Journal of Virological Methods 208 (2014) 138–143

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A new antiviral screening method that simultaneously detects viral replication, cell viability, and cell toxicity

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Article history:
Received 24 April 2014
Received in revised form 7 August 2014
Accepted 12 August 2014
Available online 22 August 2014

Keywords:
β-Galactosidase
Plant antiviral activity
Cyotpathic effect
Luciferase
Toxicity
Viral infectivity

A B S T R A C T

Viruses cause a variety of illnesses in humans, yet only a few antiviral drugs have been developed; thus, new antiviral drugs are urgently needed. Plants could be a good source of antiviral drugs, they do not have mobility and can only defend themselves by producing compounds against pathogens such as viruses in their own fix environment. These compounds may have the potential to inhibit animal and human viruses as well.

In this study, a fast and reliable method for screening plant extracts for specific antiviral activity against Herpes simplex virus type-1 (HSV-1) was developed. This method distinguishes between host cell death due to infectivity and multiplicity of the virus *versus* toxicity of the plant extract. Extracts from 80 plant and plant organs were screened using this approach. Six plant extracts showed potential to exert specific HSV-1 growth inhibition activity. In two cases, different organs from the same plant showed similar active results. With this method it is possible to screen a large number of extracts in a rapid and accurate way to detect antiviral substances against HSV-I and other viruses.

1. Introduction

Herpes simplex virus type-1 (HSV-1) is one of the most common human pathogens, infecting 40–80% of people worldwide (Kelly et al., 2009). It most commonly causes mucocutaneous infections, resulting in recurrent orolabial lesions (Kelly et al., 2009), and in adults HSV infection can cause ocular herpes and/or encephalitis. However, HSV-1 also has the capability of infecting and establishing latency in neurons, and in neonates it can cause encephalitis and severe disseminated infection with neurological impairment and high mortality (Kelly et al., 2009).

HSV-I virions consist of an inner nucleoprotein core comprising a linear dsDNA genome of 152 kbp that is enclosed in an icosahedral capsid shell. The nucleocapsid is surrounded by a layer of proteinaceous material designated the tegument, which is enclosed in an envelope of host cell-derived lipids containing virus-encoded glycoproteins (Kelly et al., 2009).

After primary infection, the virus establishes lifelong latency in sensory neurons of the peripheral nervous system. Sporadic reactivation of latent virus triggered by exogenous factors can lead to virus replication followed by recrudescent infection (Gescher et al., 2011).

There is an ever-increasing need for novel and improved antiviral agents as alternatives to the popular drug acyclovir (ACV) and other nucleoside derivatives used worldwide against HSV-1 and many other viruses. This need is dictated by several drawbacks associated to the commonly used antiherpetic drugs, including development of anti-ACV resistant mutant viruses as well as side effects such as nausea, vomiting, and headache, and the fact that ACV is not highly effective in recurrent virus attacks (Yarmolinsky et al., 2010).

Plants are known to be self-sustaining chemical chameleons capable of synthesizing different compounds according to their needs. It is well known that plants defend themselves based on the challenges encountered (Raskin and Rippol, 2004). Many plants synthesize antimicrobial compounds as a mechanism of protection against a number of pathogenic agents. Plants do not have mobility and can only defend themselves by producing compounds against all the different enemies they encounter. Some plants are tolerant to various plant viruses, and might produce antiviral compounds that confer this resistance (Lindbo et al., 1993). These compounds may have the potential to inhibit animal and human viruses (Yarmolinsky et al., 2010).

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http://dx.doi.org/10.1016/j.jviromet.2014.08.007
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To date, many natural plant compounds have demonstrated antiviral effects in clinical studies, in animal experiments, and *in vitro* (Knipping et al., 2012). *Ficus benjamina* is known as a plant with anti-HSV-1 effect that effectively inhibits infection of Vero cells by HSV-1 (Yarmolinsky et al., 2010). Similarly, *Mentha longifolia*, *Origanum syriacum*, and *Melissa officinalis* have been vigorously promoted as having antiviral agents that are effective against several viruses (Allahverdiyev et al., 2004; Geuenich et al., 2008; Vimalanathan and Hudson, 2012). However, a fast and reliable method for detecting potential new antiviral products in plants is required.

Viruses multiply within cells; thus, a direct method that examines the presence of a virus in cells is required to be able to track viral cultures accurately. Since plant extracts with potential anti-HSV-1 effect may also be toxic to animal cells, it is important to use an approach that distinguishes between antiviral and animal cell toxicity characteristics, since death of cells carrying target viruses could be related to cell toxicity of the extract as well as, or in lieu of, any antiviral effect.

In this study a two-step method was developed for screening plant extracts for their specific antiviral activity against HSV-1. The first step detects the toxicity of the extract to the infected cells while the second step identifies inhibition of viral replication. After confirming the validity of the technology on several plant extracts, the antiviral activity was screened in extracts derived from 80 different plants.

2. Materials and methods

2.1. Cell lines

Monkey kidney Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma–Aldrich), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37 °C in 5% CO_2. Vero cells were kindly provided by Dr. A. Panet (Department of Virology, The Hebrew University – Hadassah School of Medicine, Jerusalem, Israel).

2.2. Viruses

HSV-1 was chosen for this assay because of the importance of finding novel agents to inhibit this specific virus, and because of its ability to grow easily in cell cultures. In addition, acyclovir, a known antiviral agent could be used as a positive control. HSV 17+/pR20.5/5 was the chosen viral strain selected for this assay because it contains the β-galactosidase (β-gal) gene, which is under the control of the Rous sarcoma virus (RSV) promoter, and the green fluorescent protein (GFP) gene, which is under the control of the cytomegalovirus (CMV) promoter (Kolodkin-Gal et al., 2008). Both genes are expressed constitutively and, for purpose of the research, were inserted at the Us5 gene locus. It was previously shown that inactivation of Us5 does not otherwise affect virus growth *in vitro* or the virulence or kinetics of latency *in vivo* (Thomas et al., 1999).

Virus infectivity was detected indirectly by analyzing cell viability and directly by identifying the virus through its β-galactosidase activity using the ≡eta-Glo Assay system (Promega, Madison, WI, USA).

The virus was kindly provided by Dr. A. Panet (Department of Virology, The Hebrew University – Hadassah School of Medicine, Jerusalem, Israel). To propagate the virus, Vero cells were infected and the virus was harvested from the cell pellet, purified on a 10% sucrose cushion (Sigma–Aldrich), and titrated by plaque assay on Vero cells (Kolodkin-Gal et al., 2008).

2.3. Cell infection

Cells were seeded in 96-well plates (1×10^4 cells per well) in DMEM, for 24 h and then infected with 0.25 M.O.I. (multiplicity of infection), of HSV–(RSVβ-gal) in 50 μl DMEM, with no FCS. At 2 h post-infection, 10% FCS and DMEM were added to achieve a total volume of 100 μl, and cells were incubated for a further 46 h at 37 °C in 5% CO_2.

2.4. Plant samples

Three types of plants were selected to facilitate a sensitivity analysis of testing for lack of activity as an indication of virus viability: (1) a plant with no known antiviral activity (*Cucumis sativus* peel); (2) a plant known to be generally toxic (*Nerium oleander*) (Turan et al., 2006)); and (3) plants that have been reported as antiviral agents (*F. benjamina*, *Mentha longifiolia*, *O. syriacum*, *M. officinalis* (Allahverdiyev et al., 2004; Geuenich et al., 2008; Vimalanathan and Hudson, 2012; Yarmolinsky et al., 2010).

2.5. Preparation of plant ethanolic extracts

Ethanolic extracts were prepared from different plants and organs (leaves of *F. benjamina*, *M. longifolia*, *O. syriacum*, *M. officinalis*; peels of *C. sativus*; and others). Two grams of plant tissues with 5 ml of 60% ethanol were crushed using a mortar and pestle and clarified by centrifugation (1800 rpm for 10 min) twice. 1 ml of the supernatant was evaporated in an open 5 cm nonabsorbent plate in a biological hood for 45 min. The volume of the evaporated supernatant was measured and DMEM with no serum was added to a total volume of 1 ml. The final extract concentration was adjusted to be 400 μg/ml.

2.6. Preparation of plant extracts for Immunocytology assays

Plant powders (20 g) were dissolved in 1 ml boiling water and boiled for an additional 5 min. The extracts were vortexed for 3 min and clarified by centrifugation (3000 rpm for 10 min). The supernatant was collected and re-centrifuged under the same conditions. The supernatant was collected again and used for Immunocytology assays.

2.7. MTS cell viability assay

The MTS assay is based upon the ability of mitochondrial dehydrogenase to convert MTS substrate (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Sigma–Aldrich) to insoluble formazan, which has a purple color (Buttke et al., 1993). Vero cells were seeded in 96-well plates (1×10^4 cells per well) and infected with 0.25 M.O.I. of HSV–(RSVβ-gal) in 100 μl DMEM with various concentrations of plant extracts. After 48 h, 20 μl MTS was added to the Vero cells and the mixtures were incubated at 37 °C for 1 h. Absorbance was measured at 490 nm to assess cell metabolic activity.

2.8. Beta-Glo assay for β-gal detection

The beta-glo assay system (Promega) is a homogeneous bioluminescent assay that couples β-galactosidase activity to a luciferase reaction. β-galactosidase catalyzes a reaction in which the substrate (D-luciferin-o-β-galactopyranoside) is cleaved to release luciferin. This luciferin serves as a substrate for luciferase that is present in the reagent. As a result of the luciferase activity, oxyluciferin is formed and light is emitted (Hannah et al., 2003).

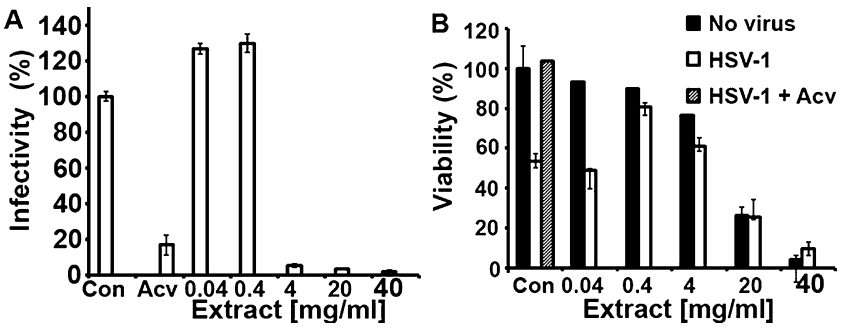

Fig. 1. Effects of *Mentha longifolia* extract on virus infectivity and cell viability. (A) Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.25 in the absence or presence of increasing doses (0.04, 0.4, 4, 20, 40 mg/ml) of *M. longifolia* extracts or 10 μM of acyclovir (Acv). Infected cells were analyzed for β-galactosidase (β-gal) enzyme-specific activity using the Beta-Glo assay as described in Section 2. (B) Mock-infected and Vero cells infected with HSV-1 at 0.25 MOI in the presence of increasing doses of *M. longifolia* extracts or 10 μM of acyclovir (Con Acv). Cell viability was analyzed by MTS assay. Data reflect means ± SD ($n = 3$ replicates). Results are representative of three independent experiments.

Vero cells were seeded in white 96-well plates (1×10^4 cells per well), infected with 0.25 M.O.I. of HSV–(RSVβ-gal) in 100 μl DMEM with various concentrations of plant extracts. After 48 h, Vero cells were incubated with 100 μl Beta-Glo solution at room temperature for 30 min. Luciferase activity was measured using a chemiluminescence imaging system (MicroChemi, Berthold Technologies, Bad Wildbad, Germany).

Varying concentrations of plant extracts were added to Vero cells during infection with HSV-1. The extracts were checked for two types of activity: toxicity of the extracts to the Vero cells, by using MTS, and infectivity of the virus, by MTS and by detecting β-Glo. The advantage of this method is the possibility to simultaneously analyze both the toxicity and the functional antiviral potential of the extracts.

2.9. Immunocytology assay

The Immunocytology assay was used to detect the expression of viral glycoproteins on the surface of infected cells using polyclonal antibodies.

Vero cells were seeded in 96-well plates (1×10^4 cells per well), infected with 0.25 M.O.I. of HSV–(RSVβ-gal) in 100 μl DMEM with various concentrations of plant extracts. After 48 h, Vero cells were fixed using 100 μl 3.8% formaldehyde in PBS, for 10 min at room temperature, washed three times with 1× PBS and blocked for 30 min at room temperature with 100 μl 1% BSA in PBS (blocking solution). Cells were washed with 1× PBS 0.02% Tween 20 and treated with 50 μl polyclonal Rabbit anti-HSV-1 glycoproteins (Biotest) diluted 1:100 with blocking solution for 1 h at room temperature. After three washes with 1× PBS 0.05% Tween 20, cells were treated for 30 min with 50 μl goat anti-rabbit IgG HRP conjugated (Biotest), and washed again. 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine; MW = 240.4) (Biotest) substrate was added and the absorbance of the soluble blue product was measured at 650 nm.

In all assays, 10 μM of acycloguanosine (Sigma–Aldrich), a known antiviral agent, was used as a positive control. Extracts were added during the adsorption stage of viral infection. Since viruses can adsorb a variety of carbohydrates and glycoproteins with no specificity, it was necessary to determine whether the plant extracts could inhibit viruses during their adsorption stage in a non-specific way. A naïve extract (*C. sativus* extract) was added before and after the adsorption stage of infection.

3. Results

Results of experiments showing viral infectivity and Vero cell viability after addition of varying concentrations of plant extracts following infection with HSV-1 are shown in Figs. 1–4. As expected, *C. sativus*, the naïve plant with no expected toxicity, showed no antiviral activity, and *N. oleander* was toxic to the Vero cells (results are not shown). Ethanol extracts of *M. longifiolia* at concentrations of 4 mg/ml or higher inhibited HSV-1 (Fig. 1A). At a concentration of 4 mg/ml there was no cytopathic effect on either infected or non-infected cells, which showed almost the same viability (Fig. 1B). However, at higher concentrations (20–40 mg/ml) *M. longifiolia* had a toxic effect and the viability of both infected and noninfected cells was reduced (Fig. 1B). Similarly, *F. benjamina*, *O. syriacum*, and *M. officinalis* showed anti-HSV-1 activity at specific concentrations, and at high concentrations all three had cell toxicity (results are not shown).

A naïve extract (*C. sativus* extract) was added before and after the adsorption stage of infection. There was no difference in the result in either case (result not shown).

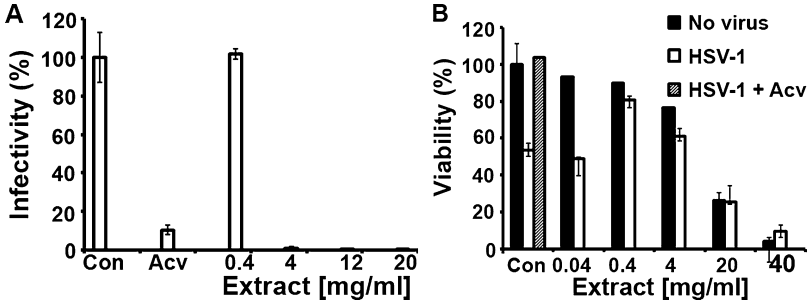

Fig. 2. Effect of toxic plant (*Bellis silvestiria*) extracts on virus infectivity and cell viability. (A) Vero cells were infected with HSV-1 at an MOI of 0.25 in the absence or presence of increasing doses (0.4, 4, 12, 20 mg/ml) of *Bellis silvestiria* extract, or 10 μM of acyclovir (Acv). Infected cells were analyzed for β-gal enzyme-specific activity by using the Beta-Glo assay. (B) Mock-infected and Vero cells infected with HSV-1 at an MOI of 0.25 in the presence of increasing doses (0.4, 4, 12, 20 mg/ml) of *Bellis silvestiria* extracts, or 10 μM of acyclovir. Cell viability was analyzed by MTS assay. Data reflect means ± SD ($n = 3$ replicates). Results are representative of three independent experiments.

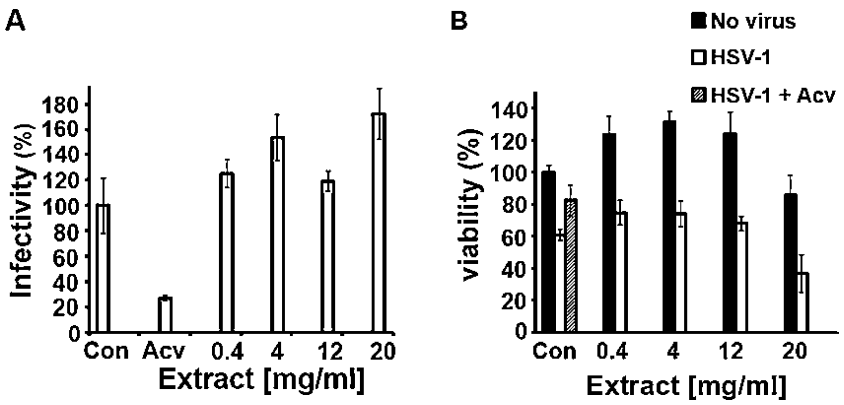

Fig. 3. Effect of naïve (*Bellevalia flexuosa*) plant extract on virus infectivity and cell viability. (A) Vero cells were infected with HSV-1 at 0.25 MOI in the absence or presence of increasing doses (0.4, 4, 12, 20 mg/ml) of *B. flexuosa* extract, or 10 μM of acyclovir (Acv). Infected cells were analyzed for β-gal enzyme-specific activity using the Beta-Glo assay. (B) Mock-infected and Vero cells infected with HSV-1 at 0.25 MOI in the presence of increasing doses of *B. flexuosa* extract, (0.4, 4, 12, 20 mg/ml) or 10 μM of acyclovir). Cell viability was analyzed by MTS assay. Data reflect means ± SD ($n = 3$ replicates). Results are representative of three independent experiments.

Following validation of the technique, a total of 80 plant samples from organs of various plants were tested. The plants were classified into three different groups: toxic plants, non-toxic/noninhibitory plants, and plants with potential growth inhibitory activity for HSV-1 without Vero cell toxicity. Fig. 2 shows illustrative results from testing with a crude extract from a toxic plant (*Bellis silverstris*). When the extract was administered in concentrations higher than 4 mg/ml, HSV-1 could not replicate (Fig. 2A) and Vero cells did not survive (Fig. 2B).

Crude extract from *Bellevalia flexuosa* is an example of an agent that was both nontoxic and noninhibitory, and it is referred to as a naïve plant (Fig. 3). HSV-1 was able to replicate at all concentrations of the extract (Fig. 3A), and the viability of infected cells was reduced due to virus growth (Fig. 3B). Fig. 4 shows an example of a plant that was not toxic to Vero cells (*Fumana thymifolia*) and has potential HSV-1 growth inhibitory activity. HSV-1 was not able to replicate in the presence of this extract (Fig. 4A), while there was no cytopathic effect on either infected or noninfected cells at concentrations of 0.4–4 mg/ml (Fig. 4B).

Among the 80 plants and plant organs screened, six extracts from several plants and plant organs showed some potential inhibitory activity against HSV-1 at a range of 400 μg/ml–12 mg/ml of crude extract (Table 1).

In order to rule out false-positive results due to a specific effect of the extracts on the reporter gene promoters, an Immunocytology assay was used as another direct anti-viral assay. This Immunocytology assay tests viral viability by checking viral glycoprotein expression on the surface of the host cell.

Table 1
Plant samples found to have ability to inhibit HSV-1 growth.

Name of plant (*Genus specie*)
Fumana thymifolia, leaves
Fumana thymifolia, flowers
Geranium rotundifolium, stems
Campanula hierosolymitana, above-ground organs
Sedum sediforme, stems
Sedum sediforme, leaves

Crude extract from 12 plants, some of them with known antiviral activity (Tareq et al., 2005; Liao et al., 2010; Zhou et al., 2014), were screened against the HSV-1 virus. As can be seen in Fig. 5, the Immunocytology assay corroborates the findings of the β-gal assay (Fig. 5A and C). Extract from the plant Flos Lonicerae showed anti-HSV effect at 10 mg/ml in both assays, while having no cytopathological effect on the host cells. At concentration higher than 15 mg/ml the extract is toxic to the cells (Fig. 5B).

4. Discussion

Despite the large number of viral diseases, there are still very few antiviral agents that effectively treat this pathogenesis. Plants can be a very valuable source of new antiviral molecules, since they combat viral infections constantly during their life cycle. The availability of a fast and reliable screening method for new potential antiviral drugs will accelerate the selection of plants with antiviral effects and stimulate the development of new plant-derived

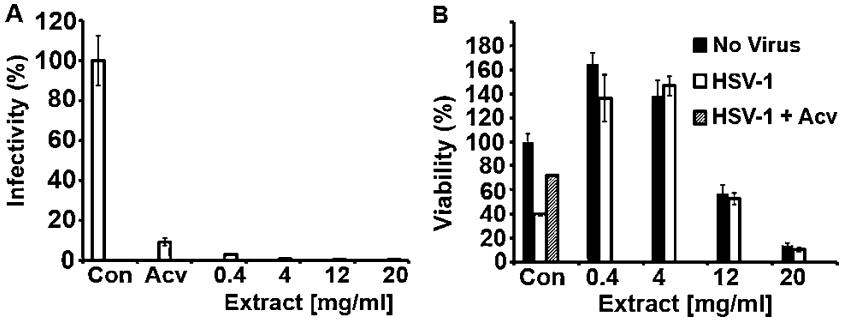

Fig. 4. Effect of a potential HSV-1 growth-inhibiting active plant extract (*Fumana thymifolia*) that affects virus infectivity but not cell viability. (A) Vero cells were infected with HSV-1 at 0.25 MOI in the absence or presence of increasing doses (0.4, 4, 12, 20 mg/ml) of *F. thymifolia* extract, or 10 μM of acyclovir (Acv). Infected cells were analyzed for β-gal enzyme-specific activity using the Beta-Glo assay as described in Section 2. (B) Mock-infected and Vero cells infected with HSV-1 at 0.25 MOI in the presence of increasing doses (0.4, 4, 12, 20 mg/ml) of *F. thymifolia* extract or 10 μM of acyclovir. Cell viability was analyzed by MTS assay. Data reflect means ± SD ($n = 3$ replicates). Results are representative of three independent experiments.

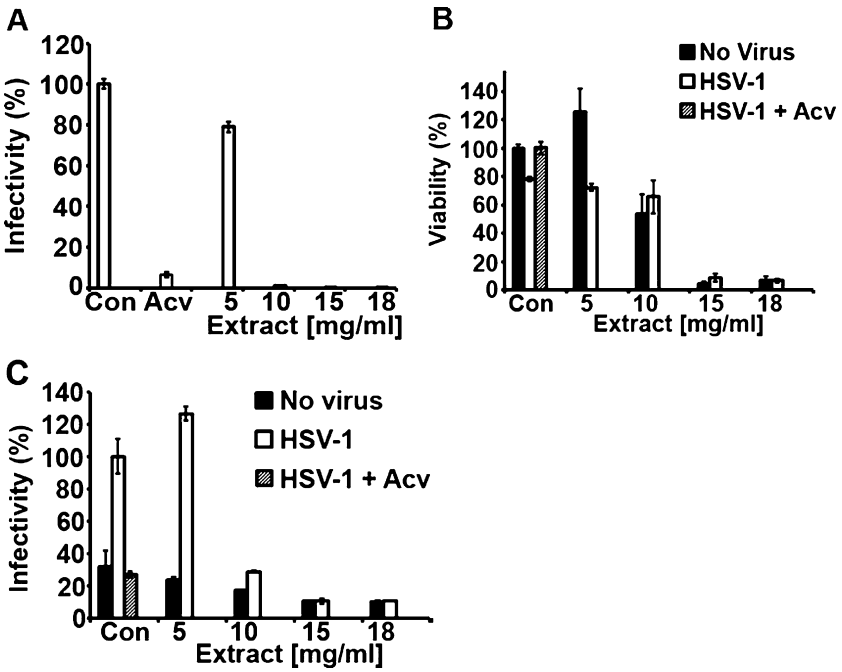

Fig. 5. Effect of a potential HSV-1 growth-inhibiting active plant extract (*Flos Lonicerae*) that affects virus infectivity (verified by β-glo assay and Immunocytology) but not cell viability. (A) Vero cells were infected with HSV-1 at 0.25 MOI in the absence or presence of increasing doses (5, 10, 15, 18 mg/ml) of *Flos Lonicerae* extract, or 10 μM of acyclovir (Acv). Infected cells were analyzed for β-gal enzyme-specific activity using the Beta-Glo assay as described in Section 2. (B) Mock-infected and Vero cells infected with HSV-1 at 0.25 MOI in the presence of increasing doses (5, 10, 15, 18 mg/ml) of *Flos Lonicerae* extract or 10 μM of acyclovir. Cell viability was analyzed by MTS assay. (C) Infected cells were analyzed by immunocytology as described in Section 2. Data reflect means ± SD ($n = 3$ replicates). Results are representative of three independent experiments.

antiviral agents. Since viruses grow within cells and are cytopathic, a differentiation should be made between cell death due to toxicity of the tested antiviral agent and death due to viral infection. Valuable antiviral activity must have the capability of inhibiting the growth of a virus without exerting toxicity on the infected cells. The method described in this study addresses this requirement and facilitates identification of plants with antiviral bioactivities that can be further studied for their potential therapeutic effects.

In the present study we developed a new method for the detection of plant extracts capable of inhibiting HSV-1 infectivity. The method described here clearly differentiates between cell death due to infectivity and multiplicity of the virus *versus* cell death due to the toxicity of the plant extract. This fast, two-step method may be used in wide screening for extracts with potential antiviral properties. Extracts that prevent viral multiplication without affecting cell viability should be considered as potential antiviral agents that may warrant further research.

Cell viability was assessed using MTS, which measures virus infectivity indirectly by detecting cell viability. Virus infectivity was measured directly using the HSV–RSV-gal virus, which is an HSV-1 recombinant virus with the reporter gene for β-galactosidase. Beta-Glo substrate products break down to luciferase enzyme substrate and cause reactionary light that can be measured accurately using a luciferase reading appliance. Adding these products enables direct detection of the presence of the virus. Analyzing the result of cell viability by MTS and the presence of the virus by beta-Glo assay enables accurate identification of extracts with the potential for virus inhibition without cell toxicity.

This detection system may be used only if two conditions exist: the virus must have a β-galactosidase reporter gene in its genome, and the reporter gene must not affect its infectivity. HSV-1 carries a reporter gene under the control of the Rous sarcoma virus (RSV) promoter, and it was previously shown that insertion of this reporter gene in HSV-1 does not affect virus growth *in vitro* or the virulence and latency kinetics of the virus *in vivo* (Thomas et al., 1999). This method is applicable for studies of other cytopathic viruses carrying a β-galactosidase reporter gene that does not affect viral infectivity.

It is possible to use other methods, such as real-time PCR (Van Elden et al., 2001) or Immunocytology assay to directly measure virus infectivity. However, the key for a successful screening technique is simultaneous measurement of cell viability and virus multiplicity. PCR techniques do not require the β-galactosidase reporter gene, but the detection stage is more difficult and less suitable for quick screening of a large number of samples.

In the β-gal assay, viral activity is detected by monitoring β-galactosidase gene expression. This gene is under the control of the Rous sarcoma virus (RSV) promoter, and the green fluorescent protein (GFP) gene, which in turn is under the control of the cytomegalovirus (CMV) promoter. A specific effect of the extract on these promoters can give false-positive results in the β-gal assay, although in such a case cell death is expected to be observed in the MTS assay. In order to confirm the results of the β-gal assay an additional independent immnocytology assay was utilized. In this assay, viral glycoproteins expressed on the surface of the host cell membrane are detected using specific antibodies. If the decrease in the protein's level correlates with the reduction in β-gal gene level this will indicate that the extracts affect virus propagation and not specifically inhibits the reporter gene promoter. Twelve extracts of plants were screened using the Immunocytology assay in parallel with the β-gal and MTS assays. In all 12 extracts there was a clear correlation between the β-gal results and the Immunocytology assay results. Extract from the plant Flos Lonicerae, with known anti-viral activity, (Zhou et al., 2014) showed potential to exert HSV-1 growth inhibition activity. The fact that all three approaches support the result expected, the use of the simpler and faster

β-gal assay for large scale screenings of potential anti-viral activity in plant extracts can be adopted.

Ethanol plays an important role in extract formulation, but has a toxic effect on cells; thus it is important to significantly reduce its concentration in extracts used for treatment. This was accomplished by evaporation and further resuspension of the dry material in buffer. Lyophilizing the extract before adding it to the cells is also recommended.

Previous studies have reported differential antiviral plant extract activity on HSV-1 in comparison with other viruses. Extracts of *M. longifolia, F. benjamina*, *Oreganum syriacum*, and *M. officinalis* were reported to exert antiviral activity against other viruses (Knipping et al., 2012; Orhan et al., 2011; Yarmolinsky et al., 2009). These plants were used as a reference for antiviral potential plants against HSV-1.

Extracts from 80 plant and plant organs were screened using this approach. As shown in Table 1, six plants extracts showed potential to exert HSV-1 growth inhibition activity. Other plants exerted cell toxicity without antiviral potential, or were naïve, with no antiviral potential and no toxicity. In two cases, different organs from the same plants showed similar active results. Both the leaves and stems of *Sedum sediforme*, and the leaves and flowers of *F. thymifolia* showed anti-HSV-1 activity. Evidence of a viral inhibitory growth effect from more than one organ of a single plant provides added evidence of the accuracy and validity of this method.

This method may be also used after fractionation and isolation of compounds from plant extract and will enable further analysis and investigation of newly anti-viral agents.

In conclusion, the new method described here enables the detection of potential antiviral agents from a large number of extracts quickly and accurately.

The advantage of this method is the possibility to independently analyze both the toxicity and the functional anti-viral potential of the extracts in a two-step process.

Acknowledgements

This study was partially funded by a grant from the European Union under the ENPI CBC MED Program for the BioXplore project (http://www.bio-xplore.org/).

The contents of this document are the sole responsibility of the BioXplore Consortium and can under no circumstances be regarded as reflecting the position of the European Union. Many thanks to Dr. Mina Faran, for collecting and identifying the plants that were screened, thereby making a huge impact on this study. Mr Ramon Velman which provided all the anti-viral plant powders and to Tal Haimov and Liana Govani for performing the immunocytology assays. Shifra Fraifeld, a senior medical writer at the Hadassah-Hebrew University Medical Center, contributed to the preparation of the manuscript.

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