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Antiviral Activity of a *Arisaema Tortuosum* Leaf Extract and Some of its Constituents against Herpes Simplex Virus Type 2

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ABSTRACT

Infections caused by HSV-2 are a public health concern worldwide, and there is still a great demand for the discovery of novel anti-herpes virus agents effective against strains resistant to current antiviral agents. In this context, medicinal plants represent an alternative source of active compounds for developing efficient antiviral therapies. The aim of this study was to evaluate the antiviral activity of *Arisaema tortuosum*, a plant used in the traditional medicine of India. A chloroform soluble fraction of the leaves exhibited anti-HSV-2 activity with a selectivity index of 758. The extract was also active against acyclovir-resistant HSV-2 and HSV-1. The mechanism of action of the extract was investigated evidencing inhibition of both early and late events of the HSV-2 replicative cycle. A HPLC-PDA-MS/MS analysis showed the presence of flavonoids including apigenin and luteolin in the chloroform extract (CE). Apigenin and luteolin showed a high inhibitory activity with EC_{50} values of 0.05 and 0.41 µg/mL, respectively. Both compounds exhibited antiviral activity when added up to 6 h post infection and were able to reduce the viral progeny production. In addition, apigenin interfered with cell-to-cell virus spread.

Introduction

Herpes simplex virus type 2 (HSV-2) is among the most prevalent sexually transmitted viruses. The Centers for Disease Control and Prevention (CDC) estimates that, annually, 776,000 people in the United States get new genital herpes infections, and more than 1 out of every 6 people aged 14 to 49 y have genital herpes [1]. Most HSV-2 infections are subclinical, but even without signs of the disease, herpes can still be spread to sex partners. Clinical manifestations are characterized by recurrent, painful lesions, named herpes sores, usually appearing on or around the genitals, rectum, or mouth. Genital herpes may cause painful genital ulcers that can be severe and persistent in immune suppressed people,

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ABBREVIATIONS

ADME-T	absorption, distribution, metabolism, excretion and toxicity
AE	aqueous extract
BE	*n*-butanol extract
CE	chloroform extract
EaE	ethyl acetate extract
EE	ethanolic extract
ESI	electrospray ionization
HE	*n*-hexane extract
HSV-1	herpes simplex virus type 1
HSV-2	HSV type 2
HSV-2	ACV-r HSV-2 strain with phenotypic resistance to acyclovir
MOI	multiplicity of infection
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium]
PFU	plaque-forming units
PIS	product ion scan
SI	selectivity index
SIM	selective ion monitoring
SRM	selected reaction monitoring

such as HIV-infected persons. Recurrent outbreaks of genital herpes are common during an individual's lifespan, since, after the primary infection, HSV-2 establishes a latency in the neurons of the sensory ganglia and it can be reactivated by several stimuli including immunosuppression, stress, and hormonal changes [2]. There is currently no cure or a commercially available vaccine for genital herpes. Antiviral medications, like acyclovir, valacyclovir, and famciclovir, can reduce the severity, shorten outbreaks, and decrease the rate of transmission to partners [3]. However, these synthetic nucleoside analogues are unable to suppress the latent infection, and their wide use favors the selection of drug-resistant HSV mutants. Genital herpes can be associated with resistance to available drugs especially in immunocompromised patients as result of extended viral replication and the reduced elimination of resistant strains [4]. Furthermore, acyclovir, the drug of choice for treating HSV infections, is characterized by a short half-life and incomplete absorption; therefore, it must be taken repeatedly throughout the day [5]. These drawbacks of available drugs prompt the discovery and development of novel anti-HSV-2 agents acting with a different mechanism of action than acyclovir and its derivatives, which are inhibitors of viral DNA replication, in order to improve the efficacy and pharmacokinetics of actual treatments. It is widely accepted that medicinal plants are rich sources of alternative molecules with large structural diversity and complexity for the treatment of infectious disorders. Natural products have been explored as HSV-2 inhibitors, which may be useful for the development of new drugs and act through different mechanisms of antiviral action [6, 7].

Arisaema tortuosum (Wall.) Schott, known as whipcord cobra lily, is a monocotyledon plant belonging to the Araceae family. It is characterized by a whip-like spadix arising from its "jack-in-the-pulpit" flower and grows to 30 cm in height [8]. This plant is widespread in rhododendron forest and alpine meadows in the Himalayas, Southern India, Myanmar, and Western China and even at elevations of 1500–2200 meters in Nepal [9, 10]. The plant tuber (dried powder and juice) has been used in traditional medicine of India for its therapeutic properties for piles, ailments related to digestive tract (constipation, indigestion, abdominal pain, and dysentery), rheumatism, snake bites, and in treating bone fracture and parasitic infections [11–13]. The tuber extract has shown antioxidant, anti-inflammatory, and antiproliferative activities; recently, *n*-hexane, chloroform, and ethyl acetate fractions of *A. tortuosum* have been investigated for their antibacterial and antifungal activities [14]. Previous works on *A. tortuosum* revealed the presence of flavonoids and lectins [9, 10]. Among the protein components, lectins purified from *A. tortuosum* tubers exerted *in vitro* antiproliferative activity against human cancer cell lines and anti-insect activity [9, 10, 15]. Recently, merged molecular docking, ADME-T, and dynamics approaches identified several *A. tortuosum* phytoconstituents as potential inhibitors of HSV-1 and HSV-2 enzymes, namely thymidine kinase and serine protease [16]. In this context, according to the need to continue searching for novel antiviral agents, this study was undertaken to explore whether *A. tortuosum* leaves and tubers extracts and their identified pure compounds, for which a commercial standard was available, exerted an effective antiviral activity against HSV-2 and ACV-r HSV-2 strains *in vitro*. Furthermore, their mode of antiviral action was investigated at different steps of the viral infection cycle.

Results and Discussion

This study investigated the antiviral activity of *A. tortuosum* extracts against HSV-2 infection *in vitro* on Vero cells. Firstly, ethanolic extracts (EE) of both leaves and tubers were prepared, and their effect on cellular viability was evaluated. Cell viability assays were performed on uninfected Vero cells, treated with 3 different concentrations (100, 10, 1 µg/mL). Since extracts were dissolved in DMSO, control wells were prepared by treating cells with equal volumes of DMSO. As shown in ▶ **Fig. 1 a**, total toxicity was evident at the highest concentration for both samples. On the other hand, partial toxicity was observed at 10 µg/mL for the tuber EE, while no toxic effect was detected for the leaf EE. Both extracts exhibited no toxicity at the lowest tested concentration. Based on these results, the EE of the leaves, which exhibit a lower toxicity compared to the tubers extract, was further fractionated using solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol). The extracts and the remaining aqueous fraction (AE) were submitted to viability assays. As reported in ▶ **Fig. 1 b**, the *n*-hexane (HE), ethyl acetate (EaE), and *n*-butanol (BE) extracts resulted highly toxic, whereas the chloroform extract (CE) and the AE did not have negative effects on cell viability at any tested concentrations. The nontoxic fractions from the leaves were selected to investigate their potential inhibitory activity against herpetic infections.

The antiviral assay was performed by treating cells in presence of decreasing concentrations of extracts before, during, and after viral infection in order to generate dose-response curves. Results
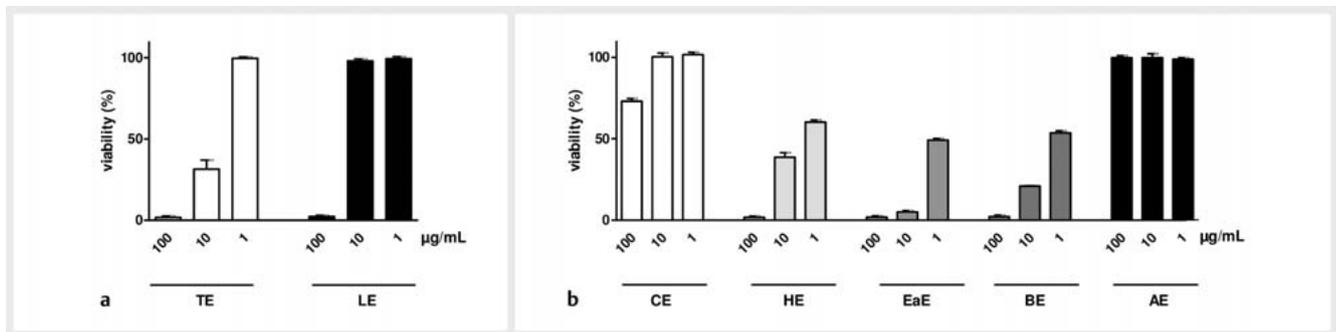
▶ **Fig. 1** Cell viability assays. Panel **a**: Evaluation of the effect of tuber (TE) and leaves (LE) EEs from *A. tortuosum* on Vero cells viability. Panel **b**: Evaluation of the effect of CE, *n*-hexane (HE), ethyl acetate (EaE), *n*-butanol (BE) fractions, and the remaining AE from LE on cellular viability. Data are reported as percentages of viable cells as determined by MTS assay for 100, 10, and 1 µg/mL concentrations. Three independent experiments were performed (n = 6).

▶ **Table 1** Antiviral activity of *A. tortuosum* extracts.

	Virus	EC$_{50}$[a] (µg/mL) (95% CI[b])	CC$_{50}$[c] (µg/mL)	SI[d]
CE leaves	HSV-2	0.53 (0.41–0.69)	402	758
	HSV-1	2.64 (0.74–9.37)	31.8	12
	HSV-2 ACV-r[e]	0.86 (0.69–1.06)	402	467.4
AE leaves	HSV-2	n. a.[f]	1852	n. a.
	HSV-1	n. a.	n. a.	n. a.
	HSV-2 ACV-r	n. a.	1852	n. a.

[a] EC$_{50}$: half maximal effective concentration, [b] CI: confidence interval, [c] CC$_{50}$: half maximal cytotoxic concentration, [d] SI: selectivity index, [e] HSV-2 ACV-r: acyclovir resistant HSV-2 strain, [f] n. a.: not assessable

of the viral plaque reduction assays showed that the CE from the leaves was endowed with anti-HSV-2 activity with EC$_{50}$ value of 0.53 µg/mL (▶ **Table 1**). The antiviral activity of CE was not a consequence of a cytotoxic effect since the active fraction showed a favorable SI value of 758. By contrast, no significant antiviral activity was reported for AE at any concentrations tested. Of note, CE exerted an inhibitory effect also against HSV-1, although with higher EC$_{50}$ than against HSV-2, indicating a broad spectrum of activity against herpes viruses. Given the emerging appearance of resistant viral strains to available drugs, the activity of CE against an HSV-2 ACV-r was assessed. Interestingly, the extract inhibited HSV-2 ACV-r infection with an EC$_{50}$ value of 0.86 µg/mL (▶ **Table 1**). These data suggested that the mode of antiviral action of the investigated fraction differs from that of acyclovir.

In order to identify the major mechanism of action, the CE was subjected to further studies. First, a virus inactivation assay was performed to explore whether the extract directly inactivates HSV-2 particles. To this end, the virus was incubated with a high concentration of extract that reduces virus infection almost completely at 37 °C for 0 or 2 h and at 4 °C for 2 h, and then the viral titer was measured at dilutions corresponding to concentrations that are not antiviral when added on cells. Results depicted in ▶ **Fig. 2** demonstrate that CE did not inactivate significantly the virus particles at any incubation conditions tested. Therefore, we
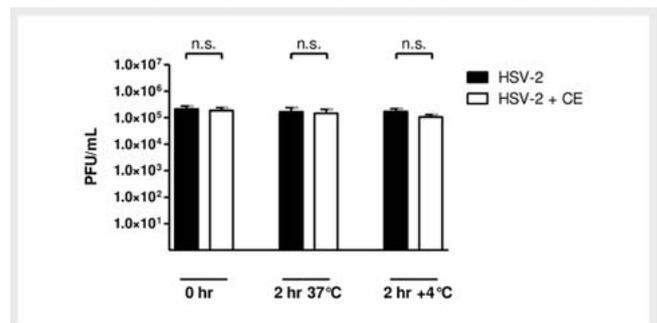

▶ **Fig. 2** Evaluation of inactivation of HSV-2 particles by *A. tortuosum* CE at 37 °C for 0 or 2 h, and at 4 °C for 2 h. On y-axis, the infectious titers are expressed as PFU per mL. Error bars represent standard error of the mean for 3 independent experiments. (n = 6).

investigated if the active extract targets cell surface or intracellular events involved in essential steps of the viral replicative cycles. To explore this hypothesis, time of addition assays were performed by treating cells with serial dilutions of the extract 2 h before infection or during infection or post-infection. As shown in ▶ **Fig. 3** (panel A), CE did not exhibit anti-HSV-2 activity when Vero cells were pretreated with scalar doses of the fraction prior
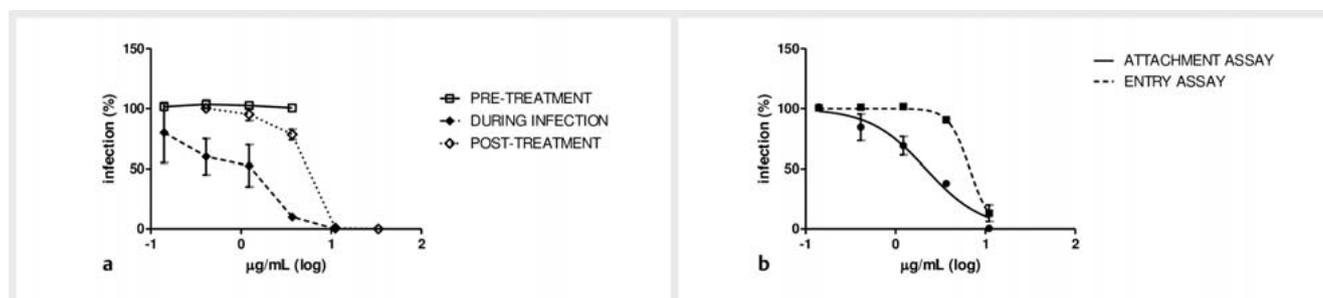
► **Fig. 3** Investigation of mechanism of action of *A. tortuosum* CE on replicative cycle of HSV-2. Panel **a**: Time of addition assays for the extract. Panel **b**: Attachment and entry assay for the extract. Error bars represent the standard error of the mean for 3 independent experiments. (n = 6).
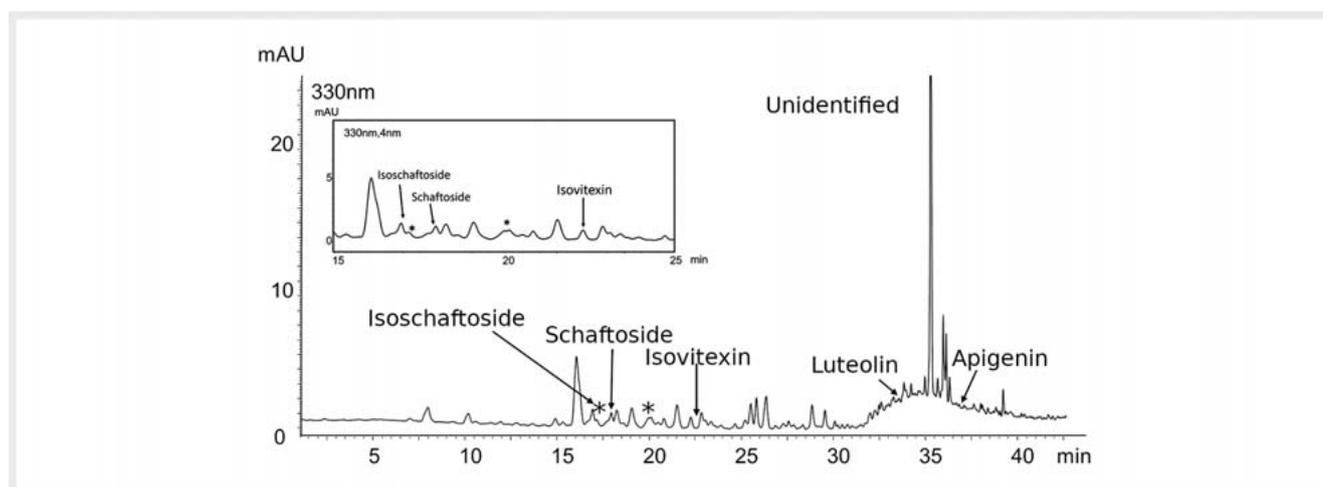
► **Fig. 4** HPLC-PDA representative profile (λ = 330 nm) of the CE of the leaves of *A. tortuosum*. (*) indicates the tentative identification of apigenin *C*-hexoside-*C*-pentoside (MW = 564) isomers.

to viral infection excluding a stable interaction of the extract with cellular components. Treatment for 2 h concomitant to HSV-2 infection showed that CE exerted net antiviral activity with an EC_{50} value of 0.77 µg/mL. These data suggest a probable activity of CE on the early steps of HSV-2 infection. Having excluded the viral particle as a target of the antiviral activity, more stringent tests (*i.e.*, the attachment and entry assays) were performed to assess if the putative target of CE was the virus-cell interactions. This hypothesis was confirmed as reported in ► **Fig. 3b**: CE inhibited both the viral attachment and entry in the cell generating dose-response curves with EC_{50} values of 2.17 and 6.69 µg/mL, respectively. When it was added to cells immediately after the HSV-2 infection, CE showed relevant anti-HSV-2 activity, with an EC_{50} value of 4.75 µg/mL (► **Fig. 3a**), indicating that later steps of the virus replicative cycle are inhibited. Taken together, the results evidenced that CE exerts its antiviral activity by inhibiting both early and late events of the HSV-2 replicative cycle.

The phytochemical composition of the CE of *A. tortuosum* leaves was investigated through HPLC-PDA-MS/MS (► **Fig. 4**). Several flavonoids, in particular luteolin and apigenin derivatives, were detected. Identification was based on the UV, MS, and MS/MS spectral information and comparison to authentic standards, when available, or to literature data (► **Table 2**). When a corre-

spondence between the positive and negative pseudo molecular ions in ESI+ and ESI− was present, a product ion scan analysis (PIS) of the pseudo molecular ions (in both ESI+ and ESI− modes) was performed, providing diagnostic fragments for each molecule. Isoschaftoside, schaftoside, isovitexin, luteolin, and apigenin were confirmed by the co-injection of the corresponding authentic standards, while other peaks were tentatively assigned as apigenin *C*-hexoside-*C*-pentoside isomers, apigenin *C*-dihexoside, and luteolin/kaempferol *O*-hexoside, based on the UV, MS, and MS/MS spectral information [17, 18]. As an example, the UV maximum absorptions at 330/271 nm, the molecular weight of 564, and the fragments obtained in the PIS analysis, similar to those of isoschaftoside and schaftoside standards, support the tentative identification of apigenin *C*-hexoside-*C*-pentoside isomers (► **Table 2**). A main unidentified peak with a UV maximum absorption at 313 nm and a molecular weight of 308 amu was detected. The UV and MS spectra together with the PIS analysis of the positive (*m/z* 309) and negative (*m/z* 307) pseudomolecular ions are provided as supporting information (Fig. 1S, Supporting Information). The presence of apigenin C-glycoside derivatives, in particular schaftoside and isoschaftoside, had been previously reported in Arisaema species [19].

▶ **Table 2** List of identified and putatively-identified compounds in *A. tortuosum* extracts.

Compound	UV max	MW	PIS ESI$^+$	PIS·ESI$^-$
Isochaftoside* (apigenin 6-*C*-arabinosyl-8-*C*-glucoside)	333/271	564	427/391/349/325/295	473/443/383/353
Schaftoside* (apigenin 6-*C*-glucosyl-8-*C*-arabinoside)	337/271	564	469/433/397/379/349/325/295	473/443/412/383/353/323
Apigenin *C*-hexoside-*C*-pentoside	330/271	564	445/379/325/391/295	473/383/353
Luteolin/kaempferol *O*-hexoside	267/330	448	287	285
Isovitexin*(apigenin 6-*C*-glucoside)	330/271	432	313/309/295/283	341/309/294/283/280.8/194/121
Luteolin*	349/252/266	286	153/133/117	151/133/107
Unidentified	313	308	309:166/145/117/107	307:215/197/169/121
Apigenin*	337/266	270	163/153/121/119	156/117

For each compound, the UV spectrum, pseudomolecular ions, molecular weight fragments obtained by PIS mode, and identified or tentatively-identified compound names, are indicated. *Compound identification confirmed by the co-injection of authentic standards

▶ **Table 3** Antiviral activity of constituents from *A. tortuosum*.

| Constituents	Virus	EC$_{50}$[a] (µg/mL) (95% CI[b])	CC$_{50}$[c] (µg/mL)	SI[d]
Isoschaftoside	HSV-2	>33	>33	>1
	HSV-1	>33	>33	>1
	HSV-2 ACV-r[e]	>33	>33	>1
Schaftoside	HSV-2	>33	>33	>1
	HSV-1	>33	>33	>1
	HSV-2 ACV-r	>33	>33	>1
Isovitexin	HSV-2	>33	>33	>1
	HSV-1	>33	>33	>1
	HSV-2 ACV-r	>33	>33	>1
Apigenin	HSV-2	0.05 (0.03–0.07)	5.12	102.4
	HSV-1	7.04 (0.61–81.1)	12.9	1.8
	HSV-2 ACV-r	2.33 (1.65–3.29)	5.12	2.1
Luteolin	HSV-2	0.41 (0.35–0.45)	7.35	17.9
	HSV-1	n.a.[f]	n.a.	n.a.
	HSV-2 ACV-r	1.55 (0.89–2.67)	7.35	4.7
Acyclovir[g]	HSV-2	0.1 (0.07–0.27)	>33	>330
	HSV-1	n.a.	n.a.	n.a.
	HSV-2 ACV-r	>33	>33	>1

[a] EC$_{50}$: half maximal effective concentration, [b] CI: confidence interval, [c] CC$_{50}$: half maximal cytotoxic concentration, [d] SI: selectivity index, [e] HSV-2 ACV-r: acyclovir resistant HSV-2 strain, [f] n.a.: not assessable, [g] acyclovir was used as positive control

Commercially available standards of those components identified in the extracts as isoschaftoside, schaftoside, isovitexin, apigenin, and luteolin were tested for their antiviral activity against HSV-2 infection. Among them, the apigenin *C*-glycosides isoschaftoside, schaftoside, and isovitexin did not exert a significant antiviral activity against HSV-2 infection (▶ **Table 3**). Conversely, apigenin and luteolin showed a high inhibitory activity with an EC$_{50}$ of 0.05 and 0.41 µg/mL, respectively, which confirmed the anti-HSV-2 effect reported in literature, although we observed a more favorable SI of apigenin with a value of 102.4 than that re-

ported by Chiang et al. and Visintini et al. [20–23]. ▶ **Table 3** showed that apigenin was active not only against HSV-2 but also against HSV-1 (EC$_{50}$, 7.04 µg/mL). By contrast, although luteolin has been identified as a promising inhibitor of HSV-1 using *in silico* approaches [16], no anti-HSV-1 activity was observed in cell-based assay here reported. Interestingly, the HSV-2 ACV-r strain's infectivity was inhibited by apigenin and luteolin with EC$_{50}$ values of 2.33 and 1.55 µg/mL, respectively. According to literature, the mode of antiviral action of apigenin has never been investigated, whereas a study reported the modulation of the NF-κB signaling

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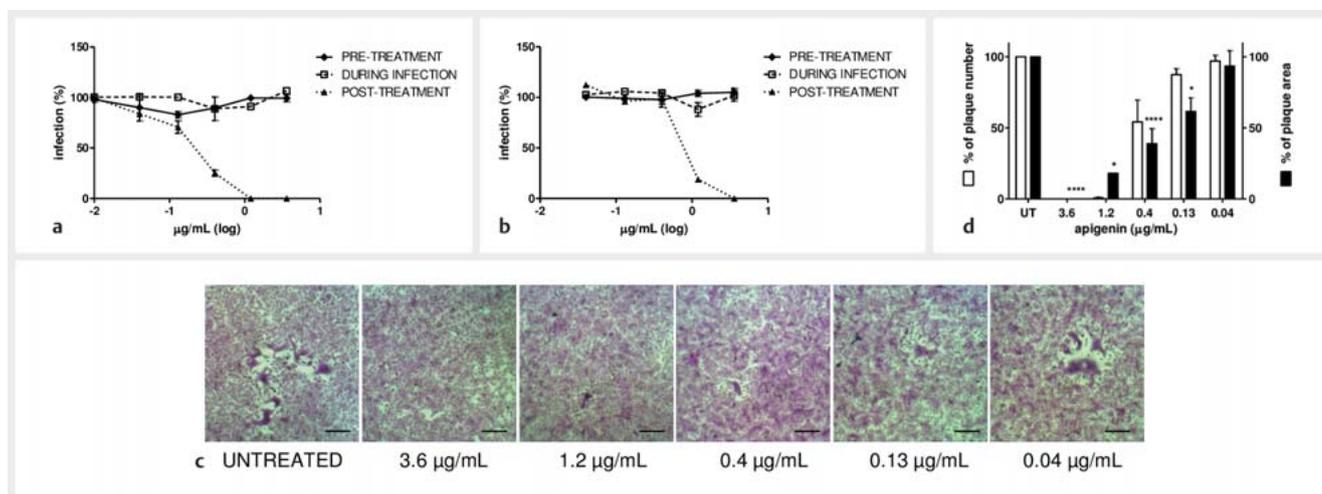
▶ **Fig. 5** Time of addition assays for apigenin and luteolin. Vero cells were treated with apigenin (panel **a**) or luteolin (panel **b**) prior to HSV-2 infection (pre-treatment), during the infection period (during infection), or after the infection (post-treatment). Error bars represent the standard error of the mean for 3 independent experiments. (n = 6). Panel **c**: Representative HSV-2 plaques in Vero cells are reported according to the concentrations of apigenin in the post-treatment assay. Scale bar, 150 µm. Panel **d**: The histograms show the percentage of HSV-2 plaque areas (black) and plaque numbers (white) of treated wells in comparison to that of untreated wells as a function of the concentration of apigenin in the post-treatment assay. The pictures and histograms reported are representative of ≥ 10 plaques per condition. *p < 0.05; **** p < 0.0001, as compared to the untreated by one-way analysis of variance. UT, untreated.
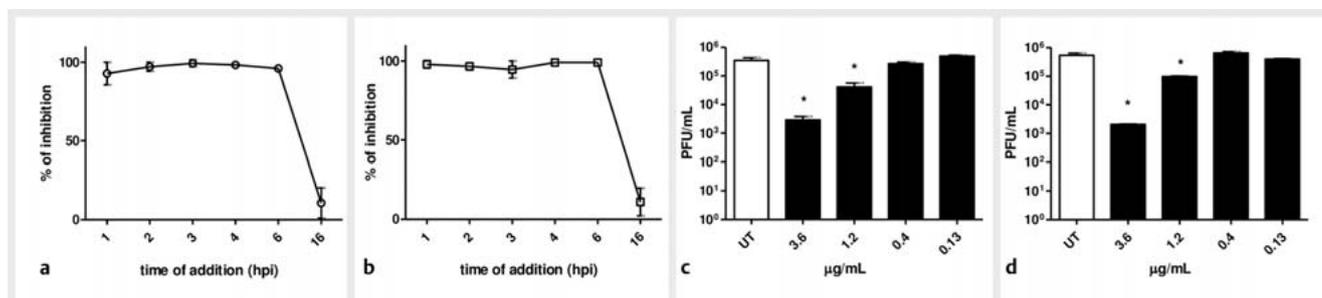
▶ **Fig. 6** Post entry assays for apigenin and luteolin. Vero cells were treated with apigenin (panel **a**) or luteolin (panel **b**) at EC_{90} concentrations at 1, 2, 3, 4, 6, 16 h post HSV-2 infection (hpi). Data are reported as percentages of HSV-2 plaques in comparison to control. Error bars represent the standard error of the mean for 3 independent experiments. (n = 6). Effects of apigenin (panel **c**) and luteolin (panel **d**) on multiple cycles of HSV-2 replication by yield reduction assay. Viral titers are expressed as PFU/mL and shown as mean ± standard error for 3 independent experiments. *p < 0.05 as compared to the untreated (UT) by one-way analysis of variance.

pathway by luteolin in infected cells [23]. We therefore explored which steps of HSV-2 replicative cycle were inhibited by these 2 active compounds and their contribution to the anti-HSV-2 activity of the CE of *A. tortuosum* leaves. Virucidal assays demonstrated that both apigenin and luteolin did not inactivate the virus particles (**Fig. 2S**, Supporting Information) and showed inhibition (EC_{50} 0.408 and 0.246 µg/mL, respectively) only when added to the cells after virus inoculum in time-of-addition assays (▶ **Fig. 5 a, b**). The absence of inhibitory activity by treating cells with compounds before infection or during infection excludes a probable activity on the early steps of HSV-2 infection. To substantiate this interpretation, we demonstrated by specific assays that apigenin and luteolin did not inhibit the attachment of the virus to the cell surface and the entry in Vero cells (**Fig. 3S**, Sup-

porting Information). By contrast, Ojha et al. evidenced that luteolin isolated from *Pedilanthus tithymaloides* exerted inhibitory effect when the drug was added 2 h prior to infection [23]. Our results of time of addition assays indicated that both compounds affect late steps of the replicative cycle of HSV-2. Furthermore, when apigenin was added on cells after infection, not only a dose response reduction of viral plaques was observed, but also the area of the remaining plaques decreased in a dose-dependent manner (▶ **Fig. 5 c, d**), suggesting that apigenin interferes with cell-to-cell virus spreading. Moreover, we tested the inhibitory activity of apigenin and luteolin by viral plaque reduction assays when they were added to the cells at different time points, from 1 to 16 h after removal of the virus (▶ **Fig. 6 a, b**, respectively). Interestingly, we observed that around 90% inhibitory activity was

still present 6 h post-infection at the EC_{90} concentration tested of both compounds. To evaluate whether the apigenin and luteolin treatment lead to a decrease in viral progeny production, post entry assays measuring the virus yield were performed. Under these experimental conditions, the 2 pure compounds significantly reduced HSV-2 titers at high tested concentrations (3.6 and 1.2 µg/mL) as expression of their ability to inhibit multiple cycles of viral replication and limit the production of infectious virus particles (▶ **Fig. 6 c, d**).

Taken together, our data indicate that apigenin and luteolin exert an inhibitory effect on post entry steps of the virus replicative cycle, suggesting a contribution to the *A. tortuosum* CE antiviral activity against the late steps of HSV-2 replication. The 2 compounds were quantified in the extract by HPLC-MS/MS, by applying 2 acquisition methods (SRM, SIM) to confirm the accuracy of the obtained data. Quantification was expressed as the mean of the results obtained in both acquisition methods (RSD% of 12%) and showed similar apigenin and luteolin contents in the extract (0.025 µg/mg ± 0.003 and 0.026 µg/mg ± 0.003 for apigenin and luteolin respectively; n = 3). Although apigenin's and luteolin's structures differ only in the presence of the 3′-OH, a higher inhibitory activity of apigenin compared to luteolin was observed against HSV-2 (▶ **Table 3**). In this context, the higher hydrophobicity of apigenin (logP 3.07) compared to luteolin (logP 2.73) and, consequently, the expected greater ability of crossing the cell membrane should be pointed out. It is noteworthy that none of the tested pure apigenin *C*-glycosides were active. Apigenin and luteolin are minor constituents of the CE and may not account alone for the antiviral activity of the extract. Therefore, further studies will be required to investigate the antiviral role of other components of *A. tortuosum* CE involved in the inhibition of early events of the HSV-2 infection.

Overall, this study reports for the first time an inhibitory activity of *A. tortuosum* leaves extract against a viral infection, in particular against herpetic infections. Among the detected components, apigenin is an interesting candidate given its antiviral activity and its ability to prevent cell-to-cell virus spread and to reduce the viral progeny production. These promising results should prompt *in vivo* studies to further assess its potential for the treatment of HSV-2 infection.

Materials and Methods

Chemicals

Acyclovir, used as a positive control for HSV-2 antiviral activity, acetonitrile (LC-MS grade), formic acid (≥ 98% HPLC purity), and apigenin (≥ 99% HPLC purity) were purchased from Sigma-Aldrich. Luteolin (≥ 99% HPLC purity), isovitexin (≥ 99% HPLC purity), schaftoside (≥ 90% HPLC purity), and isoschaftoside (≥ 95% HPLC purity) were from Extrasynthese. Deionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore).

Plant material

Leaves and tubers of *A. tortuosum* were collected in Bagsiad Village (31.561, 77.123), Mandi, Himachal Pradesh, India, in August and September 2016. A specimen was authenticated at Raw Materials Herbarium & Museum, Delhi (RMHD) CSIR-NISCAIR, India. Voucher specimens were deposited at the herbarium (NISCAIR/RHMD/Consult/2013/-2249/30). The plant parts were cleaned, shade dried, and properly grounded to coarse powder.

Extracts preparation

Powdered leaves and tubers (1 kg each) were extracted with ethanol: water (95 : 5, v/v) with a 1 : 10 ratio of herbal substance to extraction solvent. The ethanol solutions were combined and dried in a rotary evaporator at 40 ± 5 °C to provide the crude extracts (77 and 68 g for the leaves and tubers, respectively). The EE from the leaves was further partitioned using different solvents to yield the HE (26 g), CE (17.4 g), EaE (9.2 g), and BE (12 g) extracts (**Fig. 4S**, Supporting Information). The extracts were then lyophilized and kept in the dark at + 4 °C until further use. For the viability and antiviral assays, the extracts were dissolved in DMSO (except for the AE fractions, which were dissolved in water) to a final concentration of 25 mg/mL, and used as the stock solutions.

Phytochemical analysis

For HPLC-PDA-MS/MS analysis, a 10 mg/mL stock solution of the leaf CE was prepared in acetone 100%. A 2.5 mg/mL working solution was then prepared in methanol:H_2O 50:50, filtered through a 13 mm diameter, 0.22 µm pore diameter, hydrophilic PTFE syringe filter, and subjected to HPLC-PDA-MS/MS analysis. The working solution (5 µL) was analyzed in duplicate on a Shimadzu Nexera X2 system equipped with a photodiode array detector SPD-M20A and coupled in series to a triple quadruple Shimadzu LCMS-8040 system with ESI source. The separation was performed with an Ascentis Express RP-Amide column (10 cm × 2.1 mm, 2.7 µm, Supelco) using water (A) and acetonitrile (B) both containing 0.1% formic acid as mobile phase. The flow rate was 0.4 mL/min, and the column temperature was maintained at 30 °C. The gradient program was as follows: 5% B for 3 min, 5–15% B in 17 min, 15–25% B in 10 min, 25–75% B in 12 min, 75–100% B in 10 min, 100% B for 1 min. The total separation time including pre-run and post-run was 60 min. UV spectra were acquired over the 220–450 nm wavelength range. MS operative conditions were as follows: heat block temperature: 200 °C; desolvation line (DL) temperature: 250 °C; nebulizer gas flow rate: 3 L/min; drying gas flow rate: 15 L/min. Mass spectra were acquired both in positive and in negative full-scan mode over the range 100–1000 *m/z*, event time 0.5 s. PIS mode (collision energy: − 35.0 V for ESI+ and 35.0 V for ESI−, event time: 0.2 s) was applied to compounds for which a correspondence between the pseudomolecular ions [M + H]+ in ESI+ and [M – H]− in ESI− was confirmed. Luteolin and apigenin were quantified in the extract through SRM acquisition in ESI− (collision energy: 35.0 V, dwell time: 5 msec) on specific ion products derived from precursor ion fragmentation and SIM acquisition (dwell time: 5 msec) of precursor ions. The transitions and the ions selected, based on the PIS analysis of each standard, were as follows: apigenin (SRM: *m/z* 269 → 117; SIM: *m/z* 269), luteolin (SRM: *m/z* 285 → 133; SIM: *m/z* 285) (**Fig. 5S**, Supporting Information). The calibration curves were prepared with 5 different concentrations in the range of 0.001–0.5 µg/mL and with 4 different concentrations in the range of 0.01–0.5 µg/mL for the SRM and SIM acquisition mode, respec-

tively. The calibration curve equations were as follows: apigenin SRM: $y = 1\,277\,518.75\,x + 6432.02$, $R2 = 0.9996$; SIM: $y = 11\,702\,570.51x + 89\,688.49$, $R2 = 0.9998$; luteolin SRM: $y = 1\,143\,071.98x + 3815.38$, $R2 = 0.9996$; SIM: $y = 12\,392\,796.81x + 85\,938.53$, $R2 = 0.9998$. All quantification results are reported in µg/mg of extract and correspond to the mean of the contents determined with SRM and SIM, respectively. All analyses were carried out in triplicate and data expressed as mean values ± standard deviation.

Cell culture

African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81) were grown as monolayers in Eagle's Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic solution (penicillin-streptomycin, Sigma-Aldrich) in humidified 5% CO_2 atmosphere at 37 °C.

Viruses

Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV strains were propagated and titered as reported in Cavalli et al. [24]. A HSV-2 ACV-r was generated as previously described [25].

Viability assay

Cell viability was measured using an MTS assay as described in Donalisio et al. [25]. The 50% cytotoxic concentrations (CC_{50}) and the 95% confidence intervals were determined using Prism software (Graph-Pad Software).

Virus plaque reduction assay

Antiviral activity of extracts and pure compounds was determined by viral plaque reduction as reported in Cagno et al. [26]. Acyclovir was tested in parallel as a reference. Results are reported as percentages of viral plaques in comparison to controls for each concentration challenged. The concentration of extracts and pure compounds that reduced viral infectivity by 50% (half maximal effective concentration, EC_{50}) and concentrations that reduced viral infectivity by 90% (effective concentration-90, EC_{90}) were calculated by the Prism program to fit a variable slope-sigmoidal dose-response curve. The results reported are for 3 experiments performed in duplicate. The SI was calculated by dividing the CC_{50} by the EC_{50} value.

Virus inactivation assay

Extracts and pure compounds were incubated with HSV-2 (1×10^5 PFU) at concentrations corresponding to EC_{90} values (µg/mL) as obtained in the viral plaque reduction assay. The mixtures were incubated for 0 h at 37 °C, and for 2 h at 4 °C and 37 °C. Dilutions from both treated- and untreated-HSV-2 aliquots were titrated to non-inhibitory concentrations. Viral infectivity was determined microscopically by PFU quantification. Viral titers were reported as PFU per milliliter, and compared.

Time-addition assay

Serial dilutions of extracts and pure compounds were added to cells: 2 h before infection, during infection, or post-infection. After the incubation time, viral plaques were counted.

Attachment assay

The attachment assay was performed as reported in Cagno et al. with several modifications [26]. After infection, unpenetrated HSV-2 virions were inactivated with acidic glycine (0.1 M glycine, 0.14 M NaCl, pH 3) for 1 min at room temperature.

Entry assay

The entry assay was performed as reported in Cagno et al. [26]. The virus was adsorbed for 2 h at 4 °C on cells. After 3 washes to remove unbound virus, cells were treated with extract or pure compound for 3 h at 37 °C. Unpenetrated viruses were inactivated with acidic glycine.

Post entry assay

Pre-chilled Vero cells were infected with HSV-2 (MOI 0.005) for 2 h at +4 °C. After 2 washes, fresh 2% FBS MEM was added, and the monolayers were moved to 37 °C to allow virus internalization. Unpenetrated HSV-2 virions were inactivated with acidic glycine; cells were then washed with PBS and pure compounds at concentrations corresponding to EC_{90} values (µg/mL) were added at 0, 1, 2, 3, 4, 6, and 16 hpi in MEM 2% FBS, 1.2% methylcellulose, and incubated at 37 °C for 24 h.

Virus yield reduction assay

The assay was performed as reported in Cagno et al. [26]. Cells were treated with compounds, infected in presence of compounds, and incubated until control cultures displayed extensive cytopathology. Supernatants and cells were harvested, and cell-free virus infectivity titers were determined.

Statistical analysis

Data were analyzed using Student's t-test, ANOVA analysis of variance, or F-test, as reported in the legends of tables and figures, on GraphPad Prism version 5.00 software. Significance was reported for p-value < 0.05. The Student's t-test was used to compare HSV-2 titers in virus inactivation assays; analysis of variance followed by Bonferroni multiple comparison test was used to compare HSV-2 titers in virus yield reduction assays and to compare the percentages of plaque area in the post-treatment assay.

Supporting information

UV spectrum, mass spectrum, and PIS analysis of the main peak in the extract (**Fig. 1S**); evaluation of inactivation of HSV-2 particles by apigenin and luteolin (**Fig. 2S**); HSV-2 attachment and entry assays for apigenin and luteolin (**Fig. 3S**); schematic representation of the extraction procedure adopted for *A. tortuosum* leaves and tubers (**Fig. 4S**); and SRM profiles of luteolin and apigenin (**Fig. 5S**) are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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