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RESEARCH ARTICLE

Investigation of the efficiency of pomegranate (punica granatum l.) Peel extract on herpes simplex virus-1

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Nar (punica granatum l.) kabuğu ekstratının herpes simplex virus-1 üzerine etkinliğinin araştırılması

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Öz

Amaç: Bu çalışmada nar kabuğu (Punica granatum L.) ekstraktının in vitro olarak HSV-1 üzerine antiviral etkinliğinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Antiviral etkinlik değerlendirilmeden önce, nar kabuğu ekstratının saf su ile dilue edilmiş farklı konsantrasyonlarının Vero hücre kültüründe sitotoksik aktivitesi belirlendi. Bu hücre kültüründe 0,87 mg/mL konsantrasyondan daha düşük konsantrasyonlarda sitotoksiteye neden olmadığı tespit edildi. Antiviral etkinlik MTT testi ve mikroskobik analizler sonrasında sitopatojenik etkinin (CPE) varlığıyla belirlendi.

Bulgular: Nar kabuğu ekstratının 0,87 mg/mL ila 0,87x2-5mg/mL arasındaki konsantrasyonlarda HSV-1'e karşı önemli bir antiviral etkinliği olduğu belirlendi. Bu etkinin virusun hücreye girişine engel olmasıyla sağlamış olabilir.

Öneri: Nar kabuğu ekstraktının HSV-1'e karşı in vitro olarak antiviral etkinliğe sahip olduğu ve elde edilen sonuçlar doğrultusunda Punica granatum L. ekstratının antiviral etkinliğinin daha detaylı belirlenebilmesi için in vivo denemelere ihtiyaç olduğu düşünüldü. Ayrıca Punica granatum L. Ekstratının Herpes viruslar ve diğer viruslara karşı etkinliğinin detaylı bir şekilde belirlenmesi gelecekte antiviral ilaç denemelerine katkı sağlayacaktır.

Anahtar kelimeler: HSV-1, in vitro, punica granatum l., vero, antiviral aktivite

Abstract

Aim: This study, it was aimed to determine the antiviral activity of pomegranate peel (Punica granatum L.) extract on HSV-1 in vitro.

Materials and Methods: Before evaluating the antiviral efficacy, the cytotoxic activity of different concentrations of pomegranate peel extract diluted with distilled water in Vero cell culture was determined. It was determined that this cell culture did not cause cytotoxicity at concentrations lower than 0.87 mg/mL. Antiviral efficacy was determined by cytopathogenic effect (CPE) after the MTT test and microscopic analysis.

Results: It was determined that pomegranate peel extract had significant antiviral activity against HSV-1 at concentrations between 0.87 mg/mL and 0.87x2-5mg/mL. This effect may have been achieved by preventing the virus from entering the cell.

Conclusion: It was thought that pomegranate peel extract has antiviral activity against HSV-1 in vitro, and in line with the results obtained, in vivo trials are needed to determine the antiviral activity of Punica granatum L. extract in more detail. In addition, a precise determination of the effectiveness of Punica granatum L. Extract against Herpes viruses and other viruses will contribute to antiviral drug trials in the future.

Keywords: HSV-1, in vitro, punica granatum l. vero, antiviral activity

Introduction

Infection with Herpes Simplex Virus (HSV) can occur because of to Herpes Simplex Virus type 1 (HSV-1) or Herpes Simplex Virus type 2 (HSV-2). HSV-1 is a highly transmitted infection that is widespread and endemic worldwide (Morris et al 2002, Wolf et al 2003). HSV-1 is usually transmitted by infected secretions and can cause serious problems such as keratitis, encephalitis, or gingivostomatitis (Mustafa et al 2016). Some synthetic nucleoanalogues have been applied as anti-herpetic agents in the primary stages of infection, but drug resistance has been found mostly in immunocompromised patients (Field 2001, Piret and Boivin 2011). Accordingly, new antiviral agents with antiviral activity against HSV strains of pure plant-derived compounds need to be discovered. It turns out that the mechanisms of action of these substances are either by inhibiting viral genome synthesis or viral replication (Kapoor et al 2017). HSV-1 (Peerboom et al 2017) as well as many viruses (Lee and Lobigs 2002) use glycosaminoglycan (GAG) as the first binding receptors during host cell infection. It was observed that polyphenols target HSV-1 glycoproteins that interact with GAGs, thereby preventing these glycoproteins from associating with GAGs and subsequently binding receptors (El-Toumy et al 2018). This inhibitory effect was demonstrated against acellular virus, during the viral attachment and fusion steps, and at the intercellular junction of HSV-1 through its glycoproteins (Abad et al 2000, Lin et al 2011). Phytochemicals with antiviral activity against HSV types have been reported as Flavonoids (Lyu et al 2005), Alkaloids (Ren et al 2010), Saponins (Kinjo et al 2000), Terpenes (Soares et al 2012), Quinones (Gumenyuk et al 2018), Lignans (Mu Xia et al 2009), Polysaccharides (Saha et al 2012), Tannins (Lin et al 2011), Thiosulfinate (Lengbiye et al 2020), Steroid glycoside (Arthan et al 2002) and Proanthocyanidins.

Many studies have indicated that pomegranate (Punica granatum L.) is also rich in phenolic compounds (Nahar et al 2014, Wang et al 2010). In the study of Li et al (2006) on pomegranate peels, it was determined that phenolic substances were higher in the peel and were more effective than the pulp in terms of antioxidant activity. Quantitative analysis of phenolic compounds of the pomegranate fruit and its peel by high-pressure liquid chromatography (HPLC) revealed that they contain anthocyanins, gallotannins, hydroxycinnamic acid, hydroxybenzoic acids, ellagitannins, and gallagylesters (Fischer et al 2011). In the studies conducted, it was demonstrated that pomegranate peel has antioxidant (Mphahlele et al 2016, Türkyılmaz et al 2017), anti-inflammatory (Brusselmans et al 2005, Lansky et al 2005), antimicrobial (Al-Zoreky 2009, Türkyılmaz et al 2017), antidiabetic (Jain et al 2012), antiviral (Karimi et al 2020, Neurath et al 2004) and even anticancer (Adams et al 2006, Panth et al 2017) effects.

The purpose of this study is to evaluate the in-vitro antiviral activity of pomegranate peel extract on HSV-1 by MTT test and cytopathogenic effect (CPE).

Material and Methods

In the current study, Vero cells (African green monkey kidney cell, ATCC-CCL81) to which HSV-1 was sensitive were used as cell culture. HSV-1 HF strain (ATCC-VR-260) used in Vero cell culture and antiviral activity test was obtained from Selcuk University Veterinary Faculty Virology Department. The titer of the virus was determined from the cytopathological effect (CPE) in Vero cells and expressed 50% tissue culture infective dose (DCID50 0.1 mL-1) per 0.1 mL. The titer of HSV-1 was determined as 10−5 DCID50 0.1 mL-1. The virus suspension was stored at −80°C until use.

Preparation of the Pomegranate (Punica granatum L.) peel extract

Pomegranate (Punica granatum L.) peels were extracted according to the method by Demir et al. (2019). Pomegranate (Punica granatum L.) peels were obtained from fresh pomegranate fruit, dried at 40-50°C, then ground to powder form, and stored at +4°C until analysis. Dried pomegranate peels were kept in a water bath at 78°C for 113 minutes in 33% ethanol with a density of 200:20 (mL solvent/g plant). The obtained extract was centrifuged at 5000 rpm for 10 minutes, then filtered through filter paper at room temperature. The obtained filtrate was concentrated in the rotor evaporator to obtain a dense and viscous material. This extract was stored at -18°C until use. The pomegranate peel extraction product was filtered through 0.45µm (Sartorius, Germany) filters before being used in cell culture and made ready for use.

Evaluation of cytotoxicity effect of Pomegranate (Punica granatum L.) peel extract

For cytotoxicity assay, 96-well plates were coated with Vero cells at a concentration of 2×10^5 cells/mL. Stock solution of Pomegranate (Punica granatum L.) peel extract were diluted in 10-fold diluted pure water and the different concentrations of the extract were added in the 96-well plate. The plate was incubated in 5% CO_2 at 37°C for 48 h. After the incubation period, the non-toxic concentration of the extract was determined for cells by commercially MTT (M2003 Sigma) and applied as according to the procedure.

Evaluation of antiviral effect by MTT Test

Thiazolyl blue test [MTT; The 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Mosmann 1983) was used for evaluation of cells in terms of proliferation and viability. MTT test was used to evaluate antiviral activity. 100 µL of the suspension containing 2×10^5/mL cells was added to 4 wells

in the microplate (Corning, USA) and incubated for 24 hours in an incubator at 37 °C and 5% CO_2. After the cells were surface coated, the cell growth medium was aspirated from the wells, and two-fold dilutions of Punica granatum L. Extract were prepared to start at a concentration of 0.87 mg/mL. HSV-1 diluted at 100 $TCID_{50}$ was added together with Punica granatum L. CPEs grown in cell cultures were evaluated at 24 hours intervals under an inverted microscope (Olympus, Tokyo). After 48 hours, MTT solution prepared with 5mg/ml PBS (Phenol-free) was filtered through a 0.2 µm filter and made ready for use. 20 µg of MTT solution was added to all wells. After incubation at 37 °C for 4 hours, the MTT solution from all wells was aspirated. Dimethyl sulfoxide (DMSO, Sigma 67-68-5) was added to all wells to dissolve 100 µl of formazan crystals. After 30 minutes of incubation, spectrophotometric OD values were calculated by reading with an ELISA reader (Rayto RT-2100C, China) at a wavelength of 540 nm. All stages were evaluated together with cell control, virus control, and Punica granatum L. Extract control.

Statistical analysis

The data obtained in the study were evaluated with the SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) program. Since the data did not show normal distribution, the non-parametric Kruskal-Wallis and post hoc Dunn-Bonferroni tests were applied. Obtained data are presented in tables as medians (quartiles). A p-value of <0.05 was considered statistically significant (Table 1).

Results

As a result of the cytotoxicity test, Punica granatum L. concentrations higher than 0.87 mg/mL were determined to be cytotoxic on Vero cells. Therefore, 2-fold dilution doses of stock (0.87 mg/mL) Punica granatum L was used. HSV-1 with a titer of 10^{-5} was added to all plate wells except cell control and Punica granatum L. control wells. After 48 hours of incubation, no CPE was observed in Punica granatum L. control and cell control wells, while CPE was observed in virus control wells. Besides, with stock (0.87 mg/mL) Punica granatum, the onset of CPE was observed in wells with 2-5 dilutions, while no CPE was observed in wells with 2-4 dilutions. CPEs were determined in all eyes with other dilutions of Punica granatum (2^{-6}, 2^{-7}, and 2^{-8}). Also, the OD data obtained from the MTT test were evaluated statistically.

Tablo 1. Antiviral effect of Punica granatum L. concentrations on HSV-1

	CC	VC	EC (0,87 mg/mL)	(0,87 mg/mL)+V	2^{-1} +V	2^{-2} +V	2^{-3} +V	2^{-4} +V	2^{-5} +V	2^{-6} +V	2^{-7} +V	2^{-8} +V
Antiviral effect	2,03 (1,62-2,28)	0,94* (0,73-0,99)	1,90 # (1,86-2,02)	1,72 # (1,64-1,72)	1,70# (1,59-1,79)	1,78# (1,47-1,99)	1,73# (1,68-1,84)	1,61# (1,33-1,87)	1,53# (1,48-1,82)	1,40* (1,20-1,73)	1,08* (0,89-1,50)	1,31* (1,11-1,53)

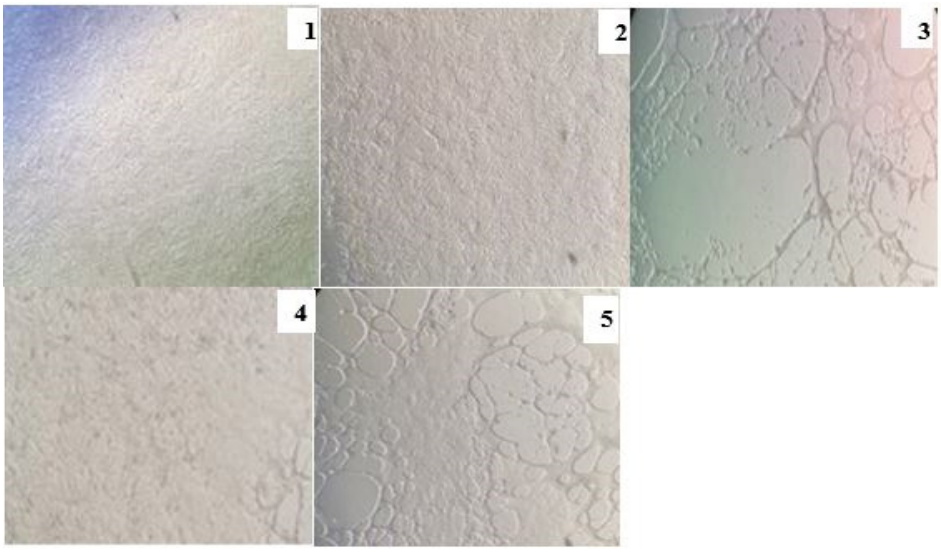

Figure 1. 1: Cell control, 2: Punica granatum control (0.87), 3: Virus control, 4:2^{-1} dilution of Punica granatum+virus, 5: 2^{-6} dilution of Punica granatum+virus

Discussion

There are eight human herpesviruses (including HSV-2, varicella-zoster virus (VZV; HHV-3), epstein–barr virus (HHV-4), cytomegalovirus (HHV-5), HHV-6, HHV-7, and HHV-8), and HSV-1 is one of them. HSV-1 is one of eight human herpes viruses. Herpesviruses are large, double-stranded DNA viruses that spread easily from person to person, remain latent for life, cause genital and oral mucocutaneous lesions, and rarely lead to life-threatening conditions such as fulminant encephalitis (Arshad et al 2019, Bradshaw and Venkatesan 2016).

As various viral infections pose a great danger to the human population, the need for new antiviral agents is increasing globally. Since prehistory medicinal plants have been used to control various microbial infections (Angamuthu et al 2019). It has been determined that many plants extracts such as Euphorbia coopire (Euphorbiaceae), Morus alba (Moraceae) (El-Toumy et al 2018), Andrographis paniculata (Wiart et al 2005), Azadirachta indica (Tiwari et al 2010), etc. have antiherpetic properties.

Pomegranate has been used as a medicinal plant from past to present. Also, its antibacterial, antifungal, antiparasitic, antiproliferative, apoptotic and antiviral activities have been investigated recently (Guerrero-Solano et al 2020, Howell and D'Souza 2013, Kim et al 2002). When the antiviral studies conducted on pomegranate were evaluated, when Neurath et al (2004) evaluated the effectiveness of fruit juices obtained from various fruits against the human immunodeficiency (HIV-1) virus, they reported that pomegranate juice prevented infection by blocking the binding of the virus to CD4 and CXCR4/CCR5. In their study evaluating the anti-HSV-2 efficacy of bioactive compounds isolated from the fruit peel of P. granatum and lyophilized extracts, they reported that the peel extracts of P. granatum had significant activity against HSV-2 (Arunkumar and Rajarajan 2018). (Arunkumar and Rajarajan 2018). In a study investigating the inhibitory effect of the crude extract, major phenolic constituents of pomegranate peel and fractions on adenovirus (ADV) in the Hep-2 cell line, it was reported that the antiviral activity of pomegranate peel was confirmed, and its fractions and constituents exhibited antiadenoviral activity (Karimi et al 2020). In the study conducted by Suručić et al (2021), they stated that punicalagin and punicalin, the peel extracts of pomegranate ingredients against different protein targets of the SARS-CoV-2 virus, have a very promising potential for important interactions. Pomegranate ingredients are considered suitable nominees for further in vitro and in vivo evaluation.

Methanol extracts of pomegranate are very rich in hydrolyzable tannins (punicalagins and punicalins), ellagic acid, and ingredients of ellagitannins, ingredient of gallotannins and gallic acid (Reddy et al 2007). Both the antibacterial and antiviral effects of pomegranate are thought to be probably related to hydrolyzable tannins and anthocyanins (Aviram et al 2008). It was stated that pomegranate peel extract is more effective in studies carried out by applying different extraction processes with different parts of the pomegranate (Arunkumar and Rajarajan 2018).

As a result of the current research, the cytopathogenic effect (CPE) was evaluated after the MTT test and microscopic analyzes to determine the antiviral activity of the pomegranate peel extract. It was determined that pomegranate peel extract had a significant antiviral activity against HSV-1 at concentrations between 0,87 mg/mL to 0,87x2-5 mg/mL. It was thought that this effect might have been achieved by preventing the virus from entering the cell. Acyclovir is a drug frequently used to treat Herpes virus infections, but it cannot control viral delay and recurrent infection, and long-term use can lead to drug resistance. Therefore, there is a search for alternatives in treating herpes viruses. Plant-based bioactive compounds are important as potential antiviral agents with reduced toxicity. As a result of studies conducted with pomegranate and the current study, it was thought that pomegranate has positive effects in treating herpesviruses.

Looking at previous antiviral studies, the anti-HSV-2 activity of P granatum peel aqueous extract using Vero cells was reported by Jadhav et al (2012) with 51-75% inhibition at a concentration of 200µg/ml. In another study (Arunkumar and Rajarajan 2018) 100% inhibition was detected at a lower concentration of 125µg/ml. It is thought that this significant difference may be due to the extraction method used. In another study (Houston et al 2017), although the potential anti-HSV-2 property of boiled peel extract is shown at a very low concentration of 0.05µg/ml, it may be noted that punicalagin in pomegranate peel has a relatively higher concentration of 216.94µg/ml in Vero cells, and thus is more effective. The anti-HSV-1 activity of Punikalagin was reported by Lin et al (2011) at a concentration of 46.26µg/ml. Demir et al (2019) identified the phenolic compounds of pomegranate peel extract by HPLC and stated that among 14 phenolic compounds, punigalagin contained the most, followed by caffeic acid, epicatechin and ferulic acid. The inhibitory effects of these flavonodes, which are abundant in pomegranate, on many virus types such as HCV, H1N1 influenza, HIV, Rhesus Rotavirus (Ninfali et al 2020), SARS-CoV-2 (Du et al 2021) have been demonstrated by studies.

In the current study, it is thought that the extract has a partial effect since a complete purification process is not applied, and its antiviral activity can be increased by applying different purification processes. Besides, it is thought that the elucidation of the active ingredients of this extract with high Phyto-ingredients will give a leg up to the development of new and effective antiviral agents.

Conclusion

It was thought that pomegranate peel extract has antiviral activity against HSV-1 in vitro. In line with the results obtained, in vivo trials are needed to determine the antiviral activity of Punica granatum L. extract in more detail. The fact that the pomegranate peel is easily available and has positive effects due to various studies has revealed the necessity of determining its effectiveness against Herpes viruses and other viruses in detail. It is also thought to contribute to antiviral drug trials in the future.

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Conflict of Interest

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Data Collection and/or Processing: Irmak Dik
Analysis and / or Interpretation: Irmak Dik
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Ethical Approval

This study was carried out with the permission of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2021/91 on 01/07/2021).

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