

Anti-Influenza Virus Activity and Phenolic Content of Pomegranate (*Punica granatum* L.) Peel Extract and Fractions

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Abstract

Background: Influenza virus, associated with high level of morbidity and mortality, has been recently considered a public health concern while the choices for the control and treatment of the disease are limited. The present study was conducted to evaluate activity of pomegranate peel extract and its fractions against *Influenza A* virus *in vitro*.

Methods: In this research, ethyl alcohol extract of pomegranate peel was prepared and subjected to fractionation with different polarities. The potential *in vitro* anti-influenza A virus activity of the extract and fractions was assessed using Cytopathic Effect (CPE) reduction assay, Hemagglutinin Assay (HA), and 50% Tissue Culture Infectious Doses (TCID₅₀) method in Madin-Darby Canine Kidney (MDCK) cells.

Results: The crude pomegranate peel extract and its n-butanol and ethyl acetate fractions had the highest inhibitory effect against influenza A virus with IC₅₀ value of 6.45, 6.07 and 5.6 $\mu\text{g/ml}$ in MDCK cells, respectively. Our results also showed that, the production of virus was significantly reduced upon treatment with crude extract, n-butanol and ethyl acetate fractions in a dose-dependent manner ($p < 0.05$).

Conclusion: Based on our results, the ethyl alcohol extract and its polar fractions of pomegranate peel can inhibit influenza A virus replication *in vitro*. Therefore, further characterization of its active ingredients and the mechanism of action should be carried out.

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Introduction

Pomegranate (*Punica granatum* L., belonging to the Punicaceae family) is one of the oldest edible fruits and is widely cultivated in many tropical and subtropical countries ¹, including Iran, Egypt, Russia, Spain, France, China, Japan, Argentina, USA, and India. Pomegranate has been used extensively in the folk medicine of Iranians and many other countries. Many studies have indicated the antioxidant, antiatherogenic, anticancer, anti-inflammatory, antimicrobial and anti-infective effects of pomegranate peel and fruit extracts ²⁻⁶.

Although pomegranate peel is sometimes considered an agro-waste, it is indeed as a source of different flavonoids with antibacterial, antiviral, antioxidant, anti-inflammatory and antineoplastic bioactivities ^{7,8}.

One of the most common human respiratory tract pathogens, associated with high morbidity and mortality,

namely, influenza virus, has been recently considered a public health concern. Although vaccination is a suitable approach to prevent influenza, this method should be updated to be effective on new subtypes due to constant changes in virus surface ⁹. Currently, there are two groups of anti-influenza agents available for the management of influenza infection. One class includes amantadine and rimantadine which are matrix protein (M2) ion-channel inhibitors and interfere with viral uncoating within the host cells. They are effective only against influenza virus A with the risk of widespread drug resistance. The other group includes oseltamivir and zanamivir which are Neuraminidase (NA) inhibitors and are widely used in the treatment of both seasonal and pandemic influenza virus infections ¹⁰. However,

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oseltamivir resistant H1N1 strains were found to be circulated since 2007-08^{11,12}. As the options for the control and treatment of the disease are limited, use of herbal extracts such as pomegranate seems to be an alternative. This research was conducted to evaluate the anti-influenza A virus activity of crude hydro alcoholic extract and the four corresponding fractions of *Punica granatum* L. peel *in vitro*.

Materials and Methods

Plant collection, extraction and fractionation

The pomegranate (*Punica granatum* L.) was from the Malas variant obtained from Shahreza, a central region of Iran (October 2015). Then, in the Herbarium of Medical Plants Research Center of the Shahrekord University of Medical Sciences (Iran), genus and species of the plant were identified and confirmed. The peel powder was dissolved in 80% ethanol and kept at room temperature for 96 hr. Then, the mixture was filtered and concentrated under nearly vacuum pressure at 40°C in the rotary evaporator. Four fractions of the crude extract, with different polarities were prepared by in-solution isolation and using the difference in various secondary metabolites' polarities (Figure 1). The crude extract was dissolved in ethyl alcohol/H₂O and fractionated by consecutive liquid/liquid partitioning with n-hexane (Merck, Germany), and then with chloroform (Merck,

Germany), ethyl acetate (Merck, Germany) and n-butanol (Merck, Germany) with increasing order of polarity¹³.

Determination of total phenolic content

The total phenolic content of pomegranate peel extract and its fractions was determined using Folin-Ciocalteu method¹⁴. Gallic acid was used as a standard reference for plotting calibration curve. Briefly, the dried extract/fractions or gallic acid were dissolved in 80% methanol for preparing 1 mg/ml of solutions. 0.2 ml of the diluted extract/fractions or standard solution of gallic acid (250, 125, 62.5, 31.2, and 15.6 µg/ml) were added to 1 ml of 10% (v/v) Folin-Ciocalteu reagent and kept at room temperature for 3-8 min. Next, 0.8 ml of 7.5% (w/v) sodium carbonate solution was added to the mixture. After the reaction solution was kept in total darkness for 30 min, its optical absorbance was measured at 765 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). Total phenolic content was calculated using a gallic acid calibration curve. The results were expressed as mg gallic acid equivalents per g of dry plant extract/fractions (mg GAE/g).

Determination of total flavonoid content

The total flavonoid content of pomegranate peel extract and its fractions was measured according to previously described methods¹⁵. Rutin was used as a standard

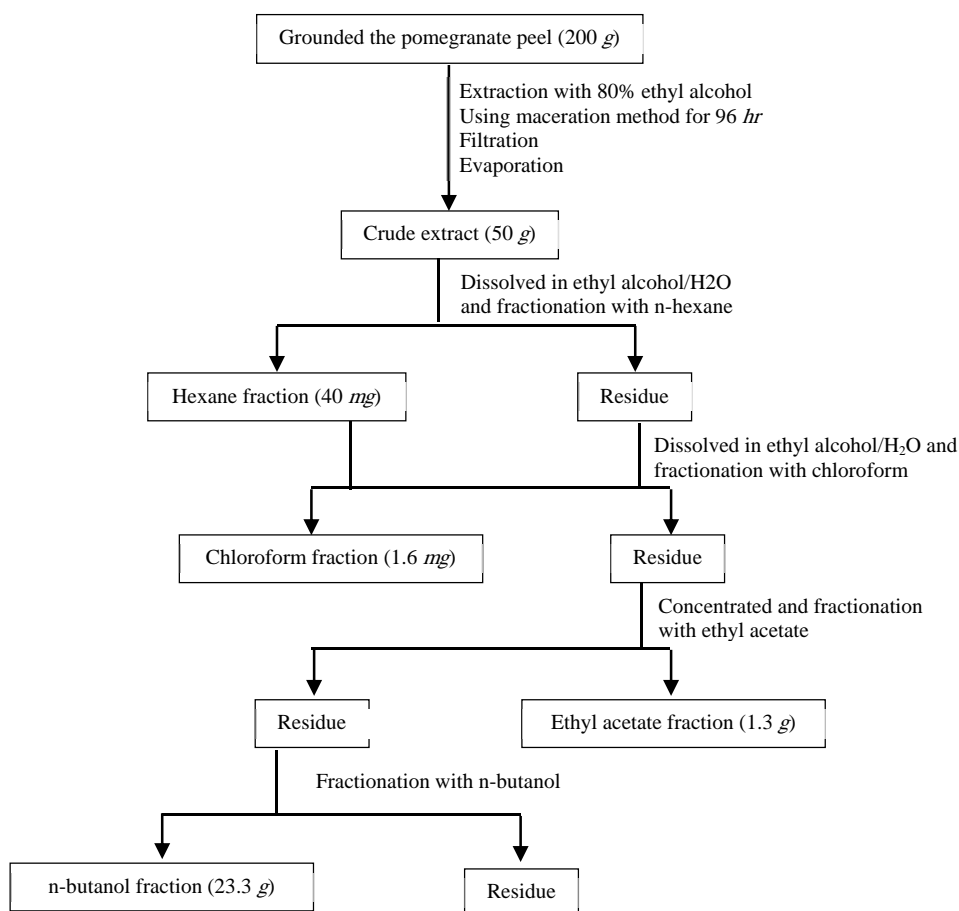


Figure 1. Flow chart for the extraction and fractionation of pomegranate peel

reference for plotting calibration curve. Briefly, 0.2 ml of the diluted extract/fractions (1 mg/ml) or standard solution of rutin (125, 62.5, 31.2, 15.6, and 7.8 µg/ml) were separately mixed with 0.2 ml of 2% (w/v) aluminum chloride and 1.2 ml of 5% (w/v) potassium acetate. After the reaction solution was incubated at Room Temperature (RT) for 40 min, its optical absorbance was read at 415 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). The results were expressed as mg of rutin equivalents per g of dry plant matter (mg RUT/g) in comparison with the standard curve, which was developed under the same conditions.

Cell culture and influenza virus propagation

Madin-Darby Canine Kidney (MDCK) cell line and influenza virus A/Puerto Rico/8/34 (H1N1; PR8) were obtained from Influenza Unit, Pasteur Institute of Iran. MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% penicillin streptomycin (Gibco, USA) at 37°C in a 5% CO₂ atmosphere and humidified incubator.

Cytotoxicity assay

The effect of pomegranate peel extract and its fractions on the viability of MDCK cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma, USA) assay, by a previously described method¹⁶ with some modifications. Briefly, when the cell monolayer was confluent, the cells were incubated with 200 µl/well of various concentrations of the extract/fractions (200, 100, 50, 25, 12.5, 6.25 and 3.1 µg/ml) in 96-well plates for 48 hr. Afterwards, cell monolayers were incubated with 50 µl of 1 mg/ml MTT in Phosphate-Buffered Saline (PBS) at 37°C for 4 hr, and then treated with 100 µl of acidic isopropanol (0.05 N HCl in absolute isopropanol). After the plates were shaken for 15 min, the absorbance was read using a reference filter at 640 nm using microplate reader (StataFax2100, USA).

Cytopathic effect (CPE) reduction assay

When the cell monolayer was confluent in 96-well plates, the cell culture medium was aspirated and washed with PBS and infected with 100TCID₅₀ of *Influenza A* (H1N1) virus for 1 hr, and then the virus was removed and the cells were treated with serial two fold dilutions of nontoxic concentration of the extract/fractions (200 µl/well) in serum-free DMEM containing 2 µg/ml of TPCK-trypsin and 0.3% BSA. 48 hr post infection, cell viability was also determined using previously described MTT assay¹⁶. Various concentrations of oseltamivir (10, 5, 2.5, 1.25, 0.62 and 0.31 µmol) (Sigma, USA) were used as positive controls. The procedure was carried out in triplicate. The 50% cytotoxic concentration (CC₅₀) and 50% inhibitory concentration (IC₅₀) were calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Selectivity index (SI) was calculated as ratio of CC₅₀ to IC₅₀.

Hemagglutination assay

MDCK cells in 24-well plates were infected with PR8 virus at 100 TCID₅₀, incubated with virus for 1 hr at 37°C and cultured in DMEM and TPCK trypsin (0.5 µg/ml; Sigma, USA) either with or without extract/ fractions treatment. The cell culture supernatants were harvested 24 and 48 hr post infection. Fifty µl of the two fold serial dilutions of the cell culture supernatants were mixed with the same volume of 0.5% chicken Red Blood Cells (RBCs) in U-bottomed 96-well plate for 45 min at room temperature. The HA was performed by measuring the dilution factor of the samples required for complete HA-mediated chicken RBC agglutination¹⁷.

TCID₅₀ virus titration

Confluent MDCK cells monolayer in 24-well plates were infected with PR8 virus (100 TCID₅₀) in the presence of the extract/fractions or control compounds for 24 hr at 37°C. Standard 50% Tissue Culture Infectious Doses (TCID₅₀) were used for virus titration in culture supernatants¹⁸. Briefly, when 90%-confluent MDCK cells were prepared in 96 well plates, the cell culture medium was aspirated and washed with PBS twice, and then 100 µl of a series of 10-fold dilutions was added into the wells and left to incubate for 2 days. After the incubation, virus replication was detected by HA¹⁸⁻²⁰. TCID₅₀ calculated based on the Reed and Muench method was expressed as log₁₀²¹.

Statistical analysis

The data were analyzed using Kruskal-Wallis test to compare differences between the groups. The IC₅₀ and CC₅₀ values were calculated by regression analysis using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Results

Total phenolic and flavonoid content

The results on total phenolic and total flavonoid content in the extract and fractions are presented in table 1. The results showed that pomegranate peel extract was the richest source of phenolic (233 mg GAE/g) and flavonoid (60.6 mg RUT/g) content. The highest phenolic (692 mg GAE/g) and flavonoid (93.7 mg RUT/g) content was obtained for ethyl acetate fraction (Table 1).

Cytotoxicity and anti-influenza A virus activity

Based on the CPE reduction assay results and probit analysis, the CC₅₀ of crude extract and n-butanol and ethyl acetate fractions was 55.66, 55.61, and 29.7 µg/ml, respectively (Table 2). The analysis showed that there was a direct, significant relationship between the concentration of the extract/fractions and cell death (p < 0.05, Figure 2). The antiviral activities of the extract and the four fractions against the influenza A/PR/8/34 virus were investigated 48 hr after treatment using an MTT-based CPE reduction assay. Results indicated that the

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Table 1. Total phenolic and flavonoid values of pomegranate peel crude extract and its fractions

Sample	Total phenolics (mg GAE/g)*	Flavonoid content (mg RUT/g)**
Crude extract	233±2.4	60.6.1±1.4
n-Hexan fraction	22.1±1.5	11.5±2.75
Choloroform fraction	221.9±3.5	83.2±1.9
Ethyl acetate fraction	476±4.2	60.6±2.7
n-Butanol fraction	199±1.8	77.1±2.5
p-value#	< 0.05	< 0.05

* mg gallic acid equivalent/g of extract/fractions, ** mg rutin equivalent/g of extract/fractions; # according to Kruskal-Wallis; all results are presented as mean (± standard error of the three measurements).

Table 2. Cell cytotoxicity and anti-influenza virus activity of pomegranate peel extract and fractions

Extract/fractions	CC ₅₀ ^a µg/ml (CI95%)	IC ₅₀ ^b µg/ml (CI95%)	SI ^c
Crude extract	55.6 (48.4-64)	6.4 (4.5-9.2)	8.63
n-hexane fraction	238.2 (142.4-398.4)	>238.2	-
Chloroform fraction	34.1 (30.1-38.6)	>34.1	-
Ethyl acetate fraction	29.7(24.9-35.3)	5.6 (3.9-7.9)	5.3
n-butanol fraction	55.61 (47.1-65.6)	6.1 (4.5-8.13)	9.16
Oseltamivir (µmol) ^d	539.4 (378.9-768.5)	0.87 (0.55-1.4)	617.8

^aCC₅₀: 50% cytotoxic concentration (MDCK cell); ^bIC₅₀: 50% inhibitory concentration (PR8 influenza virus); ^cSI: Selectivity index, i.e., the ratio of CC₅₀ to IC₅₀; CI95%: 95% confidence interval; ^d oseltamivir used as positive control.

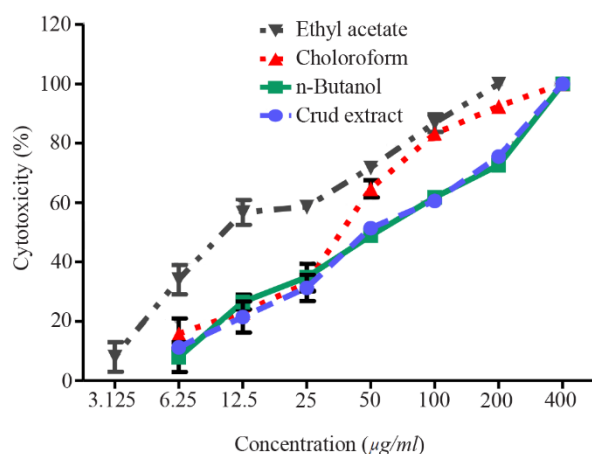


Figure 2. Cytotoxicity of pomegranate peel extract and its fractions on MDCK cells. Confluent MDCK cells were exposed to different concentrations of crude extract and its fractions for 48 hr. Cytotoxicity was measured in MTT assay; experiments were carried out in triplicate.

crude extract and n-butanol and ethyl acetate fractions produced antiviral effect against influenza virus with the SIs of 8.63, 9.16 and 5.3, respectively (Table 2).

Inhibition of influenza virus replication

Based on the CPE reduction assay results (Table 2), the crude extract and the n-butanol and ethyl acetate fractions underwent additional antiviral assays. The HA titers of the extract and the fractions on influenza virus were assessed by hemagglutination endpoint test. According to the results, the viral titer decreased dose dependently after treatment with the crude extract and the n-butanol and ethyl acetate fractions (Table 3).

To investigate whether pomegranate peel extract and n-butanol fraction and ethyl acetate fraction could have inhibitory effect on infectious virus yield, the virus was titrated by TCID₅₀ method. Consistent with the results of the HA, the production of virus was significantly reduced upon treatment with pomegranate peel extract and n-butanol and ethyl acetate fractions in a dose-dependent manner ($p < 0.05$; Figure 3).

Discussion

Pomegranate is a highly active and important medicinal plant in folk medicine and its antibacterial, antiparasitic, apoptotic, antifungal, antiproliferative, and antiviral activities have recently been studied²²⁻²⁴.

Although few studies reported the inhibitory effects of pomegranate fruit against herpes virus, influenza virus, poxviruses, and human immunodeficiency virus^{24,25}, this is the first report on the antiviral activity of corresponding fractions of pomegranate peel. Our aim, therefore, was to study the anti-influenza activity of pomegranate peel extract and its fractions in the MDCK cell line.

In the present study, the crude extract inhibited influenza A PR8 virus replication in the MDCK cell line [IC₅₀: approximately 6.45 (4.5-9.23)]. According to the results of antiviral assays to measure the titers of HA or infectious viral particles in the culture supernatants, it was observed that pomegranate peel could suppress the amplification of the infectious influenza viruses. Because the IC₅₀ of an herbal extract for infectious diseases is conventionally less than 100 µg/ml²⁶ and SI over 4²⁷, pomegranate peel extract with IC₅₀ of 6.45 and SI of 8.63 can be considered a potent agent to

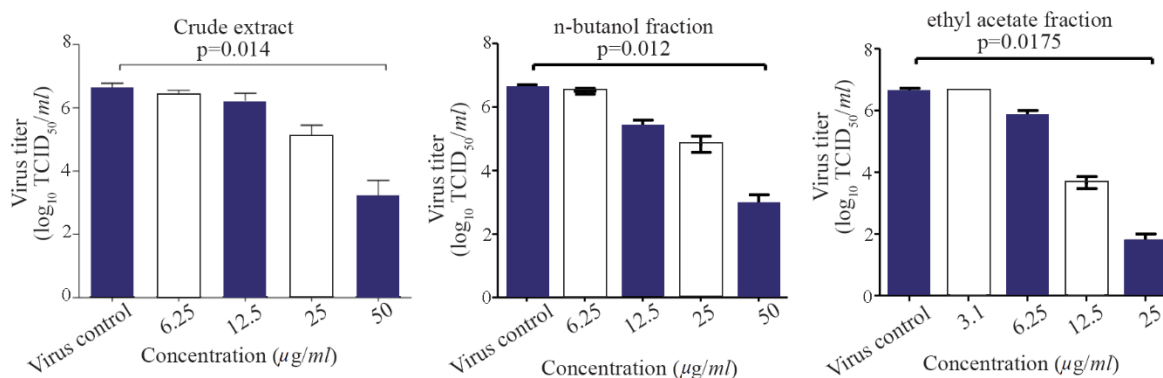


Figure 3. Reduction of influenza viral titers in the culture supernatants by the pomegranate peel extract and its more effective fractions. PR8-infected MDCK cells were incubated with different concentrations of the extract/fractions for 24 hr and the supernatants were used for TCID₅₀ titration. The data are the mean values of three independent experiments (mean±SEM). p-values were calculated against virus control (untreated sample) using Kruskal-Wallis test.

Table 3. Hemagglutination titers of PR8-infected MDCK cell supernatants in the presence of the pomegranate peel extract and its more effective fractions

Extract/fractions	Concentration ($\mu\text{g/ml}$)	Log ₂ HA titer/50 μl supernatant	
		24 hr ^a	48 hr ^a
Crude extract			
	50	0	0
	25	0.67±1.15	4±3.46
	12.5	1.67±1.15	6.33±0.58
	6.25	2.67±1.15	6.67±1.15
	virus control	5±1.4	7.33±0.58
n-butanol fraction			
	50	0	0
	25	0	2.5±0.71
	12.5	1.5±0.71	6
	6.25	3.5±0.71	6.5±0.71
	virus control	6	7.5±0.71
Ethyl acetate fraction			
	25	0	1±1.73
	12.5	0.33±0.58	4±1.43
	6.25	1.67±1.53	6.33±0.58
	3.12	2.67±0.58	6.67±1.15
	virus control	4±1.73	7.33±0.58
Oseltamivir (μmol)^b			
	5	0	0
	2.5	0	0.5±0.71
	1.25	0	4
	virus control	5±1.4	8

a: Hours post-infection; b: Oseltamivir used as positive control.

fight influenza virus.

Our results showed that the crude extract and n-butanol and the ethyl acetate fractions exerted more potent antiviral effects than other fractions. Other studies have also shown that the antiviral property of pomegranate extract may be due to hydrolysable tannins and polyphenols, especially punicalagin and gallagic acid, which have also been found in this extract²³. A study indicated that out of the four flavonoids of pomegranate, *i.e.* ellagic acid, caffeic acid, luteolin, and punicalagin, only punicalagin had inhibitory effect against influenza virus

²⁵. Pomegranate peel's polyphenol compounds such as punicalagin, ellagic acid, and hydroxy-benzoic acid that are extracted from both n-butanol and ethyl acetate fractions are probably associated with antiviral activity of pomegranate peel.

Conclusion

Based on our results, both n-butanol and ethyl acetate fractions of pomegranate peel, with high inhibitory effect against influenza virus replication, could be a new promising anti-influenza agent. More understanding of

the mechanism of action and the natural components of these fractions seems necessary. The results of this study also showed the presence of high amounts of polyphenols in the fractions. As a result, the antiviral activity of the plant could be partly attributed to its polyphenol content.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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