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

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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*.

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## 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 mountain 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

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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 is 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: previous *in vitro* studies have shown that humic substances and synthetic humic acids, derived from polyphenolic compounds, are effective against both naked and enveloped viruses In particular the oxidation product of caffeic acid, KOP, was active against HSV-1 *in vitro* and in a rabbit model of infection. These preliminary studies on humic acids-like polymers suggested a specific effect against the virus attachment to cells, an early stage of virus replication. Furthermore, an anti *in vitro* anti-HIV-1 activity has been shown for a low-molecular weight (1500) humic acid-like polymer, synthesized from hydroquinone (Klöcking and Helbig, 2005). Furthermore, Kotwal et al. (2005) described the broad-spectrum antiviral properties of a formulation called Secomet V, whose active ingredient is fulvic acid, the other major component of Shilajit with humic acid. 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, hydrobenzoin, carboxy ethane, ammonium benzoate, homocatechol, orcinol, β eudesmol, isodamascol, juniper camphor, and stearol. Shilajit was dissolved in bi-distilled sterile water to make a 25 mg/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-antimycotic 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 1 mM 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 min 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 $10 \times 10^4$ 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

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