Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(8):82-88

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

In vitro anti-herpes simplex type-1 activity, antioxidant potential and total phenolic compounds of pomegranate (*Punica granatum* L.) peel extract

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ABSTRACT

Drug resistant strains of herpes simplex virus-1(HSV-1) have been recently increased. Thus, new antiviral agents derived from medicinal plants which are effective against HSV-1 infections are urgently required. Therefore, this research was aimed to evaluate in vitro anti-HSV-1 activity of pomegranate (Punica granatum L.) peel extract and the investigation of antioxidant properties of these plant materials. In this research, crude ethanol extract of pomegranate peel was prepared. Anti-HSV-1 activity of the extract was evaluated on Vero cell line using MTT (3-[4,5-dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide) assay. The 50% inhibitory concentration (IC_{50}) and 50% Cytotoxicity Concentration (CC_{50}) of the extract were determined using regression analysis. Its inhibitory effect on adsorption and/or post-adsorption stages of HSV-1 replication cycle was determined. 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. The CC_{50} and IC_{50} of the extract were 293.5±10/1 and 37.7±6/7µg/ml, respectively. The selectivity index (SI), the ratio of CC_{50} and IC50, was 7.78. This extract inhibited HSV-1 replication in adsorption stage ($p<0.05$). The IC_{50} of DPPH radical was 7.7±1.21 µg/ml, compare with butylated hydroxytoluene (BHT), with IC_{50} of 25.41±1.89 µg/ml. The total phenol and Flavonoid contents were 282.9 mgGAE/g and 136.6mg/g, respectively. This study revealed that the pomegranate (Punica granatum L.) peel extract exhibited anti-HSV activity, probably by inhibiting the adsorption stage, with SI value of 7.7, suggesting its potential use as anti-HSV agents. Also this extract with high phytoconstituents could be a promising source of medicinally important natural compound.

Keywords: Antioxidant activity, Antiviral activity, *Punica granatum* L, pomegranate peel, Herpes simplex virus

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INTRODUCTION

Medicinal plants have been used for many years for the treatment of human diseases [1] and a number of herbal medicines have been developed into therapeutic agents or have had promising results [2-5]. Plants have the ability to synthesize a wide 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 [6-8]. 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 [9]. The compounds with natural origin, especially herbal medicines, have been shown to be reliable source for new drugs [10-12].

Pomegranate (*Punica granatum* L.; Family Punicaceae) is a popular edible fruit which is widely used in traditional medicine [13-15]. Several lines of evidence have indicated the therapeutic efficacy of pomegranate against different types of disorders [14, 16-18]. Pomegranate has also been shown to have antimicrobial, antioxidant [19], and anti-inflammatory [20] properties and to decrease the blood pressure [21]. 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 [22, 23]. It has been shown that plants flushed of flavones, tannin [24-26] and alkaloid have antiviral, antibacterial, antifungal and antiparasite effects [27].

Infections caused by herpes simplex virus (HSV) are public health concern worldwide. These infections range from inaparent to severe life threatening infections such as encephalitis [28]. During the past two decades, the mechanisms of replication and pathogenesis of HSV-1 and thus the potential antiviral targets in this virus have been widely understood and leads to development of antiviral compounds that target this virus [28]. A category of nucleoside analogues, Acyclovir (ACV), is widely used to treat HSV-1 infections as a drug of choice [29]. However, a major problem associated with use of ACV, is the development of drug resistant HSV strains, particularly in AIDS patients [30]. This kind of drug resistance may occur after long term treatment and is mainly due to mutations in the HSV-1 thymidine kinase and/or DNA polymerase genes [31]. Therefore, antiviral agents from medicinal plants with new effective compounds exhibiting different modes of action against viral 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 ethnopharmacological 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-HSV-1 activity of Pomegranate (*Punica granatum* L.) peel extract and the investigation of antioxidant properties of this plant material.

EXPERIMENTAL SECTION

Preparation of methanolic extract

Pomegranates was 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% ethyle alcohole (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 [32].

Determination of the free-radical scavenging activity

The free-radical scavenging activity was measured by the 2,2 diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, with some modification [33]. 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 (%) = (Acontrol – Asample)/Acontrol×100. The IC_{50} 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 [34]. The experiment was carried out in triplicate and the results are average values.

Determination of total phenolic content

The total phenolic content of the Pomegranate peel extract was determined using Folin-Ciocalteu method [35]. 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.

Determination of total flavonoid content

The total flavonoid content of the extract was measured as previously reported method [36]. 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 1M 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 rutin equivalents of dry plant matter by comparison with the standard curve, which was made in the same condition.

Cell and Virus

Vero (African green monkey kidney) 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 UI/ml of penicillin and 0.25 µg/mL amphotericin B (Gibco, USA), at 37°C and 5% CO2. The same medium containing 2% PBS was used for cytotoxicity and antiviral assays. HSV-1 (HSV-1, KOS strain) was kindly provided by University of Tarbiat Modares, Tehran, Iran. Virus stock was prepared by infection of confluent monolayer Vero cells in 75 cm^2 culture flasks using DMEM medium with 2% FBS, at 37°C in 5% CO_2. Virus titer was determined by cytopathic effect (CPE) of HSV-1 in Vero cells and was expressed as the 50% Tissue Culture Infective Dose ($TCID_{50}$) per ml.

Cytotoxicity assay

Prior to the investigation of anti-HSV-1 activity, the cytotoxic effect (CPE) of the extract was determined. Briefly, Vero cells 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_2 for 24 h, when the cell monolayer was confluent, the cell culture medium of cells aspirated and washed with PBS. Cells were incubated with 100 µL/well of various concentrations of ethanolic extract (in triplicates) and incubated at 37°C with 5% CO_2 for further 3 days. cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumbromide), (Sigma, USA), by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product and the procedure was followed as described earlier [37]. Briefly, the supernatants were removed from the wells and 50 µL of an MTT (Sigma, USA) solution (1mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 100 µL of DMSO (Samchun korea) 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 (STATA 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 [32]. The 50% cytotoxic concentration (CC_{50}) was defined as the cytotoxic concentration of the crude extract by regression analysis.

Antiviral assay

Antiviral activity of the extract was evaluated by inhibitory activity assay using MTT method, as described previously [32]. Briefly, Non-cytotoxic concentrations of the extract below the CC_{50} value were used to test the ability of them to inhibit CPE of HSV-1 in tissue culture. To confluent Vero cell monolayer in a 96-well plate, 100µl ($100TCID_{50}$) virus suspension was added and incubated at 37^0C 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_2 atmosphere for 3 days. DMSO with 0.1% concentration and a solution of Aciclovir (9-(2-hydroxyethoxymethyl; Sigma, USA) were used as negative and positive controls, respectively.

Cell viability was also determined using previously described MTT assay [32]. 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 [32]. The procedure was carried out three times. The 50% inhibitory concentration (IC_{50}) 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 CC50 to IC50.

Time-of-addition assay

The time-of-addition effect of the Pomegranate peel extract was evaluated with minor modifications in previously described method [38]. To assess the effect of the extract on the adsorption and post-adsorption events of HSV-1

replication, the virus was treated with one time IC90 of the extract (89µg/ml) in three different manners, 1) only during the adsorption period (adsorption); 2) after adsorption and until the end of the experiment (post-adsorption); and 3) during and after the adsorption (throughout) (**Figure 1**). To carry out these experiments, 90% confluent cells were chilled at 4°C for 1 hour followed by infection with100 µL/well of HSV-1 (100$TCID_{50}$) in the presence or absence of extract and further incubated at 4°C for 1 h allowing only the adsorption step of the viral particles to the cells (Adsorption). Subsequently, the supernatant was removed, the cells were washed twice with ice-cold PBS and the medium with or without the extract was replaced with an equal volume of DMEM and 1% PBS, and incubated for three days at 37°C /5% CO2. Using MTT assay, cell viability and the percentage of viral inhibition was evaluated compared with the control. Data represented as the percentage of virus inhibition compare with untreated control as mean ± SD (n=3). Statistical analysis was carried out by statistical software using one way analysis of variance and the post-hoc Tukey's test.

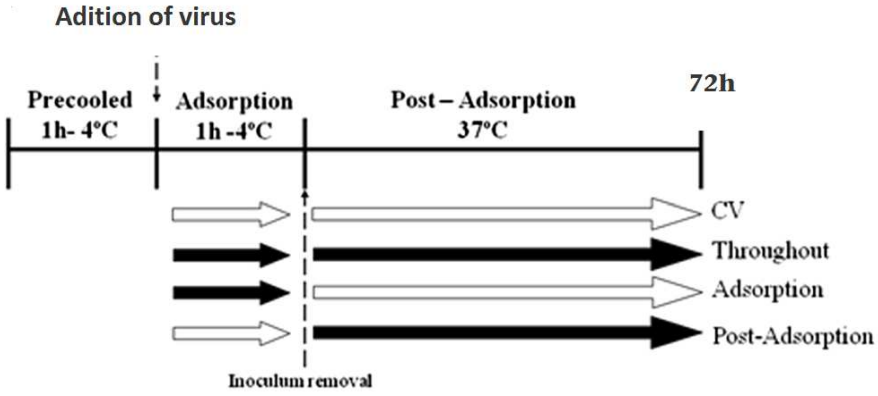

Figure 1: Scheme of addition of Pomegranate peel extract in the adsorption and post-adsorption stages of HSV-1[38]. Open and black arrows indicate the absence and presence of extract, respectively

Statistical analysis

All experiences were carried out in triplicate. The IC_{50} and CC_{50} values were calculated using dose-response analyses and related models with probit procedure using SPSS program. A p-value of less than 0.05 was considered statistically significant

RESULTS AND DISCUSSION

Antioxidant capacity, flavonoids, and phenolic compounds

Total amount of phenolic and flavonoid compounds of Pomegranate peel extract was 282/9 mg/g galic acid equivalent and 136.6 mg/g, respectively.

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

Sample	Concentration (µg/ml)	Scavenging of DPPH radical activity inhibition (%) (main ± SEM)	DPPH-radical scavenging activity IC_{50} / (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

Our results showed that the scavenging effect of the extract increases as the concentration increases. The crude extract had IC50 values of 7.7±1.21µg/ml. The results are expressed compared with butylated hydroxytoluene (BHT), a reference standard with IC50 of 25.41±1.89 µg/ml (table1)

Cytotoxicity of the extracts on Vero cells

Based on MTT analysis results, The CC_{50} value of crud extract and Acyclovir on Vero cells was 293.5 and 177.5µg/ml, respectively. 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 ($P<0.01$).

Anti-HSV-1 activity

Treatment of the Vero cells with different concentrations of crude extract and Acyclovir at the same time of inoculation by HSV-1 was done based on the method mentioned in materials and methods section. Based on the results, we understood that 5µg/ml of the extract did not show any antiviral effect while the 80 µg/ml of that extract could inhibit the performing of cytopathic effect completely due to HSV-1 replication in Vero 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_{50} of pomegranate peel extract and Acyclovir on HSV-1 was 37.7 and 1.3µg/ml, respectively. The SI value of pomegranate peel extract and acyclovir on HSV-1 was 7.78 and 136.5, respectively. Based on these results, pomegranate peel extract showed strong activity against HSV-1.

Characterization of antiviral activity

To determine whether the inhibitory effect of the crude extract occur during the adsorption and/or post-adsorption steps of HSV-1 replication cycle, different experiments were carried out with one time IC_{90} of this plant material (Figure 1). Our results showed that the extract inhibit HSV-1 replication in adsorption stage ($p<0.05$). In contrast, Acyclovir inhibited HSV-1 replication in post-adsorption stage ($p<0.001$). There was no significance difference in the percentage of viral inhibition exhibited by pomegranate peel extract and Acyclovir both in post-absorption stage and during the experiment (Throughout) (Figure 2).

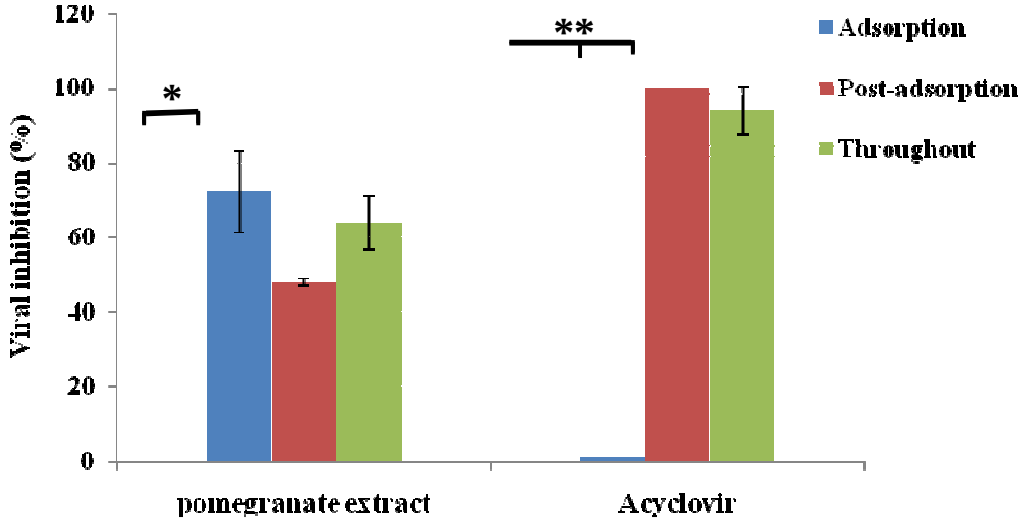

Figure 2: The effect of pomegranate (*Punica granatum* L.) peel extract and Acyclovir on the adsorption and post-adsorption of HSV-1 to the Vero cell

*Data represent the percentage of virus inhibition compared with untreated control as mean ±SD (n = 3). One time IC_{90} was used in this experiment. Statistical analysis was carried out using statistical software, Using this analysis, * P<0.05 adsorption versus post-adsorption, ** P<0.001adsorption versus post-adsorption and throughout (one way analysis variance and the post-hoc Tukey's test)*

There is currently increasing interest to use natural products in treating and preventing medical problems. At present, due to induction of resistance of pathogens to chemical drugs and the prevalence of the fatal different infections, the search for biological active extracts based traditionally used plants is extensively carried out [39]. This study was aimed to evaluate in vitro anti-HSV-1 activity of pomegranate peel extract. In this study, we have

shown that the ethanol crude extract of pomegranate (Punica granatum L.) peel have high antiviral effect against HSV-1.

Pomegranates (*Punica granatum* 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 [40]. 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 [41-43].

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 [44]. Both antibacterial and antiviral effects of pomegranate most probably are associated with the hydrolyzable tannins and anthocyanins [45].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 [46].

In our study, to understand the mechanism of action of pomegranate peel extract, HSV-1 was treated with one time IC_{90} of the extract (89µg/ml) in three separate experiments; 1) adsorption (only during the adsorption period); 2) post-adsorption (after adsorption and until the end of the experiment) and; 3) throughout (during and after the adsorption). Our results showed that the extract inhibit HSV-1 replication in adsorption stage.

Both cranberry juice, and cranberry-pomegranate have been reported to reduce the binding of human noroviruse strains specifically to certain types of human histoblood group antigens (HBGAs) [47]. Based on the results, it was suggested that the interaction of plant polyphenolic compounds with the viral capsid protein may cause irreversible damage or reversible blocking of certain regions/areas of the capsid protein [47]. It was shown that components of pomegranate juice, which adsorbed on corn starch, block HIV-1 bind to CD4 and CXCR4/CCR5 host cell receptors [43]. This published results, together, may indicate anti-HIV potential of these natural safe food sources. One study found that proanthocyanidin A-1 inhibited herpes simplex virus type 2 (HSV-2) attachment and penetration and also affected the late stage replication of the virus [48].

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 anti-HSV-1 activity through purification of the extract.

CONCLUSION

Our results showed that pomegranate (*Punica granatum* L.) peel extract exhibited anti-HSV activity, probably by inhibiting the adsorption stage, with SI value of 7.7. This may suggest the extract as a potential anti-HSV agent. Also this extract with high phytoconstituents could be a promising source of medicinally important natural compounds.

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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