IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

In vitro evaluation of the antiviral properties of Shilajit and investigation of its mechanisms of action

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1521394 since 2017-05-30T15:35:31Z

Published version:

DOI:10.1016/j.jep.2015.03.019

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

09 September 2019



UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on**:*
Questa è la versione dell'autore dell'opera:
J Ethnopharmacol, 166, 2015, doi: 10.1016/j.jep.2015.03.019

The definitive version is available at:
La versione definitiva è disponibile alla URL:
[http://www.elsevier.com/]

*Manuscript
Click here to view linked References

In vitro evaluation of the antiviral properties of Shilajit and investigation of its mechanisms of action

Valeria Cagno[§1], Manuela Donalisio[§1], Andrea Civra[1], Cecilia Cagliero[2], Patrizia Rubiolo[2], David Lembo*[1]

[1]*Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Torino, Italy.*[2]*Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, I-10125 Torino, Italy*

Running title: Antiviral activity of Shilajit

[§]VC and MD contributed equally to this work

* Corresponding author: Prof. David Lembo

Department of Clinical and Biological Sciences

University of *Torino*, S. Luigi Gonzaga Hospital

Regione Gonzole, 10

10043, Orbassano, *Torino*, Italy

Phone: +39 011 6705484

Fax: +39 011 2365484

E-mail: david.lembo@unito.it

ABSTRACT

ETHNOPHARMACOLOGICAL RELEVANCE: Shilajit, a herbomineral substance exuded from rocks in steep mountainous regions, has been used for thousands of years by the Indian Ayurvedic and Siddha systems of traditional medicine to relieve ailments and enhance quality of life. Although a large number of therapeutic properties have been ascribed to Shilajit, its therapeutic potential is still largely unexplored by modern research and many of its claimed bioactivities lack scientific validation. The present study was undertaken to investigate the antiviral activity of Shilajit against a panel of viruses including herpes simplex type 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HCMV), human respiratory syncytial virus (RSV), human rotavirus (HRV), and vesicular stomatitis virus (VSV).

MATERIALS AND METHODS: The antiviral activity of Shilajit was assayed *in vitro* by plaque reduction and virus yield assays and the major mechanism of action was investigated by virucidal and time-of-addition assays.

RESULTS: Shilajit exhibited a dose-dependent inhibitory activity against HSV1, HSV2, HCMV, and RSV infectivity *in vitro* (EC_{50} values: 31.08 μg/ml, 12.85 μg/ml, 34.54 μg/ml, and 30.35 μg/ml, respectively), but was inactive against HRV and VSV. Humic acid, a constituent of Shilajit, displayed the same spectrum of activity. Partial virus inactivation and interference with virus attachment were both found to contribute to the antiviral activity of Shilajit.

CONCLUSIONS: The results of the present study demonstrate that Shilajit is endowed with broad, yet specific, antiviral activity *in vitro* and constitutes a natural source of antiviral substances. Further work remains to be done to assess its efficacy *in vivo*.

1. INTRODUCTION

The global impact of viral infections, the development of antiviral drug resistance, and the emergence of new viruses are all driving the incessant search for new compounds endowed with antiviral activity, with the aim of developing novel safe and effective antiviral treatments.

In this context, natural products originating from botanical, animal or mineral sources traditionally used in ethnomedicine may provide leads for modern antiviral drug development once their pharmacological potential is verified by scientific investigation.

This paper focuses on Shilajit, a herbomineral substance that has been used for thousands of years by the Indian Ayurvedic and Siddha systems of traditional medicine to relieve ailments and enhance the quality of life. It is a blackish-brown matter exuded from rocks in steep mountainous regions, such as the Himalaya mountains between India and Nepal, as well as mountains in Russia, Tibet, Afghanistan, and Norway (Agarwal et al., 2007). The chemical characterization of Shilajit has revealed that it consists of three major components: 1) low and medium molecular weight non-humic organic compounds; 2) medium and high molecular weight DCPs (dibenzo-α-pyrones-chromoproteins), containing trace metal ions and coloring matter such as carotenoids and indigoids; and 3) metallo-humates, like humic acids, fulvic acids and fusims with dibenzo-α-pyrones in their core nuclei (Ghosal 2006). Shilajit has been considered a panacea by many traditional systems of oriental medicine, which have ascribed a vast array of therapeutic properties to this natural substance (Agarwal et al., 2007, Wilson et al., 2011). Preclinical studies have pointed to the potential use of Shilajit in various pathological conditions due to its numerous properties/actions, which include: antiulcerogenic properties, antioxidant properties, complement activator in the immune system and immunomodulator, antidiabetic properties, anxiolytic and antistress properties, anti-inflammatory and antiallergic properties, and memory and learning enhancer (Agarwal et al., 2007, Wilson et al., 2011). However, a recent systematic review of early studies revealed that the full therapeutic potential of Shilajit is still largely unexplored by modern research and many of its claimed bioactivities lack scientific validation and remain unproven (Wilson et al., 2011). The

antiviral potential of Shilajit has yet to be explored, with only its anti-HIV action having received research attention to date (Ghosal, 2006, Gupta et al., 2010, Rege et al., 2012). The antiviral activity of one of its components, humic acid has been partially explored (Klöcking et al., 2005).

The present study was undertaken to investigate the antiviral activity of Shilajit against a panel of viruses, consisting of herpes simplex types 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HCMV), human respiratory syncytial virus (RSV), human rotavirus (HRV), and vesicular stomatitis virus (VSV); these viruses were selected as they encompass a range of viral characteristics, including the presence or absence of a lipid envelope, different forms of genome (DNA or RNA), and different tissue/organ tropisms (Collins and Crowe, 2007, Cox and Christenson, 2012, Landolfo et al., 2003, Roizman et al., 2007). Here, we report on the cytotoxicity, the antiviral potency, and the probable mechanisms of antiviral action of Shilajit.

2. MATERIALS AND METHODS.

2.1 Compounds. Shilajit was purchased from Dekha Herbals (Lalitpur, Nepal). On the basis of the certificate of analysis provided by the manufacturer, humic acid and fulvic acid represents the 9.5% and the 26.6 % of Shilajit composition respectively. The following components are also present: 4-methyl catechol, benzoic acid, benzamide, ethyl benzoate, hybdrobenzoin, carboxy ethane, ammonium benzoate, homocatechol, orcinol, β eudesmol, isodamascol, juniper camphor, and stearol. Shilajit was dissolved in bi-distilled sterile water to make a 25mg/ml stock solution prior to each experiment. Humic acid, heparin, acyclovir and foscarnet were purchased from Sigma (St. Louis, Mo., USA).

2.2 Cells. African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81), human epithelial cells (Hep-2) (ATCC CCL-23), A549 (ATCC CCL-185), and African green monkey kidney epithelial (MA-104) cells (ATCC CRL-2378.1) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% antibiotic-anitmycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany). Low-passage human embryonic lung fibroblasts (HELFs) were grown as monolayers in Eagle's minimal essential medium (Gibco-BRL) in the same conditions as described above with the addition of 1mM sodium pyruvate.

2.3 Viruses. Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV-1 and HSV-2 strains were propagated and titrated by plaque assay on Vero cells. A HSV-2 strain with phenotypic resistance to acyclovir was generated by serial passage in the presence of increasing concentrations of acyclovir, as previously described (Donalisio et al., 2013). HCMV strain Towne was kindly provided by Prof. W. Brune, Heinrich Pette Institut, Hamburg, Germany; it was propagated and titrated by plaque assay on HELF cells. RSV strain A2 (ATCC VR-1540) was propagated in Hep-2 and titrated by the indirect immunoperoxidase staining

procedure using an RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom), as described previously (Donalisio et al., 2012). Human rotavirus strain Wa (ATCC VR-2018) was activated with 5 µg/mL porcine pancreatic trypsin type IX (Sigma, St. Louis, Mo.) for 30 minutes at 37°C and propagated in MA104 cells using MEM containing 0.5 µg trypsin per mL, as described previously (Graham et al., 2004). Virus stocks were maintained at -80 °C.

2.4 Cell viability. Cell viability was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell cultures seeded in 96-well plates were incubated with different concentrations of Shilajit or humic acid in triplicate under the same experimental conditions described for the antiviral assays. Cell viability was determined using the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The effect on cell viability at different concentrations of the compound was expressed as a percentage, by comparing absorbances of treated cells with those of cells incubated with culture medium alone. The 50% cytotoxic concentrations (CC_{50}) and 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA).

2.5 HSV inhibition assays. The effect of Shilajit on HSV infection was evaluated by plaque reduction assay. Vero cells were pre-plated 24 h in advance in 24-well plates at a density of 10 x 10^4 cells. Increasing concentrations of compounds were added to cells for 2 h; a mixture of the compound plus HSV-1, HSV-2, or acyclovir resistant HSV-2 (MOI 0.0003 pfu/cell) was subsequently added to the cells, which were then incubated at 37 °C for 2 h. The virus inoculum was then removed and the cells washed and overlaid with a medium containing 1.2% methylcellulose (Sigma) and serial dilutions of Shilajit or humic acid. After further incubation at 37 °C for 24 h (HSV-2) or 48 h (HSV-1), cells were fixed and stained with 0.1% crystal violet in

20% ethanol and viral plaques counted. The effective concentration producing 50% reduction in plaque formation (EC_{50}) was determined using Prism software by comparing drug-treated with untreated wells. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value.

2.6 HCMV inhibition assay. HELF cells were pre-plated in a 96-well plate. The following day serial dilutions of Shilajit or humic acid were added to cells and incubated for 2 h at 37°C. Virus (MOI 0.005) and compound were then added and the cells incubated for a further 2 h; monolayers were then washed and overlaid with 1.2% methylcellulose medium supplemented with 3% FCS and 1mM sodium pyruvate. After five days incubation, cells were fixed with cold methanol and acetone for 1 min and subjected to HCMV-specific immunostaining using an anti-HCMV IEA monoclonal antibody (11-003; Argene, Verniolle, France).

2.7 RSV inhibition assay. Serial dilutions of the compound were added to A549 cells grown as monolayers in a 96-well plate and incubated for 2 hours at 37°C. Mixtures of the compounds and virus (MOI 0.01) were then added to the cells and incubated for a further 3 hr at 37°C to allow viral adsorption; the monolayers were then washed and overlaid with 1.2% methylcellulose medium supplemented with Shilajit or humic acid. Three days post-infection, cells were fixed with cold methanol and acetone for 1 min and subjected to RSV-specific immunostaining. Immunostained plaques were counted, and the percent inhibition of virus infectivity determined by comparing the number of plaques in treated wells with the number in untreated control wells.

2.8 Rotavirus inhibition assay. To assess the compound's ability to inhibit rotavirus infectivity, assays were carried out using confluent MA104 cell monolayers plated in 96-well trays. Virus infectivity was activated by adding 5µg porcine trypsin (Sigma)/mL for 30 minutes at 37°C. Cells were pre-treated for 1 hours with serial dilutions of Shilajit and subsequently activated virus (MOI 0.02 pfu/cell) and Shilajit were added to cells for 1 h at 37°C; cells were then washed and fresh medium containing Shilajit added. After 16 h, cells were fixed with cold acetone-methanol and viral titers were determined by indirect immunostaining using the monoclonal antibody mab-0036

(specific for human 41 kDa inner capsid protein - VP6 - of Rotavirus) purchased from Covalab (Villeurbanne, France) and the UltraTech HRP Streptavidin-Biotin Detection System (Beckman Coulter).

2.9 VSV inhibition assay. Vero cells were pre-plated 24 h in advance in 24-well plates at a density of 10 x 10^4 cells/well. Increasing concentrations of compound were incubated with cells for 2 h at 37°C, then a mixture of VSV (MOI 0.0005 pfu/cell) and compound added and incubated for a further 2 h at at 37°C. The virus inoculum was then removed and the cells washed and then overlaid with a medium containing 1.2% methylcellulose (Sigma) and serial dilutions of Shilajit. After 24 h of incubation at 37 °C, cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques were counted.

2.10 Time-of-addition assay. Serial dilutions of Shilajit were added to cells: 2 h before infection, during infection, or post infection. After the incubation time, viral plaques were counted.

2.11 Investigation of the mechanism of action. Vero and A549 cells were plated as described above and subjected to 3 different kinds of assay:

2.11.1 Attachment assay. Serial dilutions of Shilajit were mixed with HSV2 or RSV, added to cooled cells, and incubated for 2 h at 4°C to ensure viral attachment but not entry. After two gentle washes, cells were overlaid with 1.2% methylcellulose medium and shifted to 37°C for 24 or 72 h. At the end of incubation, plaques were counted.

2.11.2 Entry assay. For entry assays, HSV-2 and RSV at a MOI of 0.001pfu/cell and 0.01 pfu/cell, respectively, were adsorbed for 2 h at 4°C on pre-chilled confluent Vero or A549 cells. Cells were then washed with cold MEM three times to remove unbound virus, treated with different concentrations of extract, and incubated for 3 h at 37°C. Unpenetrated viruses were inactivated with acidic glycine for 2 min at room temperature, as previously

described (Shogan et al., 2006). Cells were then washed with warm medium 3 times and treated as described above for plaque reduction assay.

2.11.3 Virus inactivation assay. Approximately 10^5 PFU of HSV2 or 10^4 PFU of RSV plus 100 µg/ml of Shilajit were added to MEM and mixed in a total volume of 100 µl. The virus-compound mixtures were incubated for 2 h at 37 °C or 4°C then diluted serially to the non-inhibitory concentration of test compound; the residual viral infectivity was determined by viral plaque assay.

2.12 Virus yield reduction assay. For the virus yield reduction assay, cells were pre-treated with Shilajit for 2 h at 37°C then infected in duplicate with HSV-2 at an MOI of 0.007 pfu/cell or RSV at an of MOI 0.005 pfu/cell in presence of Shilajit; following virus adsorption (2 or 3 h at 37°C), the viral inoculum was removed and cultures were exposed to Shilajit and incubated until control cultures displayed extensive cytopathology. Supernatants and cells were harvested and cell-free virus infectivity titers were determined in duplicate by plaque assay in Vero or A549 cell monolayers. The same experiment was also conducted with Shilajit only being added post-infection. Percent inhibition was determined by comparing the titer measured in the presence of the compounds to that measured in untreated wells.

2.13 Data analysis. All results are presented as the mean values from three independent experiments. The EC_{50} values for inhibition curves were calculated by regression analysis using the software GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, U.S.A.) by fitting a variable slope-sigmoidal dose–response curve.

3. RESULTS AND DISCUSSION.

To test whether Shilajit can affect HSV-1, HSV-2, HCMV, RSV, human rotavirus, and VSV infectivity *in vitro*, we used a complete protection assay in which the compound was added to the cell culture before, during, and after the infection. Shilajit exhibited dose-dependent inhibitory activity against HSV1, HSV2, HCMV, and RSV infectivity; the EC_{50} values are shown in Table 1 in comparison with those of reference antiviral compounds (i.e. acyclovir for HSV, foscarnet for HCMV, and heparin for RSV). Of note, Shilajit did not affect cell viability at concentrations as high as 1500 μg/mL, demonstrating that the antiviral effect was not a consequence of cytotoxicity. Although Shilajit proved active against three viruses belonging to the *Herpesviridae* family (i.e. HSV-1, HSV-2, and HCMV) and one of the *Paramyxoviridae* family (RSV), the lack of inhibitory activity against rotavirus (*Reoviridae*) and VSV (*Rhabdoviridae*) indicates that the spectrum of Shilajit's activity is specific to definite viruses.

Table 1. Antiviral activity of Shilajit and of reference compounds

	virus	**EC_{50} μg/mL*** **(95% C.I.)****	**SI*****
Shilajit	HSV-1	31.08 (23.64-40.87)	> 48.26
	HSV-2 acyclovir sensitive strain	12.85 (6.60-25.02)	> 116.7
	HSV-2 acyclovir resistant strain	14.39 (10.18-20.34)	> 104.24
	HCMV	34.54 (23.96-49.79)	> 43.43
	RSV	30.35 (18.41-50.04)	> 49.42
	Rotavirus	-	-
	VSV	-	-
Acyclovir	HSV-1	0.037 (0.025-0.055)	>2702

	HSV-2 acyclovir sensitive strain	0.14 (0.098-0.21)	>714
	HSV-2 acyclovir resistant strain	71.84 (41.3-101.4)	>1.41
Foscarnet	HCMV	5.17 (3.91-6.93)	>24.5
Heparin	RSV	0.048 (0.028-0.082)	> 685

* EC_{50}: 50% effective inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Moreover, as humic acid is a major component of Shilajit, we tested its antiviral activity. The results presented in Table 2 demonstrate that humic acid is endowed with antiviral activity against HSV-1, HSV-2 HCMV, and RSV with EC_{50} values that are lower than those calculated for Shilajit, with HCMV as the only exception. These results indicate that humic acid may contribute to the overall antiviral activity of Shilajit.

Table 2. Antiviral activity of humic acid

	virus	**EC_{50} μg/mL* (95% C.I.)****	**SI*****
Humic Acid	HSV-1	4.83 (2.87-8.13)	>311
	HSV-2 acyclovir sensitive strain	2.41 (1.89-3.08)	>622
	HCMV	38.3 (33.8-43.5)	24.6
	RSV	12.34 (10.41-14.63)	>39.2

* EC_{50}: 50% inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

To investigate Shilajit's mechanism of action we performed a series of time-of-addition assays, in which the compound was added to the cells only before, or during, or after infection. As shown in Figure 1, Shilajit exerted dose-dependent inhibitory activity only when added during infection with EC_{50} values of 26.74 μg/mL for HSV-2, 57.58 μg/mL for HSV-1, 41.73 μg/ml for HCMV, and 61.44 μg/mL for RSV. By contrast, inhibition was limited to the higher doses tested or was absent altogether in the pre-treatment or post-treatment assays, thus EC_{50} values could not be determined.

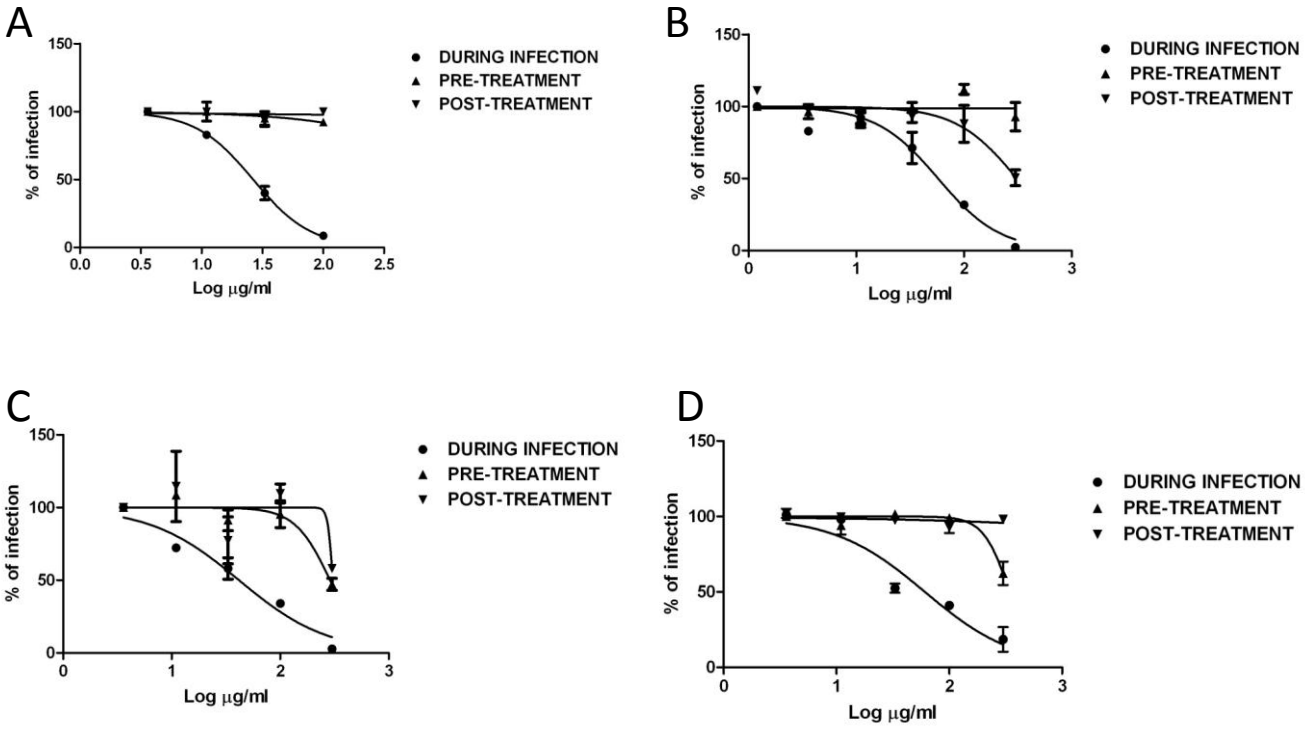

Fig.1 Time-of-addition assay. Vero (A-B), HELF (C), or A549 (D) cells were treated with Shilajit prior to virus infection (pre-treatment), during the infection period (during infection), or after infection (post-treatment) with HSV-2 (A), HSV-1 (B), HCMV (C), or RSV (D). Data are presented as % of control. Values are means ± SEM of three independent experiments.

Results from the time-of-addition assays suggest that Shilajit may either target the early steps of the virus replicative cycle (i.e. virus attachment or entry into cells) or act as a virucide by irreversibly inactivating the viral particles. To investigate these hypotheses, HSV-2 and RSV were chosen for

further experiments as representative viruses of the *Herpesviridae* and *Paramyxoviridae* virus families, respectively. First, we carried out an attachment assay, an experimental condition in which the virus is allowed to bind to the surface of the host cells, in the presence or absence of Shilajit, but not undergo cell entry. As shown in Fig. 2A, Shilajit inhibited HSV-2 and RSV infectivity with EC_{50} of 14.20 μg/ml and 25.70 μg/ml, respectively; values that are comparable to those reported in Table 1. This result indicates that the antiviral activity of Shilajit depends, at least in part, on its capacity to prevent the attachment of the viruses to the cell surface. By contrast, when the antiviral activity of Shilajit was tested using an entry assay in which Shilajit was added immediately after virus attachment to assess its ability to prevent entry, only a weak inhibition was observed at high doses (Fig. 2B).

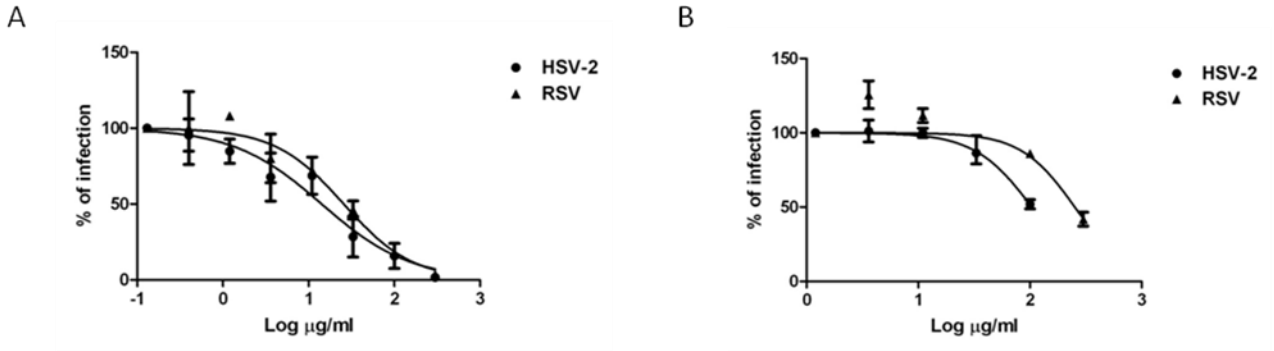

Fig.2 Attachment and entry assay. In the attachment assay (A), Vero cells were infected with HSV-2 and A549 cells were infected with RSV in presence of serial dilutions of Shilajit; inoculated cultures were then kept for 2 h at 4°C to allow virus attachment but not entry, and then tested in plaque reduction assays. In the entry assay (B), cells were infected in the absence of Shilajit and once again kept at 4°C for 2 h to allow virus attachment; serial dilutions of the compound were added to washed cells and the temperature then shifted to 37°C to allow entry. After a single wash with acidic glycine to remove virus particles from the cell surface, cells were overlaid with medium containing methylcellulose. Data are presented as % infectivity of control. Values are mean ± SEM of three separate determinations.

To explore the possibility that Shilajit also exerts direct virus-inactivating activity, a virucidal assay was performed using the effective concentration that reduced virus infection almost completely

(EC_{90}) in the standard assay. To this end, HSV-2 and RSV aliquots were incubated with 100 μg/ml Shilajit at 4°C or 37°C for 2 h. After incubation, the samples were titrated on Vero or A549 cells using high dilutions at which Shilajit was no longer active as an antiviral. When the incubations were carried out at 37°C, Shilajit produced a significant loss of both HSV-2 (71.2% inhibition) and RSV (74.4% inhibition) titers, although the treatment did not completely abrogate infectivity. This result indicates that partial virus inactivation contributes to the overall antiviral activity of Shilajit. By contrast, no virucidal activity was observed when the incubation was carried out at 4°C. This latter result rules out the possibility that the antiviral activity observed in the attachment assay, performed at 4°C, was due to a direct virus inactivation.

Some preliminary conclusions can be drawn on the main mode of antiviral action of Shilajit. Time-of-addition and virucidal assays indicate that the inhibitory effect mainly depends on the capacity of Shilajit to interact with the virus particles, rather than with cell components, thereby preventing virus attachment to the cell surface. This view is supported by several lines of evidence. First, cells pre-treated with Shilajit remained fully susceptible to virus infection, thus excluding the possibility that this compound could act by stably interacting with a cellular component(s) and thereby prevent its(their) interaction with viral glycoproteins. Second, the results of attachment and entry assays demonstrate that Shilajit blocks the adsorption of HSV-2 and RSV virions to the cell surface, but not entry. Third, pre-incubation of HSV-2 and RSV virions with Shilajit resulted in loss of infectivity, suggesting that both partial virus inactivation and interference with virus attachment contribute to the antiviral activity.

To complete the *in vitro* analysis of the antiviral potential of Shilajit against HSV-2 and RSV, the compound was also evaluated by viral yield reduction assay – a more stringent test which allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. The assay was conducted under two different conditions: 1) Shilajit was added before, during, and after infection (Fig 3 A-C); and 2) only after infection (Fig 3 B-D). In both cases, Shilajit was

active, with the strongest inhibition occurring in the first condition. This result indicates that besides preventing initial viral infection, Shilajit can also limit ongoing infection *in vitro*. This feature might be relevant for *in vivo* infections, characterized by the continuous release of virions by infected cells that promptly interact with neighboring cells, often resulting in direct cell-to-cell spread and syncytia formation. Of note, an acyclovir-resistant HSV-2 strain was as susceptible to Shilajit as the acyclovir-sensitive strain (Table 1), suggesting that the mode of antiviral action is different from that of acyclovir - a widely used inhibitor of the viral DNA polymerase. This latter feature, along with Shilajit's low cytotoxicity and favorable selectivity index make this natural compound a promising starting material for bioguided fractionation, with the aim of identifying anti-HSV-2 or anti-RSV compounds with novel mechanisms of action, which might also be used against acyclovir-resistant HSV-2 strains.

Overall, the results of the present study demonstrate that Shilajit constitutes a natural source of antiviral substances endowed with broad, yet specific, antiviral activity *in vitro* and are in line with the use of Shilajit as antiseptic and germicide in traditional medicine (Wilson et al., 2011). In this work, we demonstrated that humic acid, a major component of Shilajit, is endowed with antiviral activity, however, further work remains to be done to isolate the active constituents and elucidate their mechanism(s) of action.

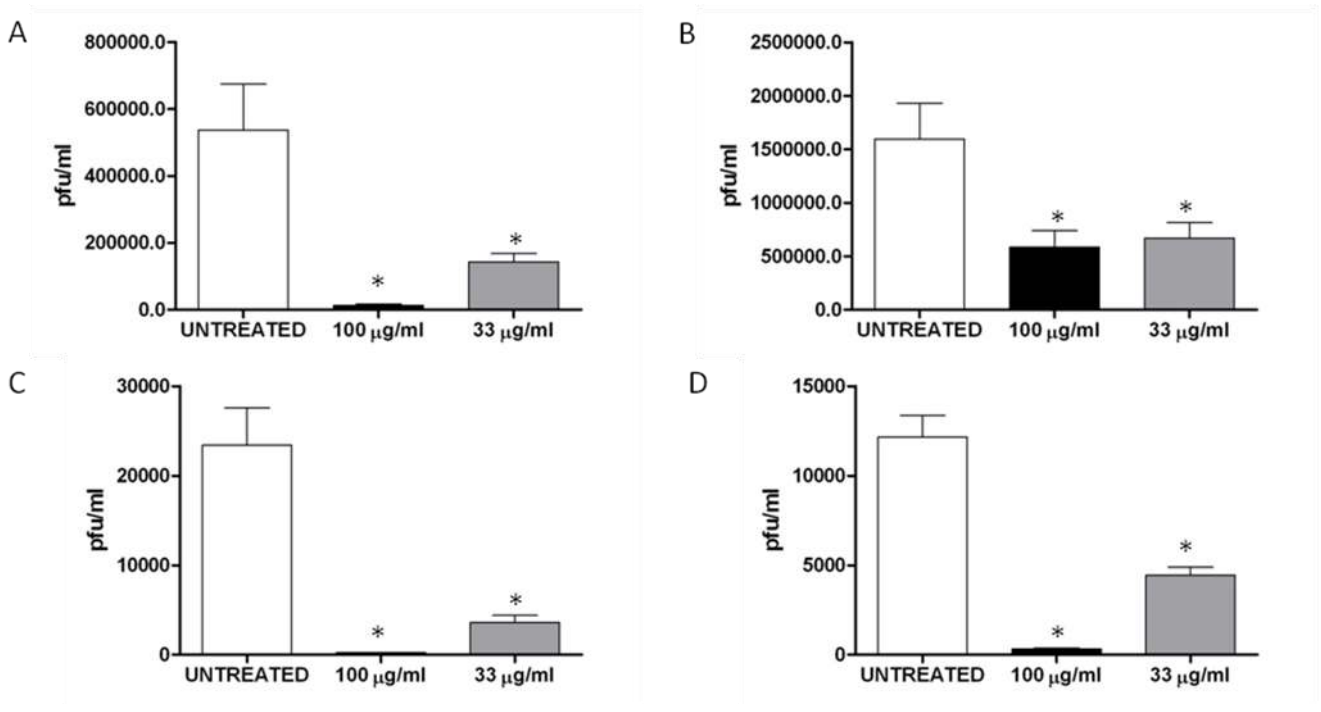

Fig. 3 Viral yield reduction. Panels A and B refer to Vero cells infected with HSV-2; while panels C and D refer to Hep-2 cells infected with RSV. Shilajit was added before, during, and after infection (A, C), or only after infection (B, D). When the cytopathic effect involved the whole monolayer of untreated cells, the supernatant was harvested and titrated. Plaques were counted and percent infection calculated by comparing treated with untreated (control) wells. Viral titers (expressed as PFU/ml) are shown as means plus SEM for three independent experiments. *, $P<0.05$.

ACKNOWLEDGEMENTS

We thank Mirella Grosso and Roberto Rubiolo for having provided the inspiration for this study.

We are grateful to Mr. Surya Krishna Shrestha (Production Department, Dekha herbals Lalitpur, Nepal) for having provided the chemical composition of the Shilajit analyzed in this study.

This work was supported by a grant from **"**Ricerca finanziata dall'Università degli Studi di Torino (ex 60%) 2012".

REFERENCES.

Agarwal, S.P., Khanna, R., Karmarkar, R., Anwer, M.K., Khar, R.K., 2007. *Shilajit*: a review. Phytotherapy Research 21, 401-405.

Collins, P.L., Crowe, J.E. Jr., 2007. Respiratory syncytial virus and metapneumovirus, in: Knipe, D.M., Howley, P.M. (Eds.) Fields Virology. 5th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1601–1646.

Cox, E., Christenson, J.C., 2012. Rotavirus. Pediatrics in Review 33, 439-445.

Donalisio, M., Rusnati, M., Cagno, V., Civra, A., Bugatti, A., Giuliani, A., Pirri, G., Volante, M., Papotti, M., Landolfo, S., Lembo, D., 2012. Inhibition of human respiratory syncytial virus infectivity by a dendrimeric heparan sulfate-binding peptide, Antimicrobial Agents and Chemotherapy 56, 5278-5288.

Donalisio, M., Nana, H.M., Ngono Ngane, R.A., Gatsing, D., Tiabou Tchinda, A., Rovito, R., Cagno, V., Cagliero, C., Boyom, F.F., Rubiolo, P., Bicchi, C., Lembo, D., 2013. In vitro anti-Herpes simplex virus activity of crude extract of the roots of *Nauclea latifolia Smith* (Rubiaceae). BMC Complementary and Alternative Medicine 13, 266.

Ghosal, S., 2006. Shilajit in perspective. first ed. Alpha Science International Limited. Oxford.

Graham, K.L., Zeng, W., Takada, Y., Jackson, D.C., Coulson, B.S., 2004. Effects on rotavirus cell binding and infection of monomeric and polymeric peptides containing alpha2beta1 and alphaxbeta2 integrin ligand sequences. Journal of Virology 78, 11786-11797.

Gupta, G.D., Sujatha, N., Dhanik, A., Rai, N.P., 2010. Clinical evaluation of *Shilajatu Rasayana* in patients with HIV infection. Ayu Journal 31, 28–32.

Klöcking, R., Helbig, B., 2005. Medical aspects and applications of humic substances. Biopolymers for medical and pharmaceutical applications.

Landolfo, S., Gariglio, M., Gribaudo, G., Lembo, D., 2003. The human cytomegalovirus. Pharmacology & Therapeutics 98, 269-297.

Rege, A.A., Ambaye, R.Y., Deshmukh, R.A., 2012. Evaluation of *in vitro* inhibitory effect of selected plants and *Shilajit* on HIV-Reverse Transcriptase. Indian Journal of Natural Products and Resources 3, 145-151.

Roizman, B., Knipe, M.M., Whitley, R.J., 2007. Herpes simplex viruses, in: Knipe, D.M., Howley, P.M. (Eds.) Fields Virology. 5th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 2501-2601.

Shogan, B., Kruse, L., Mulamba, G.B., Hu, A., Coen, D.M., 2006. Virucidal activity of a GT-rich oligonucleotide against herpes simplex virus mediated by glycoprotein B. Journal of Virology 80, 4740–4747.

Wilson, E., Rajamanickam, G.V., Dubey, G.P., Klose, P., Musial, F., Saha, F.J., Rampp, T., Michalsen, A., Dobos, G.J., 2011. Review on *Shilajit* used in traditional Indian medicine. Journal of Ethnopharmacology 136, 1-9.

FIGURE CAPTIONS

Fig.1 Time-of-addition assay. Vero (A-B), HELF (C), or A549 (D) cells were treated with Shilajit prior to virus infection (pre-treatment), during the infection period (during infection), or after infection (post-treatment) with HSV-2 (A), HSV-1 (B), HCMV (C), or RSV (D). Data are presented as % of control. Values are means ± SEM of three independent experiments.

Fig.2 Attachment and entry assay. In the attachment assay (A), Vero cells were infected with HSV-2 and A549 cells were infected with RSV in presence of serial dilutions of Shilajit; inoculated cultures were then kept for 2 h at 4°C to allow virus attachment but not entry, and then tested in plaque reduction assays. In the entry assay (B), cells were infected in the absence of Shilajit and once again kept at 4°C for 2 h to allow virus attachment; serial dilutions of the compound were added to washed cells and the temperature then shifted to 37°C to allow entry. After a single wash with acidic glycine to remove virus particles from the cell surface, cells were overlaid with medium containing methylcellulose. Data are presented as % infectivity of control. Values are mean ± SEM of three separate determinations.

Fig. 3 Viral yield reduction. Panels A and B refer to Vero cells infected with HSV-2; while panels C and D refer to Hep-2 cells infected with RSV. Shilajit was added before, during, and after infection (A, C), or only after infection (B, D). When the cytopathic effect involved the whole monolayer of untreated cells, the supernatant was harvested and titrated. Plaques were counted and percent infection calculated by comparing treated with untreated (control) wells. Viral titers (expressed as PFU/ml) are shown as means plus SEM for three independent experiments. *, $P<0.05$.

Table 1. Antiviral activity of Shilajit and of reference compounds

	virus	**EC_{50} μg/mL*** **(95% C.I.)****	**SI*****
Shilajit	HSV-1	31.08 (23.64-40.87)	> 48.26
	HSV-2 acyclovir sensitive strain	12.85 (6.60-25.02)	> 116.7
	HSV-2 acyclovir resistant strain	14.39 (10.18-20.34)	> 104.24
	HCMV	34.54 (23.96-49.79)	> 43.43
	RSV	30.35 (18.41-50.04)	> 49.42
	Rotavirus	-	-
	VSV	-	-
Acyclovir	HSV-1	0.037 (0.025-0.055)	>2702
	HSV-2 acyclovir sensitive strain	0.14 (0.098-0.21)	>714
	HSV-2 acyclovir resistant strain	71.84 (41.3-101.4)	>1.41
Foscarnet	HCMV	5.17 (3.91-6.93)	>24.5
Heparin	RSV	0.048 (0.028-0.082)	> 685

* EC_{50}: 50% effective inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Table 2. Antiviral activity of humic acid

	virus	**EC_{50} μg/mL*** **(95% C.I.)****	**SI*****
Humic Acid	HSV-1	4.83 (2.87-8.13)	>311
	HSV-2 acyclovir sensitive strain	2.41 (1.89-3.08)	>622
	HCMV	38.3 (33.8-43.5)	24.6
	RSV	12.34 (10.41-14.63)	>39.2

* EC_{50}: 50% inhibitory concentration

** 95% CI: 95% confidence interval

*** SI: selectivity index

Figure 1

Click here to download high resolution image

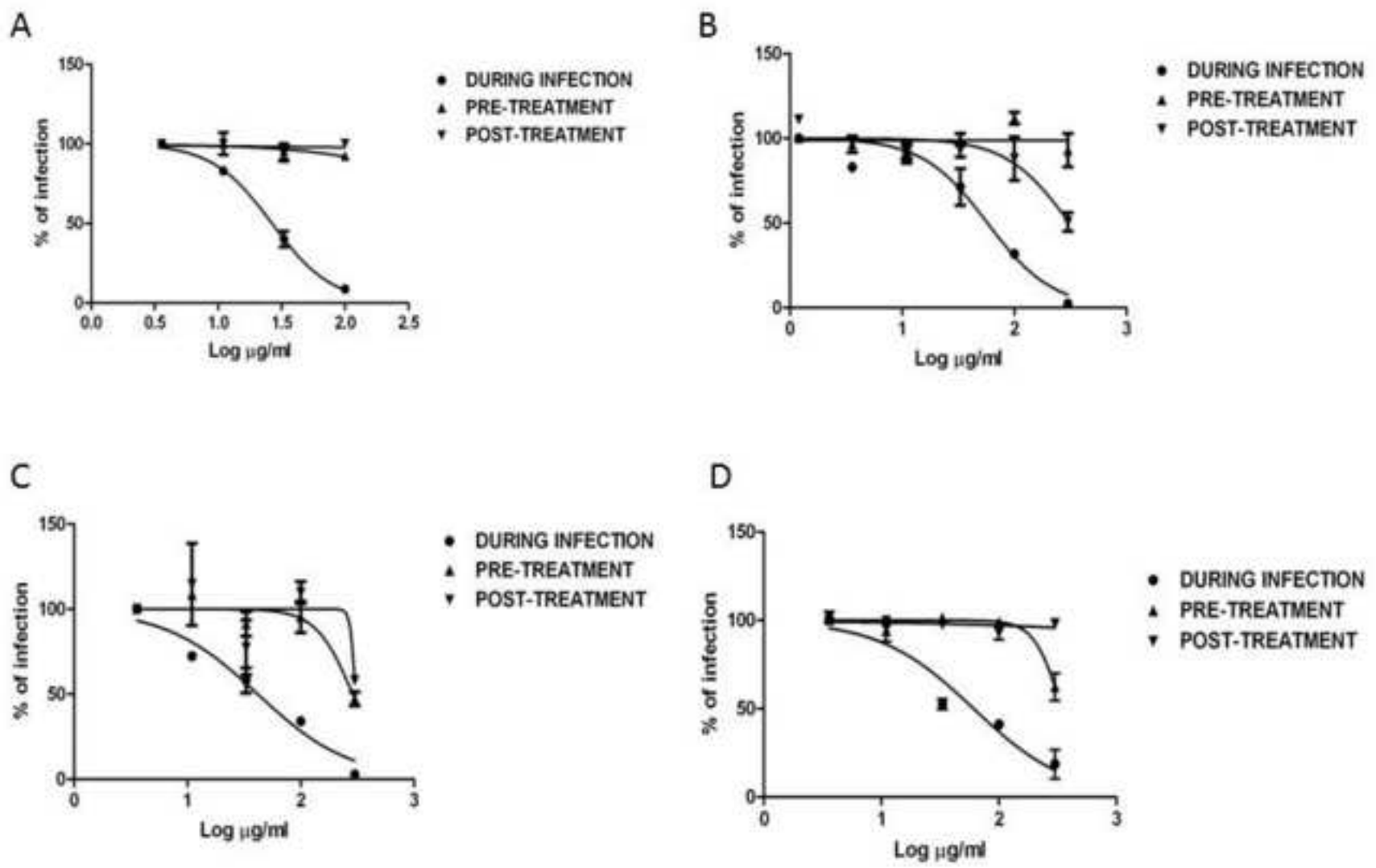

Figure 2

Click here to download high resolution image

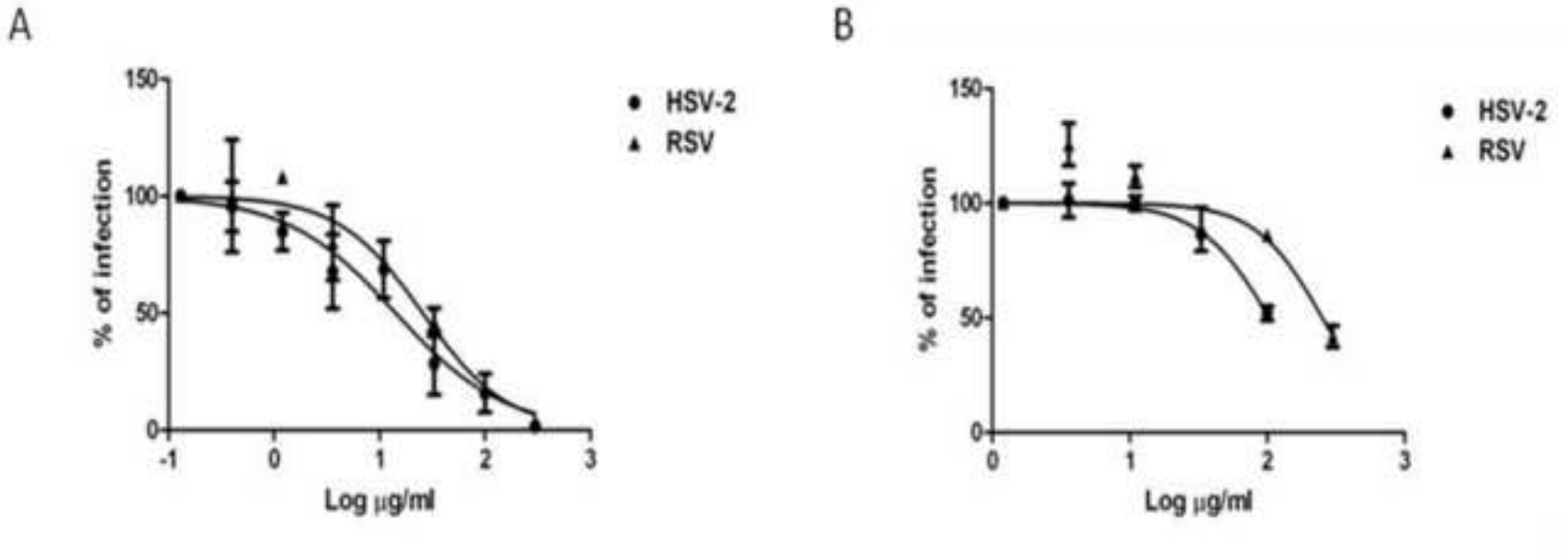

Figure 3

Click here to download high resolution image

