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BRIEF REPORT

# *Ficus religiosa* L. bark extracts inhibit infection by herpes simplex virus type 2 *in vitro*

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**Abstract** *Ficus religiosa* extracts have been used in traditional Indian medicine to treat sexually transmitted infections such as gonorrhea and genital ulcers. The aim of this study was to investigate the antiviral activity of *F. religiosa* extracts against herpes simplex virus type 2 (HSV-2), the main causative agent of genital ulcers and sores. Water and chloroform bark extracts were the most active against HSV-2, and also against an acyclovir-resistant strain. We demonstrate that the water extract has a direct virus-inactivating activity. By contrast, the chloroform extract inhibits viral attachment and entry and limits the production of viral progeny.

**Keywords** HSV-2 · Antiviral · Plant extract · *Ficus religiosa*

*Ficus religiosa,* a member of the genus *Ficus* (*Moraceae*) [1], is the most sacred tree of South Asia, and its different parts have been extensively used in traditional systems of medicine such as Ayurveda and Unani, alone or in combination with other herbs, for various disorders [2]. Ethnomedical uses include treatment of diabetes, inflammation, anxiety, convulsion, epilepsy, menstrual irregularities, diarrhea, gastric problems, respiratory system, sexually transmitted infections and disorders of bacterial, fungal, viral, and protozoal origin [3, 4]. In the past few years, different studies have been carried out to confirm the antibacterial and antifungal potential of *F. religiosa,* but few findings have supported its traditional use for viral disorders. Kusumoto et al. [5] studied the protease inhibitory activity of aqueous and methanolic bark extracts against the human immunodeficiency virus 1. Choudhari et al. [4] investigated their antineoplastic potential *in vitro* against human-papillomavirus-induced cancers. We have recently reported the antiviral activity of *F. religiosa* against two respiratory viruses, namely human rhinovirus (HRV) and respiratory syncytial virus (RSV) [6]. *F. religiosa* burnt bark applied topically has also been used as an ethnic remedy for the cure of ulcers and sores in venereal diseases, [7]. One of the main causes of these genital lesions is herpes simplex virus 2 (HSV-2) [8]. Genital herpes is a widespread sexually transmitted infection, characterized by ulcerative lesions, which are often very painful and can lead to substantial psychological morbidity [9]. After a lifelong latent infection in lumbosacral sensory ganglia, HSV-2 can be reactivated periodically by external conditions (stress, hormonal changes, or UV light) and causes either asymptomatic episodes or more-severe complications of the central nervous system that manifest with acute encephalitis and meningitis in neonates and immunocompromised patients [8]. Of note, HIV infection is often attributable to pre-existing HSV-2 infection, since genital ulcers damage the mucosa and induce local inflammation, which may increase the risk of HIV acquisition [10]. Even if antiviral drugs targeting the viral DNA polymerase (acyclovir, penciclovir and their derivatives) are available against HSV infection, plants and herbs used in traditional medicine may represent additional sources of antiviral compounds. In this context, the present study was undertaken to explore whether the ethnomedical use of *F. religiosa* to treat genital lesions is substantiated by its antiviral activity against

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HSV-2. We report on the anti-HSV-2 potency and mechanisms of antiviral action of *F. religiosa* extracts. To this aim, fresh bark and leaves of *F. religiosa* were prepared as described by Cagno et al. [6] and resuspended in water or DMSO to a final concentration of 25 mg/mL before use. The plant extracts were subjected to preliminary phytochemical screening, and flavonoids, tannins, saponins, alkaloids and steroids/triterpenoids were detected (see Supplementary Table S1). HSV-2 strain MS, which is sensitive to acyclovir, was propagated in Vero cells and titered as described previously [11]. The anti-HSV-2 activity of *F. religiosa* extracts was examined by plaque reduction assay in Vero cell monolayers grown in 24-well microplates. For this analysis, a complete-protection assay was used in which serial dilutions of extracts, ranging from 100 μg/mL to 0.13 μg/mL, were added to the cell culture before, during, and after the infection. After 2 h of incubation, the medium was removed, and infection was performed at a multiplicity of infection (MOI) of 0.001 pfu/cell for 2 h at 37 °C. The cells were then washed twice with medium and overlaid with a medium containing 1.2% methylcellulose. Treatment of control samples with equal volumes of DMSO or water, depending on the solvent used to dissolve the extract, was performed in order to rule out the possibility of any cytotoxic effect ascribable to the solvent. After an incubation of 24 h at 37 °C, the plates were fixed and stained with 0.1 % crystal violet in 20 % ethanol, and viral plaques were counted. Cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay on confluent cell cultures seeded in 96-well plates as described by Donalisio et al. [12]. The 50 % cytotoxic concentration ($CC_{50}$) and the half-maximal effective concentration ($EC_{50}$) values for inhibition curves were calculated by regression analysis using the program GraphPad Prism, and the selectivity index (SI) was calculated by dividing the $CC_{50}$ by the $EC_{50}$ value. Acyclovir was tested in parallel as a reference drug for HSV. Table 1 shows that all extracts exerted an antiviral activity, although to a different extent, generating dose response curves. In particular, a notable inhibition of HSV-2 (MS) infection was exerted by water, chloroform and methanol bark extracts, with $EC_{50}$ values of 9.76 μg/mL, 6.75 μg/mL and 5.20 μg/mL, respectively. A potential antiviral effect due to the extraction process can be excluded since the extracting solvents are volatile in nature and were completely removed by vacuum evaporation, leading to completely dried powdered extracts. The antiviral effect was not a consequence of cytotoxicity, since the $CC_{50}$ value of all extracts was above 800 μg/mL, with the exception of methanol bark extract, with a $CC_{50}$ value of 161.8 μg/mL. The water and chloroform bark extracts showed the most favorable selectivity index values (> 100) and were thus selected for further studies.

We first assessed whether the antiviral activity of the two extracts is independent of the sensitivity of the virus to acyclovir using HSV-2 strain MS with phenotypic resistance to acyclovir [13, 14]. As expected, the resistant strain exhibited an elevated $EC_{50}$ value for acyclovir (60 μg/mL). By contrast, it was susceptible to the water and chloroform extracts, with $EC_{50}$ values of 6.28 μg/mL, and 13.5 μg/mL, respectively (Table 1).

This finding stimulated us to perform a set of experiments aimed at elucidating the major mechanism of action of the extracts. A virucidal assay was used to investigate a possible direct virus-inactivating activity of extracts at a concentration that almost completely prevented virus infection (>$EC_{90}$) in the previous assay. To this aim, the extract (33 μg/mL) and a virus aliquot containing $10^5$ pfu were

**Table 1** Antiviral activity of *F. religiosa* extracts against HSV-2

| Virus | Source | Extract | $EC_{50}$* (μg/mL) ± SD# | $EC^{\S}_{90}$ (μg/mL) ± SD# | $CC^{\dagger}_{50}$ (μg/mL) | SI‡ |
|---|---|---|---|---|---|---|
| HSV-2 (MS) | Leaves | Water | 26.91 ± 0.98 | 80.81 ± 1.31 | >2500 | >92.9 |
| | | Methanol | 13.29 ± 0.25 | 26.54 ± 2.50 | 1058 | 79.6 |
| | Bark | Ethyl acetate | 14.26 ± 0.31 | 19.95 ± 1.72 | 1057 | 74.1 |
| | | Water | 9.76 ± 0.59 | 18.39 ± 3.35 | 1530 | 156.8 |
| | | Chloroform | 6.75 ± 0.57 | 30.98 ± 2.73 | 809.6 | 119.9 |
| | | Methanol | 5.20 ± 0.96 | 9.15 ± 2.14 | 161.8 | 31.1 |
| | Reference drug | Acyclovir | 0.64 ± 0.18 | 3.17 ± 1.90 | >300 | >468 |
| HSV-2 acyclovir-resistant strain | Bark | Water | 6.28 ± 0.04 | 23.05 ± 2.06 | 1530 | 243.6 |
| | | Chloroform | 13.50 ± 0.50 | 23.74 ± 2.24 | 809.6 | 59.97 |
| | Reference drug | Acyclovir | 60.00 ± 1.23 | >500 | >300 | >5.00 |

Results are presented as the mean values from three independent experiments (two replicates per experiment)

$EC_{50}$* half maximal effective concentration

SD# standard deviation

$EC^{\S}_{90}$ 90 % effective concentration

$CC^{\dagger}_{50}$ half maximal cytotoxic concentration

SI‡ selectivity index

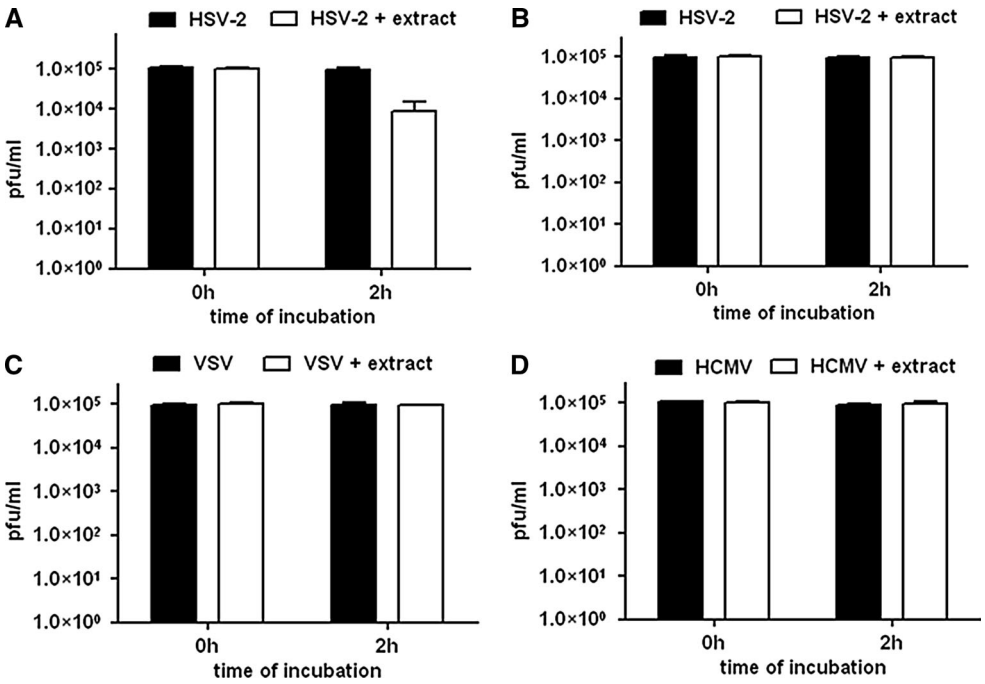


**Fig. 1** Evaluation of HSV-2 (MS) inactivation by *F. religiosa* water bark extract (panel A) and chloroform bark extract (panel B). VSV inactivation by *F. religiosa* water bark extract (panel C) and HCMV inactivation by *F. religiosa* water bark extract (panel D) at 37 °C for 0 or 2 h. On the *y*-axis, the infectious titers are expressed as plaque-forming units per mL (PFU/mL). Error bars represent the standard deviation of three independent experiments

mixed and incubated at 37 °C for 0 or 2 h, and viral samples were then titrated on Vero cells at high dilutions at which extracts were not active. Figures 1a and 1b show that if virus/extract mixtures were added promptly to cells without incubation, water and chloroform extracts had no time to act on the virus, and no inhibition was observed. By contrast, when the incubation was carried out for 2 h at 37 °C, water bark extract significantly reduced HSV-2 (MS) titers by 91 % ($P < 0.05$) (Fig. 1a). These data indicate that the major mode of antiviral action of water bark extract is direct virus inactivation. By contrast, the virus titers of samples treated with the chloroform extract did not significantly differ from those determined for untreated samples ($P < 0.05$), indicating that the compound does not inactivate extracellular virus particles (Fig. 1b).

To assess whether virucidal activity of bark extract was virus-specific, the assay was repeated with other enveloped viruses: vesicular stomatitis virus serotype Indiana (VSV) and human cytomegalovirus strain Towne (HCMV), which were kindly provided by Prof. W. Brune, Heinrich Pette Institut, Hamburg, Germany. VSV and HCMV were propagated and titrated by plaque assay on Vero and HELF cells, respectively. No activity was observed against VSV and HCMV at either incubation time (Fig. 1c and d). Of note, we previously reported that the water extract produced a modest but significant loss of RSV titer only when the incubations were carried out for 2 h at 37 °C [6]. No virucidal effect of chloroform extract against VSV and HCMV was observed, as was reported against HSV-2 (data not shown).

The results of the virucidal assays indicate that the chloroform extract most probably targets cell-surface or intracellular components involved in essential steps of the HSV-2 replicative cycle.

To obtain more insight into the nature of the antiviral activity of the chloroform extract, we first investigated its effect on HSV-2 (MS) protein expression by immunoblotting using the viral glycoprotein D (gD) as a marker [15]. To this end, total protein cell extracts were prepared from infected Vero cells (MOI of 1 pfu/cell) treated with extract or DMSO for 3, 6 or 24 h after infection. At 3 h gD was not detectable (data not shown), while its expression was completely inhibited at 6 h and significantly reduced at 24 h postinfection by the treatment as shown in Fig. 2a. This result, along with the absence of direct virus inactivation activity, indicates that the chloroform extract inhibits the HSV-2 replicative cycle. To investigate the mechanism of action of the chloroform extract, we performed a set of time-of-addition assays in which the sample was added to the cells only before, during, or after HSV-2 (MS) infection, and after an incubation time of 24 h, viral plaques were counted. As shown in Fig. 2b, the extract did not exert an inhibitory activity at any of the tested concentrations when it was added 2 h prior to virus infection and then washed out before virus inoculum was added (pre-treatment assay). By contrast, the results exhibited dose-dependent effects when the extract was added during of after infection, with $EC_{50}$ values of 6.35 μg/mL and 14.04 μg/mL, respectively.

Next, we investigated whether the extracts could interfere with the early steps of the HSV-2 replicative cycle, at the virus attachment or entry stage. The attachment assay is an experimental condition in which the virus is allowed to

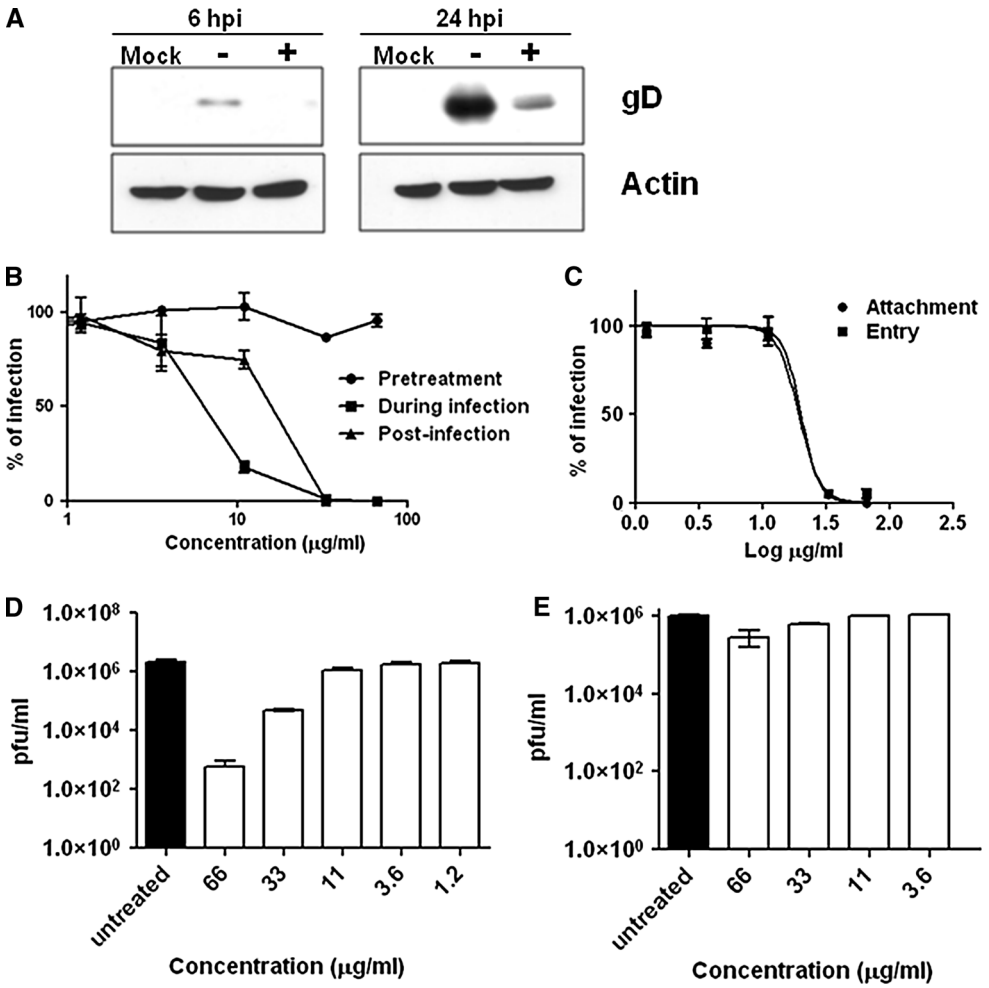


**Fig. 2** Mode of anti-HSV-2 (MS) activity of chloroform bark extract. a) Effect on HSV-2 protein expression. Vero cells were mock infected or infected with HSV-2 (MS) at an MOI of 1 and incubated for 6 or 24 h in the presence of DMSO (-) or chloroform bark extract (+). Total cell extracts were fractionated by 8.5 % SDS-PAGE (15 μg protein/lane), and analyzed by immunoblotting with anti-HSV gD. Immunodetection of actin with a MAb served as an internal control. b) Time-of-addition assays. Cells were treated with chloroform bark extract for 2 h before infection (pre-treatment), for 1 h during infection, or by adding the extracts immediately after infection (post-infection). The virus titers in the treated samples are expressed as a percentage of the titer obtained in the control (DMSO treated). c) HSV-2 entry and attachment assays. The chloroform bark extract of *F. religiosa* was added to the cell culture during virus-cell binding (attachment assay) or penetration of the cell by the virus (entry assay). The number of infected cells in the treated samples is expressed as a percentage of control (DMSO-treated). Each point represents the mean and SEM for triplicate assays. d-e) Viral yield reduction assay. The chloroform bark extract of *F. religiosa* was added to cell culture before, during, and after infection (panel d), or only after infection (panel e). When the cytopathic effect involved the whole monolayer of untreated cells, the supernatant was harvested and titrated. Viral titers (expressed as pfu/mL) are shown as the mean plus standard deviation for three independent experiments

bind to the cell surface but does not undergo cell entry. Briefly, serial dilutions of extracts were mixed with HSV, added to cooled cells and incubated for 2 h at 4 °C. After two gentle washes, cells were overlaid with 1.2 % methylcellulose medium, and then shifted to 37 °C for 24 h, and thereafter, plaques were counted. The entry assay was conducted to assess whether the extract prevents viral penetration into the host cells. For this assay, HSV at an MOI of 0.01 pfu/cell was allowed to adsorb for 2 h at 4 °C to pre-chilled confluent cells. Cells were then washed three times with cold MEM to remove unbound virus, treated with different concentrations of extract, and incubated for 3 h at 37 °C. Viruses that did not penetrate the cell were inactivated with acidic glycine for 2 min at room temperature as described previously [16]. Cells were then washed three times with warm medium and treated as described above for plaque reduction assay. As shown in Fig. 2c, the chloroform extract inhibited both attachment and entry of virus with similar $EC_{50}$ values of 19.94 μg/mL and 19.21 μg/mL, respectively.

Finally, we investigated the ability of the chloroform bark extract to limit the production of viral progeny, using a viral yield reduction assay – a more stringent test that allows multiple cycles of viral replication to

occur before measuring the production of infectious viruses. The assay was performed under two different experimental conditions: adding the extract to cell culture before, during, and after the infection (Fig. 2d), and adding the extract only after the infection (Fig. 2e).

Cells were infected with HSV-2 (MS) at an MOI of 0.01 pfu/cell and, after infection, cultures were exposed to the extract in medium and incubated until control cultures displayed extensive cytopathology. Supernatants from duplicates were pooled as appropriate 48 h after infection, and cell-free virus infectivity titers were determined in duplicate by the plaque assay in Vero cell monolayers. The degree of inhibition was determined as a percentage by comparing the titer measured in the presence of the compounds to that measured in untreated wells, analyzing as described by Civra et al. [17]. Under both experimental conditions, the results exhibited dose-dependent effects with $EC_{50}$ values of 11.7 μg/mL, and 41.65 μg/mL respectively, indicating the ability of the extracts to limit an ongoing infection.

Although a crude extract may contain several components with antiviral activity, each acting through a different mechanism, some preliminary conclusions can be drawn on the main mode of antiviral action of the two extracts. The virus inactivation assay demonstrated that the water bark extract directly inactivates the infectivity of HSV-2 (MS) virus particles. Interestingly, Yarmolinsky et al. reported indirect evidence for interactions between ethanol leaf extract of *Ficus binjamina* and HSV-1 and HSV-2 [18]. On the other hand, the chloroform extract of *F. religiosa* did not inactivate extracellular virus particles but targeted early steps of the viral replicative cycle such as virus attachment and/or entry. Moreover, a significant reduction in the number of viral plaques was also observed when the extract was added to methylcellulose medium after infection. This finding suggests that the virus was blocked in some of the initial infections. Similar to *F. religiosa* chloroform bark extract, the ethanol extract of *F. binjamina* showed the greatest anti-HSV-2 effect when added to cells at the time of infection and showed a partial inhibitory effect when added post-infection [18].

Together, these findings provide a basis for the identification of bioactive metabolites of *F. religiosa* as therapeutic natural compounds to treat genital herpetic ulcers caused by acyclovir-resistant HSV-2 strains. Moreover, they support the traditional use of *F. religiosa* bark applied externally for the treatment of ulcers and sores in genital diseases [7]. Further work remains to be done in order to assess its clinical potential.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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