

## In vitro Anti-adenovirus activity of pomegranate (*Punicagranatum L.*) peel extract

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Received: 1/Nov/2015 Accepted: 2/Dec/2015

### ABSTRACT

**Background and aims:** Human adenoviruses can cause a diversity of clinical diseases, but there is no antiviral therapy formally approved by adenovirus infections. Thus, antiviral agents derived from medicinal plants which are effective against adenoviruses infections are urgently required. Therefore, this research was aimed to evaluate in vitro anti-adenovirus activity of pomegranate (*Punicagranatum L.*) peel extract.

**Methods:** In this research, crude ethanol extract of pomegranate peel was prepared. Anti-adenovirus activity of the extract was evaluated on Helacell line using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The 50% inhibitory concentration (IC<sub>50</sub>) and 50% Cytotoxicity Concentration (CC<sub>50</sub>) of the extract were determined using regression analysis. To determine antioxidant activity, total phenol content, and flavonoids content of the extract, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay; Folin-Ciocalteu method and aluminum chloride colorimetric method was used, respectively.

**Results:** The CC<sub>50</sub> and IC<sub>50</sub> of the extract were 165±10.1 and 18.6±6.7µg/ml, respectively. The selectivity index (SI), the ratio of CC<sub>50</sub> and IC<sub>50</sub>, was 8.89. The IC<sub>50</sub> of DPPH radical was 7.7±1.21 µg/ml, compared to Butylated hydroxytoluene (BHT), with IC<sub>50</sub> of 25.41±1.89 µg/ml. The total phenol and Flavonoid contents were 282.9 mgGAE/g and 136.6mg/g, respectively.

**Conclusion:** Peel extract exhibited anti-adenovirus activity, with SI value of 8.9, suggesting its potential use as anti-adenovirus agents. Also, this extract with high phytoconstituents could be a promising source of medicinally important natural compound.

**Keywords:** Antioxidant activity, Antiviral activity, *Punicagranatum L.*, Pomegranate peel, Antiadenovirus.

### INTRODUCTION

Medicinal plants have been used for many years for the treatment of human diseases<sup>1</sup> and a number of herbal medicines

have been developed into therapeutic agents or have had promising results.<sup>2-5</sup>

Plants have the ability to synthesize a wide

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array of compounds and have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of drugs.<sup>6-8</sup> Incidence of toxic side effects, development of resistance, and sensitivity of individuals are several reasons for the need to substitute these synthetic drugs with new ones.<sup>9</sup> The compounds with natural origin, especially herbal medicines, have been shown to be reliable source for new drugs.<sup>10-12</sup>

Pomegranate (*Punicagranatum* L., Family Punicaceae) is a popular edible fruit which is widely used in traditional medicine.<sup>13-15</sup> Several lines of evidence have indicated the therapeutic efficacy of pomegranate against different types of disorders.<sup>14,16-18</sup> Pomegranate has also been shown to have antimicrobial, antioxidant,<sup>19</sup> and anti-inflammatory<sup>20</sup> properties and to decrease the blood pressure.<sup>21</sup> The pomegranate has considerable amounts of biologically active phytochemicals including flavonoids (e.g. anthocyanins, catechins, quercetin, and rutin), other types of polyphenols, ellagitannins, and antioxidant vitamins.<sup>22,23</sup> It has been shown that plants flushed of flavones, tannin<sup>24-26</sup> and alkaloid have antiviral, antibacterial, antifungal and antiparasite effects.<sup>27</sup>

Human adenoviruses (HAdVs) are classified in the genus *Mastadenovirus*, which contains seven known HAdV species HAdV-A to HAdV-G.<sup>28</sup> Adenoviruses can cause an array of clinical diseases, including conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia. Most of these occur in children younger than the age of 5 years and are generally self-limiting illnesses. However, severe and life-threatening adenovirus infections have been reported in some patients, including immunocompromised patients.<sup>29,30</sup> Although, some antiviral drugs are reported to inhibit adenovirus infection in vitro and

used for empirical therapy in severe adenovirus infections, such as nucleoside analogues and even protease inhibitors, usefulness of them had not been approved.<sup>30</sup> Therefore, antiviral agents from medicinal plants with new effective compounds exhibiting different modes of action against Adenoviruses infections are urgently needed.

Iran is endowed with rich and diverse local health tradition, which is equally matched with rich and diverse plant genetic source. A detailed investigation and documentation of plants used in local health traditions and ethno pharmacological evaluation to verify their efficacy and safety can lead to the development of invaluable herbal drugs or isolation of compounds of therapeutic value. Therefore, this research was aimed to evaluate in vitro Anti-adenoviruses activity of Pomegranate (*Punicagranatum* L.) peel extract and the investigation of antioxidant properties of this plant material.

## METHODS

Pomegranates were purchased from a local market. Peels were first prepared and dried for a week at room temperature (RT) in darkness. Then the dried peels were separately ground to obtain uniform powders. The peel powder (100 g) was dissolved in 70% ethylealcohol (400ml) and kept at RT for 96 h. Subsequently, the mixture was filtered and concentrated under nearly vacuum pressure and at 40°C using rotary evaporator. The extracts were kept in sterile bottles under refrigerated conditions until further use. The extracts were suspended at 37°C in dimethylsulphoxide (DMSO) to give a stock solution of 25mg/mL, dissolved in culture medium, filtered (Millipore® 0.22 µm) and stored (4°C) until use. The remaining DMSO in the wells (maximal 0.2%) did not affect the experiment results.<sup>31</sup>

The free-radical scavenging activity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, with some modification.<sup>32</sup> Different amounts of the extract and methanol were added to a solution of 0.3 mg/mL methanolic solution of DPPH to make up a total volume of 3.0 mL. After standing for 15 min at room temperature, the absorbance was measured at 517 nm using UV-Vis spectrophotometer (UNICO 2100: USA). High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxytoluene (BHT) was used as positive control. Inhibition of free radical by DPPH was calculated as follows: Antiradical activity (%) =  $(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$ . The IC<sub>50</sub> value defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and was calculated based on linear regression of plots of the percentage antiradical activity against the concentration of the tested compounds.<sup>33</sup> The experiment was carried out in triplicate and the results are average values.

The total phenolic content of the Pomegranate peel extract was determined using Folin-Ciocalteu method.<sup>34</sup> Briefly, 0.1 ml of the diluted sample was added to 0.5 ml of 10% (v/v) Folin-Ciocalteu reagent and kept at room temperature (RT) for 3-8 min. Subsequently, 0.4 ml of 7.5% (w/v) sodium carbonate solution was added to the mixture. After being kept in total darkness for 30 min, the absorbance of the reaction mixture was measured at 765 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). Amounts of total phenolic were calculated using a gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) g/g of dry plant matter.

The total flavonoid content of the extract was measured as previously reported method.<sup>35</sup> Briefly; 0.5 ml of diluted plant material was independently

mixed with 1.5 ml of methanol, 0.1 ml of 10% (w/v) aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. Following incubation at room temperature (RT) for 40 min, the absorbance of the reaction mixture was read at 415 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). The results were expressed in mg of routine equivalents of dry plant matter by comparison with the standard curve, which was made in the same condition.

Hela (cervix adenocarcinoma) cells was kindly provided by Pasteure Institute of Iran. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco, USA), 100 µg/mL of streptomycin, 100 IU/ml of penicillin and 0.25 µg/mL amphotericin B (Gibco, USA), at 37°C and 5% CO<sub>2</sub>. The same medium containing 2% FBS was used for cytotoxicity and antiviral assays. Adenovirus (type 5) was kindly provided by Health Faculty, Tehran University of medical sciences, Tehran, Iran. Virus stock was prepared by infection of confluent monolayer Hela cells in 75 cm<sup>2</sup> culture flasks using DMEM medium with 2% FBS, at 37°C in 5% CO<sub>2</sub>. Virus titer was determined by cytopathic effect (CPE) of adenovirus in Helacells and was expressed as the 50% Tissue Culture Infective Dose (TCID<sub>50</sub>) per ml.

Prior to the investigation of Antiadenovirus activity, the cytotoxic effect (CPE) of the extract was determined. Briefly, Helacells were seeded onto 96-well plates with a concentration of 10000 cells/well with final volume of 100 µl per well. After incubation at 37°C with 5% CO<sub>2</sub> for 24h, when the cell monolayer was confluent, the cell culture medium of cells aspirated and washed with Phosphate-Buffered Saline (PBS). Cells were incubated with 100 µL/well of various concentrations

of ethanolic extract (in triplicates), and incubated at 37°C with 5% CO<sub>2</sub> for further 5 days. Cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), (Sigma, USA), by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product and the procedure was followed as described earlier<sup>31</sup>. Briefly, the supernatants were removed from the wells and 50 µL of an MTT (Sigma, USA) solution (1 mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 100 µL of DMSO (Samchunkorea) was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and the absorbance were read on an enzyme-linked immunosorbent assay (ELISA) reader (STAT FAX 2100, USA) at 492 nm. Data were calculated as the percentage of toxicity using the following formula: toxicity (%) = [100 - (At/As) × 100] %, where At and As refer to the absorbance of the test substance and the solvent control, respectively.<sup>31,36</sup> The 50% cytotoxic concentration (CC<sub>50</sub>) was defined as the cytotoxic concentration of the crude extract by regression analysis.

Antiviral activity of the extract was evaluated by inhibitory activity assay using MTT method, as described previously.<sup>31</sup> Briefly, Non-cytotoxic concentrations of the extract below the CC<sub>50</sub> value were used to test the ability of them to inhibit CPE of adenovirus in tissue culture. To confluent Helacell monolayer in a 96-well plate, 100 µl (100TCID<sub>50</sub>) virus suspension was added and incubated at 37 °C for about one hour to allow virus adsorption. Thereafter, serial twofold dilutions prepared from non-toxic dose of the extract was added and tested in triplicate. As positive control,

cells were infected with the same concentration of virus but without addition of extract. As a negative or cell control, only 1% DMEM was added to the cells. The plates were incubated at 37 °C in a humidified CO<sub>2</sub> atmosphere for 5 days.

Cell viability was also determined using previously described MTT assay.<sup>31</sup> Data were calculated as the percentage of inhibition using the following formula: Antiviral activity (%) = (Atv - Acv) / (Acd - Acv) × 100%, where Atv, Acv, and Acd are the absorbance of the test compounds on virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively.<sup>31</sup> The procedure was carried out three times. The 50% inhibitory concentration (IC<sub>50</sub>) was determined from a curve relating inhibition to the concentration of the extract. Selectivity index (SI), as a marker of antiviral activity, was determined as the ratio of CC<sub>50</sub> to IC<sub>50</sub>.

All experiences were carried out in triplicate. The IC<sub>50</sub> and CC<sub>50</sub> values were calculated using dose-response analyses and related models with probit procedure using SPSS program. P < 0.05 was considered statistically significant.

## RESULTS

Total amount of phenolic and flavonoid compounds of Pomegranate peel extract was 282.9 mg/g garlic acid equivalent and 136.6 mg/g, respectively.

Our results showed that the scavenging effect of the extract increases with the concentration increases. The crude extract had IC<sub>50</sub> values of 7.7 ± 1.21 µg/ml. The results are expressed compared with Butylated hydroxytoluene (BHT), a reference standard with IC<sub>50</sub> of 25.41 ± 1.89 µg/ml (Table 1).

**Table 1:** DPPH radical-scavenging activity of the pomegranate peel extract

Sample	Concentration (µg/ml)	Scavenging of DPPH radical activity inhibition (%) (mean ± SEM)	DPPH-radical scavenging activity IC <sub>50</sub> / (µg/ml)
Pomegranate peel extract	20	91.2±0.3	7.7±1.21
	10	71.9±1	
	5	44.15	
	2.5	30.12	
	1.25	0	
BHT	50	90.8±1.5	25.41±1.89
	40	78.3±1.2	
	30	55.5±0.7	
	20	40.09±1.7	
	10	22±1.06	

All results are presented as mean ± standard mean error of three assays; DPPH: 1,1-Diphenyl-2-picrylhydrazyl, BHT: Butylated hydroxytoluene.

Based on MTT analysis results, The CC<sub>50</sub> value of crud extract on Helacells was 165±10.1µg/ml. The analysis showed significant relationship between the concentration of the extract and cell death with the more increase the extract concentration, the more cell death was shown (P<0.01).

Treatment of the Helacells with different concentrations of crude extract at the same time of inoculation by adenovirus was done based on the method mentioned in materials and methods section. Based on the results, we understood that 3 µg/ml of the extract did not show any antiviral effect while the 30 µg/ml of that extract could inhibit the performing of cytopathic effect completely due to adenovirus replication in Hela cells. According to the model, with the increasing of the extract concentration, the percentage of inhibition of the cytopathic effect (CPE) was increased (P<0.05). Therefore, in Probit analysis, IC<sub>50</sub> of pomegranate peel extract on adenovirus was 18.6±6.7 µg/ml. The SI value of pomegranate peel extract was 8.9on adenovirus.

## DISCUSSION

Based on MTT analysis results, The CC<sub>50</sub> value of pomegranate peel extract on Helacells was 165±10.1 µg/ml and the IC<sub>50</sub> value on adenovirus was 18.6±6.7 µg/ml.

The SI value of pomegranate peel extract on adenovirus was 8.9. Based on these results, pomegranate peel extract showed strong activity against adenovirus.

Pomegranates (*Punicagranatum* L.) have been used for a long time as an antibacterial agent in traditional medicine. The bactericidal effect of pomegranates on a number of highly pathogenic and drug-resistant bacterial strains was investigated in different areas of the world. Methanol extract of its fruit, and especially the peel, exhibited the broadest antibacterial activity.<sup>37</sup> The fruit of pomegranate and its extracts has been reported to be effective against influenza virus, herpes simplex virus, poxviruses, and human immunodeficiency (HIV-1) virus.<sup>38-41</sup>

Methanol extracts of pomegranate are highly rich in hydrolyzable tannins (punicalins and punicalagins), ellagic acid, a component of ellagitannins, and gallic acid, a component of gallotannins.<sup>42</sup> Both antibacterial and antiviral effects of pomegranate most probably are associated with the hydrolyzable tannins and anthocyanins.<sup>43</sup> There is one published report indicating out of four flavonoid compounds pomegranates (ellagic acid, caffeic acid, luteolin, and punicalagin) of pomegranates only punicalagin had inhibitory effect on influenza virus.<sup>44</sup>

For using in this study, the extract was prepared with 70% ethanol, and after drying, tested without further purification. It has been suggested that more purification of the extract might increase its antiviral activity. As the extract was not completely purified, it probably showed its partial antiviral activity and it should be possible to increase the antiviral activity through purification of the extract.

## CONCLUSION

Our results showed that pomegranate (*Punicagranatum* L.) peel extract exhibited Anti-adenovirus activity, with SI value of 8.9. This may suggest the extract as a potential Anti-adenovirus agent. Also this extract with high phytoconstituents could be a promising source of medicinally important natural compounds.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

## ACKNOWLEDGMENT

Authors are thankful to the Director of Medical Plants Research Center and to the Deputy of Research and Technology of Shahrekord University of Medical Sciences, Shahrekord, Iran for financial support.

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**How to cite the article:** Moradi MT, Karimi A, Alidadi S, Saedi-Marghmaleki M, Salehian M. In vitro Anti-adenovirus activity of pomegranate (*Punicagranatum* L.) peel extract. *Adv Herb Med.* 2015; 1(4): 1-8.