Pomegranate (Punica granatum) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir

Mehran Haidari a,b, Muzammil Ali a, Samuel Ward Casscells III a,b, Mohammad Madjid a,b,*

* University of Texas Health Science Center at Houston, USA
† Texas Heart Institute, 6770 Bertner Ave., MC 2-255, Houston, TX 77030, USA

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ABSTRACT

Influenza epidemics cause numerous deaths and millions of hospitalizations each year. Because of the alarming emergence of resistance to anti-influenza drugs, there is a need to identify new naturally occurring antiviral molecules. We tested the hypothesis that pomegranate polyphenol extract (PPE) has anti-influenza properties. Using real time PCR, plaque assay, and TCID 50% hemagglutination assay, we have shown that PPE suppresses replication of influenza A virus in MDCK cells. PPE inhibits agglutination of chicken red blood cells (cRBC) by influenza virus and is virucidal. The single-cycle growth conditions indicated that independent of the virucidal effect PPE also inhibits viral RNA replication. PPE did not alter virus ribonucleoprotein (RNP) entry into nucleus or translocation of virus RNP from nucleus to cytoplasm in MDCK cells. We evaluated four major Polyphenols in PPE (ellagic acid, caffeic acid, luteolin, and punicalagin) and demonstrated that punicalagin is the effective, anti-influenza component of PPE. Punicalagin blocked replication of the virus RNA, inhibited agglutination of chicken RBC’s by the virus and had virucidal effects. Furthermore, the combination of PPE and oseltamivir synergistically increased the anti-influenza effect of oseltamivir. In conclusion, PPE inhibited the replication of human influenza A/Hong Kong (H3N2) in vitro. Pomegranate extracts should be further studied for therapeutic and prophylactic potential especially for influenza epidemics and pandemics.

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Introduction

Influenza virus is documented to cause epidemics and pandemics in human population over several centuries. Influenza A virus has several zoontite hosts, therefore cannot be eradicated from human populations. Despite widespread access to vaccines and antiviral therapies, influenza continues to be a major cause of morbidity and mortality. About 31,000 deaths each year in the US are associated with influenza A infections (Thompson et al. 2003).

Frequent antigenic drifts necessitate a change in vaccine composition almost every single year. Vaccines with a good antigenic match have an efficacy of 60% to 80% in healthy adults; however, the rate of protection is lower in certain high risk groups particularly the elderly and the immuno-compromised. Furthermore, vaccines are even less effective when they are not matched with new strains which were not expected at time of vaccine development. Examples are H5N1 strains that caused an outbreak in Hong Kong in 1997 and emerging avian influenza strains causing epidemic in 2006.

Annual vaccination is the mainstay strategy for preventing influenza infections and antiviral drugs offer additional preventive and therapeutic benefits (Hayden 2006a). Currently, there are two classes of anti-influenza agents available for influenza management and are under consideration for stockpiling in the event of an influenza pandemic; one class targets the M2 ion channel (e.g., amantadine and rimantadine) and the other inhibits neuraminidase (e.g., oseltamivir and zanamivir). In the absence of an abundant supply of an effective vaccine, neuraminidase inhibitors will be considered the drug of choice for controlling an emerging influenza pandemic until vaccine supplies become available. Post-Exposure use of oseltamivir has an efficacy of 70% to 90% and can shorten the duration of illness by 1.5 days when used within the first 48 hours (Hayden et al. 1999). Due to continuous genetic variations in influenza virus genome, development of resistance against anti-influenza drugs has been a serious problem. Usage of amantadine and rimantadine, are limited by a lack of inhibitory effect against influenza B viruses, side effects, and a rapid emergence of antiviral resistance (Hayden 2006b).

Emergence of resistance to oseltamivir in human influenza A viruses (Ison et al. 2006) and the H5N1 subtype in Vietnam (de Jong et al. 2005) is a cause for concern. However, resistance has not been reported for the other neuraminidase inhibitor, Zanamivir (Moscona 2005). Nevertheless, expanding the range of...
antiviral drugs that effectively inhibit replications of the influenza A virus, or potentially act in synergy with neuraminidase inhibitors is a matter of urgency.

Several novel agents that may be effective against influenza virus, specifically the avian flu virus strain, are currently under development. Naturally occurring anti-viral nutrients may be of special interest because they are widely available and may be used as part of the diet to combat diseases, including influenza infection. Plant-derived flavonoids found in fruits, leaves, and vegetables have recently been the focus of many studies because of their beneficial health effects in several disease models (Williamson and Manach 2005). Among fruits, pomegranate (Punica granatum, Punicaeae) is a rich source of anthocyanins and other phenolic compounds that have strong antioxidant activity (Gil et al. 2000). Pomegranate, native to Persia, is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, and the United States (California). Edible parts of pomegranate fruit (about 80% of total fruit weight) comprise 80% juice and 20% seed. Pomegranate fruit extract is a rich source of 2 types of polyphenolic compounds: anthocyanins (derived from delphinidin, cyanidin and pelargonidin), which give red color to the fruit and juice, and hydrolysable tannins (i.e. punicalin, pedunculagin, punicalagin, gallocatechin and ellagitannin esters of glucose), which account for 92% of the antioxidant activity of the whole fruit (Gil et al. 2000).

In an HPLC fingerprint analysis, pomegranate-specific polyphenol classes were identified as punicalin (2%), punicalagins (15.7%), oligomers (77%) and ellagic acid (3%). (Numbers in parenthesis are percent area on HPLC peak area distribution) Punicalagin and punicalin are considered pomegranate-specific and can be used as a standard of pomegranate product authenticity (Martin et al. 2008).

Pomegranate has been widely used for several centuries in traditional medicine for a wide variety of diseases including upper respiratory tract infections and influenza (Nonaka et al. 1990). The potential effect of pomegranate on the replication of the influenza virus has not been systemically studied before and very little data is available in English language literature on this subject. In the present study, we have tested the efficacy of a purified flavonoid-rich extract of pomegranate fruit (e.g. POMx compound) against influenza A virus.

Material and methods

Reagents

Pomegranate juice, commercially available for human consumption was used in a concentrate form (70% Brix Extract). Pomegranate polyphenol extract (PPE), punicalagin (in powder form), and liquid pomegranate concentrate were provided by POM Wonderful (LLC, Los Angeles, CA). PPE and punicalagin powders were dissolved in DMSO (100 mg/ml stock). MDCK cells were purchased from ATCC (Manassas, VA). Hockeist 33342, ellagic acid, luteolin and caffeic acid were purchased from Sigma Aldrich (St. Louis, MO). Oseltamivir carboxylic acid (active form of oseltamivir) was purchased from Toronto Research chemicals (Toronto, Ontario, Canada). Tyramide signal amplification kits (TSA, Fluorescein) were obtained from PerkinElmer (Waltham, Massachusetts). Anti-influenza monoclonal antibody against nucleoprotein (NP) protein was purchased from Chemicon International Inc (Temecula, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary and anti-FITC–HRP antibodies were purchased from Abcam (Cambridge, MA). Chicken red blood cells (RBCs) were obtained from Lampire Biological Laboratories (Pipersville, PA).

Viral stock and cell culture

Influenza virus A/Hong Kong/2/68; H3N2 [A/HK (H3N2)] was used as the primary influenza virus strain in our experiments. Influenza A/HK (H3N2), A/USSR/90/77 (H1N1) and influenza B/Harbin/07/94 were kindly provided by Dr. Philip Wyde (Baylor College of Medicine, Houston, Texas). MDCK cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Cell viability assay

The effect of PPE and other chemicals on the viability of MDCK were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) assay. The cells were plated in 200 μl of complete culture medium containing serial dilutions of the chemicals in 96-well microtiter plates for 48 h. After incubating the cells for specified times at 37 °C in a humidified incubator, we added (MTT) (5 mg/ml in PBS) to each well and incubated the cells for 2 h. The absorbance was recorded on a microplate reader at the wavelength of 540 nm.

Viral infection

When 90% confluent, MDCK cells were washed twice with phosphate-buffered saline (PBS) to remove residual FBS and infected with virus at an MOI of 10 or 0.05 to allow single-cycle (evaluated 4–12 h after infection) or multicyle (24 h after infection) replication, respectively. Viral stock was used in serum-free DMEM for 60 minutes in 4 °C to inoculate the cells (absorption phase). Cells were then washed with PBS and cultured in DMEM+2% FBS and TPK (trypsin) (4 μg/ml, Worthington Biochemicals, Tryp-MEM) either with or without drug treatment, for 12–48 h (post infection phase).

Extracellular virus yield reduction assay

The extracellular virus yield reduction assay was performed in 24-well plates containing confluent MDCK monolayers. Three experiments were conducted for each assay. Drugs, alone or in combination, were added to cells in 24-well plates, and the plates were incubated for 16 h at 37 °C. The cells were then inoculated with virus first for 1 h at 4 °C, and after washing for 24 h at 37 °C in DMEM+2% FBS, and TPK trypsin. The medium was removed and centrifuged at 3,200 g for 5 min to remove the floating cells and used for RNA extraction and quantification of virus using RT-PCR technique. The media were also used for tissue culture infectious dose (TCID) 50%, or plaque assay. To determine TCID 50%, we titrated the supernatant by adding serially diluted samples to four wells (each) in 96-well plates of MDCK cells (4 °C, DMEM). The medium was replaced with DMEM+2% FBS, and TPK trypsin 60 minutes after the virus inoculation to remove residual compound. Virus replication was detected by a hemagglutination assay 72 h after the inoculation and titers were expressed as log10 of the 50% TCID (Reed and Muench 1938). To perform hemagglutination assays Chicken RBC’s were added to the medium to make a final dilution of 0.5% for RBCs. After 60 min incubation at room temperature, chicken RBCs in negative wells settled to form red button pellets, whereas positive wells had a diffuse appearance with no sedimentation.

Cell-associated virus yield assay

Cell-associated virus yield was quantified by real time PCR assay. After removing the supernatant, we washed the cells twice.
in PBS, and used the RNeasy mini kit (Qiagen: Cat # 74104) to extract virus RNA from the cells.

**Real time PCR assay**

We used TaqMan real time PCR to quantify the presence of virus in the media and monolayers of MDCK cells after infection with influenza virus. Virus RNA was extracted from the media by QiAamp viral RNA mini kit (Qiagen, cat # 52906). Following extraction of RNA, cDNA was synthesized using MultiScribe reverse transcriptase and random hexamers (both from PE Applied Biosystems, Foster City, CA). Each 50-µl reaction mixture contained 10 µl of eluted RNA, 5 µl of RT buffer, 5.5 mM MgCl₂, 500 mM each deoxynucleoside triphosphate (dNTP), 2.5 mM random hexamer, 40 U of Multiscribe reverse transcriptase and 0.4 U of RNase inhibitor per ml (all from PE Applied Biosystems).

After incubation for 10 min at 25 °C, RT was carried out for 30 min at 48 °C, followed by RT inactivation for 5 min at 95 °C. The cDNA was stored at -20 °C before further use. Real time PCR reactions were performed using the default PCR cycle on a sequence detection system (ABI Prism 7000 HT; Applied Biosystem). The primer and probe sequences for detection of influenza A and B were already optimized (Ward et al. 2004). A TaqMan-based real time technique, was used to amplify a 104- nucleotide fragment from a matrix protein of influenza A as described previously (Ward et al. 2004). The primers (forward) 5’- AAG ACC AAT CCT GTC ACC TCT GA 3’ and (reverse) 5’- CAA AGC GTC TAC GCT GCA GTC C 3’ amplify a 104-base pair fragment in the M₁ gene of influenza A. The influenza A specific probe FAM (5-carboxyfluorescein)-5’TGT GTC TAC CTC ACC GT 3’ TAMRA (6-carboxytetramethylrhodamine) annealed to part of the sequence amplified by the two primers.

The expression of a cellular housekeeping gene GADPH was used to control for amount of RNA loaded into the assay. The amplified DNA was detected by measurement of fluorogenic signal from FAM-labeled TaqMan MGB probes. The amount of influenza virus was determined by the cycle number at which the amount of fluorescence reached 10 standard deviations over the background (designated the CT). To determine the level of influenza RNA, we normalized the results from each sample by background (designated the CT). To determine the effect the inhibitors had upon influenza RNA levels, the ΔCT values of the inhibitor-treated samples were subtracted from the ΔCT values in the presence of solvent alone to yield a ΔΔCT value. To understand the significance of these values more intuitively, we converted the ΔΔCT values to fold difference

**Plaque assay**

MDCK cells were cultured in 6-well plates and infected with virus at 10-fold serial dilutions. After removing the unadsorbed virus by washing with PBS, we overlaid the cells with PPE diluted in DMEM with 2% sea plaque agar pre-warmed to 42 °C. The agar solidified, the plates were inverted and transferred to a 37 °C incubator. After blocking, the cells were incubated with the antibodies for 45 min and with Tyramide substrate for 10 min and then mounted with Antifade kit (Molecular Probes, Eugene, OR). Viral nucleoprotein (NP) protein was detected using mouse monoclonal antibody (Chemicon International Inc., Temecula, CA). The secondary antibody was FITC-conjugated goat anti-mouse IgG. Tyramide amplification system was used in conjunction with anti-influenza antibody. After quenching the remaining HRP activity with 1% H₂O₂, 1µg/ml anti-FITC-HRP (for 30 min) and FITC-Tyramide were used to reveal FITC–anti-influenza staining. Hoechst 33342 (0.5 µg/ml for 5 minutes) was used as a nuclear counter-stain.

**Hemagglutination inhibition assay**

Hemagglutination inhibition assay was used to test the effect of PPE on agglutination of chicken RBC by influenza virus. PPE solutions (25 µl) with two-fold serial dilutions in PBS were mixed with an equal volume of influenza virus solution. The mixture was incubated for 30 min at room temperature, and then 50 µl of the solution was mixed with an equal volume of 1% chicken erythrocyte suspension and incubated for 60 min at room temperature.

**Virucidal experiments**

Viral stock with an MOI of 10 was used in serum-free DMEM and incubated with 0-40 µg/ml of PPE for 30 minutes. The media containing virus were first diluted 1/10000 and then used to infect MDCK cells. The virus yield in supernatant was measured by real time PCR after 24 h of incubation.

**Drug combination studies**

Possible interactions between PPE and oseltamivir were determined by real time PCR as described above. The final drug concentrations were 15 to 500 nM for oseltamivir and 0.62-40 µg/ml for PPE. The monolayers of cells were subjected to assays after 48 h incubation. The extent of the effect of the combination treatment was analyzed by the isobole method (Berenbaum 1989; Wagner and Ulrich-Merzenich 2009). For a combination of drugs A and B by the equation: \( Ac/Ae + Be = D \), where \( Ac \) and \( Be \) correspond to the concentrations of A and B when used in combination, and \( Ae \) and \( Be \) correspond to the concentrations able to produce an effect of the same magnitude if used alone. If \( D \) (Interaction Index) >1, the effect of the combination is synergistic, whereas if \( D = 0 \), the effect is additive or antagonistic, respectively.

**Results**

**Pomegranate extracts dramatically inhibit proliferation of influenza A virus**

Cytotoxicity studies demonstrated that PPE, ellagic acid, caffeic acid, luteolin and punicalagin did not induce significant cytotoxicity in MDCK cells until concentrations exceeded 100 µg/ml, 50 µM, 20 µM, 10 µM and 100 µg/ml in medium, respectively. Thus, subsequent in vitro studies were performed with PPE, ellagic acid, caffeic acid, luteolin, and punicalagin concentrations ranging from 0 to 40 µg/ml, 0 to 20 µM, 0 to 20 µM, 0 to 10 µM, and 0 to 40 µg/ml, respectively. First, we studied the effect of PPE on the proliferation of influenza A/HK (H3N2) virus. When MDCK cells were pre-treated with PPE 24 h before infection and not during the absorption phase, no significant antiviral effect was detected (Fig. 1). In a series of multiple-cycle growth conditions,
Fig. 1. Addition of PPE during or after viral absorption inhibits replication of influenza A/HK (H3N2) virus in MDCK cells (multiple cycle experiment). MDCK cells were infected with influenza A virus (MOI, 0.05) and exposed to serial dilution of PPE 24h before infection (dotted line), during the viral absorption phase (broken line, 1 h post infection, 4°C) and after the viral absorption phase (solid line). The media collected and used for RT-PCR technique 24h after the viral absorption phase.

Fig. 2. Inhibition of influenza A replication by Pomegranate Extract Powder (multiple cycle experiments). MDCK cells were infected with influenza A/HK (H3N2) virus (MOI, 0.050) and incubated at 4°C for 1 h. The cells were washed and exposed to 2 fold serial dilutions of PPE in DMEM with 2% FBS. After incubation for 24 h at 37°C the media were collected and used for measurement of virus yield (2A, 2B and 2C). 2A- The media was titrated by adding serially diluted samples to four wells (each) in 96-well plates of MDCK cells. Virus replication was detected by a hemagglutination assay 72 h after the inoculation. 2B- MDCK cells were cultured in 6-well plates, infected with virus and exposed to DMSO or 20μg/ml of PPE. Viral infection resulted in opaque plaques, which were readily visible after 2 days with a solution of 0.1% crystal violet, 10% formaldehyde in PBS. 2C- The cells were washed and used for RNA extraction using QIAamp RNeasy mini kit (Qiagen). RT-PCR technique was used to quantify the percent of released virus compared to DMSO-treated cells. 2D- MDCK cells were infected with influenza A virus H1N1 strain (A/USSR/90/77, MOI, 0.05) and were exposed to serial dilution of PPE during the viral absorption phase (1 h post infection, 4°C). After viral absorption phase the cells were washed twice with PBS and the media were replaced with 2% DMEM. The media collected and used for RT-PCR technique 24h after the viral absorption phase.

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we found that exposure of MDCK cells to PPE during the viral absorption phase or post infection (24 h) significantly suppressed viral propagation using RT-PCR technique (Fig. 1). In experiment that the cells were exposed to PPE only during viral absorption phase the viruses diluted in the PPE containing media and added to cells at 4°C for 1 h and then the cells were washed and the media were replaced with 2% DMEM. In addition to RT-PCR technique we used TCID 50% and plaque assay to assess the effect of PPE on influenza proliferation. As shown in Figs. 2 A and B, the proliferation of virus was blocked when MDCK cells were exposed to PPE only during the viral absorption phase, detected by TCID 50% hemagglutination (Fig. 2 A) and plaque assay (Fig. 2 B). The cell-associated virus proliferation in MDCK cells was also suppressed in multiple-cycle growth conditions (Fig. 2 C). Furthermore, liquid pomegranate concentrate blocked virus proliferation (data not shown). In addition to influenza A/HK (H3N2), PPE inhibited the proliferation of influenza A/USSR/90/77 (H1N1) (Fig. 2 D) and influenza B/Harbin/07/94 (data not shown).

The suppression of viral propagation when MDCK cells were exposed to PPE (only during the viral adsorption phase) suggests that either the virus attachment to the cells is inhibited or PPE has virucidal capability. As a way to test if the attachment of virus to the cells is the point of inhibition, we performed the hemagglutination inhibition assay, which showed that pomegranate inhibits agglutination of chicken RBC’s by influenza A virus (Fig. 3). However, further experiments indicated that PPE has virucidal effects against influenza virus (Fig. 4). This virucidal effect might explain why PPE inhibited agglutination of RBC by influenza virus. Furthermore, in the multiple cycle growth condition the PPE-inhibited virus release when the MDCK cells

![Fig. 3. Pomegranate inhibits agglutination of chicken RBC’s by influenza A/HK (H3N2) virus. PPE solutions (25 µl) with two-fold serial dilutions in PBS were mixed with an equal volume of influenza virus solution (MOI, 10). The mixture was incubated in a 96 well plate for 30 min at room temperature. 50 µl of the solution was mixed with an equal volume of 1% chicken erythrocyte suspension and incubated for 30 min at room temperature. The percent of hemagglutination inhibition was compared with DMSO as no (zero) inhibition.](image1)

![Fig. 4. Virucidal effects of PPE on influenza A/HK (H3N2) virus. A viral stock with MOI of 10 was exposed to serial dilution of PPE for 30 minutes. The virus was diluted to make a 1/100000 dilution and used for infection of MDCK cells. The media collected after 24 h and were used to quantify virus by PR-PCR techniques.](image2)
were exposed to PPE only after the viral absorption phase, suggesting that independent to virucidal effect PPE inhibits influenza virus replication. To test this we investigated the effect of PPE on proliferation of influenza virus in a single-cycle growth condition.

Pomegranate inhibits the virus proliferation in single-cycle growth conditions

To probe the molecular mechanism underlying the antiviral activity of PPE, we used immunofluorescence staining to evaluate the chronology of the intracellular trafficking of virus ribonucleoprotein (RNP) in a single-cycle growth condition. In parallel, we used real time PCR technique and assessed the virus RNA production and release under the same conditions. Immunofluorescence staining showed that influenza RNP can be detected in the nucleus of infected MDRK cells as early as 2 h post-infection (pi) and can stay there 4 h pi. The RNP appeared in the cytoplasm at 4-5 h pi and translocation of the RNP from the nucleus was completed at 8-10 h pi. Real time PCR confirmed that intracellular virus RNA dramatically increased 4-5 h pi. In addition, the virus was detected in the medium of infected MDCK cells 8-10 h following the infection. Based on the above experiments and the findings from previous studies, we concluded that a single-cycle growth of influenza A virus from the attachment to the exit of virus is 8-10 h following the infection. We used immunofluorescence staining to test the hypothesis that entry of virus RNP into nucleus or translocation of virus RNP from nucleus to cytoplasm is affected by exposure of MDCK cells to PPE. Our experiments showed that exposure of infected MDCK cells to PPE did not suppress the virus genome entry into nucleus or translocation of the virus RNP from nucleus to cytoplasm (Fig. 5). To test if the replication of virus RNA is inhibited after exposure of infected MDCK cells to PPE, we used real time PCR to measure the intracellular level of virus RNA in a single-cycle growth condition. MDCK cells were infected with 10 MOI of influenza virus and incubated at 4°C for 1 h. Unbound virus was removed by washing, and cells were exposed to 2-fold serial dilutions of PPE or DMSO in DMEM+2% FBS, and TPCK trypsin. The level of cell-associated virus was quantified at 3, 6, 9 and 12 h after infection using real time PCR techniques. As depicted in Fig. 6, the replication of virus RNA was inhibited in single-cycle growth conditions, independent of the virucidal effect of PPE.
To study which component is responsible for the anti-influenza effect of PPE, we tested four major flavonoid compounds of pomegranate known to have antioxidant properties: ellagic acid, caffeic acid, luteolin, and punicalagin. To control the possible role of pH alteration on influenza proliferation the pH of working solution of each compound was adjusted to 7.4 before the addition of chemicals to the cells. From these four compounds, punicalagin potently inhibited the growth of influenza virus. As shown in Fig. 7, punicalagin has a virucidal effect (Fig. 7 A) and inhibits agglutination of chicken RBC’s by influenza virus (Fig. 7 B). In addition, punicalagin inhibited the proliferation of influenza A virus in both multiple cycle and single-cycle growth conditions. Furthermore, the antiviral effect of punicalagin sustained when the MDCK cells were exposed to the compound only after the absorption phase (Fig. 7 C), indicating that the inhibition of virus proliferation is independent of the virucidal effects. The other tested compounds (ellagic acid, caffeic acid, and luteolin) did not show any anti-influenza activities (Fig. 7 D).

The combination of oseltamivir and PPE dramatically improved the inhibitory effect of oseltamivir on release of virus from infected MDCK cells (Fig. 8 A). Using the isobole method, a synergistic effect was demonstrated for the combination of pomegranate and oseltamivir (Fig. 8B). The interaction index for a concentration of 0.007 nM for oseltamivir and 0.62 mg/ml for pomegranate is 0.17, indicating a synergistic effect.

**Discussion**

Our study provides strong evidence that pomegranate has an anti-influenza effect. We have demonstrated that punicalagin is the active anti-influenza component of PPE. Punicalagin/PPE inhibits agglutination of chicken RBCs by influenza virus and has virucidal properties. Furthermore, the single-cycle growth experiments indicated that Punicalagin/PPE, independent of their virucidal effects, inhibit replication of the viral RNA. Moreover, the combination of PPE and oseltamivir synergistically increases the anti-influenza effect of oseltamivir.

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Pomegranate juice has many biological effects, some of which may have important clinical implications. The potent anti-atherogenic effects of pomegranate juice have recently been demonstrated in atherosclerotic mice and healthy humans (Aviram et al. 2000; Kaplan et al. 2001). In addition, pomegranate has been reported to show anti-oxidant and anti-tumor activity (Su et al. 1988; Kashiwada et al. 1992). Because of its rich concentration of diverse, free-radical–scavenging bioflavonoid, pomegranate has been recommended in the treatment of acquired immune deficiency syndrome (AIDS) (Lee and Watson 1998). The interest in identifying naturally occurring antiviral molecules has recently been intensified (Jung et al. 2000).

In our study, the mechanisms underlying the virucidal effect of PPE have not been determined. However, our experiments indicate that the effect is independent of pH alterations (data not shown). Pomegranate has been used in phage amplification assays as a virucidal compound (Stewart et al. 1998; de Siqueira et al. 2006). These studies are in line with our findings that pomegranate has virucidal activity. In addition, pomegranate extract has been reported to have microbicidal effects on HIV-1.

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Combination of oseltamivir and PPE increases the inhibitory effects of oseltamivir on influenza A/HK (H3N2) virus replication. 8A- MDCK cells were exposed to serial dilution of oseltamivir carboxylate (dotted line) PPE (broken line) and combination of oseltamivir carboxylate and PPE (full line). The media collected 48 h after the infection with influenza A/HK (H3N2) virus, MOI of 0.05 and used for detection of virus by RT-PCR technique. 8B- Isobologram showing interactions between pomegranate and oseltamivir. Each point is a geometric average of three to five separate experiments. The solid diagonals in the isobolograms represent the theoretical line of additivity (i.e., no interaction), while the values below this line indicates a synergistic effect between the two compounds.

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(Neurath et al. 2005). Anti-influenza virucidal activity has also been associated with other flavonoid compounds (Song et al. 2005). Furthermore, plant polyphenols, including punicalagin, have antibacterial (causing food-borne illness) and anti-tuberculosis properties (Asres et al. 2001; Taguri et al. 2004).

The inhibitory effect of PPE on hemagglutination of chicken RBC’s by virus can be attributed to its virucidal effect. However, the possible PPE-induced aggregation of viruses might be another mechanism for the inhibition of agglutination of chicken RBC’s by PPE.

In our studies, exposure of MDCK cells to PPE resulted in the reduction of release of virus particles into media in the single-cycle growth experiments. This inhibitory effect was independent of the attachment of virus to MDCK cells because PPE was added to the cells after the virus absorption phase (1 h post-infection). We performed an experiment to eliminate the possibility that PPE destroyed the viral structure when the virus exited the cell at the end of a single growth cycle. In a single-cycle experiment, we exposed MDCK cells to PPE only after the absorption phase and quantified the intracellular virus RNA level with the use of real time PCR (Fig. 7). Our results showed that the level of cell-associated virus RNA was significantly lower by the end of one growth cycle (9-12 h) in PPE-treated cells than in MDCK cells exposed to DMSO. These findings suggest that PPE, independent of its virucidal effect, inhibits the replication of influenza A virus. However, the possibility of an intracellular virucidal effect of PPE must be considered when interpreting the data.

We studied the anti-viral properties of four major flavonoid components of pomegranate. Our results indicate that punicalagin has virucidal capability and inhibits influenza virus RNA proliferation independent of the virucidal effect. Commercially-available pomegranate juices show potent antioxidant and anti-atherosclerotic properties. These properties are attributed to its high content of polyphenols, including ellagitannins, ellagic acid and other flavonoids (quercetin, kaempferol, and luteolin glycosides) (Gil et al. 2000; Cerda et al. 2003; Aviram et al. 2004). The most abundant of these polyphenols is punicalagin, an ellagitanin implicated as the bioactive constituent responsible for more than 50% of the juice’s potent antioxidant activity. Punicalagin is a high molecular weight (1108), water-soluble polyphenolic antioxidant molecule isolated from pomegranate fruit waste-pith and capillary membrane (Kulkarni et al. 2004). Antioxidant activity may be responsible, at least in part, for punicalagin’s remarkable pharmacological activities, including anti-inflammatory (Lin et al. 1999), hepato-protective (Lin et al. 2001), anti-genotoxic activities and the observed inhibitory effects on influenza virus proliferation. The inhibitory effect of antioxidants on influenza virus has been reported by others (Cai et al. 2003), and our experiments have shown that two commonly used antioxidants (N-acetyl-cysteine and glutathione) also have anti-influenza activities (data not shown). However, further studies are necessary to understand the mechanisms underlying the inhibitory effect of punicalagin on proliferation of influenza virus.

Our study demonstrates that addition of PPE increases the inhibitory effect of oseltamivir on influenza A virus. Oseltamivir inhibits neuraminidase in the influenza virus, thereby preventing new viruses from entering infected cells. The synergistic effects of natural products such as polyphenols with antimicrobial agents have been reported previously (Han 2007; Hemaissarya et al. 2008). Therapy with synergistically active antiviral drugs that target different viral proteins and have different mechanisms of action may provide several advantages over single-agent treatment. The advantages of a multi-drug regimen include potentially greater potency, better clinical efficacy and dose-sparing, less toxicity and side effects, and greater cost-effectiveness. Furthermore, the combined use of two or more drugs with different mechanisms of action can potentially reduce the incidence of drug resistance (Hemaissarya et al. 2008).

The physiologic relevance of our in vitro data should be interpreted with caution. We have shown that pomegranate inhibits two reference subtypes of influenza A virus. However, examining the inhibitory effect of pomegranate on clinical isolates of influenza A virus, in addition to in vivo studies, is necessary to determine the role, if any, of pomegranate in the prophylaxis and treatment of influenza infection. Determining the activity of pomegranate against the highly pathogenic H5N1 subtype may be helpful in adding to the limited antiviral stockpile for use in a pandemic.

In conclusion, our studies show that pomegranate inhibits the replication of human influenza A virus in vitro. Our findings encourage the need for further clinical studies to explore the therapeutic and prophylactic potential of pomegranate products in treating seasonal or pandemic influenza.

Potential Conflict of Interest:
Dr. Madjid has received research support and honoraria from POM Wonderful, LLC.

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