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

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/1634686 since 2022-01-26T11:41:15Z

Published version:
DOI:10.1007/s00705-016-3032-3

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

(Article begins on next page)
This is the author's final version of the contribution published as:

Ghosh, Manik; Civra, Andrea; Rittà, Massimo; Cagno, Valeria; Mavuduru, Siva Ganesh; Awasthi, Preeti; Lembo, David; Donalisio, Manuela. Ficus religiosa L. bark extracts inhibit infection by herpes simplex virus type 2 in vitro. ARCHIVES OF VIROLOGY. 161 (12) pp: 3509-3514. DOI: 10.1007/s00705-016-3032-3

The publisher's version is available at:

When citing, please refer to the published version.

Link to this full text:
http://hdl.handle.net/
## Abstract

Ficus religiosa, a member of the Moraceae family, is the most sacred tree of South Asia and its bark extracts have been used in the traditional Indian medicine to treat sexual infections like 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, the main causative agent of genital ulcers and sores. Water and chloroform bark extracts were the most active with selectivity index of 156.8 and 132.9 respectively. Of note, they were active also against an acyclovir-resistant HSV-2 strain, suggesting that their mechanism of action could be different from that of the reference drug. To this regard, we demonstrated that water bark extract has a direct virus-inactivating activity; by contrast, chloroform bark extract inhibited different steps of the viral replicative cycle, such as virus attachment and entry into cells and the cell-to-cell spread of the virus. Furthermore, chloroform bark extract limited the production of viral progeny by multiple cycles of viral replication. Our study demonstrates that F. religiosa bark extracts exert inhibitory activities against HSV-2 and support the traditional use of the plant for the treatment of genital ulcers and sores.
Dear Editor,

please find herewith attached the manuscript entitled: “Ficus religiosa L. bark extracts inhibit infection by Herpes Simplex Virus type 2 in vitro” by Ghosh et al., to be considered for publication in Archives of Virology as “Original Article”.

To support the traditional Indian medicinal use of F. religiosa extracts for the cure of ulcers and sores in venereal diseases, the present study reports for the first time a remarkable anti-HSV-2 activity of F. religiosa bark extracts. The main mechanism of action of extracts was also investigated. Of interest, they were active also against an acyclovir-resistant strain of HSV-2 suggesting that their mode of antiviral action is different from that of acyclovir, the prototype of conventional anti-HSV drugs. This latter feature, along with a low cytotoxicity and a favourable selectivity index make F. religiosa bark extracts promising starting materials for a bioguided-fractionation aimed at identifying anti-HSV-2 compounds with a novel mechanism of action that can be used against acyclovir-resistant HSV-2 strains.

For this reasons, we believe the enclosed manuscript to be suitable for publication in Archives of Virology.

Yours sincerely,

Dr. Manuela Donalisio, Ph.D.
Department of Clinical and Biological Sciences
University of Torino, S. Luigi Gonzaga Hospital
Regione Gonzole, 10
10043, Orbassano, Torino, Italy
Phone: +39 011 6705427
Fax: +39 011 2365427
**Ficus religiosa** L. bark extracts inhibit infection by Herpes Simplex Virus type 2 in vitro

Manik Ghosh¹, Andrea Civra², Valeria Cagno², Siva Ganesh Mavuduru¹, Preeti Awasthi¹, David Lembo², Manuela Donalisio²*

¹Department of Pharmaceutical Sciences & Technology, Birla Institute of Technology, Mesra, Ranchi 835215, India
²Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Torino, Italy.

Running title: Anti-HSV-2 activity of *Ficus religiosa* L. bark extracts

* Corresponding author: Dr. Manuela Donalisio, Ph.D.

Department of Clinical and Biological Sciences
University of Torino, S. Luigi Gonzaga Hospital
Regione Gonzole, 10
10043, Orbassano, Torino, Italy
Phone: +39 011 6705427
Fax: +39 011 2365427
E-mail: manuela.donalisio@unito.it
Abstract

*Ficus religiosa*, a member of the *Moraceae* family, is the most sacred tree of South Asia and its bark extracts have been used in the traditional Indian medicine to treat sexual infections like 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, the main causative agent of genital ulcers and sores. Water and chloroform bark extracts were the most active with selectivity index of 156.8 and 132.9 respectively. Of note, they were active also against an acyclovir-resistant HSV-2 strain, suggesting that their mechanism of action could be different from that of the reference drug. To this regard, we demonstrated that water bark extract has a direct virus-inactivating activity; by contrast, chloroform bark extract inhibited different steps of the viral replicative cycle, such as virus attachment and entry into cells and the cell-to-cell spread of the virus. Furthermore, chloroform bark extract limited the production of viral progeny by multiple cycles of viral replication. Our study demonstrates that *F. religiosa* bark extracts exert inhibitory activities against HSV-2 and support the traditional use of the plant for the treatment of genital ulcers and sores.

**Key words:** HSV-2; antiviral; plant extract; *Ficus religiosa*
Introduction

*Ficus religiosa* is the most popular member of the genus Ficus (*Moraceae*), a native of the sub-Himalayan tract, Bengal, and central India and extensively distributed worldwide through cultivation [1]. It is the most sacred tree of South Asia and its different parts have been extensively used in the traditional Hindu and South-Asian traditional systems of medicine like Ayurveda and Unani, alone or in combination with other herbs for various disorders [2]. Ethnomedical uses included treatment of diabetes, inflammation, anxiety, convulsion, epilepsy, menstrual irregularities, diarrhea, gastric problems, respiratory system, and disorders of bacterial, fungal, viral, and protozoal origin [3]. Of note, fresh plant materials or crude extracts showed some effects in treating microbial diseases associated to the respiratory tract (cough, measles, tuberculosis) gastrointestinal (ulcers, stomatitis, dysentery) and sexually transmitted infections (gonorrhea, cervical cancer) [3, 4]. In last years, different studies have been carried out to validate the antibacterial and antifungal potential of *F. religiosa*, but few findings supported its traditional use in viral disorders. Kusumoto et al [5] studied the human immunodeficiency virus-I protease inhibitory activity of the aqueous and methanolic bark extracts. Choudhari et al [4] investigated the antineoplastic potential of the aqueous extract of *F. religiosa* bark by reducing the expression of HPV oncoproteins E6 and E7 in cervical cancer cell lines and inducing cell cycle arrest and apoptosis. 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* has been also used as an ethnic remedy for the cure of ulcers and sores in venereal diseases, applying burnt bark applied externally [7]. The main responsible agent of these genital lesions is the Herpes Simplex virus (HSV). Two types of HSV are known, HSV-1, and HSV-2, which are transmitted mainly through close contact. Both types may infect the genital mucosa causing genital herpes, even if HSV-1 is more frequently associated with oral and labial lesions [8]. Genital herpes is a widespread sexually transmitted infection, characterized by ulcerative lesions, often very painful and can lead to substantial psychological morbidity [9]. After a lifelong latent infection in lumbosacral sensory ganglia, periodically HSV-2 can be reactivated by external conditions (stress, hormonal changes and 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 ulcer disease, damaging the mucosa, induces local inflammation that may increase the risk of HIV acquisition [10]. Even if antiviral drugs targeting the viral DNA polymerase (acyclovir, penciclovir and their derivates) are available against HSV infection, plants and herbs used in traditional medicine may represent additional sources of antiviral compounds. In this context, our study wanted to explore whether the ethnomedical use of *F. religiosa* L. to treat genital lesions is substantiated by its antiviral activity against HSV-2.
Materials and Methods

Plant material

The bark and leaves of *F. religiosa* L. were collected from medicinal plant garden of Birla Institute of Technology, Mesra, Ranchi and authenticated by Botanical Survey of India, Central National Herbarium, Botanical Garden, Howrah (Letter No. CNH/11/2014/Tech.II/ dated 26-03-2014). A specimen has been deposited at the herbarium (voucher no, C-130176: PA-8).

Preparation of extracts

The fresh bark and leaves of *F. religiosa* L. were prepared as per the method described by Cagno et al [6]. Before use for antiviral assays, extracts were resuspended in a final concentration of 25 mg/mL. Water extracts were resuspended in sterile water while other extracts were resuspended in sterile DMSO. Every experiment was performed with freshly resuspended extracts.

Phytochemical investigations of *F. religiosa* L. extracts

The plant extracts were submitted to preliminary phytochemical screening (see Table 1). Briefly, flavonoids, tannins, saponins, alkaloids and steroids/triterpenoids, were detected by Shinoda test, FeCl3, Frothing test, Dragendorff’s reagent and Salkowski test respectively [6].

<table>
<thead>
<tr>
<th>Source</th>
<th>Extract</th>
<th>% Yield</th>
<th>Saponins</th>
<th>Carbohydrate</th>
<th>Alkaloids</th>
<th>Flavonoids</th>
<th>Tannins</th>
<th>Steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Water</td>
<td>6.23</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>8.41</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bark</td>
<td>Ethylacetate</td>
<td>5.27</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>9.26</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>8.14</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>10.24</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Cells

African green monkey kidney cells (Vero) (ATCC CCL-81) were cultured in Eagle’s minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat inactivated 10% fetal calf serum (FCS) (Gibco/BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37°C in an atmosphere of 5% of CO2.

Viruses

HSV-2 strain (ATCC VR-540), sensitive to acyclovir, was used for in vitro studies. HSV-2 strain with phenotypic resistance to acyclovir was generated by serial passage in the presence of increasing concentrations of acyclovir as previously described by Field et al [11]. The resistant virus was then plaque purified, and the antiviral susceptibility was tested as described in Donalisio et al [12]. Viral strains were propagated in Vero cells at 37°C in a humidified 5% CO2 incubator. When the full cytopathic effect (CPE) developed, cells and supernatants were harvested, pooled, frozen and
thawed three times, clarified and aliquoted. Viruses were stored at −70°C. Viral titers were determined by the standard plaque method as described previously [13].

**Cell viability**

Cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell cultures seeded in 96-well plates were incubated with different concentrations of extracts in triplicate under the same experimental conditions described for the antiviral assays. Cell viability was determined as described in Donalisio et al [14]. The effect on cell viability at different concentrations of the compound was expressed as a percentage, by comparing absorbances of treated cells with the ones of cells incubated with culture medium alone.

**Virus inhibition assay**

Inhibition of HSV replication was first evaluated with plaque reduction assay. Vero cells were seeded at 10 × 10⁴ cells/well in 24 well plates. The medium was removed from the plates before treatment with different concentrations of extracts (from 100 to 0.13 µg/mL). After 2h of incubation (37°C, 5% CO₂), the medium was removed and infection was performed at a multiplicity of infection (MOI) of 0.001 PFU/cell in the presence of serial dilutions of the test extract for 2 hours at 37°C. After incubation, cells were washed with medium twice and overlaid with a medium containing 1.2% methylcellulose (Sigma) and the plant extract. Treatment of control samples with equal volumes of solvent was performed in order to rule out the possibility of any cytotoxic effect ascribable to the solvent. The plates were incubated at 37°C for 1 day. After incubation, the plates were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques were counted.

Virus yield reduction assay, was performed in the same conditions previously described in plaque reduction assay but cells were infected with HSV-2 at 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 hours after infection and cell-free virus infectivity titers were determined in duplicate by the plaque assay in Vero cell monolayers. Percents of inhibition were determined by comparing the titer measured in the presence of the compounds to that measured in untreated wells.

**Virus inactivation assay**

Approximately 10⁵ PFU of HSV-2 and 33 µg/mL of water or chloroform or methanol extract were added to medium and mixed in a total volume of 100 µL. The virus-compound mixtures were incubated for 0 or 2h at 37°C then diluted serially to the non-inhibitory concentration of test compound, and the residual viral infectivity determined by standard plaque method as described above.

**Time of addition assay**

Serial dilutions of extracts were added on cells 2h before infection or during infection or post-infection. After the incubation time described before, viral plaques were counted. A virus yield reduction assay was performed adding extract on cells post infection, as described previously.

**Attachment assay**

Serial dilutions of extracts were mixed with HSV and added to cooled cells and incubated for 2h at 4°C to ensure viral attachment but not entry. After two gentle washes, cells were overlaid with 1.2% methylcellulose medium, shifted to 37°C for 24h and successively plaques were counted.

**Entry assay**

For entry assay, HSV at MOI of 0.01 PFU/cell was adsorbed for 2h at 4°C on pre-chilled confluent cells. Cells were then washed with cold MEM three times to remove unbound virus, treated with different concentrations of extract, and
incubated for 3h at 37°C. Un-penetrated viruses were inactivated with acidic glycine for 2 min at room temperature, as previously described [15]. Cells were then washed with warm medium three times and treated as described above for plaque reduction assay.

**Data analysis**

All results are presented as the mean values from three independent experiments. The 50% cytotoxic concentrations (CC\textsubscript{50}) and the half maximal effective concentration (EC\textsubscript{50}) values for inhibition curves were calculated by regression analysis using the program GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve. A selectivity index (SI) was calculated by dividing the CC\textsubscript{50} by the EC\textsubscript{50} value. Virucidal assays and virus yield reduction assays were analyzed as described in Civra et al [16].
Results

Different *F. religiosa* extracts from leaves and bark were examined *in vitro* for their antiviral activity against HSV-2. For this analysis, we used a complete protection assay 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. Acyclovir was tested in parallel as a reference drug for HSV. Table 2a shows that all extracts exerted an antiviral activity, although to a different extent, generating dose response curves. In particular, a remarkable inhibition of HSV-2 infection was exerted by water, chloroform, and methanol bark extracts with EC\(_{50}\) values of 9.76 μg/mL, 6.09 μg/mL and 5.20 μg/mL respectively. 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 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.

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

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Extract</th>
<th>EC(_{50}) * (μg/mL) – 95% C.I. #</th>
<th>EC(_{90}) § (μg/mL) – 95% C.I.</th>
<th>CC(_{50}) † (μg/mL)</th>
<th>SI ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2</td>
<td>Leaves</td>
<td>Water</td>
<td>30.94 (27.73-34.53)</td>
<td>63.83 (49.52-82.28)</td>
<td>&gt;2500</td>
<td>&gt;80.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>13.29 (9.573-18.46)</td>
<td>26.54 (9.69-72.65)</td>
<td>1058</td>
<td>79.6</td>
</tr>
<tr>
<td></td>
<td>Bark</td>
<td>Ethyl acetate</td>
<td>14.26 (5.35-38.06)</td>
<td>19.95 (2.13-187.0)</td>
<td>1057</td>
<td>74.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>9.76 (7.86-12.10)</td>
<td>18.39 (9.72-34.78)</td>
<td>1530</td>
<td>156.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>6.09 (3.17-11.69)</td>
<td>22.87 (5.83-34.78)</td>
<td>809.6</td>
<td>132.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>5.20 (3.56-7.60)</td>
<td>9.15 (4.08-20.55)</td>
<td>161.8</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td>Reference drug</td>
<td>Acyclovir</td>
<td>0.64 (0.48-0.86)</td>
<td>3.17 (1.69-5.95)</td>
<td>&gt;300</td>
<td>&gt;468</td>
</tr>
</tbody>
</table>

Table 2b. Antiviral activity of *F. religiosa* extracts against an acyclovir resistant HSV-2 strain

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Extract</th>
<th>EC(_{50}) * (μg/mL) – 95% C.I. #</th>
<th>EC(_{90}) § (μg/mL) – 95% C.I.</th>
<th>SI ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2 acyclovir resistant strain</td>
<td>Bark</td>
<td>Water</td>
<td>6.70 (4.56-9.80)</td>
<td>27.01 (11.91-61.24)</td>
<td>228.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>13.50 (9.96-18.30)</td>
<td>23.74 (8.96-62.90)</td>
<td>59.97</td>
</tr>
</tbody>
</table>

Firstly, we assessed whether the antiviral activity of the two extracts is independent on the virus sensitivity to acyclovir (Table 2b). As expected, the resistant strain exhibited elevated EC\(_{50}\) for acyclovir (60 μg/mL, data not shown in Table
2b). By contrast, it was susceptible to the water and chloroform extracts with EC50 values of 6.7 µg/ml and 13.5 µg/ml respectively.

This finding stimulated us to perform a set of experiments aimed at elucidating the major mechanism of action of the extracts. The virucidal assay explored a possible direct virus-inactivating activity of extracts at a concentration that reduced almost completely virus infection (>EC50) in the previous assay. To this aim, the extract (33 µg/mL) and a virus aliquot containing 10⁵ PFU were mixed and incubated at 37°C for 0 or 2h and then, viral samples were titrated on Vero cells at high dilutions at which extracts were no active. Fig. 1 shows that if virus/extracts mixtures were added promptly on cells without incubation, extract had no time to act on the virus and no inhibition was observed. By contrast, when the incubation was carried out for 2 hours at 37°C, water bark extract reduced significantly HSV-2 titers by 91% (P<0.05). These data indicate that the main 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. This result indicates that the chloroform extract most probably targets cell-surface or intracellular events involved in essential steps of the HSV-2 replicative cycle.

To investigate the mechanism of action of chloroform extract, we performed a series of time-of-addition assays, in which the sample was added to the cells only before, or during, or after HSV-2 infection. As shown in Fig. 2, the extract did not exert an inhibitory activity at any tested concentrations when it was added 2 h prior to virus infection and then washed out before virus inoculum (pre-treatment assay). By contrast, it generated dose-response curves when added either during and post infection, with EC50 values of 6.35 µg/mL and 14.04 µg/mL, respectively.

Next, we investigated whether it could interfere with the early steps of the HSV-2 replicative cycle, as virus attachment or entry. The attachment assay is an experimental condition in which the virus is allowed to bind to the surface of the host cells but do not undergo cell entry. The entry assay was conducted to assess whether the extract prevents the viral penetration into the host cells. As shown in Fig. 3, the chloroform extract inhibited both attachment and entry of virus with similar EC50 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 by a viral yield reduction assay - a more stringent test which allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. The assay was performed in two different experimental conditions: firstly adding the extract to cell culture before, during, and after the infection (Fig. 4A) and secondly only after the infection (Fig. 4B). In both cases, the extract generated dose response curves with EC50 values of 11.7 µg/mL and 41.65 µg/mL respectively, indicating its ability to limit an ongoing infection.
Discussion

Ficus religiosa L. (Moraceae) has been extensively used in traditional medicine for a wide range of ailments, including infectious disorders [3]. Here, we report the first study of antiviral activity of F. religiosa leaves and bark extracts against HSV-2, the main causative agent of genital ulcers and sores. Results of plaque reduction assays demonstrated that all extracts generated dose response curves with EC_{50} < 30.94 \mu g/ml. Of note, leaves water and methanol extracts of F. religiosa exerted an higher antiviral activity than water extract from the leaves of Ficus carica [17]. Phytochemical investigations demonstrated the presence of saponins and flavonoids in both water and methanol of F. religiosa leaves extracts. Further investigations are required to elucidate which fractions and pure compounds are endowed with antiviral activity. Interestingly, a new anti-HSV-1 triglycoside flavones was isolated from the leaves of Ficus ischnopoda [18] and flavones glycosides from Ficus binjamina leaves inhibited HSV-1, HSV-2 and Varicella Zoster virus cell infection in vitro [19]. Our results pointed out a stronger inhibitory activity of water and chloroform bark extracts of F. religiosa against HSV-2 infections than leaves extracts and therefore they were selected for further studies. Of note, water and chloroform bark extracts also generated dose response curves against HSV type 1, a member of alpha-herpesvirinae subfamily usually associated with oro-labial infections but also capable of infecting anogenital mucosal sites (data not shown). As reported in table 2, both extracts were able to inhibit the infection of acyclovir-resistant strain with SI of 228.36 and 59.97 for water and chloroform bark extracts, respectively, suggesting that the mechanism of action of the extracts could be different from that of acyclovir, a known inhibitor of the viral DNA polymerase. Although a crude extract may contain several components endowed 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 virucidal assay demonstrated that only the water bark extract has a direct virus-inactivating activity when HSV-2 was preincubated with the extract for 2 hours at 37°C. Interestingly, Yarmolinsky et al. published also indirect evidence for interactions between ethanol leaf extract of Ficus binjamina and HSV-1 and HSV-2 [20].

Since chloroform extract of F. religiosa did not inactivate extracellular virus particles, we explored if it could interfere with the early stages of viral infection. Time of addition assays showed that preincubation of cells with extract had no significant effect on the development of infection induced by virus, whereas when the cells were treated at the time of infection or post-infection, inhibition of HSV-2 was highest. The antiviral activity observed when the extract and the virus inoculum were added simultaneously to the cells, indicated that the extract may target early steps of viral replicative cycle such as virus attachment and/or entry. Performing specific assays, we demonstrated the ability of chloroform extract to inhibit both attachment and entry of virus. A significant reduction of the number of viral plaques was also observed when the extract was added to methylcellulose medium after infection. This finding suggests that the chloroform extract could able to inhibit the cell-to-cell spread of virus. Similarly to F. religiosa chloroform bark extract, ethanol extract of F. binjamina showed a greatest anti-HSV-2 effect when it was added to cells at the time of infection and a partial inhibitory effect when added post-infection [20].

Summarizing the results, F. religiosa water bark extract inactivates extracellular virus particles, instead chloroform bark extract exerts multiple inhibitory activities on HSV-2 replicative cycle including the virus attachment to the cell surface, the virus entry into cells, and the viral plaque formation. Furthermore, it was also able to limits the development of viral cytopathic effect during ongoing infection.

All together these findings provide a basis for the identification of bioactive metabolites of F. religiosa as therapeutic natural compounds to treat genital herpetic ulcers also 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].

**Acknowledgments**

This study was supported by a donation from Silvana Legnani

**Compliance with ethical standards** *Conflict of interest* The authors declare that they have no conflict of interest
References


LEGENDS OF FIGURES

**Fig. 1** Evaluation of virus inactivation by *F. religiosa* water bark extract (panel A) and chloroform bark extract (panel B) on infectious HSV-2 particles at 37°C for 0 or 2h. On the y axis, the infectious titers are expressed as plaque-forming units per mL (PFU/mL). Error bars represent the SEM of 3 independent experiments.

**Fig. 2** Time-of-addition assays. Cells were treated with chloroform bark extract for 2h before infection (pre-treatment), for 1h 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).

**Fig. 3** HSV-2 entry and attachment assays. The chloroform bark extract of *F. religiosa* L. was added to the cell culture during virus-cell binding (attachment assay) or virus-cell penetration (entry assay). The number of infected cells in the treated samples is expressed as a percentage of control (DMSO treated). Each point represents mean and SEM for triplicates.

**Fig. 4** Viral yield reduction assay. The chloroform bark extract of *F. religiosa* was added to cell culture before, during, and after infection (panel A), or only after infection (panel B). 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 means plus SEM for three independent experiments.
Figure 1

A

<table>
<thead>
<tr>
<th>pfu/ml</th>
<th>HSV-2</th>
<th>HSV-2 + extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>7.5x10^5</td>
<td>6.0x10^5</td>
</tr>
<tr>
<td>2h</td>
<td>6.0x10^5</td>
<td>4.5x10^5</td>
</tr>
</tbody>
</table>

Time of incubation

B

<table>
<thead>
<tr>
<th>pfu/ml</th>
<th>HSV-2</th>
<th>HSV-2 + extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>3.0x10^5</td>
<td>2.5x10^5</td>
</tr>
<tr>
<td>2h</td>
<td>2.0x10^5</td>
<td>1.5x10^5</td>
</tr>
</tbody>
</table>

Time of incubation
Table 1. Phytochemical analysis of *F. religiosa* L. extracts

<table>
<thead>
<tr>
<th>Source</th>
<th>Extract</th>
<th>% Yield</th>
<th>Saponins</th>
<th>Carbohydrate</th>
<th>Alkaloids</th>
<th>Flavonoids</th>
<th>Tannins</th>
<th>Steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Water</td>
<td>6.23</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>8.41</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bark</td>
<td>Ethylacetate</td>
<td>5.27</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>9.26</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>8.14</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>10.24</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 2a. Antiviral activity of *F. religiosa* extracts against HSV-2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Extract</th>
<th>EC_{50}^* (µg/mL) – 95% C.I.</th>
<th>EC_{90}^§ (µg/mL) – 95% C.I.</th>
<th>CC_{50}† (µg/mL)</th>
<th>SI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2</td>
<td>Leaves</td>
<td>Water</td>
<td>30.94 (27.73-34.53)</td>
<td>63.83 (49.52-82.28)</td>
<td>&gt;2500</td>
<td>&gt;80.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>13.29 (9.57-18.46)</td>
<td>26.54 (9.69-72.65)</td>
<td>1058</td>
<td>79.6</td>
</tr>
<tr>
<td></td>
<td>Bark</td>
<td>Ethyl acetate</td>
<td>14.26 (5.35-38.06)</td>
<td>19.95 (2.13-187.0)</td>
<td>1057</td>
<td>74.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>9.76 (7.86-12.10)</td>
<td>18.39 (9.72-34.78)</td>
<td>1530</td>
<td>156.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>6.09 (3.17-11.69)</td>
<td>22.87 (5.83-89.70)</td>
<td>809.6</td>
<td>132.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>5.20 (3.56-7.60)</td>
<td>9.15 (4.08-20.55)</td>
<td>161.8</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reference drug</td>
<td>Acyclovir 0.64 (0.48-0.86)</td>
<td>3.17 (1.69-5.95)</td>
<td>&gt;300</td>
<td>&gt;468</td>
</tr>
</tbody>
</table>

Table 2b. Antiviral activity of *F. religiosa* extracts against an acyclovir resistant HSV-2 strain

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Extract</th>
<th>EC_{50}^* (µg/mL) – 95% C.I.</th>
<th>EC_{90}^§ (µg/mL) – 95% C.I.</th>
<th>SI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2 acyclovir resistant strain</td>
<td>Bark</td>
<td>Water</td>
<td>6.70 (4.56-9.80)</td>
<td>27.01 (11.91-61.24)</td>
<td>228.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>13.50 (9.96-18.30)</td>
<td>23.74 (8.96-62.90)</td>
<td>59.97</td>
</tr>
</tbody>
</table>

EC_{50}^* half maximal effective concentration
C.I. ‡ confidence interval
EC_{90}^§ 90% effective concentration
CC_{50}† half maximal cytotoxic concentration
SI‡ selectivity index