Journal of Virology & Antiviral Research

Research Article

Effect of Extracts of *Passiflora edulis* Leaves on Herpes Viruses Infection

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Abstract

Passiflora edulis (Pe) is considered as a traditional medicinal plant with different therapeutic effects. In the present study we examined, *in vitro*, the antiviral potential of aqueous and ethanolic leaf extracts of *Passiflora edulis* on herpes simplex viruses 1 and 2 (HSV-1, HSV-2), and on Varicella-Zoster virus (VZV). Viral infection development was evaluated by plaque assay. Our results showed significantly higher antiviral activity of the ethanolic extract against all tested viruses, when compared to the aqueous extract. The 80%-MeOH fraction of the ethanolic extract offered the highest activity against these viruses, with 50% inhibitory concentration (IC50) of 4 ± 0.18, 7 ± 0.30 and 2.3 ± 0.15µg/ml against HSV-1, HSV-2, and VZV respectively, without any significant cytotoxicity. Most prominent antiviral activity was obtained when the 80%-MeOH fraction was added during the infection, or when it was incubated with the virus particles prior to infection. Also, a significant inhibition of the viral infection was obtained when this fraction was added after viral entry into the host cells, probably as a result of a significant delay in the production of infective viral particles inside the infected cells. A synergistic antiviral effect against all tested viruses was also observed when cells were treated with a combination of acyclovir (ACV) and 80%-MeOH fraction of Pe. Further study is required for the isolation and purification of the anti-virally active component/s of this Pe fraction.

Keywords

Passiflora edulis; Ethanolic extract; Herpes virus; Acyclovir

Abbreviations

HSV-1: Herpes Simplex Virus Type 1; HSV-2: Herpes Simplex Virus Type 2; VZV: Varicella-Zoster Virus; p.i: Post Infection; SI: Selectivity Index; Pe: *Passiflora edulis*

Introduction

Despite the continuous progress made in the field of antiviral drugs, viral diseases have become the leading cause of death worldwide. Herpes simplex viruses are responsible for many serious disorders in humans and animals. The Herpes family of viruses includes several members, such as herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and varicella-zoster virus (VZV). These viruses share many characteristics, including DNA homology, antigenic determinants, tissue tropism, and disease symptoms [1,2]. HSV

infections can cause serious systemic illnesses in immune-comprised patients and neonates [3]. Although acyclovir (ACV) is the most prescribed drug for use against herpes viruses [4-6], the search for new and effective anti-herpetic drugs is still important due to (a) development of ACV- resistant herpes viruses mutants [7], (b) side effects such as nausea, vomiting, headache, rash, and diarrhea, which are associated with the used drugs, and (c) ACV is not highly effective in recurrent HSV attacks [8]. Therefore, there is a need for novel anti-herpetic agents with high efficacy, low toxicity, and a different mode of action from ACV and other nucleoside derivatives.

Natural products have been used in medicine since ancient times. Approximately half of all pharmaceuticals in use today are derived from natural products [9,10], and many are known bases of antiviral and anticancer drugs [10]. A World Health Organization report has indicated the dependence of over 80% of world's population on traditional plants for their health treatments [11]. Although anti-herpetic activities of plant extracts were previously reported in many reviews [11-17] and although many antiviral compounds were identified, such as flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furil compounds, alkaloids, polyines, thiophenes, and different sugars, their antiviral mechanism of action is still poorly understood.

Passiflora edulis, the common, hardy, and edible passion fruit vine, is a self-clinging climbing plant that grows around the world and produces tart-tasting egg-sized fruit [18]. It is known in folk medicine and has proven medical qualities such as its activity against depression, anxiety, and insomnia [18-21]. It is used also for treatment of breathing disorders such as asthma and severe bronchitis, high blood pressure and cholesterol accumulation in blood vessels and others [22-25]. Flowers and fruits were found to be responsible for most of the known therapeutic activities of Pe [22,23]. The main components that were involved in these therapeutic activities are flavonoids, alkaloids, and maltol [19,26]. Very little is known about antiviral activity of *Passiflora edulis*, although root extracts of *Passiflora edulis* were found to be effective against herpes simplex virus type 1(HSV-1) and rabies virus [18,27].

In this study the antiviral activity of aqueous and ethanolic extracts of *Passiflora edulis* leaves against various herpes viruses was examined in order to elucidate their mechanism of action. Our results showed promising antiviral activity of the tested extracts against the tested viruses.

Materials and Methods

Material

Passiflora edulis was obtained from a nursery and grown in a controlled greenhouse at the Ben Gurion University, Beer-Sheva, Israel.

Acyclovir (ACV) was purchased from Sigma, Israel.

Preparation of plant aqueous and ethanolic extracts

Aqueous and ethanolic extracts were prepared from young fresh leaves of *Passiflora edulis*. Leaves were smashed in either distilled water

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(ddH$_2$O) or 95% ethanol solution, incubated at room temperature for 48 h, centrifuged at 2000 rpm for 10 min, and the supernatant was evaporated by lyophiliser. The resulting pellet was dissolved in a minimal amount of ddH$_2$O or 95% ethanol. The extracts were diluted to the appropriate concentrations with a medium containing 2% Newborn calf serum (NBCS).

Fractionation of ethanolic plant extract

The obtained plant ethanolic extracts were separated into different fractions by reverse phase chromatography (RP-C18 Sepack) with a stepwise methanol gradient: 0%, 20%, 40%, 60%, 80%, and 100%.

Cells and viruses

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were grown in RPMI medium supplemented with 10% NBCS and penicillin, streptomycin, and nystatin antibiotics. They were then incubated at 37°C in a humidified air containing 5% CO$_2$.

HSV-1 was obtained from the ATCC (VR-735), HSV-2 and VZV were obtained from the virology laboratory at Soroka University Medical Center, Beer-Sheva, Israel.

Cytotoxicity examination

Vero cells were treated with various concentrations of plant extracts and the toxicity of the extracts was tested by the following methods:

- Direct count - The cells were counted by Neubauer hemacytometer indicating their replication rate.

- Morphological changes were daily observed by optical inverted microscope

- MTT assay was performed as previously described [28].

Briefly, Vero cells were incubated with a 50 μg/ml MTT solution at 37°C for 5h. This solution was converted by mitochondrial succinate dehydrogenase enzyme into insoluble formazan (purple). The MTT solution was removed and replaced with an SDS solution to dissolve the formazan. After overnight incubation at 37°C, absorbance was measured at 570 nm, indicating the metabolic activity of the cells.

Virus infection

Vero cells were seeded at 0.15 x 10^6 cells/well in 24-well culture plates, in RPMI with 10% NBCS and antibiotics. Following overnight incubation, medium was removed and each well was infected at a multiplicity of infection (m.o.i.) of 1 PFU/cell for two hours at 37°C. The infection development was evaluated by plaque assay as previously described [29]. Briefly, the unabsorbed virus was removed and cells were overlaid with a layer of 0.6% carboxymethylcelleluse (CMC). Two to three days post-infection (p.i.) the CMC overlay was removed, cell monolayers were fixed with 10% formalin in saline, stained with crystal violet, and plaques were counted.

Elucidation of the antiviral mechanism of action of tested products

To elucidate the antiviral mechanism of the plant extracts, infected cells were treated with increasing concentrations of the extracts at various periods of time before, during, or after infection.

To study a possible direct effect of the tested antiviral agent on virus particles infectivity, 10^3 PFU of the appropriate virus particles were pre-incubated with appropriate doses of the tested extracts at 22°C for 30 min. These mixtures were then diluted 10^3-fold with fresh medium (to minimize the concentration of the antiviral agent at the time of infection) and cell monolayers were infected with the diluted mixture.

The influence of the tested compounds on intracellular HSV-1 production was estimated. Vero cells were infected by HSV-1 without treatment with the tested compound at the time of infection. At the end of a two hour infection the medium was removed, the cell culture was washed twice with physiological saline solution, and fresh medium was added. The tested extract was added at different time p.i. points (1, 2, 4, 6, and 8 hours) and the infected cells were removed from the wells by treatment with trypsin at 18 hrs. p.i. The obtained cells were placed in a centrifuge at 1500 rpm for 5 minutes, to create pellets, which were then washed three times with saline solution. Each pellet was resuspended with 100 μl of physiological saline solution and the cells were broken by freezing and thawing them. Cell debris was removed by centrifugation at 1500 rpm for 5 minutes and the mixture containing the endogenous virus was then used for infecting Vero cell monolayers.

Results and Discussion

Cytotoxicity of extracts

Aqueous and ethanolic extracts were prepared from leaves of *Passiflora edulis*. Different concentrations of each extract were added to Vero cell monolayers for 3 days and the cytoxicity of these extracts was evaluated by different assays, as described in the materials and methods section. The concentrations that were found to cause 50% toxicity (CC$_{50}$) of the aqueous and the ehanolic extracts were 400 and 500 μg/ml respectively.

Antiviral activity of extracts against herpes viruses

Vero cell monolayers were treated with increasing concentrations of the appropriate extracts at the time of infection with 1 m.o.i. of HSV-1, HSV-2, or VZV. The treatment was continued until the end of the experiment and the antiviral activity was evaluated by plaque assay. The results demonstrated significant and reproducible antiviral activity of both extracts against HSV-1, HSV-2, and VZV with the respective IC$_{50}$ (extract concentration required to confer a 50% plaque inhibition) of 70, 80, and 50 μg/ml in the case of the aqueous extract (Figure 1A) and 15, 20, and 10 μg/ml in the case of the ethanolic extract (Figure 1B). These results are in agreement with our previous studies that showed that ethanolic extracts of *F. binjamina and L. candidum* leaves effectively blocked HSV1, HSV-2, and VZV infection [15]. Although HSV-1, HSV-2, and VZV belong to the same *Alphaherpesvirinae* subfamily and have many similar characteristics, they have different clinical symptoms, as well as different biochemical and serological properties [30,31]. In contrast to our results, some previous studies have reported differential antiviral activity of plant extracts on HSV-1, HSV-2, and VZV. For instance, an aqueous extract of *Gardenia jasminoids* inhibited HSV-2 infection more effectively than HSV-1 *in vitro* [32]. Flavonoids from *Capparis spinosa* buds and excoecarianin from acetone extraction of *P. urinaria* were active against HSV-2, but did not show activity against HSV-1 [33,34]. Biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*
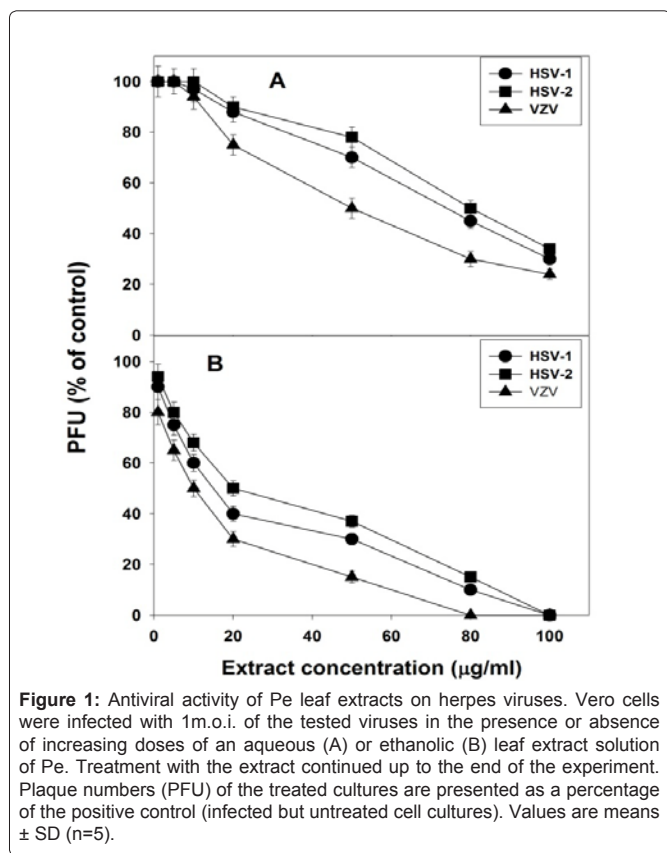
Figure 1: Antiviral activity of Pe leaf extracts on herpes viruses. Vero cells were infected with 1m.o.i. of the tested viruses in the presence or absence of increasing doses of an aqueous (A) or ethanolic (B) leaf extract solution of Pe. Treatment with the extract continued up to the end of the experiment. Plaque numbers (PFU) of the treated cultures are presented as a percentage of the positive control (infected but untreated cell cultures). Values are means ± SD (n=5).

Antiviral activity of Passiflora edulis ethanolic extract's fractions

The ethanolic extract was separated into several fractions as detailed in the Materials and Methods section. Their cytotoxicity and antiviral activity against different herpes viruses was examined. The obtained results (Table 1) showed that while fraction 80% - MeOH strongly inhibited HSV-1, HSV-2, and VZV, and while fraction 60% - MeOH moderately inhibited all tested viruses, the rest of the fractions offered only low antiviral activity against the examined viruses. It can also be seen that the 80% - MeOH fraction had significantly higher SI compared to the crude extract (Table 1). This result is consistent with our assumption that isolation and purification of the active antiviral component will yield significantly higher SI values. These results are in contrast to previous studies showed that 80% MeOH fraction of both *F. binjamina* and *L. candidum* leaves ethanolic extracts almost have no effect on VZV infection [15].

Mechanism of antiviral activity of Passiflora edulis 80%-MeOH fraction

(a) Effect on early steps of the viral infection: It is known from previous studies that natural products primarily affect early stages of viral infection such as virus adsorption and/or penetration into the host cells [34,36,37]. In order to examine which step of the viral infection is inhibited by the 80%-MeOH fraction of *Passiflora edulis*, Vero cells were infected with 1 m.o.i. of the tested herpes viruses and treated with 10 μg/ml of this fraction for different periods of time as follows:

- Only before infection- the cell monolayers were incubated with medium containing the fraction for 2 h., then the medium was removed and the cells were infected with 1 m.o.i. of the appropriate virus without further treatment with the fraction

- Only during infection

- Only p.i. cells were treated with the fraction immediately after 2 h infection with the virus. The treatment was continued up to the end of the experiment

- During and p.i.

Our results showed that when the cells were treated only before the infection, a slight non-significant inhibition in the viral infection of all tested viruses was obtained (p>0.1) (Figure 2). It seems that there is no strong interaction between the tested *Passiflora* fraction and Vero cells. However, treatment at the time of infection or both at

demonstrated moderate activities against HSV-1 and HSV-2, while they did not affect VZV infection. Acetone, ethanol, and methanol extracts of *Phyllanthus urinaria* inhibited HSV-2 but not HSV-1[35].

The selectivity index (SI) (determined as CC_{50}/IC_{50}) of the ethanolic leaf extract against the examined herpes viruses was significantly higher than that of the aqueous extract (not shown results). Therefore, we decided to focus only on the ethanolic extract. Although the SI values of these extracts were markedly lower than that of the positive control ACV (Table 1), it is still worthwhile to continue examining their antiviral potential due to the fact that this comparison was made between an extract containing a variety of components and ACV, which is a pure component. Future isolation and purification of the active antiviral component of this extract will most likely improve its potential efficacy.

Table 1: Antiviral activity of *Passiflora edulis* MeOH fractions against Herpes viruses.

Passiflora edulis Fractions	CC_{50} (μg/ml)	HSV-1		HSV-2		VZV	
		IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI
Ethanolic extract	500 ± 19	15 ± 0.8	33.3	20 ± 1.5	25	10 ± 0.4	50
0%-MeOH	800 ± 37	Inactive	-	Inactive	-	Inactive	-
20%-MeOH	700 ± 30	80 ± 4.1	8.6	110 ± 4.1	6.3	100 ± 5.1	7
40%-MeOH	850 ± 36	90 ± 4.7	9.4	120 ± 6.1	7	110 ± 7.3	7.7
60%-MeOH	800 ± 16	40 ± 1.8	20	60 ± 3.5	13.3	32 ± 1.8	25
80%-MeOH	900 ± 43	4 ± 0.18	225	7 ± 0.3	128.5	2.3 ± 0.15	391
100%-MeOH	600 ± 22	60 ± 3.1	10	70 ± 3.7	8.5	85 ± 3.9	7
ACV	7 ± 3	0.1 ± 0.005	700	0.25 ± 0.01	280	0.15 ± 0.007	466.7

Vero cell monolayers were treated with different doses of the *Passiflora edulis* purified fractions at the time of infection. The CC_{50} and the IC_{50} values of these fractions were determined by MTT and plaque assay as detailed in "Material and Methods" section. Values are means ± SD (n=5).

the time and p.i. caused the highest inhibition of all viruses (Figure 2). Treatment of the infected cells only p. i. caused partial inhibition of the viral infection (Figure 2). These results support the possibility that the tested fraction exerts its anti-herpetic effect mainly by blocking early steps of the viral infection before or during the penetration of the virus into the host cells. These results are in agreement with previous findings indicating that different plant extracts are thought to exert their inhibitory action at a very early stage in the viral infection cycle, that is, at the time of virus adsorption onto and/or penetration into the host cell [36,37].

In order to examine possible interactions between viral particles and the examined fraction, the tested viruses were pre-incubated with 100 µg/ml of the fraction and then these mixtures were diluted 10^3 times with a fresh medium to anti-virally inactive concentrations of the fraction as detailed in the materials and methods section. The diluted mixtures were used for infecting Vero cell monolayers. Our results showed considerable inhibition of HSV-1 and HSV-2 infection by the highly diluted extracts mixtures, while they did not show any effect on VZV infection (Figure 2). These results suggested a strong and direct interaction between HSV-1 and HSV-2 particles and the examined fraction, which seems to abrogate the infectivity of the viruses, while there was no such interaction with VZV.

(b) Effect on late steps of the viral infection: As mentioned above, when the infected cells were subjected only p. i. treatment, this also caused significant inhibition of the viral infection with the different examined viruses (Figure 2). This inhibition may be a result of a further inhibitory effect of the tested fraction on internal steps during the viral replication cycle after the penetration of the virus into the host cells, or may well be a result of reinfection inhibition. In order to examine these possibilities, Vero cell monolayers were infected with 1 m.o.i. of HSV-1, HSV-2, or VZV. The medium containing the infecting virus was removed at the end of 1 hour p.i. and replaced with a fresh medium containing 50 µg/ml of the tested fraction or 1 µg/ml ACV. At 20, 25, or 35 hours p.i. the cells of each culture were broken by freezing and thawing in liquid nitrogen and the obtained mixtures containing the endogenous viruses were examined for the virus infectivity by infecting new Vero cell monolayers. Our results (Figure 3) showed that all treated cell monolayers with Pe fraction.
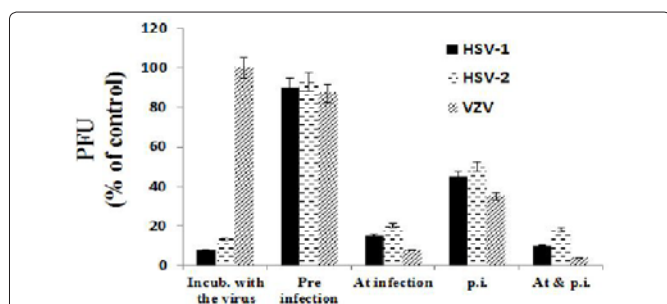
Figure 2: Effect of addition time of Pe 80%-MeOH fraction on infection development of herpes viruses. Vero cells were infected with 1 m.o.i. of the tested herpes viruses and treated with 10µg/ml of Pe 80%-MeOH fraction for different periods of time as follows: (a) incubated with the virus *, (b) only for 2h before infection, (c) only during infection, (d) only p.i., or (e) during and p.i. PFU of the treated cultures are presented as a percentage of the positive control. Values are means ± SD (n=5).

The (a) cells * were infected with viruses (10^3 m.o.i.) that were pre-incubated with 100 µg/ml of the fraction and then these mixtures were diluted 10^3 times with a fresh medium to anti-virally inactive concentrations of the fraction.
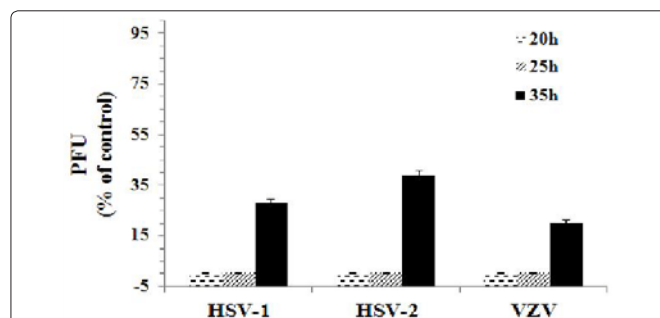
Figure 3: Effect of Pe 80%-MeOH fraction on endogenous herpes virus production. Vero cell monolayers were treated with 1 m.o.i. of HSV-1, HSV-2 or VZV and the medium containing the infecting virus was removed at the end of a 1 hour infection and replaced with a fresh medium containing 50 µg/ml of the tested fraction. At 20, 25, or 35 hours p.i. the cells of each culture were broken by freezing and thawing in liquid nitrogen and the obtained mixtures containing the endogenous viruses were examined for the virus infectivity by infecting new Vero cell monolayers. Plaque forming units were evaluated by the standard plaque assay and presented as means ± SD (n=5).
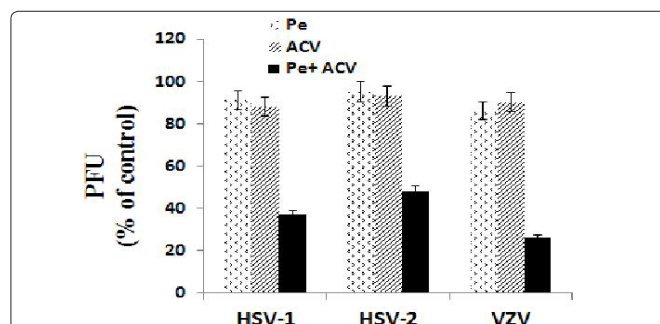
Figure 4: Synergistic activity between Pe 80%-MeOH fraction and ACV. Vero cells were treated separately with 0.1 µg/ml of a Pe fraction or 0.01µg/ml of ACV at the time, and p.i. with the different examined viruses or with a mixture of both 0.1 µg/ml of Pe fraction and 0.01µg/ml ACV. PFU of the treated cultures are presented as a percentage of the positive control. Values are means ± SD (n=5).

They were also examined for the production of infective endogenous viruses at 20 or 25h p.i. No plaque formation was shown in any of the tested viruses. However, when tested at 35h p.i. all cell monolayers infected with either of the examined viruses and treated with Pe fraction showed only partial inhibition of plaque formation (Figure 3). Furthermore, the cells treated with ACV did not show any plaque formation of any of the tested viruses at the different tested times (not shown results). It seems that this Pe fraction caused a significant delay in the production of infective viral particles production inside the host cells.

It is well established that the 80%-MeOH fraction of various plant leaves is rich with flavonoids [15]. Flavonoids are known to have antiviral activities [11,38,39]. The flavonoids quercetin, quercetin 3-rhamnoside, and quercetin 7-rhamnoside, which are found in a range of plant species, were reported to inhibit several viruses [40,41].

Synergistic activity with ACV

A significant synergistic antiviral activity between ACV and the 80%-MeOH fraction of Pe was found (Figure 4). When the cells were treated separately with 0.1 µg/ml of Pe fraction or 0.01µg/ml ACV at the time of infection and p.i. with the different examined viruses, a low antiviral activity (10-20%) was observed. However, when the

cells were treated with a mixture of both 0.1 µg/ml of Pe fraction and 0.01µg/ml ACV, a significant inhibition (~75 %) of the viral infection was obtained. This synergistic activity between the commercial anti-herpetic drug and the examined fraction is highly important. Such future combinations might contribute to a significant reduction in both the side effects and the costs of ACV.

Conclusions

1. The Pe leaves ethanol extract effectively inhibited the infection of Vero cells by HSV-1, HSV-2, and VZV *in vitro*, compared to the aqueous extract of its leaves.

2. Although all the purified fractions of Pe leaves ethanol extract had lower cytotoxicity compared to the crude extract, only the 80%-MeOH fraction gave higher SI value compared to the crude extract.

4. The highest antiviral activity of the extracts against all tested viruses was obtained when the cells were treated with the 80%-MeOH fraction at the time of infection.

5. Our results suggest that the antiviral activity of the examined fraction might be due to its effect on different stages during the viral infection cycle: (a) a strong interaction between the virus and the fraction in the case of HSV-1 and HSV-2, (b) an inhibition of viral adsorption/ penetration into the host cells and (c) a significant delay in the production of infective viruses inside the infected host cells.

6. The effect of the Pe 80%-MeOH fraction on the different stages during the replication cycle of the examined herpes viruses might be caused by different components of the fraction.

7. A promising synergistic antiviral effect of the 80%-MeOH fraction of *Passiflora edulis* and ACV was found.

8. Further study is required for the isolation and purification of the anti-virally active components of the Pe 80%-MeOH fraction.

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