses derived from avian isolates. The loss of influenza infectivity was frequently accompanied by loss of hemagglutinating activity. PP treatment decreased Ab binding to viral surface molecules, suggesting some coating of particles, but this did not always correlate with loss of infectivity. Electron microscopic analysis indicated that viral inactivation by PPs was primarily a consequence of virion structural damage. Our findings demonstrate that the direct anti-influenza activity of pomegranate PPs is substantially modulated by small changes in envelope glycoproteins.

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1. Introduction

The control of influenza A virus infections in humans continues to present major challenges. Seasonal influenza epidemics account for an annual average of approximately 36,000 deaths and more than 200,000 hospitalizations in the United States. In addition, occasional influenza pandemics with extensive worldwide morbidity and mortality are a feature of human history (Salomon and Webster, 2009). The ongoing influenza threat is a consequence of the periodic emergence of variant viruses with diminished susceptibility to pre-existing Ab-mediated immunity in human populations (Subbarao and Joseph, 2007). Vaccines provide an effective strategy for protection against seasonal and pandemic influenza, but vaccine formulation and manufacture is always playing "catch-up" to emerging viruses. Furthermore, influenza has a demonstrated capacity to develop resistance to the small number of available anti-influenza medications (Weinstock and Zuccotti, 2009). There is clearly a need for the continued development of anti-influenza strategies that, ideally, are effective against antigenically diverse viruses.

The long history of traditional use of the pomegranate fruit as a folk remedy for multiple ailments, including respiratory infections, is supported by a growing number of studies (Lansky and Newman, 2007). A variety of medical benefits have now been associated with constituents of the fruit, particularly the high levels of polyphenolic compounds, including hydrolysable tannins (predominantly punicalagins) and plant flavonoids such as the anthocyanins (Gil et al., 2000; Tzulker et al., 2007). Cardiovascular benefits, antiatherosclerotic effects, and inhibition of tumor initiation and growth have been attributed to potent antioxidative activity mediated primarily by the polyphenols (PPs) in pomegranates (Adams et al., 2006; Aviram et al., 2008; de Nigris et al., 2005; Ignarro et al., 2006; Malik et al., 2005). Pomegranate components also have growth inhibiting activity against diverse microorganisms. Direct antibacterial, antifungal, and antiplasmodial activity was demonstrated for pomegranate PP fractions (Reddy et al., 2007). Binding by a

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doi:10.1016/j.antiviral.2010.06.014

pomegranate component to the viral envelope glycoprotein of HIV-1 blocked interaction with cell receptors and inhibited infection, but the chemical nature of the active component was not defined (Neurath et al., 2004).

Studies of polyphenolic compounds derived from a range of plant species have demonstrated antiviral effects against DNA and RNA viruses, indicating the potential for broad spectrum antiviral activity (Ehrhardt et al., 2007; Nakayama et al., 1993; Schnitzler et al., 2008; Serkedjieva and Manolova, 1992; Weiss et al., 2005). There is evidence that plant PPs exert an antiviral effect by interacting directly with viral particles (Ehrhardt et al., 2007; Nakayama et al., 1993; Schnitzler et al., 2008; Song et al., 2005), although the extent of PP binding to viral surface components may be influenced by the nature of the virus (Ehrhardt et al., 2007; Serkedjieva, 2003). Plant PPs may also exert antiviral effects during intracellular replication (Palamara et al., 2005). In part, this may be due to PPs opposing the pro-oxidant state induced in cells by the replication of some viruses (Fraternale et al., 2009).

The high level of diverse polyphenolic compounds in pomegranates suggests that the fruit may be a rich source of molecules that exert antiviral activity via multiple mechanisms. Recently, evidence was presented that pomegranate PPs are directly virucidal for influenza viruses and also act at the intracellular level to inhibit influenza virus replication (Haidari et al., 2009). The current study focused on the direct anti-influenza virus activity of pomegranate extracts. We demonstrate that influenza viruses representing diverse subtypes were susceptible to rapid inactivation by components of the PP fraction of pomegranates. However, susceptibility to inactivation differed between reassortant H5N1 viruses, indicating modulation by relatively small changes in virion surface glycoproteins. Our findings also indicate that inactivation is primarily a consequence of PP-induced virion structural damage.

2. Materials and methods

2.1. Pomegranate extracts

Extracts were prepared from California-grown and processed Wonderful variety pomegranates and supplied by POM Wonderful (Los Angeles, CA). Three forms of commercially available extracts were used: pomegranate juice (PJ), a pomegranate liquid extract (POMxl), and a pomegranate PP powder extract (POMxp). Preparation of the pomegranate extracts and the colorimetric determination of phenolic content (predominantly pomegranate PPs) is described elsewhere (Aviram et al., 2008; Heber et al., 2007). Total phenolic content of the extracts (expressed as μg gallic acid equivalents) was estimated as 3600 μg/ml (0.36%) for PJ, 130,000 μg/ml (13%) for POMxl, and 930 μg/mg (93%) for POMxp (Aviram et al., 2008).

2.2. Viruses and viral titration

Experiments were performed using the following influenza A viruses:

(i) A/Puerto Rico/8/34 (H1N1) (PR8) and the reassortant virus A/HK/X31 (H3N2) (X31), which expressed the H3 and N2 of A/Aichi/2/68 on the PR8 background (Kilbourne et al., 1971).
(ii) Reassortant H5N1 viruses generated by reverse genetics (identified by the prefix rg) from A/Vietnam/1203/04 (VN/04), an isolate from a fatal human case, and from the avian isolates A/Duck/Hunan/795/02 (Dk/HN/02), A/Duck/Laos/3295/06 (Dk/LS/02), and A/Japanese White Eye/Hong Kong/1038/06 (JWE/HK/06). The reassortant H5N1 viruses were constructed with PR8 and expressed H5 (from which polybasic amino acids that are associated with high virulence were removed) and N1 of the original isolates (Forrest et al., 2009).
(iii) A reassortant (PR8 background) expressing the H1 and N1 of A/California/7/09 (CA/09), a human isolate of the novel swine-origin A(H1N1) virus that spread globally in 2009 and generated a pandemic alert (Maines et al., 2009).
(iv) A/New Caledonia/20/99 (H1N1) (NC/99), a strain that circulated in human populations in the United states for approximately the past 10 years (Wood, 2002).

Influenza virus stocks were grown in the allantoic cavity of embryonated hen's eggs and titers of infectious virus were measured by 50% tissue culture infective dose ($TCID_{50}$) titration. Madin Darby canine kidney (MDCK) cells for viral titration were grown in MEM with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (10 μg/ml), and supplemented with 10% FBS. Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum-free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% BSA and 1 μg/ml L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ) were prepared in replicate wells of MDCK monolayers (200 μl total volume per well) and incubated for 2–3 days at 37 °C with 5% CO_2. Wells positive for virus growth were identified by the presence of hemagglutinating (HAg) activity in the supernatant, and $TCID_{50}$ titers were calculated by the method of Reed and Muench.

Mouse hepatitis virus (MHV) A59 obtained from Dr. David Brian, University of Tennessee, Knoxville, TN, was grown in rat lung epithelial (RLE) cells cultured in DMEM with L-glutamine (2 mM), HEPES (4 mM), and gentamicin (10 μg/ml), and supplemented with 10% FBS. To determine viral titers by plaque assay, serial 10-fold dilutions of virus in cell culture medium were added to washed RLE cell monolayers in 6-well tissue culture plates (200 μl/well). Plates were incubated for 2 h at 37 °C to allow viral adsorption, wells were washed, and an agarose overlay (1% agarose in MEM supplemented with L-glutamine, gentamicin, nonessential amino acids, and 10% FBS) was added and allowed to set. Plates were incubated inverted at 37 °C with 5% CO_2, and then cell monolayers were fixed with 1% formaldehyde and stained with Coomassie Blue to visualize viral plaques.

2.3. Treatment of virus with pomegranate extracts and evaluation of antiviral activity

PJ and POMxl were tested at a range of concentrations in PBS. POMxp was dissolved in PBS at a PP concentration of 4.0 mg/ml (=4.3 mg/ml POMxp), passed through a 0.2 μm diameter filter, and diluted in PBS for testing. Influenza viruses at 5×10^7 $TCID_{50}$/ml unless specified were combined with different concentrations of PJ and POMxl and incubated for 5 min at room temperature. Immediately following incubation, titers of infectious virus were determined by $TCID_{50}$ assay in MDCK cells. To control for antiviral effects due solely to pH, virus was treated in parallel with citrate/phosphate buffers matching the pH of test solutions of PJ and POMxl. A similar strategy was used to evaluate the anti-influenza activity of different concentrations of POMxp (expressed as PP concentration). To control for possible effects of low PP concentrations in cell culture medium during incubation of the $TCID_{50}$ assay, untreated influenza viruses were titrated in wells containing PP concentrations that matched those in wells during titration of POMxp-treated viruses. A range of concentrations of POMxp (up to 1600 μg/ml PP) were diluted 1 in 10 in cell culture medium over MDCK cell monolayers in replicate wells. Influenza viruses at 5×10^7 $TCID_{50}$/ml were then diluted 1 in 10 in the same wells. The media in these wells were then immediately serially diluted

10-fold in replicate wells of MDCK cell monolayers. Plates were then incubated and $TCID_{50}$ titers were determined as described above. The activity of POMxp against MHV A59 at 2.5×10^7 PFU/ml was tested as described for influenza virus, with the exception that infectious viral titers after treatment were determined by plaque assay.

The effect of POMxp on MDCK cell viability was evaluated using the MTT cell proliferations assay kit (ATCC, Manassas, VA) according to the manufacturer's instructions. Briefly, confluent MDCK cell monolayers were washed, and culture medium containing POMxp concentrations up to 160 μg/ml PPs were added to triplicate wells (200 μl/well). After 24 h incubation, cell monolayers were washed and 100 μl culture medium containing 10 μl of MTT reagent was added to each well. Plates were held in a 37 °C incubator for 3 h, and 100 μl/well of detergent reagent was added. After overnight incubation at room temperature, absorbance at 570 nm was read using a BioTek Synergy 2 microplate reader.

The HAg activity of influenza viruses was also evaluated immediately after treatment with different concentrations of POMxp as described above. Serial 2-fold dilutions of treated virus were prepared with PBS in 96-well round-bottom plates (50 μl/well). An equal volume of 0.5% chicken RBC resuspended in PBS with 0.3% BSA was added to each well, plates were briefly agitated, and hemagglutination (HAg) was scored after 30 min incubation at room temperature. To investigate whether PPs present in the HAg assay interacted with RBC surface molecules and inhibited virus-mediated HAg, the efficacy of POMxp-treated RBCs in the HAg assay was assessed. RBCs were mixed with a range of concentrations of POMxp (up to 1600 μg/ml PP) and incubated at room temperature for 30 min. After a single gentle wash, RBCs were resuspended at a concentration of 0.5% in PBS with 0.3% BSA. POMxp- and PBS-treated RBCs were used to determine the titer of influenza X31 as described above. POMxp alone at a PP concentration of 4 mg/ml had no HAg activity when the assay was performed in the presence of 0.15% BSA, as was the case in all experiments. In the absence of BSA, PP concentrations greater than approximately 50 μg/ml had inherent HAg activity.

2.4. Inhibition ELISA

A modified ELISA (Sangster et al., 2000) was used to evaluate the inhibition of binding of virus-specific Abs by PPs. Briefly, 96-well ELISA plates were coated with 0.2 μg/well of sucrose-gradient purified, detergent-disrupted virus, blocked with 3% BSA in PBS, and washed. Serial 2-fold dilutions of a POMxp solution starting at 4 mg/ml were prepared in PBS and added to the plates in triplicate wells (100 μl/well). Plates were incubated at room temperature for 1 h and washed 3× with PBS. Virus-specific Abs were diluted in PBS–Tween (0.05%)–BSA (0.5%) and added to appropriate wells (50 μl/well). The Ab preparations were used at concentrations that gave an absorbance of approximately 1.0 in the absence of PP inhibition. Plates were incubated for 2 h and washed. Bound Abs were detected with alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL), followed by *p*-nitrophenyl phosphate substrate. Absorbance at 405 nm was read using a microplate reader. The viruses used were X31, rg-VN/04, and rg-Dk/HN/02 influenza viruses and MHV A59. Virus-specific Abs were the influenza-specific Mabs X31 14-4 (H3-specific), GY-14 (N2-specific), and VN-10 (H5-specific), and a rabbit antiserum (Vu-14) raised against MHV A59 and kindly provided by Dr. Mark Denison, Vanderbilt University, Nashville, TN. To control for non-specific Ab adherence to PP-treated viruses, the analysis was also performed using plates coated with influenza PR8 (H1N1).

2.5. Electron microscopy

The X31, PR8, and rg-Dk/HN/02 influenza viruses and MHV A59 were treated for 5 min at room temperature with 1600 μg/ml PPs and negatively stained for examination by transmission electron microscopy. Untreated control virus was incubated with PBS. Immediately after treatment, samples were adsorbed to freshly glow-discharged collodion and carbon films on 400 mesh copper grids, stained with 0.5% phosphotungstic acid, and examined with a Hitachi H-800 electron microscope at 75 kV.

2.6. Statistical analysis

Group means were compared using the two-tailed Mann–Whitney U test for unpaired samples. Contingency table analyses involving proportions of normal or aberrant virions were performed using the two-sided Fisher's exact test. Tests were performed using GraphPad software (San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Pomegranate PPs eliminate influenza virus infectivity and hemagglutinating activity

Initial experiments tested PJ and POMxl for direct antiviral activity against influenza X31. The virus was treated for 5 min at room temperature with different concentrations of PJ and POMxl and titers of infectious virus were determined immediately thereafter. Titers were consistently lower after PJ and POMxp treatment compared with the pH control, suggesting antiviral activity that was distinct from the effect of pH alone. However, differences were significant only for 2.5% POMxl (Fig. 1A and B). An identical approach was used to evaluate the anti-influenza activity of PP-enriched POMxp. Exposure of influenza X31 to >100 μg/ml PPs rapidly reduced infectivity, with titers being lowered by more than 4 logs at 800 μg/ml (Fig. 2A). To determine whether pomegranate PPs had antiviral activity against other influenza subtypes, we evaluated the activity of POMxp solutions against PR8 (H1N1). As was the case for X31 (H3N2), infectivity was rapidly eliminated by treatment with >100 μg/ml PPs (Fig. 2B). The titers of X31 and PR8 were not decreased after incubation with control buffers matching the pH of 1600, 400 and 100 μg/ml PPs (6.5, 6.8, and 6.9, respectively) (data not shown).

The $TCID_{50}$ assay that we routinely applied to measure titers of infectious influenza virus after POMxp treatment was performed by incubating serial 10-fold dilutions of treated virus for 2–3 days in replicate wells of MDCK cell monolayers. Low concentrations of PPs were therefore present in wells during incubation of the assay and could potentially affect viral titers by blocking viral replication at the cellular level (Haidari et al., 2009) or by reducing MDCK cell viability (Seeram et al., 2005). In one set of experiments to control for this, the pre-incubation of X31 and PR8 with different POMxp concentrations prior to $TCID_{50}$ titration was omitted; instead, the viruses were first combined with POMxp as 10-fold dilutions were prepared over MDCK cell monolayers for $TCID_{50}$ titration. Essentially, this approach titrated untreated viruses in wells containing PP concentrations that matched those in wells during titration of POMxp-treated viruses. $TCID_{50}$ titers were identical to those measured using PP-free culture medium (data not shown), indicating that the antiviral activity that we describe for POMxp does not reflect effects during $TCID_{50}$ titration. Furthermore, analysis by the MTT assay indicated that 24 h incubation of MDCK cells with 160 μg/ml PPs (the maximum PP concentration in wells during $TCID_{50}$ titration of POMxp-treated viruses) had no

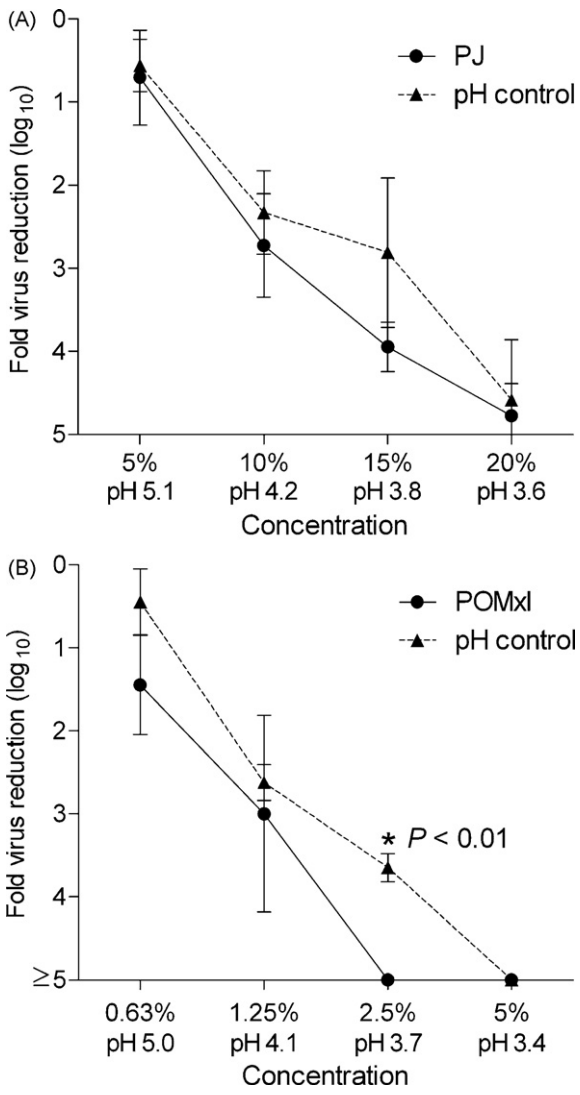

Fig. 1. Antiviral activity of PJ and POMxl against influenza virus. Influenza X31 (H3N2) at 5×10^7 $TCID_{50}$/ml was treated for 5 min at room temperature with different concentrations of PJ (A) or POMxl (B), or with a pH-matched buffer. Infectious virus titers after treatment were determined by $TCID_{50}$ assay. Results are shown as the fold reduction in titer relative to untreated virus (incubated with PBS). Data are mean ± SD for 3–6 separate experiments. $^*P < 0.01$.

adverse effect on cell viability. In a strategy to minimize the effects of PPs during $TCID_{50}$ titration, POMxp-treated X31 and PR8 were incubated on MDCK monolayers for 1 h to allow viral adsorption. Plates were then washed, and PP-free culture medium was added for the duration of the assay. Results matched those shown in Fig. 2A and B. Taken together, our findings demonstrate that a direct interaction between pomegranate PPs and viral particles is responsible for potent antiviral activity against X31 and PR8.

Influenza virus-induced HAg results from binding of the viral hemagglutinin (HA) to sialic acid residues on RBC surface molecules and reflects RBC cross-linking by intact virions. To determine the effect of POMxp treatment on the HAg activity of X31 and PR8, viral preparations were treated as described above for the analysis of effects on viral infectivity. HAg titers were measured immediately following incubation of the virus with POMxp solutions. A loss of HAg activity accompanied the loss of infectivity of X31 and PR8 (Fig. 2A and B), raising the possibility of PP interactions with the viral HA or loss of virion integrity. To investigate whether PPs present in the HAg assay bound to and masked RBC-associated sialic acids and rendered RBCs resistant to virus-induced HAg, the HAg titer of X31 was determined using RBCs that had been pre-treated with POMxp. The POMxp-treated RBCs gave the same HAg titer for X31 as did PBS-treated RBCs. In total, results indicate that the loss of viral HAg activity after POMxp treatment reflects a direct and preferential interaction between PPs and viral particles.

The activity of POMxp against an unrelated RNA virus was evaluated using a coronavirus, MHV A59. Titers of infectious MHV A59 were reduced by PP concentrations ≥200 μg/ml (Fig. 2C), but the antiviral effect was markedly less than that against influenza virus. Treatment of comparable viral concentrations with 1600 μg/ml PP reduced X31 and PR8 titers by approximately 6 logs, but the reduction in MHV A59 titer was only approximately 2.5 logs, suggesting that the antiviral activity is virus-specific.

3.2. *Influenza virus variants differ in susceptibility to pomegranate PPs*

In our analysis, POMxp demonstrated potent antiviral activity against X31 and PR8, two long-established and now standard laboratory strains of influenza A virus. An important question was whether antiviral activity would be evident against influenza viruses displaying a diversity of envelope glycoproteins, since changes in these molecules typify emerging influenza viruses that threaten human health. To investigate this, POMxp activity was tested against reassortant influenza viruses expressing the HA and neuraminidase (NA) molecules of H5N1 isolates and an isolate of the recent pandemic H1N1 virus of swine origin. The non-HA and NA components of the reassortants were derived from PR8.

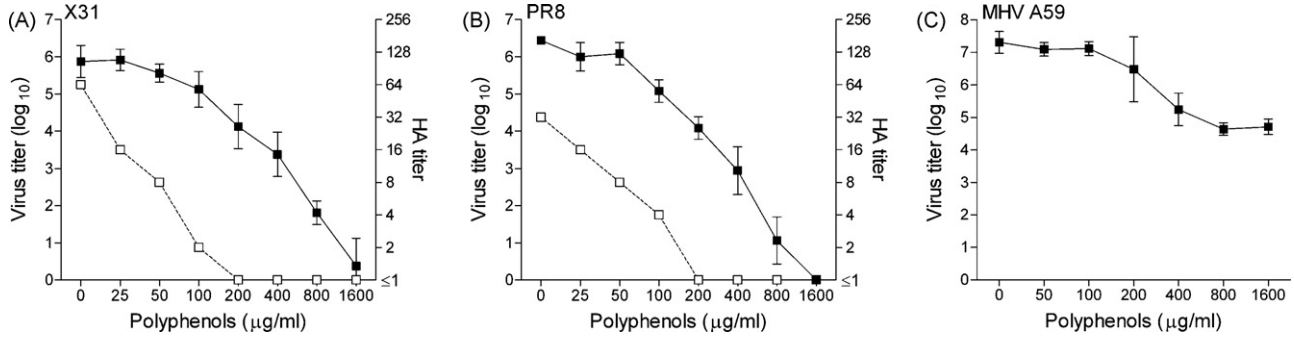

Fig. 2. Antiviral activity of POMxp against influenza viruses X31 and PR8 and the coronavirus MHV A59. Influenza X31 (H3N2) (A) and PR8 (H1N1) (B) at 5×10^7 $TCID_{50}$/ml were treated for 5 min at room temperature with different concentrations of POMxp (expressed as PP concentration). Infectious virus titers measured by $TCID_{50}$ assay (filled squares, left axis) and HAg titers (open squares, right axis) were determined after treatment. MHV A59 (C) at 2.5×10^7 PFU/ml was treated with POMxp as for influenza virus and infectious virus titers were determined by plaque assay. Infectious virus titers are mean ± SD for 3–4 separate experiments. HAg titers are representative of three separate experiments that gave similar results.

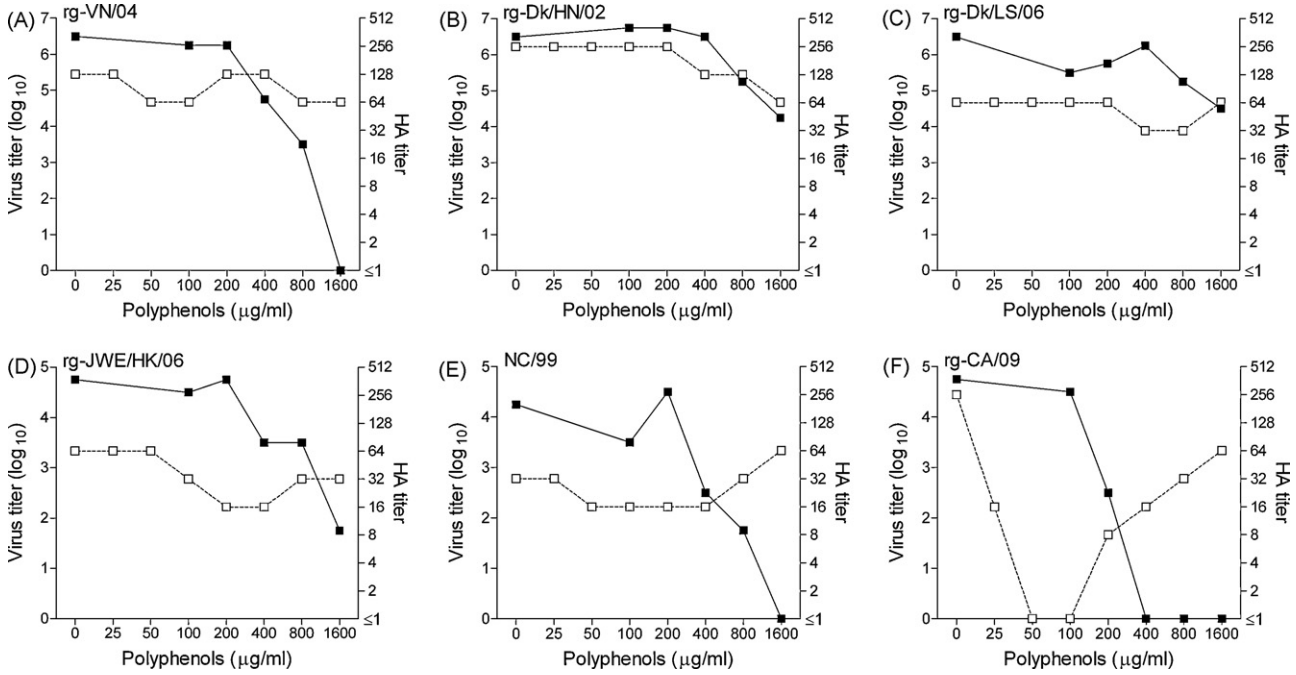

Fig. 3. Antiviral activity of POMxp against reassortant H5N1 and H1N1 influenza viruses and influenza NC/99 (H1N1). The following influenza viruses at the indicated titers were treated for 5 min at room temperature with different concentrations of POMxp (expressed as PP concentration): rg-VN/04 (H5N1) at 5×10^7 $TCID_{50}$/ml (A), rg-Dk/HN/02 (H5N1) at 5×10^7 $TCID_{50}$/ml (B), rg-Dk/LS/02 (H5N1) at 2.8×10^7 $TCID_{50}$/ml (C), rg-JWE/HK/06 (H5N1) at 1×10^7 $TCID_{50}$/ml (D), NC/99 (H1N1) at 2.5×10^5 $TCID_{50}$/ml (E), and rg-CA/09 (H1N1) at 7.4×10^5 $TCID_{50}$/ml. Infectious virus titers measured by $TCID_{50}$ assay (filled squares, left axis) and HAg titers (open squares, right axis) were determined after treatment. Results are representative of at least two separate experiments that gave similar results.

Compared with the effect against X31 and PR8 (Fig. 2A and B), POMxp treatment resulted in only a minor reduction in the infectivity of rg-Dk/HN/02, rg-Dk/LS/02, and rg-JWE/HK/06. However, the infectivity of rg-VN/04 was markedly reduced, resembling the effect of POMxp on X31 (Fig. 3A–D). Thus, the susceptibility of variant H5N1 subtype viruses to PP treatment is modulated by the nature of the expressed HA and NA. Treatment with sufficiently high concentrations of POMxp completely eliminated the infectivity of the H1N1 viruses NC/99 and rg-CA/09 (Fig. 3E and F). Rg-CA/09 was particularly susceptible and titers were reduced by >4 logs at 400 μg/ml PPs.

POMxp treatment had little effect on the HAg activity of rg-Dk/HN/02, rg-Dk/LS/02, and rg-JWE/HK/06, a result consistent with the minor effect of POMxp on the infectivity of these viruses. However, the HAg activity of rg-VN/04 and NC/99 was also not lost after treatment with PP concentrations that completely eliminated infectivity, suggesting that effects on infectivity were not simply a consequence of PP coating of viral particles. Surprisingly, the HAg activity of rg-CA/09 was lost after treatment with 50 and 100 μg/ml PPs, but not after treatment with higher PP concentrations that eliminated infectivity. Overall, our findings indicate that the effect of PP exposure on HAg activity is not a predictor of effects on infectivity. However, a cautious interpretation is warranted because POMxp itself has HAg activity when the assay is performed in the absence of BSA, and it is not possible to predict the consequences of potential multidirectional interactions when PP-treated viruses are combined with RBC in a BSA solution. In experiments described in this section, X31 was tested in parallel to ensure the reproducibility of earlier findings. The results for X31 matched those shown in Fig. 2A.

3.3. Viral inactivation by pomegranate PPs primarily reflects damage to virion integrity

An ELISA-based Ab inhibition assay was used to investigate whether POMxp treatment resulted in a coating of virion surface components. POMxp treatment of plate-bound influenza X31 (H3N2) inhibited the binding of mAbs specific for the H3 and N2 (Fig. 4A). Interestingly, the inhibitory effect plateaued at high PP concentrations and complete inhibition of mAb binding was not achieved. This residual binding was due to specific mAb–ligand interactions, since the mAbs were not bound in control experiments using plate-bound PR8 (H1N1) instead of X31. POMxp treatment also inhibited the binding of an H5-specific mAb to plate-bound rg-VN/04 (Fig. 4B) and rg-Dk/HN/02 (Fig. 4C). Notably, this analysis did not clearly discriminate between X31 and rg-Dk/HN/02, two influenza viruses that were susceptible and resistant, respectively, to the effects of POMxp treatment as assessed by infectivity and HAg assays (compare Figs. 2A and 3B). Thus, the impression is that PP effects on influenza virus infectivity and HAg activity do not simply reflect the extent of masking of viral surface components by bound PPs. POMxp treatment of plate-bound MHV A59 resulted in some inhibition of binding of a virus-specific polyclonal antiserum (Fig. 4D). However, this was less marked at high PP concentrations compared with the analysis of influenza viruses described above, suggesting that the relative resistance of MHV A59 to the antiviral activity of POMxp may in part reflect decreased PP binding to viral components.

To gain further insights into the mechanism of antiviral activity, viruses were examined by transmission electron microscopy after treatment with 1600 μg/ml PPs. This analysis focused on X31 and PR8, which were highly susceptible to inactivation at this PP concentration, and rg-Dk/HN/02 and MHV A59, which were relatively resistant. PP treatment of X31, PR8, and rg-Dk/HN/02, but not MHV A59, significantly increased the proportion of aberrant virions (Table 1). A high proportion of X31 and PR8 particles showed aberrant morphology after treatment. Interestingly, the morphological changes differed for X31 and PR8. Treated X31 particles featured widespread loss of the ordered arrangement and definition of envelope glycoproteins, as well as envelope irregularities and breaks. The appearance of the envelope glycoproteins of X31 suggested interactions with extraneous material (Fig. 5A and B). In contrast,

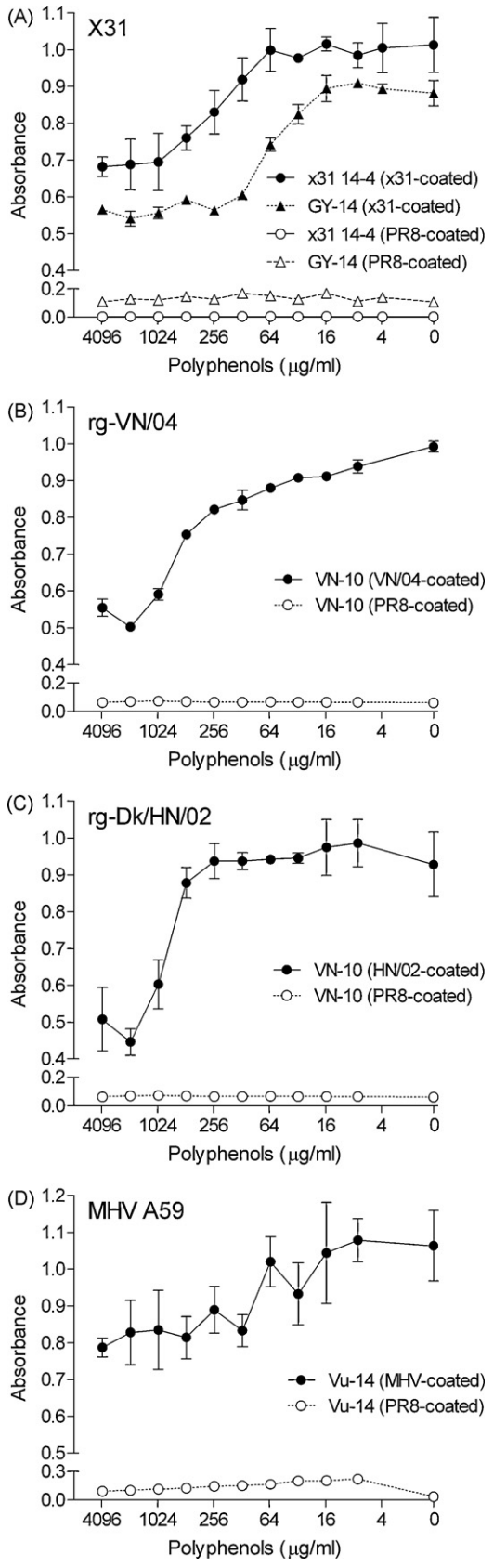

Fig. 4. Inhibition of virus-specific Ab binding after POMxp treatment. ELISA plates coated with the influenza viruses X31 (A), rg-VN/04 (B), or rg-Dk/HN/02 (C), or with MHV A59 (D) were incubated with serial dilutions of POMxp (expressed as PP concentration) prior to the addition of Abs specific for the coating Ags (mAbs specific for influenza H3 (X31 14-4), N2 (GY-14), and H5 (VN-10), and a rabbit antiserum (Vu-14) raised against MHV A59). Color development reflecting Ab binding was generated with enzyme-conjugated secondary Abs and substrate. The analysis was also performed using plates coated with influenza PR8 (H1N1) to control for non-specific adherence of Abs. Each point represents the mean ± SD for triplicate wells. Results are representative of two separate experiments that gave similar results.

Table 1
Electron microscopic analysis of virion morphology after POMxp treatment[a].

Virus	Proportion of aberrant virions[b]	
	Untreated	Treated[c]
X31	1/35 (2.9%)	42/46 (91.3%)
PR8	7/55 (12.7%)	102/137 (74.5%)
rg-Dk/HN/02	10/108 (9.3%)	71/120 (59.2%)
MHV A59	1/61 (1.6%)	1/73 (1.4%)

[a] High titer viral preparations were examined after 5 min incubation with PBS (untreated) or 1600 μg/ml PPs (treated).
[b] Criteria for scoring aberrant virions, based on comparison with the majority of untreated virions, were envelope breaks and irregularities, and (for influenza viruses) disorganization and loss of definition of envelope glycoproteins. Results were pooled from at least 2 separate experiments for each virus.
[c] Treated X31, PR8, or rg-Dk/HN/02 vs. untreated, $P < 0.0001$; treated X31 vs. treated rg-Dk/HN/02, $P < 0.0001$; treated PR8 vs. treated rg-Dk/HN/02, $P < 0.02$.

treated PR8 particles showed varying degrees of fragmentation, but the envelope glycoproteins on unaffected parts of the virion remained ordered and relatively well-defined (Fig. 5C and D). The proportion of aberrant virions after PP treatment was significantly smaller for rg-Dk/HN/02 compared with X31 or PR8 (Table 1), correlating with the relative resistance of rg-Dk/HN/02 to inactivation by PPs. Morphological changes in aberrant rg-Dk/HN/02 virions generally resembled those in PR8 virions (Fig. 5E and F). Treatment of MHV A59 had no effect on virion structure based solely on the appearance of the virion envelope. However, the surface glycoprotein spikes of MHV A59 were occasionally less abundant after PP treatment (Fig. 5G and H). Generally, there was a tendency for PP-treated virions to aggregate, and this may have contributed to some reduction in infectivity. Overall, our electron microscopic analysis indicates that the antiviral effects of PPs are mediated in different ways depending on the nature of the virus. In the case of influenza virus, our observations suggest that the elimination of infectivity by PPs is primarily a consequence of damage to virion integrity, rather than simply a coating of viral particles.

4. Discussion

Our analysis adds to the growing body of literature describing the antiviral activity of PP preparations derived from plants. We show that the PP component of pomegranates rapidly inactivates influenza virus through a direct effect on the viral particle. The antiviral activity of pomegranate PPs was potent against H1N1 and H3N2 influenza viruses and against the reassortant H5N1 virus rg-VN/04. Previous studies demonstrating the anti-influenza activity of PPs from other plant sources have provided evidence for a direct interaction of PPs with the viral HA and NA (Ehrhardt et al., 2007; Nagai et al., 1992; Nakayama et al., 1993; Roschek et al., 2009; Serkedjieva and Manolova, 1992; Song et al., 2005). This is also likely for pomegranate PPs, since the treatment of influenza viruses with POMxp reduced the binding of HA- and NA-specific mAbs. It is unclear whether PP interactions with viral surface glycoproteins significantly modify molecular structure and antigenic determinants or simply establish a coating that impedes accessibility. Numerous studies that evaluated the effects of a variety of PP preparations on influenza viruses demonstrated an association between loss of infectivity and loss of HAg activity, consistent with PP interactions with the viral HA (Ehrhardt et al., 2007; Haidari et al., 2009; Nagai et al., 1992; Nakayama et al., 1993; Serkedjieva and Manolova, 1992; Song et al., 2005). This was also the case

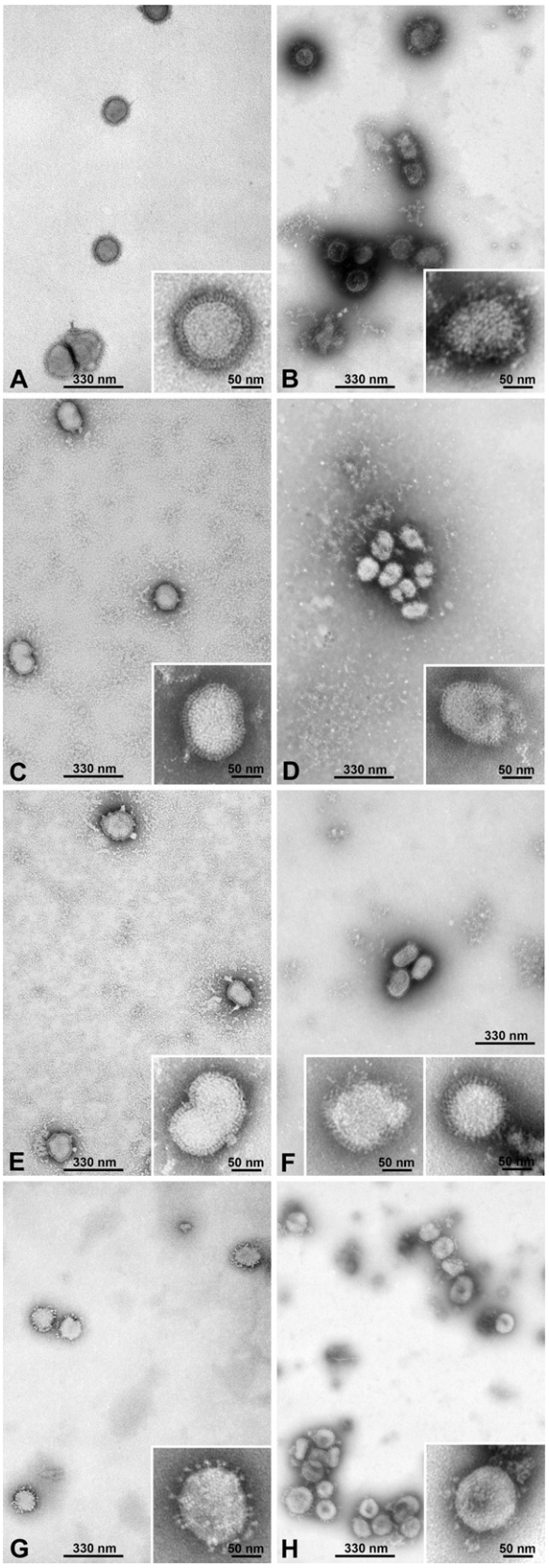

Fig. 5. Electron microscopic analysis of viruses after treatment with POMxp. Influenza viruses X31 (A and B), PR8 (C and D), and rg-Dk/HN/02 (E and F), and the coronavirus MHV A59 (G and H) at concentrations of 1×10^8 to 1×10^9 infectious units/ml were incubated for 5 min at room temperature with PBS (left-hand panels) or 1600 μg/ml PPs (right-hand panels).

in our studies using (in particular) X31 and PR8 (Fig. 2A and B). However, for NC/99 (H1N1) and the H5N1 reassortant rg-VN/04, POMxp treatment eliminated infectivity without loss of HAg activity (Fig. 3A and E), suggesting mechanisms of viral inactivation in addition to interference with HA function.

PP preparations derived from other plants have been shown to vary in activity against different influenza subtypes as well as variant viruses with a subtype (Ehrhardt et al., 2007; Serkedjieva, 2003). In our analysis, pomegranate PPs were effective in reducing the infectivity of the reassortant H5N1 virus rg-VN/04, but the effect was much smaller against three other H5N1 reassortants: rg-Dk/HN/02 and rg-Dk/LS/02 (in particular) and rg-JWE/HK/06 (Fig. 3A–D). Although POMxp treatment had little effect on the infectivity and HAg activity of rg-Dk/HN/02, it still reduced the binding of an H5-specific mAb (Fig. 4C), suggesting that PPs interacted directly with the HA of rg-Dk/HN/02 as they did with the HAs of viruses that were far more susceptible to POMxp inactivation. Electron microscopic analysis of POMxp-treated viruses identified clear morphological abnormalities in viruses that were susceptible to inactivation, whereas these changes were less frequent in the relatively resistant rg-Dk/HN/02 (Fig. 5A–F). Taken together, our findings indicate that pomegranate PPs bind to diverse influenza virus HAs, but this is not predictive of viral inactivation. Apparently, other mechanisms resulting in structural damage to the virion are responsible for the elimination of infectivity. The distinct appearances of POMxp-treated X31 and PR8 examined by electron microscopy (Fig. 5B and D) are consistent with multiple mechanisms of viral inactivation and raise the possibility that the mechanisms may vary even between relatively similar viruses. In part, our findings are consistent with a recent analysis of the antiviral activity of hydroxytyrosol, a small phenolic derivative of a major polyphenol component of the olive tree. The inactivation of different influenza virus subtypes by direct exposure to hydroxytyrosol was associated with damage to virion integrity, but HA and NA function was unaffected (Yamada et al., 2009). Our findings for the H5N1 reassortants and PR8, which have all viral components in common except the HA and NA, identify the HA and NA as key determinants of the susceptibility of influenza viruses to inactivation by pomegranate PPs. Furthermore, relatively small changes in these molecules modulate susceptibility. It is likely that changes in viral surface glycoproteins influence reactivity with PPs, but it is unclear how this may relate to damage to virion integrity. The antiviral mechanism apparently involves direct or indirect effects on the viral envelope, since enveloped viruses are considered more susceptible than non-enveloped viruses (Kotwal, 2008).

Recently, Haidari et al. (2009) analyzed the direct effect of a pomegranate PP extract on influenza virus and demonstrated inactivation associated with loss of HAg activity. In extending this work, we show that the susceptibility of influenza viruses to direct inactivation is regulated by the nature of the surface glycoproteins. Our findings also indicate that inactivation is the result of damage to virion structural integrity that may be distinct from effects on HA function. Haidari et al. (2009) also present evidence that, in addition to a direct virucidal effect, pomegranate PPs also act at an intracellular step to inhibit influenza replication. However, it is unclear whether the mechanism of the intracellular effect is distinct from that of direct antiviral activity. One possibility is that the antioxidant properties of PPs counter the pro-oxidant intracellular environment that favors influenza replication (Cai et al., 2003). It will be of interest to determine whether influenza viruses that are resistant to direct inactivation by pomegranate PPs are also resistant to intracellular antiviral effects.

PP preparations derived from plants are typically a complex mixture of molecular forms. There is evidence for differences in the antiviral activity of individual PP compounds (Haidari et al., 2009) and synergistic activity by PP mixtures (Song et al., 2005). Presum-

ably, the biochemical reactivity of PPs and their interactions with proteins and lipids play an important role (Diniz et al., 2008; Soares et al., 2007). Within a PP preparation, different PPs and perhaps also small derivative molecules (Yamada et al., 2009) may exert antiviral activity by interacting with different viral components. However, the molecular interactions that are the basis for the antiviral activity of PPs and how these relate to the characteristics of specific PPs are not well understood (Song et al., 2005). Although PP binding to cell surface molecules has been described (Williamson et al., 2006), there is evidence for some selectivity in PP reactivity and preferential binding to viral compared with host cell molecules (Nagai et al., 1992).

Rg-Dk/HN/02, rg-Dk/LS/02, and rg-JWE/HK/06 were derived from avian H5N1 isolates, whereas rg-VN/04 was derived from an isolate from a fatal human case. This raises the possibility that features of the surface glycoproteins of influenza viruses adapted to maintenance in avian reservoirs may also confer resistance to the effects of PPs. In particular, this may apply to viruses maintained in ducks and other aquatic birds, in which transmission is via the fecal/oral route and is favored by viral persistence in the aquatic environment (Breban et al., 2009). PPs and the related humic substances, which may also have antiviral activity (Anesio et al., 2004; Kotwal, 2008), are components of natural organic matter in aquatic environments (Chen et al., 2002) and may be present at sufficient concentrations to exert a selective pressure on influenza viruses. A selective pressure may also operate in the gastrointestinal tract of influenza-infected waterbirds, an environment that is likely rich in PPs. Notably, Serkedjieva (2003) demonstrated that passage of influenza virus in the presence of PPs selected for decreased sensitivity to PP inhibition.

An important consideration is whether PPs derived from pomegranates and other plant sources may be utilized to combat influenza and other viral infections. In our analysis, complete inactivation of the more susceptible influenza virus strains generally required treatment with approximately 1600 μg/ml PPs. However, it should be emphasized that the duration of treatment was only 5 min and that titer reductions of 99% or more were frequently observed at 400 μg/ml PPs. Haidari et al. (2009) treated an H3N2 influenza virus for 30 min with a pomegranate PP extract and demonstrated significant direct antiviral activity at concentrations of approximately 1 μg/ml. Little is known about the metabolism of ingested PPs and whether absorbed constituents contribute to antiviral activity in the body. The benefits of PPs as antivirals for the prophylaxis or treatment of influenza is likely to be optimal when they are applied directly at the site of infection (Droebner et al., 2007). In mouse model studies, PP administration to the lung reduced the effects of influenza infection without toxicity to the host (Droebner et al., 2007; Nagai et al., 1992; Serkedjieva et al., 2008). Interestingly, nasopharyngeal administration of pomegranate extracts is a Cuban folk remedy for influenza (Vidal et al., 2003). Continued characterization of the PP constituents and their derivatives that mediate antiviral activity may lead to inhaled or topically applied preparations that aid in the control of influenza.

Acknowledgments

This work was supported by a grant from POM Wonderful Ltd., Los Angeles, CA.

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