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ANTIVIRAL ACTIVITY OF PUNICA GRANATUM SPECIES PLENIFLORA, SAVEH BLACK LEATHER, AND SWEET ALAK AGAINST HERPES SIMPLEX VIRUS TYPE 1

Gavanji S.[1], Baghshahi H.[2], Bakhtari A.[3] , Hamami Chamgordani Z.[4], Badripour N.[2*]

[1)] Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan.Iran
[2)] Barij Medicinal Plants Research Center, Kashan, Iran
[3)] Department of Reproductive Biology, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran
[4)] Department of Adult Health Nursing, Faculty of Nursing and Midwifery, Isfahan University of Medical Sciences, Isfahan, Iran.

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Abstract

Background: *Herpes simplex virus-1, commonly known as oral herpes, is a highly contagious viral infection in humans. Various therapies and clinical management strategies have treated Herpes simplex virus-1 infection, but drug resistance is a concern, which has sparked an obsession with herpes simplex virus therapy. Therefore, interest in herbal medications with antiviral properties has increased. This research aimed to investigate the antiviral activity of pomegranate flower extracts on herpes simplex virus-1 type 1 in Vero cells under in vitro conditions.*

***Material andMethods*:** *This study evaluated the anti-herpetic effect of pomegranate flower extracts, and the plaque reduction assay was performed on Vero cells. For cytotoxicity determination of pomegranate flower extracts, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide was used. The total flavonoid and phenolic contents of three varieties of Punica granatum (P. granatum) were measured based on gallic acid (mg/g) and rutin equivalents (mg/g), respectively.*

Results: *Pomegranate flower extracts had no cytotoxic impact at doses ranging from 80 to 140 µg/ml. Our study revealed that pomegranate flower extracts prevented the growth of viral plaques, and the IC_{50} values of three P. granatum species—pleniflora, Saveh Black Leather, and Sweet Alak—were 109.63, 131.24, and 128.87 µg/ml, respectively. Also, evaluation of total phenolic and flavonoid content showed that P. granatum var. pleniflora (Golnare farsi) had the highest total phenolic and flavonoid content (17.8 mg/g of gallic acid and 2.2 mg/g of rutin equivalents, respectively).*

Conclusion: *The pomegranate flower extracts have an inhibitory impact on herpes simplex virus-1 and could be used as an anti-HSV-1 agent in further investigation.*

Keywords: *Antiviral activity, Cytotoxicity, Punica granatum, Herbal medicine, Herpes.*

Introduction

One of the most common and recurrent epitheliotropic pathogens is the Herpes simplex virus (HSV), which belongs to the family of Herpesviridae and can cause epithelial cell infection in human

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Address for Correspondence:

Nima Badripour, Doctor of Veterinary Medicine ORCID ID: 0009-0008-4051-6526
Barij Essence Medicinal Plants Research Center
78 Marzdaran Street, between Ariafar and Sarsabz, Kashan 379519116, Iran
Tel.: +989130876330
E-mail: Nimabadripour@gmail.com

populations [*Álvarez DM et al., 2020; Danaher RJ et al.,2011*]. The human herpesviruses possess a linear, double-stranded DNA (dsDNA) molecule that causes the primary and recurrent lesions [*Pebody RG, et al.,2004*]. These highly contagious viruses can cause dermatitis, encephalitis, meningitis, and herpes genitalis or genitourinary infections, as well as increase the risk of cervical cancer [*Reuven NB , et al., 2003; Kłysik K, et al., 2020; Jain A, et al.,2022*]. HSV viruses are categorized into two main serotypes: HSV-1 and HSV-2 [*Gavanji, S. 2022*]. HSV-1, also known as oral herpes, can cause cold sores or fever blisters on the face or around the mouth cavity, leaps, and skin of the loin, as well as inflammation of oral and eye cells [*Asai D, Nakashima H. 2018; Vaghela D, et al., 2021; Dhanushkodi NR, et al.,2021*]. This serotype can range from mild to severe, resulting in serious complications such as conjunctivitis [*Koujah L, et al.,2019*], herpetic stromal keratitis (HSK) [*Stuart PM, Keadle TL. 2012*], gingivostomatitis [*George AK, Anil S. 2014*], and encephalitis (HSE) (*Feola A,et al., 2018*). HSV-2, often known as genital herpes, can be transferred through sexual activity and usually affects the genital or anal areas as well as the skin of places below the loin, causing significant issues and increasing the chance of sexual human immunodeficiency virus (HIV) transmission [*Crisci E, et al.,2019* ; *Zhang X, et al., 2022*]. Various therapeutic strategies and clinical care have been established for HSV infections. Several antiviral drugs, such as penciclovir, valacyclovir, famciclovir, acyclovir, and cidofovir, have also been used to treat herpes infections [*Pasternak B, Hviid A. 2010*]. The anti-herpetic agents with systemic mechanisms ultimately target and inactivate the viral DNA polymerase enzyme and inhibit the replication and proliferation of herpes viral DNA [*Li F, et al., 2019*]. Antiviral drug resistance is a severe problem, especially among patients with immunodeficiency illnesses. Consequently, many research investigations have focused on examining and creating novel antiviral medications [*Roy S, et al., 2022; Majewska A, Mlynarczyk-Bonikowska B. 2022*; *Chuerduangphui J, et al., 2022*]. Using herbs with antiviral properties has recently received attention in various studies [*Gavanji S, et al. 2014*]. Several antiviral active substances have been shown to possess anti-HSV characteristics, including flavonoids, alkaloids, terpenes, and phenols [*Pesola JM, Coen DM. 2007*; *Tolo FM, et al.,2006*]. The suppression of HSV viruses is significantly influenced by phenolic classes [*Treml J, et al., 2020*]. *Punica granatum* var. pleniflora, a plant belonging to the Punicaceae family, is an important medicinal plant that has historically been used to treat disorders including recurrent aphthous stomatitis, wounds, bronchitis, male sex power reconstruction, diarrhea, and digestive problems. Some researchers have reported that *P. granatum* has anti-inflammatory, antioxidant, and antimicrobial properties [*Gavanji S, et al., 2014*]. The polyphenolic chemicals punicalin, punicalagin, ellagic acid, gallotannin, and ellagitannins are abundant in pomegranates [*Petiwala SM, et al.2014*]. In the current investigation, the antiviral activities of different concentrations of three varieties of *P. granatum* extracts —plentiflora, Saveh Black Leather, and Sweet Alak— were investigated on HSV type 1 in Vero cells under in vitro conditions.

Materials and methods

Plant collection and extraction: The flowers from three varieties of *P. granatum*, including pleniflora, Saveh Black Leather, and Sweet Alak, were obtained from Golestan Province, north Iran. Before extraction, the flowers were crushed by a mechanical mill and passed through a mesh sieve of 80 to 100 μm. Then, at a low temperature, they were dried in the shade (25 °C). The maceration technique was applied to the extraction process. Briefly, 750 g of powdered flowers were added to 1.5 liters of 70% ethyl alcohol, and the mixed solution was kept for 96 hours under controlled laboratory conditions on a magnetic mixer. To prevent solvent evaporation and contamination, parafilm was used to cover the Erlenmeyer flask aperture. The transparent liquid and the top portion were then separated using filtering. Extracts were dried in a vacuum (Laborota 4001, Heidolph, Germany) at 40 °C in a rotary evaporator. The dried ethanolic extract was stored in a dark place in a refrigerator at 4 °C until use [*Gavanji S, Larki B. 2017*].

Determination of total phenolics: The total phenolic content of the pomegranate flower extracts was determined using the Folin-Ciocalteu reagent. Each stock solution of flower extract was prepared at a concentration of 10 *mg/mL*, and 0.02

ml of each stock solution was diluted with 1.58 *ml* of distilled water to determine the total phenolic content. After that, 0.1 *ml* of the diluted sample was added to 0.5 *ml* of the diluted Folin Ciocalteau reagent and kept at room temperature for 5 minutes. The solution was then given 0.4 *ml* of a 7.5% (*w/v*) sodium carbonate solution and allowed to remain in a dark place for 30 minutes. The absorbance of each sample was measured at 765 nm by a UV-spectrophotometer (UNICO 2100: USA). Gallic acid was used as the reference absorbance for calculating the total phenolic content (Merck, Germany). For each stock, the results of the three tests were expressed as milligrams of gallic acid equivalents (*mg of GAE/g* of extract powder) [*Mahboubi A, et al., 2015*].

Determination of total flavonoid content: A colorimetric assay was used to calculate the total flavonoid content of the pomegranate flower extracts. The dried extract of each flower at a concentration of 1 *mg/mL* was prepared by dissolving it in 80% methanol. Then, the calibration curve standard was prepared (0.1–1 *ml* series of Rutin solution, 500 *µL* of the acetic acid solution, 2 *ml* of the pyridine solution, and 1 *ml* of the reagent aluminum chloride solution). The final volume was adjusted to 10 *ml* using 80% methanol, and the final Rutin concentration was 1–10 *g/ml*. For flavonoid qualification, 0.5 *ml* of the ethanolic extract of each pomegranate flower was transferred to a test tube. Then, 2 *ml* of the pyridine solution, 0.5 *ml* of the acetic acid solution, 1 *ml* of the reagent aluminum chloride solution, and 6 *ml* of 80% (*v/v*) methanol were added to the solution and kept at room temperature. The absorbance of each sample was determined at 420 *nm*. The assay was conducted three times, and the result of the flavonoid concentration in samples was represented as milligrams of rutin equivalents (RE) per gram of extract sample (*mg RE/g* of extract powder) [*Mahboubi A, et al., 2015*].

In vitro analyses: Cells and viruses: The African green monkey kidney (Vero) cells and a HSV-1 stock were obtained from the Institute of Traditional Medicine and Herbal Plants of Isfahan, Iran. The cells were grown in a MEM culture medium (Eagle's minimum essential medium), which was supplemented with 10% newborn calf serum, 100 *U/ml* penicillin (Gibco), and 100 *g/ml* streptomycin sulfate. Plaque-forming units per milliliter (*PFU/ml*), a measure of virus titers, were measured in Vero cells using the plaque assay method. Before use, the viruses were kept at -70° *C*.

Cytotoxicity assay determination using MTT assay: Using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the cytotoxicity of pomegranate flower extracts was assessed. This test depends on tetrazolium component conversion (MTT) into formazan crystals by a few specific enzymes in the mitochondria of live cells and shows mitochondrial activity or malfunction in cells. Its OD may be determined using a microplate reader (ELISA). Each well of the plate was filled with 180 *µl* of cell suspension (cells at a concentration of 10,000 cells/well) and 20 *µl* of various concentrations of pomegranate flower extracts (50-550 *g/ml*). Acyclovir, an antiviral medication, and 5% DMSO were also used as positive and negative controls, respectively. The plates were then kept at 37° *C* for 48 hours in a CO_2 incubator. After that, wells were filled with 20 *µl* of MTT solution and incubated for 2 hours. The absorbance of various doses of pomegranate flower extracts was measured at 560 nm after adding 100 *µl* of DMSO to dissolve Formosan crystals. The IC_{50} was determined to be the concentration of pomegranate flower extracts that caused a 50% reduction in cell viability.

$$PCS=\frac{ODt - ODb}{ODn - ODb} \times 100$$

where PCS - Percentage of cell survival, ODt - Test compound OD, ODb-Blank OD, ODn-Negative control OD.

Antiviral activity: A plaque inhibition assay was carried out on Vero cells to assess the anti-herpetic efficacy of pomegranate flower extracts. Plaque reduction is an assay that has been extensively used to evaluate the impact of synthetic and organic substances on the plaque-forming units (PFUs) of viruses in contrast to the control group. In this experiment, in each well of the plate, 400×10^3 *µl* of Vero cells were cultivated in 1 *ml* of Gibco Dulbecco's Modified Eagle Medium, which contained 3% FBS, and to shape a monolayer cell line, the plate was seeded and incubated for 24 hours. Following adding 1 *µl* of viral suspension, monolayer cells were cultured for an hour at 37 °C to allow virus adsorption before replacing the

media with 1 *ml* of Dulbecco's Modified Eagle Medium medium. The anti-HSV-1 activity of the extracts was tested after they were added at a concentration of 80-140 *g/ml*. Also, 20 *µl* of DMSO and 20 *µl* of Acyclovir were used as negative and positive controls, respectively. Plates were incubated at 37 °*C* for 48 hours. The number of plaques was measured under a microscope in each well to determine the inhibition percentage using the following formula:

$$PI = [1-\frac{NPt}{NPc}] \times 100$$

where PI - Percentage of inhibition, NPt -number of plaque (tested), NPc - Number of control plaque.

Data analysis: One-way ANOVA was used to analyze the data using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA), and Tukey's multiple comparison test was used to compare means. P-values < 0.05 were used to determine the significance of the differences.

RESULTS

VALUES OF TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT OF* P. GRANATUM *FLOWERS: The findings showed that there were variations in the phenolic and flavonoid content of the pomegranate flower extracts, with total phenolic content ranging from 9 to 18 *mg GAE/g* of dry powder and total flavonoid content ranging from 0.9 to 2.2 *mg RE/g* of dry powder. The study revealed that, in contrast to other pomegranate flower extracts, *P. granatum* var. pleniflora flower had a higher content of flavonoids and phenols (Fig 1).

CYTOTOXIC EFFECTS ON THE VIABILITY OF VERO CELLS: Our results indicated no cytotoxic effect in three varieties of *P. granatum* up to a concentration of 400 *µg/ml.* Cell viability drastically decreased with a rise in extract concentration. The respective 50% cytotoxic concentration (CC_{50}) values for pleniflora, Saveh Black Leather, and Sweet Alak were 414.60, 486.09, and 488.05 *g/ml*, respectively (Fig 2). Furthermore, no cytotoxic effects were seen in either the negative or positive groups. It was found that all the cells survived.

ANTI HSV-1 ACTIVITY: The anti-HSV-1 activity of extracts showed that they could prevent the formation of viral plaque, which increases with increasing concentration (Fig 3). Three *P. granatum* species—pleniflora, Black Sawah Leather, and Sweet Alak—had IC50 values of 109.63, 131.24, and 128.87 *g/mL*, respectively (Table 1).

Compared to all extracts, acyclovir, a positive control, demonstrated significantly more antiviral activity against herpes simplex virus type 1 with an IC_{50} value of 0.05 *g/ml.* (Fig 3). Three varieties of *P. granatum* -pleniflora, Saveh Black Leather, and Sweet Alak- had selective indices (SI) of 3.78, 3.78, and 3.70, respectively (Table 1). This crucial criterion implies that SI>4 is appropriate as an antiviral agent.

DISCUSSION

Medicinal herbs have been extensively used to cure various infectious diseases throughout history. According to research, plant compounds with different mechanisms help cure human viral infections caused by the two HSV serotypes [*Gavanji, S. 2022*]. The current study was conducted on the antiviral activity of various dosages of three vari-

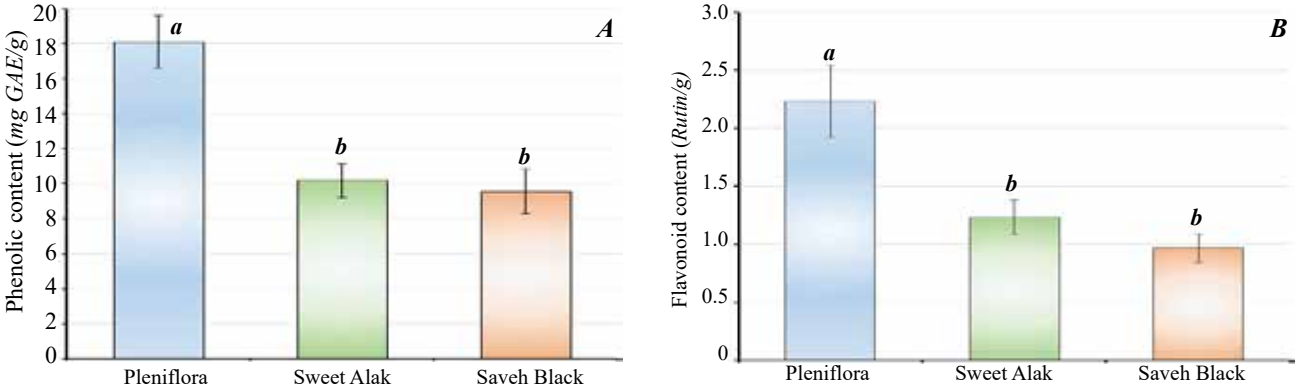

***FIGURE** 1. Phenolic (A) and flavonoid (B) content of three varieties of P. granatum var. pleniflora, Saveh Black Leather, and Sweet Alak. Each value showed the mean ± standard deviation.*
[a, b, c, d, e, f] Similar letters in each column indicate no difference at the 5% level.

eties of pomegranate flower extract on HSV-1 to compare with acyclovir. The findings showed that acyclovir outperformed all *P. granatum* extracts examined. Virus resistance or a low concentration of pomegranate flower extracts might be the reason. Various studies have indicated that different parts of *P. granatum* extracts have antimicrobial

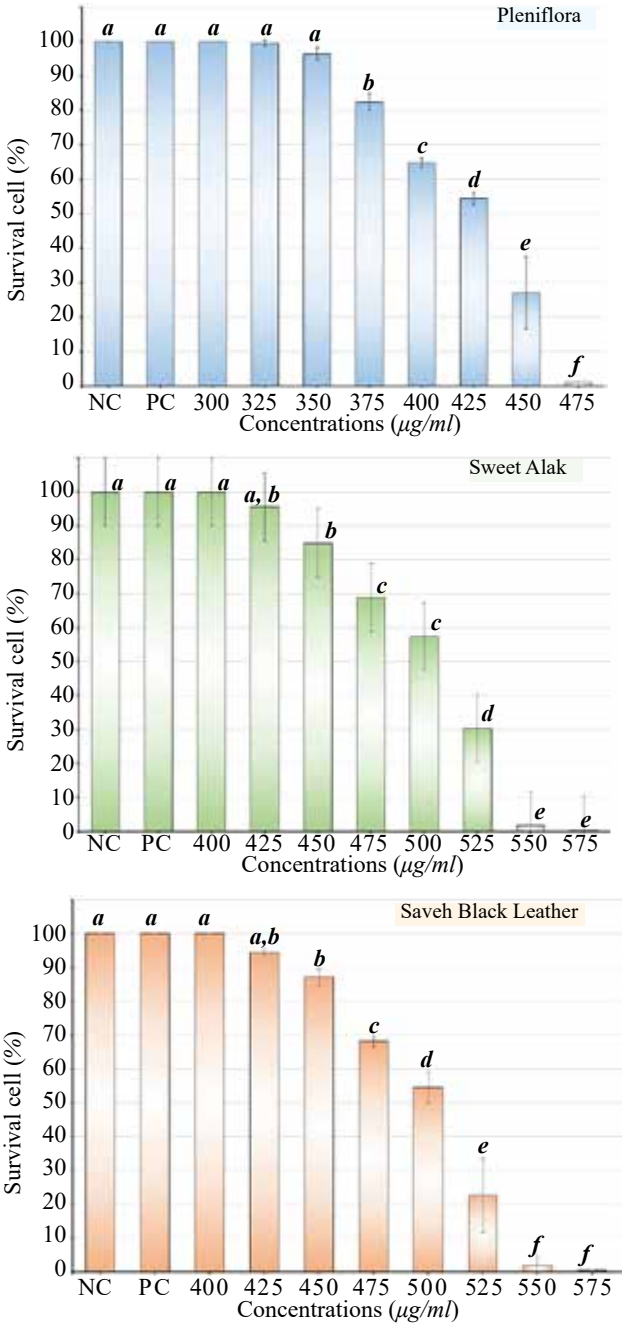

Figure 2. *Survival cells at different concentrations of Punica granatum var. pleniflora, Saveh Black Leather, and Sweet Alak. Each value showed the mean ± standard deviation.*
a, b, c, d, e, f Similar letters in each column indicate no difference at the 5% level.

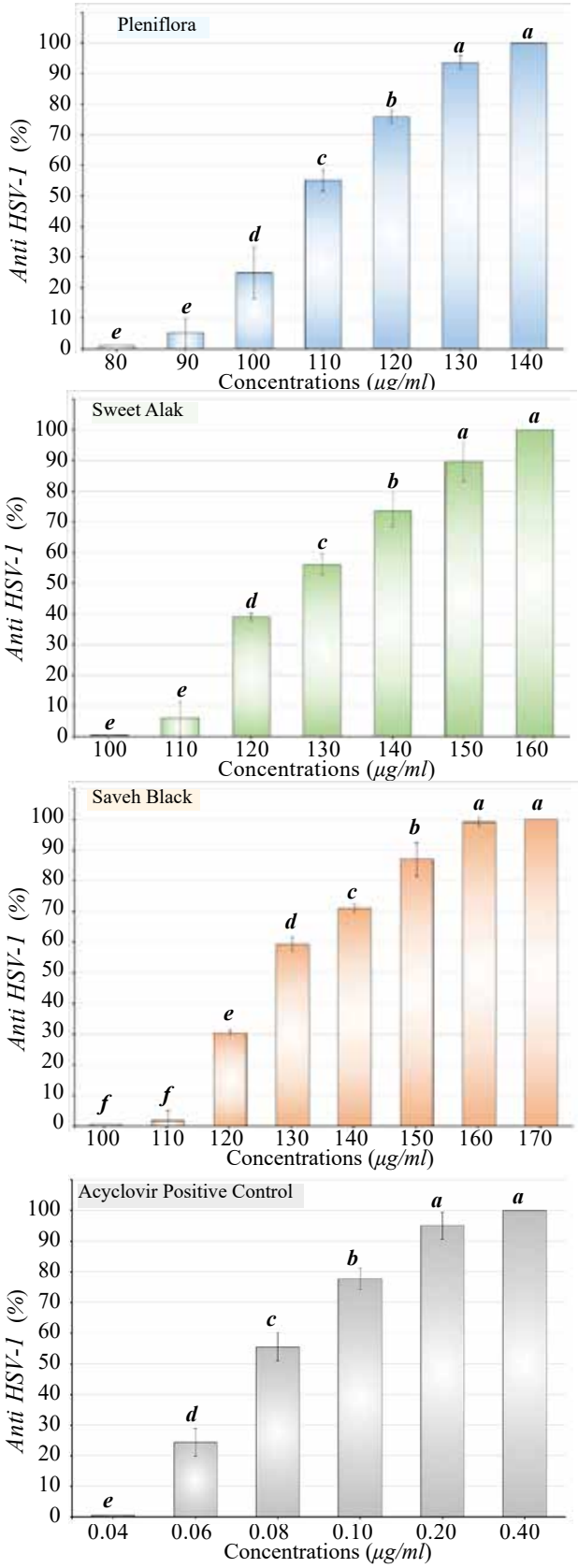

Figure 3. *Antiherpes simplex virus-1 activity of different concentrations of Punica granatum var. pleniflora, Saveh Black Leather, Sweet Alak and acyclovir. Each value showed the mean ± standard deviation.*
a, b, c, d, e, f Similar letters in each column indicate no difference at the 5% level.

and anti-inflammatory effects. Also, research stated that *P. granatum* has inhibitory effects against HIV-1, influenza virus, poxviruses, and herpes simplex viruses [*Howell AB, D'Souza DH. 2013*]. It is stated that peel extract of *P. granatum* inhibited HSV-1 replication in the adsorption stage with IC_{50} and CC_{50} values of 37.7±7.6 *μg/ml* and 293.5±1.10, respectively [*Moradi MT, et al., 2015*]. It showed that the peel extract of *P. granatum* is more potent than the flower extract. According to various research, the antiviral activity of *P. granatum* extract is related to its active components, including ellagic acid, gallic acid, hydrolyzable tannins, and anthocyanins [*Reddy MK, et al., 2007*]. Another study showed that *P. granatum* peel extract could inhibit influenza A virus replication under vitro conditions [*Moradi MT, et al., 2019*]. This comparison between our study and Moradi et al. indicated that peel extract *P. granatum* is more effective than pomegranate flower extracts. Variations in the antimicrobial properties of extracts in different concentrations could be attributable to the different amounts of phytochemical compositions. The comparison between total phenolic and flavonoid values in flower and peel extracts indicated that pomegranate peel extract contains a higher amount of phenolic and flavonoid compounds (233±2.4 *mg GAE/g* and 60.6.1±1.4 *mg RUT/g*) than flower extract that leads to higher antimicrobial and antiviral properties. A study demonstrated that pomegranate juice inhibited viral entry into the target cells by inactivating the viral particles [*Neurath AR, et al., 2005*]. In a related trial, the anti-herpetic activity of *P. granatum* methanolic and aqueous extracts against HSV-1 was evaluated. Both methanolic and aqueous extracts significantly reduced the viral infection at 68 and 64 *μg/ml* [*Nawawi A, et al., 1999*]. It is stated that the aqueous extract of the fruit cortex of pomegranates was active against HSV-1 with IC_{50} and CC_{50} values of 80.3 and 1000 *μg/ml*, respectively [*Li Y, et al., 2004*]. Mothana et al. evaluated the antiviral activity of P. protopunica leaf and fruit extract against HSV-1 [*Mothana RA, et al., 2006*]. They indicated that natural bioactive compounds like phenols, flavonoids, alkaloids, and terpenes have various anti-herpetic mechanisms against both HSV serotypes. It seems that phenolic compounds in pomegranate extract play a significant role in inhibiting HSV. These compounds also cause structural or functional abnormalities in the membrane proteins of Vero cells or the HSV-1 envelope. As a result, binding, penetration, and viral entrance into the target cells are blocked [*Gavanji S, et al., 2015*]. Microscopic observations have shown that change or damage to the viral structure is the main cause of the inactivation of viruses by polyphenols [*Lim TK. 2012*]. Another study suggested that tannins in *P. granatum* are a highly effective agent against HSV-2 by inhibiting viral replication and blocking HSV absorption into the target cells [*Zhang J, et al., 1995*].

CONCLUSSION

Our study demonstrated that pomegranate flower extracts have an inhibitory effect on HSV-1. The extraction and isolation of bioactive compounds with anti-HSV activity and toxicity potential of these agents will help determine the therapeutic characteristics.conflict of interest.

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Armen A. Muradyan

Address for correspondence:

Yerevan State Medical University
2 Koryun Street, Yerevan 0025,
Republic of Armenia

Phones:

(+37410) 582532 YSMU
(+37493 588697 Editor-in-Chief
Fax: (+37410) 582532
E-mail:namj.ysmu@gmail.com, ysmiu@mail.ru
URL:http//www.ysmu.am

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