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*Hemidesmus indicus* (L.) R. Br. extract inhibits the early step of herpes simplex type 1 and type 2 replication

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**Running title:** *Hemidesmus indicus* extract blocks HSV infection

**SUMMARY**

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) cause several clinically relevant syndromes in both adults and neonates. Despite the availability of efficient anti-HSV agents, the search for new therapeutic approaches is highly encouraged due to the increasing drug resistance of virus strains. Medicinal plants represent a source of potential bioactive compounds. In this context we evaluated the anti-herpetic activity of *Hemidesmus indicus* (L.) R. Br., a plant widely used in traditional Indian medicine. The hydroalcoholic extract prepared from roots was characterized by NMR and HPLC analysis and assayed *in vitro* by CPE reduction and virus infectivity assays to define its anti-viral effect. The extract’s mechanism of action was investigated by virucidal and time-of-addition assays and by *in vitro* \(\alpha\)-glucosidase inhibitory assay. The extract exhibited a remarkable anti-herpetic activity at 100 \(\mu\)g/mL, at non-cytotoxic concentration, through multiple mechanisms: it reduced the infectivity of viral particles released from infected cells possibly through its anti-ER \(\alpha\)-glucosidase inhibitory activity and it inhibited the beginning stage of HSV infection acting as a virucide agent and/or preventing virus attachment to the host cell surface.

*Key words:* *Hemidesmus indicus* (L.) R. Br., Herpes simplex virus, \(\alpha\)-glucosidase activity, Antiviral activity, Mechanism of action, Virus attachment prevention.

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INTRODUCTION
Herpes simplex virus types 1 (HSV-1) and type 2 (HSV-2) are enveloped DNA viruses of the Herpesviridae family. They are common latent viruses in humans, responsible for a wide spectrum of clinical manifestations ranging from asymptomatic infection or mild mucocutaneous lesions to more severe infections like aseptic meningitis and encephalitis, mainly in neonates and in immunocompromised individuals (Roizman et al., 2013). After establishing latency, HSV may reactivate causing lesions at the site of primary infection. HSV-1 is commonly connected with oral or facial infections, whereas HSV-2 is responsible for genital infections and is found to be a high risk factor for the acquisition of HIV infection and further spread (Freeman et al., 2006); HSV-2/HIV-1 co-infected individuals tend to have more severe herpetic lesions and increased HSV-2 shedding (Strick et al., 2006). These findings, together with the absence of any approved prophylactic drug and recent HSV-2 vaccine failures (Bagley et al., 2017; Belshe et al., 2012), highlight the importance of developing new HSV prevention strategies. In addition, despite the availability of several anti-HSV agents, the increasing incidence of drug-resistant virus strains requires new HSV therapeutic approaches (Piret and Boivin., 2016; Andrei and Snoeck, 2013).

Most antiviral agents approved so far for the treatment of viral diseases are based on a direct action against virus-encoded enzymes, such as DNA/RNA polymerases, integrase, proteases and neuraminidase. Recently, in view of a potential broad-spectrum and resistance-refractoriness, a host-target based approach, implying the inhibition of proteins involved in the different steps of viral infection and replication, has received considerable attention. The host cellular proteins explored as possible antiviral targets (Geller et al., 2012) include the endoplasmic reticulum (ER)-located α-glucosidases I and II. By sequentially removing the glucose residues during N-linked oligosaccharide processing, these enzymes are responsible for the proper folding, trafficking and functions of many glycoproteins (Hebert et al., 1995). Since mammalian viruses are not able to encode their own carbohydrate-modifying enzymes, they require host cellular ER α-glucosidases for a proper glycan processing of their envelope proteins, which are, in most cases, glycoproteins containing N-linked glycans. Thus, inhibition of α-glucosidase can impair the maturation and function of viral envelope glycoproteins, which may, in turn, reduce virion particle assembly, secretion and/or infectivity (Chang et al., 2013; Yu et al., 2012).

Medicinal plants represent a potential source of new antimicrobial compounds and a growing number of plant-based traditional remedies with antiviral activity are garnering evidence of experimental and/or clinical efficacy (Li and Peng, 2013). Furthermore, for many of them, the clear mode of action at the various stages of the virus replication cycle has been clarified. From the drug discovery point of view,
plant-derived active compounds are potentially better lead compounds for further chemical improvements compared to molecules identified by random screening from a standard combinatorial chemical library, due to their long-term usage. Plant extracts may contain multiple active components with more than one target, thus they may also constitute new all-in-one tools in the prevention or treatment of different infectious diseases.

*Hemidesmus indicus* (L.) R. Br., also known as Indian Sarsaparilla, is a species belonging to Apocynaceae, widely used in traditional Indian medicine. Since 1962, when diuretic activity was demonstrated for *H. indicus* roots, extensive pharmacological investigations have been carried out, with the aim of supporting the wide array of its ethnomedicinal uses. From these studies, several effects have been demonstrated, such as antidiarrhoeal (Das et al., 2003), antiasthmatic (Bhujbal et al., 2009), hepatoprotective (Baheti et al., 2006), antiulcerogenic (Austin and Jegadeesan, 2003), antimicrobial and antihelminthic (Kekuda et al., 2009). Chemical constituents of polar extracts from *H. indicus* roots are triterpenic and steroidal saponins, tannins, coumarino-lignans, alkaloids, coumarins and phenols (Ananthi et al., 2010; Darekar et al. 2008; Das et al., 1992; Zhao et al., 2013), while the volatile fraction contains 2-hydroxy-4 methoxy benzaldehyde and (-) ledol as major compounds, and over forty minor constituents (Nagarajan et al., 2001).

With the aim of discovering a new biological activity for a plant already known to possess antimicrobial and anti-HIV effects (Esposito et al., 2017), a hydroalcoholic extract from *Hemidesmus indicus* root was investigated in this study for its possible anti-herpetic activity. The inhibitory effect of the extract was assayed *in vitro* using Vero cells as a model system against both HSV types, and experiments were performed to elucidate the mechanism of antiviral action. The *in vitro* anti-α-glucosidase activity of the extract was also evaluated and the relationship between these activities is discussed.

**MATERIALS AND METHODS**

**Chemicals**
The compounds 2-hydroxy-4-methoxy-benzaldehyde (2-OH-4-OMeAld), 2-hydroxy-4-methoxybenzoic acid (2-OH-4-OMeAc) and 3-hydroxy-4-methoxy-benzaldehyde (3-OH-4-OMeAld), were purchased from Sigma-Aldrich, dissolved in methanol solution at a concentration of 30 mg/mL; acyclovir (ACV) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in sterile distilled water. Heparin sodium (5000 IU/mL) was purchased from Biologici Italia laboratories (Milan, Italy).

**Plant material and extract preparation**
Hemidesmus indicus root powder was provided by Maharishi Ayurveda Product Italy (Verona, Italy). The root was collected from Ram Bagh (Rajasthan, India), authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India, and the voucher of the crude drug was deposited at the Department of Pharmacy and Biotechnology of Bologna University (code # MAPL/20/178).

Ultrasonic-assisted extraction of the drug was performed after homogenizing 2 g root powder in 30 mL aqueous methanol solution (50/50). The suspension was sonicated in an ultrasound water bath for 30 min (Transsonic 310-H, Elma-D-78224, Singen, HTW, power 100 W, frequency 35 kHz). After filtering through a paper filter, methanol was evaporated under vacuum and the aqueous extract was freeze-dried and stored at room temperature until analysis. The extraction yield was 14.7% (w/w). For cell-based assays, a stock solution of 30 mg/mL was prepared in aqueous methanol solution.

¹H NMR analysis
The NMR samples were prepared by mixing the powdered plant material (50 mg) with 1.5 mL of the NMR solvent consisting of a phosphate buffer (90 mM; pH 6.0; Fluka Chemika, Buchs, Switzerland) in water-d₂ (Cambridge Isotope Laboratories, Andover, MA, USA) and methanol-d₄ (Sigma-Aldrich, St. Louis, MO, USA) (1:1) containing 0.1% w/w trimethylsilylpropionic-2,2,3,3-d₄ acid sodium salt (TMSP, Sigma-Aldrich, St. Louis, MO, USA). The mixture was vortexed at room temperature for 1 min, sonicated for 30 min, and centrifuged at 13000 rpm for 10 min and the supernatant was analyzed by NMR. ¹H NMR spectrum was recorded at 2°C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz operating at 1H frequency) equipped with TCI cryoprobe and Z-gradient system methanol-d₄ was used for internal lock purposes.

HPLC analysis
HPLC analysis was carried out for determination of 2-OH-4-OMeAld, its derivative 2-OH-4-OMeAc, and 3-OH-4-OMeAld in the extract, according to Sircar et al. (2007). The analytical HPLC system was composed of a Jasco 1500 Series system (Jasco Europe, Cremella, Italy) equipped with a PDA detector (MD-2010 plus). The HPLC pumps, autosampler, column oven, and diode array detector were controlled using the ChromNav software (Ver. 2.0, Jasco Europe, Cremella, Italy). Separation was achieved using a Waters Spherisorb C₁₈ reversed-phase column (250x4.6 mm, i.d. 5.0 µm) (Waters, Milford, MA, USA) coupled with a C₁₈ guard column (8.0x3.0 mm) (Phenomenex, Torrance, CA, USA) at a flow of 1 mL/min. Chromatograms were monitored at 280 nm. The peak identification of analytes in the extract was based on the comparison of retention time with those of the reference standards under the same conditions.
\( \alpha \)-glucosidase inhibitory assay

\( \alpha \)-glucosidase inhibition assay was performed according to Venditti et al. (2015). *Hemidesmus* extract was tested at concentrations ranging from 25 to 200 \( \mu \)g/mL. The IC\(_{50}\) value (concentration necessary for 50% enzyme inhibition) was calculated by constructing a logarithmic curve showing sample concentrations on x-axes and percentage inhibition on y-axes. The percentage of inhibition of enzyme activity was calculated by the following formula:

\[
\text{% Inhibition} = \frac{1 - \Delta \text{Abs/min}_{\text{sample}}}{\Delta \text{Abs/min}_{\text{neg contr}}} \times 100
\]

A negative control was performed by adding water to the reaction assay instead of samples and \( \Delta \text{Abs} \) values were calculated as \( \text{Abs}_{t10} - \text{Abs}_{t0} \) and referred to 1 min.

**Cell culture and viruses**

African green monkey kidney cells (Vero) were cultured in Eagle’s Minimal Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Carlo Erba Reagents, Milan, Italy), 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin at 37°C with 5% CO\(_2\). Clinical isolates of HSV-1 and HSV-2, available for research purposes according to the Italian privacy law, were propagated and titrated by plaque reduction assay on Vero cells, and used in the assays at 30 PFU/well (plaque forming unit). In a preliminary series of experiments the sensitivity of both clinical strains to ACV was assayed in the range of concentrations 0.016-0.5 \( \mu \)g/mL and EC\(_{50}\) values (effective concentration inhibiting at 50% the viral replication) of 0.25 \( \mu \)g/mL were obtained. That defined concentration was subsequently used as control in the antiviral assays.

**Cell viability assay**

Vero cells were seeded into 96-well plates at \( 10^4 \) cells/well, and incubated at 37°C for 24h. Following washes with phosphate buffered saline (PBS), cell monolayer was incubated with different extract concentrations (10-400 \( \mu \)g/mL), or single molecules (2-OH-4-OMeAld, 2-OH-4-OMeAc, 3-OH-4-OMeAld; 0.1-20 \( \mu \)g/mL). Both untreated cells and cells incubated with medium containing solvent dilutions were included in each experiment as controls. After 48h of incubation, cell viability was measured by the WST-8 based method, according to the manufacturer’s instructions (CCK-8, Cell Counting Kit-8, Dojindo Molecular Technologies, Rockville, MD, USA). Briefly, after washes with PBS, 100 \( \mu \)L of fresh medium and 10 \( \mu \)L of CCK-8 solution were added to each well. Following a 2h-incubation at 37°C, the absorbance was measured at 450/650 nm; data were calculated as the percentage of cell viability relative to the untreated controls and expressed as the cytotoxic concentration of the extract and of the single compounds which reduced viable cell number by 50% (CC\(_{50}\)).
HSV antiviral assay
The inhibitory effect of *H. indicus* extract and its main constituents towards HSV-1/2 replication was evaluated *in vitro* by means of a CPE (virus-induced cytopathic effect) reduction assay followed by an infectivity assay. Vero cells were seeded into 96-well plates at 3×10^4 cells/well, 24h before experiments, then infected with 30 PFU/well HSV-1/2, diluted in MEM supplemented with 2% FBS. Following adsorption (2h at 37°C), the inoculum was removed, cells were washed and cultured for 48h in a final volume of 100 µL containing the concentrations of either the extract (10-200 µg/mL) or the single constituents (1-20 µg/mL). The following controls were included: HSV-infected cells and incubated in regular medium or supplemented with the corresponding solvents dilutions or with ACV at 0.25 µg/mL, and mock-infected cells. At 48 hours post-infection (hpi), the plate was visualized microscopically for any changes in cell appearance compared with normal controls. Quantitative evaluation of the inhibition of the virus-induced CPE due to the protective effect of test samples was determined by the CCK-8 assay, as previously described. Viral inhibition rate was calculated as:

\[ \frac{(\text{Abs}_{\text{tv}} - \text{Abs}_{\text{cv}})}{(\text{Abs}_{\text{uc}} - \text{Abs}_{\text{cv}})} \times 100 \]

\text{Abs}_{\text{tv}} indicates the absorbance of the test sample (extract/compounds/solvents) with virus-infected cells. \text{Abs}_{\text{cv}} and \text{Abs}_{\text{uc}} indicate the absorbance of the virus control and the absorbance of the untreated cell control, respectively (Bag et al., 2012, Mukherjee et al., 2013). Data were considered consistent when the viral inhibition rate calculated for infected cells in the presence of 0.25 µg/mL of ACV ranged from 45% to 55%, as that concentration correspond to the previously determined EC50 value for HSV-1/2.

The cell-free supernatants recovered from the CPE reduction assay and containing the released progeny were tested to check their infectivity in fresh and untreated cells. Briefly, 100 µL of supernatants were used as virus inoculum to infect 24h monolayers for 2 h at 37°C; then cells were washed, grown in regular medium for 48h and analyzed using the CCK-8 assay.

Mode of antiviral activity
Different assays were performed with HSV-1/2 using Vero cells as model system to trace the mode of antiviral activity of the *H. indicus* extract.

The pre-treatment assay was carried out with cells seeded in a 96-well plate being treated with the extract (10-200 µg/mL) or phytoconstituents (1-20 µg/mL) for 2h at 37°C before viral infection at 30 PFU/well. The inhibition of the virus-induced CPE was determined at 48hpi as previously described.

The attachment assay was performed by mixing the extract (10-200 µg/mL) or its constituents (1-20 µg/mL), with HSV-1 or HSV-2 (30 PFU/well), and adding the mixtures on cooled cells. The attachment
was allowed to proceed for 2h at 4°C, then cells were gently washed with cold MEM to remove unbound viruses. Heparin sodium was used as positive control for inhibition of virus attachment. Cells were cultured in regular medium for 48h, and analyzed by using the CCK-8 assay.

For entry assay, HSV-1 or HSV-2 (30 PFU/well) were adsorbed for 2h at 4°C on pre-chilled Vero cells, previously seeded in a 96-well plate. Cells were washed with cold MEM to remove unbound viruses, then treated with the extract (10-200 µg/mL) or pure compounds (1-20 µg/mL) and incubated for 3h at 37°C. Virions still attached to the cell surface were inactivated by acidic glycine treatment (100 mM glycine, 150 mM NaCl, pH 3), for 2 min at room temperature; then cells were washed and cultured for 48h before evaluating the virus-induced CPE.

To determine the effect of *H. indicus* extract and its components on the direct inactivation of virus particles, HSV-1/2 (1000 PFU/100 µL) were treated with 10–200 µg/mL of extract and 1-20 µg/mL of pure molecules at either 4 or 37°C for different exposure times. After incubation, the mixture was added on Vero cells and infection was allowed to proceed. At 48 hpi, the residual virus infectivity was measured determining the viral-induced CPE with the CCK-8 assay.

**Statistical analysis**

Results are presented as the mean values ± standard deviation (SD) of at least two separate experiments, each performed in triplicate. Percentage values at the different experimental conditions were determined as relative to untreated/mock-infected sample controls. Fifty-percent cytotoxic concentration (CC$_{50}$) value was determined using the program GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) as the inflection points of the four-parameter dose-response curves obtained by plotting the percentage values of the formazan dye reduction (CCK-8 reagent) vs. the logarithm of extract concentration. Likewise, 50% effective concentration (EC$_{50}$) values were obtained by plotting the percentage values of the inhibition of induced CPE vs. the logarithm of extract concentration. t-test was used to compare HSV-1 and HSV-2 inhibition rates; one-way analysis of variance (ANOVA) was used to compare data among the different experimental conditions. Statistically significant differences were determined at *p* < 0.05.

**RESULTS AND DISCUSSION**

**Phytochemical analysis of *H. indicus* extract**

The chief chemical constituents of *Hemidesmus* roots are unusual phenolic compounds, structurally similar to vanillin, which are responsible for its fragrance (Chopra et al., 1980; Sreekumar et al., 1998), and which were also identified in another widely used Indian plant, *Decalepis hamiltonii* Wight & Arn.,
The present study characterized the phytochemical profile of the hydroalcoholic extract of *Hemidesmus indicus* by means of $^1$H NMR and HPLC analysis. In addition to several primary metabolites such as aminoacids and sugars, $^1$H NMR highlighted the secondary metabolites 3-OH-4-OMeAld and 2-OH-4-OMeAld (Table 1). HPLC analysis of *H. indicus* extract (1 mg/mL) revealed two aromatic aldehydes 3-OH-4-OMeAld and 2-OH-4-OMeAld, and its derivative 2-OH-4-OMeAc at concentrations of 0.41, 0.16, 0.60 mg/g DW, respectively (corresponding to 2.82, 1.09, 4.05 µg/g extract). Since a range of bioactivities are reported for this plant and its constituents, comprising antimicrobial (Phade et al., 1994) and insecticidal activities (George et al., 1999), the root extract and these three detected pure compounds (dissolved in methanol) were tested for their potential antiviral activity.

**Cell-based assay optimization**

The suitability of the CCK-8 assay for the assessment of Vero cell viability upon HSV infection and/or incubation with the extract or selected constituents was carefully determined in preliminary experiments. The analytical performances of the assay were evaluated by testing different numbers of Vero cells seeded on a 96-well plate and incubation times with the CCK-8 reagent solution. The optimal condition in terms of limit of detection and goodness-of-fit ($R^2 = 0.99$) was achieved for $1-3\times10^4$ cells/well and 2h of incubation (data not shown). At the defined parameters, cell number and absorbance share a linear and proportional relationship, thus validating the use of the standardized assay for the quantitative evaluation of both the cellular health and growth of Vero cells in cytotoxicity assays, and the effect of tested samples to inhibit HSV-induced cell killing. Methods based on the *in situ* reduction of a tetrazolium salt by mitochondrial enzymes in viable cells have been previously used in the evaluation of anti-HSV compounds in a Vero model system as an alternative to plaque reduction assays (Chattopadhyay et al., 2009).

**Analysis of the antiviral effect**

The potential activity of *H. indicus* extract and of 3-OH-4-OMeAld, 2-OH-4-OMeAld, and 2-OH-4-OMeAc was evaluated against HSV-1/2 replication in Vero cells. The range of extract concentrations used in the virological assays was selected basing on the cytotoxicity results, while for single molecules a much lower range was chosen, based on their concentration in the hydroalcoholic extract. HPLC results were taken into account, as studies concerning the three phenolic compounds were aimed at establishing whether, at the defined concentrations present in the extract, they could contribute to the overall antiviral activity of the extract.
The effect of the extract (from 10 to 400 µg/mL) on Vero cell metabolism was investigated following a 48h-incubation, equivalent to the time window used for HSV-1/2 in vitro infection in order to discern a selective activity on viral replication from a general cytotoxic potential. In our experimental conditions, the extract exerted a dose-dependent inhibitory effect on cell proliferation with a CC50 value of 268.53 µg/mL (95% C.I. 237.68-298.54 µg/mL) (Fig. 1) thus it was then assayed for its antiviral activity at concentrations ≤ 200 µg/mL.

The effectiveness of *H. indicus* to reduce HSV-1/2 replication, thus to lessen the virus-induced CPE, was quantitatively evaluated by measuring cell viability in two subsequent infectious cycles. Fig. 2 shows the anti-herpetic activity of the extract in a first round and in a second round of infection, when cell-free supernatants recovered from the previous infection were used as viral inocula. Data are expressed as percentages of inhibition of the induced CPE. A comparable and modest effect (<40%) was measured for the extract at 100 and 200 µg/mL following a replicative cycle of both virus types (Fig. 2A). However, while the reduction in the induced CPE at 200 µg/mL cannot be definitively ascribed to a selective antiviral activity, as cytotoxicity is at a similar extent, the extract at 100 µg/mL displayed only a negligible effect on cell viability (89.7 ± 5.1 % respect to control cells, Fig. 1) suggesting an impairment on viral replication. When cell-free supernatants were used to infect fresh and untreated cells in a second round of infection, viral progeny displayed a very limited infectivity potential, the inhibition of induced CPE being >80% when cells were initially treated with 200 µg/mL (Fig. 2B) and, most importantly, at the non-cytotoxic concentration of 100 µg/mL the inhibition still remained rather high (>50%). Indeed, the measured EC50 values were 91.3 µg/mL for HSV-1 and 86.1 µg/mL for HSV-2. It is possible to hypothesize that *H. indicus* extract, limiting the ongoing infection in vitro, could reduce direct cell-to-cell spread and glycoprotein mediated cell-to-cell fusion in the course of natural infections, blocking viral transmission at the mucosal surface.

The host cellular targets nowadays considered a potential broad-spectrum and resistance-refractory therapeutic approach for antiviral drugs include the (ER) α-glucosidases (Noble et al., 2010), enzymes that sequentially trim the three terminal glucose moieties on N-linked glycans attached to nascent glycoproteins. Studies carried out in the last three decades have demonstrated that these host enzymes represent viable drug targets for the treatment of a broad spectrum of enveloped viruses (Dwek et al., 2002 and references therein). Indeed, abrogation of normal glycoprotein trafficking and processing by inhibition of ER α-glucosidases generates misfolded glycoproteins retained in the ER and directed to undergo ER-associated degradation, or even expressed on the cell surface but with compromised functionality.
Thus, *H. indicus* extract was tested for its *in vitro* α-glucosidase inhibitory activity. At 200 µg/mL, a 96% inhibition was observed (Table 2) and IC$_{50}$ value (calculated from the logarithmic curve $y = a \ln (x) + b$, where $a = 34.09$; $b = - 85.20$, $R^2 = 0.997$) turned 53 µg/mL. This result suggests that the observed antiviral effect could be ascribed to the ability of *H. indicus* to target cellular ER α-glucosidases. It has been previously demonstrated that the inhibition of these enzymes is sufficient for antiviral activity against multiple families of enveloped viruses, including *Herpesviridae* (Chang et al. 2013; Caputo et al., 2016). As a consequence, it is conceivable that the extract, altering glycoprotein processing via inhibition of ER α-glucosidases, interferes with HSV morphogenesis, in particular reducing or modifying virion egress, fusogenicity and infectivity. Specifically, of all four viral glycoproteins, the functions of gB are more critical in viral binding and fusion, and its modifications mainly affect these steps of virus life cycle (Luo et al., 2015; Campadelli-Fiume et al., 2012). In addition, since gB belongs to a class III fusion glycoprotein with 7 potential N-linked glycosylation sites, it may be predominantly affected by inhibition of cellular ER α-glucosidases. A previous study ascribes the antiviral activity of an inhibitor of ER α-glucosidase I towards HSV-2 replication in *in vitro* infected cells to the production of abnormal gB glycoproteins with increased molecular weight related to the retention of the terminal glucose and, consequently, mannose residues associated with N-linked glycans (Ahmed et al., 1995).

Having established the anti-herpetic potential of the extract, the three phenolic compounds were investigated following the above described experimental design; in the test range (0.1-20 µg/mL), neither cell metabolism, nor HSV-1/2 replication were disrupted by a 48h treatment and/or infection cycle. However, in an attempt to elucidate the biological properties of the single pure molecules, concentrations 10-fold higher that those quantitatively evaluated within the tested extract were assayed, but only a cytotoxic effect on Vero metabolism was demonstrated (data not shown). Obviously, the extract contains many different bioactive secondary metabolites, other than those identified herein and assayed *in vitro*: some of these may work directly on the viral target, whereas others may enhance the bioavailability of the extract, indicating that complex interactions among different bioactive constituents may contribute to the demonstrated anti-herpetic activity of the whole extract.

**Mechanisms of antiviral activity**

As the herpesvirus infectious cycle is characterized by a complex sequence of steps, and each of them could represent a target for antiviral agents, a series of assays were performed to trace the mode of antiviral action. For this purpose, the extract or the components, 3-OH-4-OMeAld, 2-OH-4-OMeAld, and 2-OH-4-OMeAc, were incubated with cells before viral infection or mixed with either HSV-1 or
HSV-2 and then adsorbed on cells. Fig. 3 details morphological changes of monolayers at the different experimental conditions.

As shown in Table 3, the extract did not show an effect on HSV replication when Vero cells were treated prior to infection, whereas it displayed high inhibition on viral adsorption to target cells. Both viruses induced a quite distinct CPE in Vero cells, characterized by cell enlargement and detachment following pretreatment assays. On the other hand, microscopic examination of cells during attachment assays revealed only slight changes comparing to untreated cells (Fig. 3A and B). Indeed, the attachment assays performed on prechilled Vero cells with HSV in presence of the extract revealed that it inhibited viral attachment, leading to a significant suppression of virus multiplication. Moreover, the effect was dose-dependent, with EC50 values of 66.8 µg/mL for HSV-1 and 70.6 µg/mL for HSV-2, thus meeting the stringent criteria for defining inhibitory activities of extracts from natural source in bioassays (Cos et al., 2006). Results of the viral entry assays, where extracellular virions were inactivated by acidic glycine treatment, ruled out that the extract could interfere with this stage of virus life cycle as only a slight reduction of the CPE-induced effect was measured at the highest extract concentration and cells displayed prominent morphologic changes comparable to infected control cells (Fig. 3C).

Considering the overall data, it is possible to speculate that the antiviral activity of *H. indicus* depends, at least in part, on its capacity to prevent attachment of the viruses to the cell surface mainly by interaction with virus particles rather than a stable binding to cellular components, as target cells retained fully susceptibility to viral infection when pretreated with the extract.

To complete the *in vitro* analysis on the antiviral potential of *H. indicus* against free HSV-1 and HSV-2, virucidal assays were carried out in which the extract and HSV particles were mixed and incubated at 37°C and 4°C for different times before their adsorption on cells. After incubation, samples were ten-fold diluted to reduce the extract to a concentration unable to inhibit HSV replication and attachment, and, as a consequence, to lower the amount of HSV inoculum, to make a direct comparison possible.

As seen in Figure 4, the extract completely inhibited the induced CPE when incubated at 200-100 µg/mL with viral particles for 60 and 30 min at both temperatures. Following an incubation time of 10 min, the extract at 200 µg/mL retained its potent anti-herpetic activity at both tested temperature and most importantly, at 100 µg/mL and at physiological temperature, the inhibition of the induced CPE did not statistically differ and was measured at 84.8% for HSV-1 and 82.3% for HSV-2. As a confirmation, cell morphology of HSV-1 infected cells in this experimental condition is similar to that of mock-infected cells (Fig. 3D and F).
Extracts with this mode of action could be attractive candidates for the development of novel topical virucides to prevent HSV disease. Moreover, as the antiviral activity of the extract possibly relies even on its effect on cellular structures, *H. indicus* could represent a potential broad-spectrum therapeutic agent to prevent infections by sexually transmitted viruses. The extract displayed a remarkable activity towards HSV and, recently, the inhibition of innovative HIV-1 drug targets such as RT-associated RNase H function has also been reported for this plant (Esposito et al., 2017).

Once again the complete panel of assays was carried out with the three phenolic components at non-cytotoxic concentrations (1-20 µg/mL) to assess their contribution to the activity of the hydroalcoholic extract. Unlikely, it was not possible to define a mechanism of action for the single pure molecules.

Many extracts derived from various plant species have been reported to exert inhibitory activities on HSV replication at the first step of virus life cycle (Astani et al., 2012; Astani et al., 2014; Navid et al., 2014; Hung et al., 2015; Schnitzler et al., 2010; Wilson et al., 2009). Some proposed mechanisms of action include the interaction of the phytoconstituents with viral particles by a specific protein domain binding or general coating with virion envelope structures, thus masking viral components necessary for adsorption or by a direct damage of the virions, thereby impairing their ability to infect host cells (Terlizzi et al., 2016). Among the secondary plant substances, triterpenes have demonstrated higher anti-herpetic activity *in vitro* when incubated with viral particles prior infection compared to other time-on additions (Navid et al., 2014), as described in the present study for *H. indicus*.

**CONCLUSIONS**

This work investigated the inhibitory activity of the hydroalcoholic extract of *Hemidesmus indicus* prepared from roots against both types of HSV in a series of cell-based assays. The effects of the extract on the different stages of the infectious cycle were individually analyzed, and some conclusions can be drawn on the main mode of antiviral action. The observed antiviral activity could be ascribed (1) to the capacity of the extract to limit ongoing viral infections reducing the infectivity of the produced progeny in subsequent rounds of infection, and (2) to its direct interaction with virus particles acting as a virucide agent and/or preventing virus attachment to the host cell surface. Possibly, the first mentioned antiviral effect is related to the aberrant biosynthesis of the enveloped viruses within infected and treated cells that in turn is determined by the anti-ER α-glucosidase activity demonstrated for the extract.

As a final remark, molecules that act by blocking specific host functions required by many different viruses, rather than a specific virus target, have the additional potential to treat a wide range of viral
infections and co-infections without the risk of losing efficacy due to escape mutations in the viral genomes.

ACKNOWLEDGEMENTS
The authors are grateful Dr Paolo Scartezzini (Maharishi Ayurveda Product, Italy) for providing the plant samples.
REFERENCES


Astani A, Navid MH, Schnitzler P. 2014. Attachment and penetration of acyclovir-resistant Herpes simplex virus are inhibited by Melissa officinalis extract. Phytother. Res. 28:1547-1552


Table 1. Diagnostic signals of metabolites detected by $^1$H NMR in *Hemidesmus indicus* extract.

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<th>Metabolites</th>
<th>$^1$H-NMR data</th>
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<tr>
<td>Alanine</td>
<td>1.48 (H3, $d$ $J$ = 7.2)</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.59 ($H1$ $\beta$, $d$, $J$ = 7.8); 5.19 ($H1$ $\alpha$, $d$, $J$ = 3.8)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.15 (H3’, $d$, $J$ = 8.4); 5.38 (H1, $d$, $J$ = 3.6)</td>
</tr>
<tr>
<td>2-Hydroxy-4-methoxybenzaldehyde</td>
<td>3.88 (-OCH$_3$, s); 6.51 (H3, $d$, $J$ = 2.4 Hz, 1H); 6.66 (H5, dd, $J$ = 8.7, 2.4 Hz, 1H); 7.66 (H6, $d$, $J$ = 8.7 Hz, 1H) 9.77 (-COH, s)</td>
</tr>
<tr>
<td>3-Hydroxy-4-methoxybenzaldehyde</td>
<td>7.17 (H2, $d$, $J$ = 8.3 Hz); 7.36 (H5, $d$, $J$ = 2.0 Hz); 7.54 (H6, dd, $J$ = 8.3, 2.1 Hz); 9.73 (-COH, s)</td>
</tr>
<tr>
<td>Formate</td>
<td>8.47 (s)</td>
</tr>
</tbody>
</table>
Table 2. *In vitro* $\alpha$-glucosidase inhibitory activity of *Hemidesmus indicus* extract at different concentrations.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>$\alpha$-glucosidase inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 $\mu$g/mL</td>
<td>96.1</td>
</tr>
<tr>
<td>125 $\mu$g/mL</td>
<td>77.4</td>
</tr>
<tr>
<td>100 $\mu$g/mL</td>
<td>71.4</td>
</tr>
<tr>
<td>75 $\mu$g/mL</td>
<td>63.6</td>
</tr>
<tr>
<td>50 $\mu$g/mL</td>
<td>48.3</td>
</tr>
<tr>
<td>35 $\mu$g/mL</td>
<td>37.2</td>
</tr>
<tr>
<td>25 $\mu$g/mL</td>
<td>23.1</td>
</tr>
</tbody>
</table>
Table 3. Antiviral activity of *Hemidesmus indicus* extract.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Pre-treatment</th>
<th>Attachment</th>
<th>Entry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>HSV-1</td>
<td>HSV-1</td>
</tr>
<tr>
<td>200 µg/mL</td>
<td>&lt; 5</td>
<td>90.7 ± 0.8</td>
<td>33.5 ± 4.4</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>&lt; 5</td>
<td>83.1 ± 6.2</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>50 µg/mL</td>
<td>&lt; 5</td>
<td>22.4 ± 2.8</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

Values are the viral inhibition rates (%) measured by CPE reduction assays. Data are obtained in two independent experiments performed in triplicate.
Figure 1. Cell viability. Dose-response curve of *H. indicus* extract was obtained by plotting cell viability (expressed as percentage values of treated cells relative to untreated cells) as a function of the concentrations. The CC$_{50}$ value was 268.53 µg/mL.
Figure 2. Anti-herpetic activity of *H. indicus* measured by CPE reduction and virus infectivity assays. Panel A reports viral inhibition rates obtained in a first replicative cycle while Panel B in a second round of infection, indicating a clear reduction of infectivity of the viral progeny released from treated cells. Data are mean values ± SD of at least two independent experiments performed in triplicate.
Figure 3. Microscopic images of Vero cells following *H. indicus* treatment and/or HSV-1 infection. Morphology of cells upon treatment with 100 μg/mL of extract and/or infection with HSV-1 at 48 hpi and following different experimental conditions: (A) pretreatment assay, (B) attachment assay, (C) entry assay, (D) virucidal assay (at 37°C for 10 min), (E, F) infected and mock-infected control cells. In panels A and C, Vero cells display enlargement, granularity, and detachment comparable to that observed in HSV-1 infected cells (E); infected cells in panel B show only a slight cytopathic effect while infected cells in panel D are similar to mock-infected cells.
Figure 4. Inhibitory effect of *H. indicus* on free viral particles. Panel A reports HSV-1 inhibition rates while Panel B HSV-2 inhibition rates measured by CPE reduction assays as function of time and temperature. Data are mean values ± SD of at two independent experiments performed in triplicate. Panel A: ***p<0.0001 for 100 μg/mL at 4°C, 10 min of incubation Vs. all other experimental conditions. Panel B: **p<0.0033 for 100 μg/mL at 4°C, 10 min of incubation Vs. all other experimental conditions.