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ORIGINAL ARTICLE

# *Strychnos pseudoquina* A. St. Hil.: a Brazilian medicinal plant with promising *in vitro* antiherpes activity

L. Boff[1], I.T. Silva[1], D.F. Argenta[1], L.M. Farias[2], L.F. Alvarenga[3], R.M. Pádua[3], F.C. Braga[3], J.P.V. Leite[2], J.M. Kratz[1] and C.M.O. Simões[1]

1 Laboratório de Virologia Aplicada, Programa de Pós-graduação em Farmácia, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil
2 Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil
3 Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

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**Correspondence**
Cláudia M. O. Simões, Laboratório de Virologia Aplicada, Programa de Pós-graduação em Farmácia, Universidade Federal de Santa Catarina (UFSC), Campus Trindade, Florianópolis - SC 88040-970, Brazil.
E-mail: claudia.simoes@ufsc.br

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## Abstract

**Aims:** To investigate the anti-HSV and anti-inflammatory effects of a standardized ethyl acetate extract (SEAE) prepared with the stem bark of *Strychnos pseudoquina*, along with two isolated compounds: quercetin 3-*O*-methyl ether (3MQ) and strychnobiflavone (SBF).
**Methods and Results:** The mechanisms of action were evaluated by different methodological strategies. SEAE and SBF affected the early stages of viral infection and reduced HSV-1 protein expression. Both flavonoids elicited a concentration-dependent inhibition of monocyte chemoattractant protein-1 (MCP-1), whereas 3MQ reduced the chemokine release more significantly than SBF. Conversely, both compounds stimulated the production of the cytokines TNF-$\alpha$ and IL-1-$\beta$ in LPS-stimulated cells, especially at the intermediate and the highest tested concentrations.
**Conclusions:** SEAE and SBF interfered with various steps of HSV replication cycle, mainly adsorption, postadsorption and penetration, as well as with $\beta$ and $\gamma$ viral proteins expression; moreover, a direct inactivation of viral particles was observed. Besides, both flavonoids inhibited MCP-1 selectively, a feature that may be beneficial for the development of new anti-HSV agents.
**Significance and Impact of the Study:** The results indicated that the samples present anti-HSV and anti-inflammatory activities, at different levels, which is an interesting feature since cold and genital sores are accompanied by an inflammation process.

## Introduction

Currently, it is estimated that 60–95% of worldwide adult population is infected with at least one Herpes Simplex Virus (HSV-1 or HSV-2). HSV-1 is generally associated with orofacial infections and HSV-2 with the genitourinary tract infections; nevertheless, although this anatomical distinction is no longer as strict as it was. Hence, HSV infections are an important public health problem, especially due to HSV ability to cause acute and recurrent infections as well as to become resistant to commonly used antiherpes drugs. Furthermore, HSV genital infection increases the risk of acquiring human immunodeficiency virus (HIV), which is three times higher in people with HSV-2, indicating that it is a cofactor for HIV spreading (Reynolds and Quinn 2005; Roizman *et al.* 2013).

HSV-1 and HSV-2 are alphaherpesviruses with double-stranded DNA packed in an icosahedral capsid, which is surrounded by the tegument, and lastly by a lipid envelope containing various glycoproteins. For replication, it is necessary that the viruses adsorb to and penetrate host cells, and express $\alpha$ genes (immediate early phase) that mainly regulate the viral replication, such as ICP27; $\beta$ genes (early phase) that synthesize and package DNA, such as UL42; and $\gamma$ genes (late phase), which are mostly

responsible for the structural components of the virions, such as gB and gD. Therefore, these steps can be considered potential targets for antiviral therapy, as inhibition of any one of them could lead to the blockage of HSV replication (Roizman *et al.* 2013; Kukhanova *et al.* 2014).

Natural products provide an important source of biologically active substances, playing a key role in the research and development (R&D) of novel antiherpes therapies. Actually, natural products and natural-derived scaffolds account for 70% of the new chemical entities introduced in the pharmaceutical market (Newman and Cragg 2016). Our research group has been working in the last years with different taxa of the Brazilian biodiversity to contribute to the search for new antiviral agents (Bettega *et al.* 2004; Freitas *et al.* 2009; Silva *et al.* 2010; Cardozo *et al.* 2011; Luckemeyer *et al.* 2012; Da Rosa Guimaraes *et al.* 2013; Caon *et al.* 2014).

The *Strychnos* genus (Loganiaceae) contains about 200 species distributed through Africa, South America and Australasia (Thongphasuk *et al.* 2003; Philippe *et al.* 2004; Adebowale *et al.* 2014). *Strychnos pseudoquina* A. St. Hil., popularly known as *quina-do-cerrado*, is a native cinchona-like tree of Brazilian savanna. This plant is used in folk medicine as a bitter tonic and against fevers, as well as to alleviate spleen, liver and stomach troubles (Lorenzi and Matos 2008; De Saint-Hilaire 2009). In fact, phytochemical studies carried out with *S. pseudoquina* have demonstrated the presence of alkaloids and flavonoids (Monache *et al.* 1969; Nicoletti *et al.* 1984), while pharmacological studies have reported its antiulcer (Da Silva *et al.* 2005), antileishmanial (Lage *et al.* 2013), hypoglycaemic (Honorio-Franca *et al.* 2008) as also its genotoxic potential (Santos *et al.* 2006).

In this context, due to the popularity and medicinal potential of *S. pseudoquina*, this study was performed in order to evaluate the effects of a standardized ethyl acetate extract (SEAE) prepared with the stem bark of this plant, along with two isolated compounds obtained from SEAE, namely: quercetin 3-*O*-methyl ether (3MQ) and strychnobiflavone (SBF) against herpesviruses, as well as to elucidate the mechanism of action of the most active tested compounds by using different methodological strategies. In addition, we also evaluated the effect of 3MQ and SBF on inflammatory mediators produced during HSV infection.

## Materials and methods

### Plant material

Stem bark of *S. pseudoquina* A. St. Hil. was collected in a Brazilian savanna region, more precisely at Uberlândia (Minas Gerais State, Brazil). A voucher specimen (code HUFU 10936) was deposited in the Herbarium of the Federal University of Uberlândia (UFU). Preparation and fractionation of the ethyl acetate standardized bark extract (SEAE), and the isolation, purification and structure elucidation of its compounds quercetin 3-*O*-methyl ether (3MQ) and strychnobiflavone (SBF) (Fig. 1) were performed by Lage *et al.* (2013) (as detailed in the supplementary material).

### Viruses and cell lines

All biological assays were performed on Vero cells (ATCC: CCL81), grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, NM) and maintained at 37°C and 5% $CO_2$. Vero cells are permissive for *in vitro* replication of HSV-1 and HSV-2, and demonstrate visible cytopathic effect (Hu and Hsiung 1989).

HSV-1 (KOS and 29-R strains, which are acyclovir (ACV) sensitive and resistant, respectively; Faculty of Pharmacy, University of Rennes I, Rennes, France) and HSV-2 (333 strain; Department of Clinical Virology, Göteborg University, Sweden) viral stocks were prepared, titrated based on plaque-forming units (PFU), counted by plaque assay as previously described by Burleson *et al.* (1992), and stored at −80°C. Throughout the study, two viral passages were performed from the original viral stock suspension.

### Cytotoxicity and antiherpes evaluation

The extract and its isolated compounds were dissolved in 1% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and further diluted in culture medium prior to the biological evaluation. DMSO stock solutions were stored at −20°C. Cytotoxicity of the SEAE, 3MQ and SBF was determined by sulforhodamine B (SRB) assay (Vichai and Kirtikara 2006). In brief, Vero cells ($2{\cdot}5 \times 10^4$ cells per well) were exposed to different concentrations of the extract and isolated compounds for 48 h. The 50% cytotoxic concentration ($CC_{50}$) was defined as the concentration that reduced cell viability by 50% when compared to untreated controls.

Subsequently, the potential antiherpes activity was evaluated by plaque number reduction assay as described previously by Silva *et al.* (2010). Briefly, confluent cell monolayers ($2{\cdot}5 \times 10^5$ cells per well) were infected with approximately 100 PFU of each virus strain for 1 h at 37°C. The nonattached particles were removed by washing with phosphate-buffered saline (PBS) and the treatments were performed by adding nontoxic concentrations of the extract and isolated compounds either

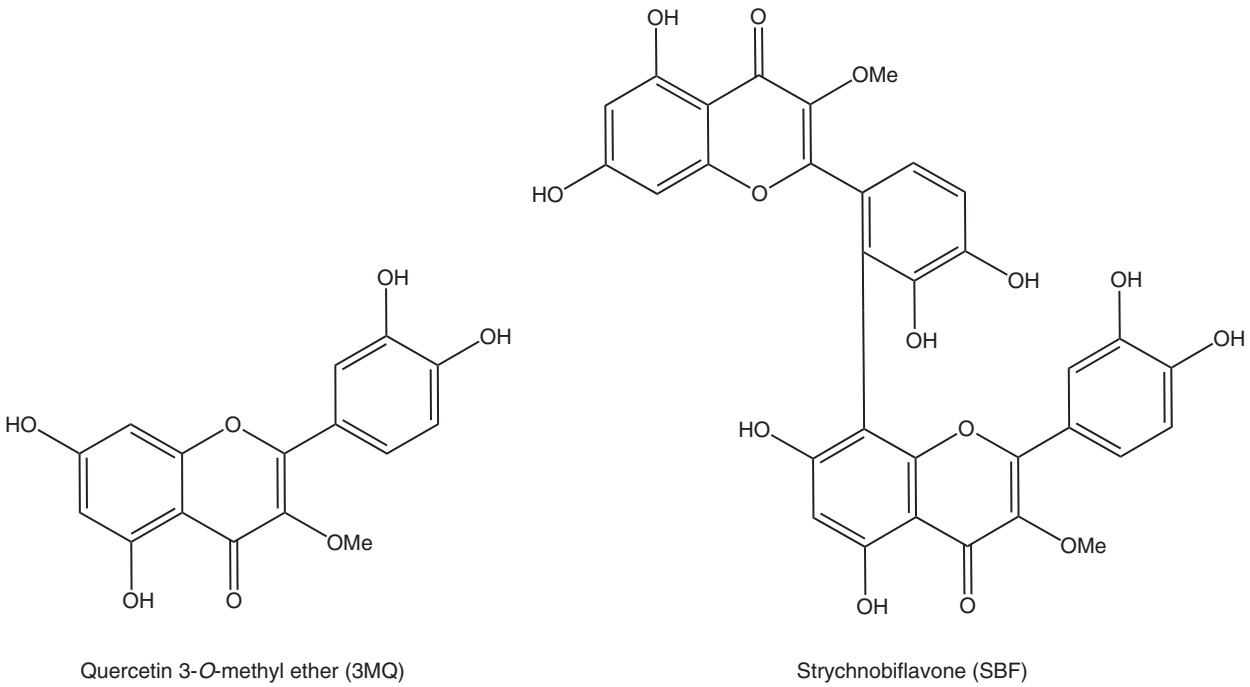


**Figure 1** Chemical structures of quercetin 3-*O*-methyl ether (3MQ) and strychnobiflavone (SBF).

simultaneously with the virus (simultaneous treatment) or after virus infection (postinfection treatment). Cells were then washed with PBS and overlaid with MEM containing 1·5% carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO) in the presence or absence of different concentrations of the SEAE, 3MQ and SBF, and incubated for 48 h at 37°C for both treatments. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich) and viral plaques were counted by using a stereomicroscope. The concentration of each sample that reduced viral replication by 50% ($IC_{50}$), when compared to untreated controls, was estimated. The ratio between $CC_{50}$ and $IC_{50}$ values was calculated to obtain the selectivity index (SI) of each sample. Acyclovir (ACV; Sigma-Aldrich) was used as positive control.

### Evaluation of antiherpes mechanism of action

The mechanisms of antiherpes action of SEAE and SBF were studied by using different methodological strategies. The KOS strain of HSV-1 and the 333 strain of HSV-2 were used since the best preliminary results were obtained with these viruses.

### Virucidal assay

This assay followed the procedures described by Silva *et al.* (2010). Mixtures of equal volumes of the extract and isolated compound at different concentrations and $4 \times 10^4$ PFU of HSV-1 or HSV-2 in serum-free MEM were co-incubated for 15 min at 4°C or 37°C. SEAE and SBF were then diluted to noninhibitory concentrations (1 : 100) to determine the residual infectivity by plaque number reduction assay as described above.

### Pretreatment

This assay was conducted as described by Bertol *et al.* (2011). Confluent cell monolayers were pretreated with different concentrations of the extract and the isolated compound for 3 h at 37°C. Then, cells were washed with PBS, infected with 100 PFU per well of HSV-1 or HSV-2 and treated as described earlier for plaque number reduction assay. ACV was used as positive control.

### Adsorption, postadsorption and penetration assays

These assays were performed following the general procedures described by Silva *et al.* (2010), and dextran sulphate (DEX-S; Sigma-Aldrich) was used as a positive control throughout the different assays. For the attachment assay, confluent cell monolayers prechilled at 4°C for 30 min were exposed to a mixture of 100 PFU per well of HSV-1 or HSV-2 in the absence (viral control) or presence of the extract and isolated compound, and incubated at 4°C for additional 2 h. Unabsorbed viruses were

removed by washing with cold PBS, cells were overlaid with CMC medium and treated as described earlier for plaque number reduction assay.

For the postattachment assay, confluent cell monolayers prechilled at 4°C for 30 min were incubated with 100 PFU per well of HSV-1 or HSV-2 at 4°C for additional 2 h in order to allow stable attachment of viruses without fusion with cell membranes. Following, SEAE and SBF were added at different concentrations, and the infected cells were incubated again at 4°C for 2 h and treated as described earlier for plaque number reduction assay.

For penetration assay, 100 PFU per well of HSV-1 or HSV-2 were adsorbed for 2 h at 4°C on confluent cell monolayers prechilled at 4°C for 30 min (at this temperature the virus can bind, but cannot penetrate the cells). Then, the temperature was shifted to 37°C for 5 min, to allow viruses penetration, and cells were treated with different concentrations of the extract and isolated compound, and incubated for 1 h at 37°C. After incubation, unpenetrated viruses were inactivated with citrate buffer (pH 3·0) for 1 min. Cells were washed with PBS and treated as described earlier for plaque number reduction assay.

### Western blot analysis

To evaluate whether the SEAE and SBF interfere with HSV-1 (KOS strain) protein expression, Western blotting experiments were performed following the procedures described by Argenta *et al.* (2015). Briefly, confluent cell monolayers were infected or not with HSV-1 at multiplicity of infection (MOI) of 0·2 for 1 h at 37°C. In next, residual viruses were removed with PBS and cells were treated with three different concentrations of SEAE and SBF (20, 40 and 60 $\mu$g ml$^{-1}$) or 20 $\mu$g ml$^{-1}$ of ACV (positive control) for 18 h (one viral replication cycle). Then, the cells were lysed and protein quantification was carried out (Bradford 1976). The protein content was separated electrophoretically in a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking, membranes were incubated overnight with anti-ICP27 (1 : 1000; Millipore, Billerica, MA), anti-UL42 (1 : 1000; Millipore), anti-gD (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-$\beta$-actin antibodies (1 : 5000; Millipore); the last one was used as a control for total protein loading. After washing, membranes were incubated with the respective secondary antibodies conjugated to horseradish peroxidase for 1 h. Protein bands were revealed using Pierce Enhanced Chemiluminescence ECL Western Blotting substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The images were acquired using a ChemiDocTM MP System (Bio-Rad, Hercules, CA, USA) and digitalized using the Image Lab program, version 4.1 (Bio-Rad, Hercules, CA, USA). Relative densitometry data for the blots were analysed with ImageJ free software (National Institutes of Health, Bethesda, USA).

### Synergistic effects in combination with acyclovir

Potential synergistic effects of SEAE and SBF with ACV were evaluated by plaque number reduction assay following the procedures described by Cardozo *et al.* (2011). Briefly, each sample, alone or in combination, was tested at fixed ratios, based on its corresponding $IC_{50}$ value (i.e. at $IC_{50}$ × 0·25, × 0·5, × 1, × 2 and × 4). The degree of interactions between SEAE, 3MQ, SBF and ACV were calculated through the combination index (CI) equation, based on the median-effect principle of the mass-action law, using the software CALCUSYN (version 2.1; Biosoft, Cambridge, UK). According to Chou (2006), CI values <1, =1, and >1 indicate synergism, additive effect and antagonism respectively.

### Assay of pro-inflammatory mediators

The potential anti-inflammatory activity of 3MQ and SBF was evaluated by quantifying TNF-$\alpha$, IL-1$\beta$ and monocyte chemoattractant protein - 1 (MCP-1) produced by LPS-stimulated THP-1 cells employing an immunoassay, as previously reported (Gusman *et al.* 2015). THP-1 cells (ATCC TIB-202) were cultivated in RPMI 1640 medium (Sigma-Aldrich) supplemented with 0·05 mmol l$^{-1}$ 2-mercaptoethanol, 10% FBS (Gibco), 100 U ml$^{-1}$ of penicillin (Sigma-Aldrich), and 100 $\mu$g ml$^{-1}$ of gentamicin (Sigma-Aldrich) at 37°C in an atmosphere containing 5% $CO_2$. The medium was replaced twice a week, when cell concentrations reached 1·0 × 10$^6$ cells ml$^{-1}$. The cells were transferred to a 96-well microplate at a density of 100 000 cells per well, incubated for 18 h and pretreated with the compounds for 3 h. LPS (Sigma-Aldrich) was administrated at 100 ng ml$^{-1}$ as inflammatory stimulus. After incubating the plate at 37°C overnight, it was centrifuged (1800 ***g***, 5 min, 16°C), the supernatant was collected and the pro-inflammatory mediators were quantified by ELISA, according to the manufacturer's instructions (TNF-$\alpha$ duo set, IL-1$\beta$ duo set and CCL2 duo set, R&D Systems, Minneapolis, MN). Cell viability was evaluated at the assayed concentrations by the MTT method (Ferrari *et al.* 1990), using untreated cells as reference for viability. The percentages of TNF-$\alpha$, IL-1$\beta$ and CCL2 inhibition were calculated by the ratio between the amount of mediators secreted by treated cells (pg ml$^{-1}$) and their baseline level (pg ml$^{-1}$) observed for solvent control (0·1% DMSO). The compounds were tested at 10, 30 and 90 $\mu$mol l$^{-1}$ and dexamethasone

(Sigma-Aldrich) was employed as positive control (0·1 $\mu$mol $l^{-1}$). For the assays, all compounds were solubilized in RPMI medium containing 0·1% DMSO.

### Statistical analysis

The mean values ± standard deviations are representative of two or three independent experiments. For the determination of $CC_{50}$ and $IC_{50}$ values, nonlinear regression of concentration-response curves was used. Statistical analyses were performed by ANOVA followed by *post hoc* tests as indicated.

## Results

### Antiherpes activity

According to the $CC_{50}$ results shown in Table 1, 3MQ exhibited the highest toxic effects on Vero cells (2·32 $\mu$g $ml^{-1}$) and SEAE presented moderate toxic effects (53·77 $\mu$g $ml^{-1}$), while SBF was well tolerated (267·23 $\mu$g $ml^{-1}$). Regarding the antiviral screening (postinfection treatment), the most promising results were obtained with SBF against HSV-2 and HSV-1 (KOS strain), presenting SI values of 42·33 and 22·61, respectively. Concerning SEAE activity, the $IC_{50}$ values were similar to SBF, but due to the higher toxicity of the extract, the SI values were significantly lower. 3MQ showed no significant antiviral action at the tested concentrations and experimental conditions employed in this study.

### Mechanism of antiherpes activity

The elucidation of the mechanism of antiherpes activity was conducted with SEAE and SBF against HSV-1 (KOS strain, ACV sensitive) and HSV-2 (333 strain), since HSV-1 (29-R strain, ACV resistant) was less sensitive to the extract and the isolated compounds considered in this study.

Firstly, the virus-inactivating activity of SEAE and SBF in the absence of cells was evaluated by the classical virucidal assay. At 37°C, the extract and its isolated compound caused complete inactivation of HSV-1 and HSV-2 infectivity, exhibiting $IC_{50}$ values of 6·07 ± 1·51 and 3·55 ± 0·56 for SEAE and SBF against HSV-1, respectively; and 5·70 ± 1·24 and 9·64 ± 2·00 $\mu$g $ml^{-1}$ for SEAE and SBF against HSV-2, respectively. On the other hand, when the temperature was reduced to 4°C, the effects of the extract and its isolated compound on the infectivity of both tested viruses were significantly reduced, with $IC_{50}$ values of 36·06 ± 4·60 and 68·08 ± 8·98 $\mu$g $ml^{-1}$ for SEAE and SBF against HSV-1, respectively; and 12·86 ± 2·70 and 7·91 ± 1·14 $\mu$g $ml^{-1}$ for SEAE and SBF against HSV-2, respectively.

**Table 1** Cytotoxicity and antiherpes activity of SEAE, 3MQ and SBF obtained from *Strychnos pseudoquina*

| Samples | $CC_{50}$ ($\mu$g $ml^{-1}$) | $IC_{50}$ ($\mu$g $ml^{-1}$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Simultaneous treatment | | | | Postinfection treatment | | | | | |
| | | HSV-1 (KOS) | | HSV-2 (333) | | HSV-1 (KOS) | | HSV-1 (29-R) | | HSV-2 (333) | |
| | Vero cells | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI |
| SEAE | 53·77 ± 14·67 | 5·29 ± 1·56 | 10·16 | 6·55 ± 0·25 | 8·21 | 14·01 ± 2·56 | 3·84 | 17·62 ± 1·53 | 3·05 | 8·64 ± 0·39 | 6·22 |
| 3MQ | 2·32 ± 0·67 | NT | NT | NT | NT | NI | – | NI | – | NI | – |
| SBF | 267·23 ± 34·55 | 2·04 ± 0·16 | 131·10 | 0·99 ± 0·13 | 270·13 | 11·82 ± 4·32 | 22·61 | 27·64 ± 1·99 | 9·67 | 6·31 ± 1·42 | 42·33 |
| DEX-S | >500 | 0·17 ± 0·06 | >2·941 | 0·19 ± 0·05 | >2·631 | NT | – | NT | – | NT | – |
| ACV | >2·000 | NI | – | NI | – | 1·38 ± 0·46 | >1·449 | NI | – | 3·23 ± 0·89 | >619 |

SI, Selectivity index (SI = $CC_{50}/IC_{50}$); NI, no inhibitory detected activity; NT, not tested; SEAE, ethyl acetate standardized bark extract; 3MQ, quercetin 3-*O*-methyl ether; SBF, strychnobiflavone; DEX-S, dextran sulphate; ACV, acyclovir.
$CC_{50}$: 50% cytotoxic concentration for Vero cells ($\mu$g $ml^{-1}$); $IC_{50}$: 50% concentration that inhibited viral replication ($\mu$g $ml^{-1}$).
These values represent the mean ± SD of three independent experiments.

To investigate the effects of SEAE and SBF directly on the Vero host cells, a pretreatment assay was performed; when this strategy was tested against HSV-2 infection, the extract and its isolated compound exhibited $IC_{50}$ values of 7·56 ± 1·89 and 3·43 ± 0·98 $\mu$g ml$^{-1}$, respectively. In contrast, the pretreatment of cells with SEAE and SBF did not affect HSV-1 viral replication.

A set of different assays was carried out to verify if the extract and its isolated compound were able to interfere with the early events of HSV infection. The results obtained demonstrated that SEAE and SBF inhibited attachment, postattachment and penetration, at different levels, of both HSV strains tested (Table 2). When the extract and its isolated compound were mixed and incubated with viral suspensions, the SEAE was more active against HSV-1 (KOS strain) than against HSV-2 (333 strain) exhibiting $IC_{50}$ values approximately 2 $\mu$g ml$^{-1}$ (HSV-1) and 6 $\mu$g ml$^{-1}$ (HSV-2). Concerning the SBF, its anti-herpetic activity was not different regarding the tested viral strain, showing $IC_{50}$ values around 3 $\mu$g ml$^{-1}$. When the cell monolayer was infected and then treated with the extract and its isolated compound, both SEAE and SBF showed no differences in intensity of antiviral action against the strains tested, exhibiting $IC_{50}$ values approximately 3 $\mu$g ml$^{-1}$ (SEAE) and 1 $\mu$g ml$^{-1}$ (SBF); the SBF affected this step more strongly than SEAE. Finally, the $IC_{50}$ values obtained from penetration assay were around 7 $\mu$g ml$^{-1}$ (SEAE) and 8 $\mu$g ml$^{-1}$ (SBF) against HSV-1, and 4 $\mu$g ml$^{-1}$ (SEAE) and 5 $\mu$g ml$^{-1}$ (SBF) against HSV-2.

The effects of SEAE and SBF on HSV-1 protein expression were evaluated by Western blot analyses. Figure 2 shows a representative blot of the results obtained after 18 h of treatment (one viral replication cycle) with the extract and its isolated biflavone (20, 40 and 60 $\mu$g ml$^{-1}$), or ACV (20 $\mu$g ml$^{-1}$). SEAE reduced the expression of ($\beta$) UL42 and ($\gamma$) gD proteins more efficiently than SBF. Regarding ($\alpha$) ICP27 protein expression, treatment with the extract and its isolated compound led to a concentration-dependent inhibition. The SEAE and SBF did not interfere with constitutive proteins, as $\beta$-actin synthesis was unaffected.

The potential synergistic effects between SEAE or SBF with ACV were evaluated by a classical combinatorial experiment. Only SBF showed CI values <1: 0·969 (HSV-1) and 0·987 (HSV-2), indicating nearly additive effects with ACV, according to the CI theorem (Chou 2006). Concerning the effects of SEAE, no synergistic effects were detected (data not shown).

### Effects on pro-inflammatory mediators

The effects of the isolated compounds 3MQ and SBF on the pro-inflammatory mediators MCP-1, TNF-$\alpha$ and

**Table 2** Effects of SEAE and SBF obtained from *Strychnos pseudoquina* on HSV adsorption, postadsorption and penetration

| Samples | $CC_{50}$ ($\mu$g ml$^{-1}$) Vero cells | $IC_{50}$ ($\mu$g ml$^{-1}$) Attachment | | | | Postattachment | | | | Penetration | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HSV-1 (KOS strain) | | HSV-2 (333 strain) | | HSV-1 (KOS strain) | | HSV-2 (333 strain) | | HSV-1 (KOS strain) | | HSV-2 (333 strain) | |
| | | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI | $IC_{50}$ | SI |
| SEAE | 53·77 ± 14·67 | 1·96 ± 0·39 | 27·43 | 6·02 ± 0·99 | 8·93 | 3·12 ± 1·42 | 17·23 | 3·15 ± 0·21 | 17·07 | 7·33 ± 0·85 | 7·34 | 4·47 ± 0·50 | 12·03 |
| SBF | 267·23 ± 34·55 | 3·05 ± 0·73 | 87·63 | 3·35 ± 0·74 | 79·72 | 1·40 ± 0·17 | 191·04 | 1·11 ± 0·16 | 242·35 | 7·92 ± 1·87 | 33·74 | 5·11 ± 1·62 | 52·29 |
| DEX-S | >500 | 0·16 ± 0·06 | >3·125 | 0·10 ± 0·04 | >5·000 | 0·21 ± 0·10 | >2·380 | 0·17 ± 0·08 | >2·941 | 1·78 ± 0·37 | >1·351 | 2·39 ± 0·55 | >909·1 |

SI, Selectivity index (SI = $CC_{50}/IC_{50}$); NI, no inhibitory detected activity; NT, not tested; SEAE, ethyl acetate standardized bark extract; SBF, strychnobiflavone; DEX-S, dextran sulphate.
$CC_{50}$: 50% cytotoxic concentration for Vero cells ($\mu$g ml$^{-1}$); $IC_{50}$: 50% concentration that inhibited viral replication ($\mu$g ml$^{-1}$).
These values represent the mean ± SD of three independent experiments.

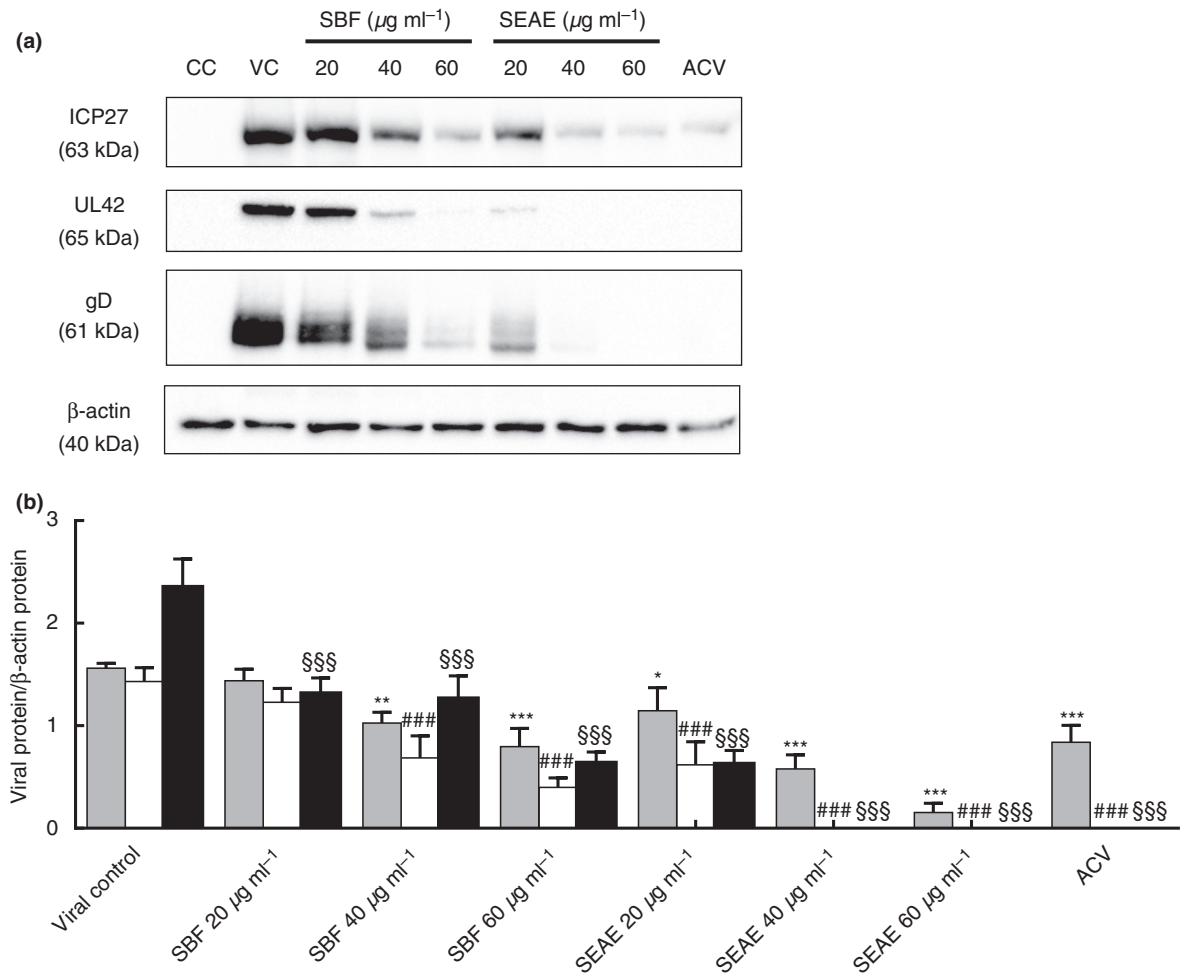


**Figure 2** Effects of SEAE and SBF obtained from *Strychnos pseudoquina* on HSV-1 (KOS strain) protein expression. (a) Confluent Vero cells were infected with HSV-1 (MOI 0·2) and treated with 20, 40 and 60 $\mu$g ml$^{-1}$ of the extract and its isolated compound or 20 $\mu$g ml$^{-1}$ of acyclovir. Lysates were collected after 18 h of incubation, run on SDS-10% PAGE gel and analysed using specific antibodies for viral (ICP27, UL42 and gD) proteins. Equal protein loading was confirmed by probing for $\beta$–actin. CC (cell control) = Vero cells uninfected and untreated; VC (viral control) = Vero cells infected but not treated; ACV (acyclovir). Equal protein loading was confirmed by probing for $\beta$-actin. (b) The graph indicates the ratio of each viral protein (▨ ICP27; □ UL42; ■ gD) to $\beta$-actin protein ($^{*}P < 0{\cdot}01$, $^{**}P < 0{\cdot}001$, $^{***}P < 0{\cdot}0001$; $^{\#\#\#}P < 0{\cdot}0001$; $^{\S\S\S}P < 0{\cdot}0001$ *vs* the respective viral controls, two-way ANOVA, *post hoc* test Dunnet).

IL-1-$\beta$ were evaluated on a human monocytic cell line (THP-1) previously stimulated by LPS. The compounds showed no toxicity to the THP-1 cells at all concentrations assayed (cell viability >90%, data not shown). Both flavonoids elicited a concentration-dependent inhibition of MCP-1, whereas 3MQ more significantly reduced chemokine release. When tested at 90 $\mu$mol l$^{-1}$, 3MQ induced a similar response to the reference drug dexamethasone (Fig. 3a). On the other hand, both compounds stimulated the production of the cytokines TNF-$\alpha$ (Fig. 3b) and IL-1-$\beta$ (Fig. 3c) in LPS-stimulated cells, especially at the intermediate and the highest tested concentrations.

## Discussion

In this study, the antiherpes activities of SEAE, a standardized extract obtained from *S. pseudoquina* stem bark, and two isolated compounds (3MQ and SBF), were investigated against HSV-1 and HSV-2 replication. SEAE and SBF showed promising *in vitro* antiherpes activity against HSV-1 (KOS strain) and HSV-2 (333 strain). The isolated compound SBF was slightly more active than the standardized extract against both viruses, with significant lower cytotoxicity. Antiherpes activity of other biflavones has been reported against HSV-1 and HSV-2 (Hayashi *et al.* 1992; Andrighetti-Frohner *et al.* 2005; Freitas *et al.*

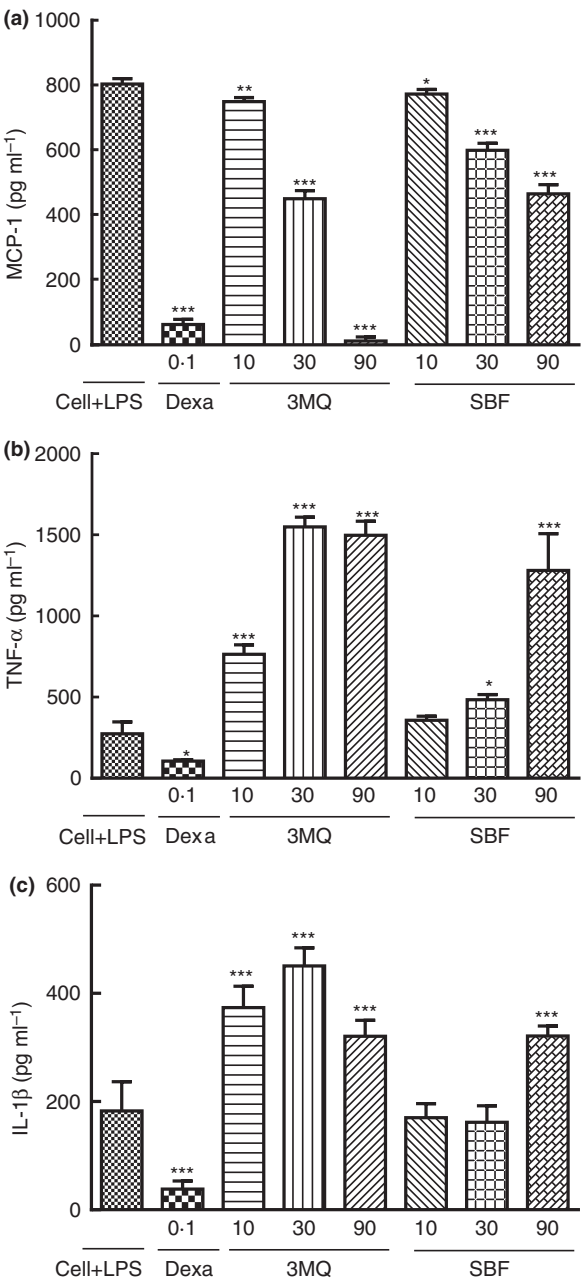


**Figure 3** Effects of 3MQ and SBF on pro-inflammatory mediators. THP-1 cells were treated with the isolated compounds 3MQ and SBF, or dexamethasone (0·1 $\mu$mol $l^{-1}$; Dexa, positive control), or solvent control (0·1% DMSO) and stimulated with LPS (100 ng $ml^{-1}$). Supernatants were collected for MCP-1, TNF-$\alpha$ and IL-1$\beta$ measurements by ELISA. Differences in the release of the mediators elicited by the compounds in comparison to the control (cell + LPS) were analysed by one-way ANOVA, *post hoc* test Newman-Keuls (*$P < 0{\cdot}01$, **$P < 0{\cdot}001$, ***$P < 0{\cdot}0001$).

2009). Although antiherpes activity of 3MQ has been reported previously (Bettega *et al.* 2004), in this work, our results detected higher toxic effects and no significant antiviral action, for all HSV strains, at the tested experimental conditions. For this reason, the study of the mechanism of antiherpes action was conducted only with SEAE and SBF.

HSV viral replication cycle can be divided into several stages: viral entry into the host cells, expression of viral genes, DNA replication, virus assembly, and egress of the new generation of viral particles (Kukhanova *et al.* 2014). Herein, the initial phases of HSV-1 replication cycle seem to be particularly affected by SEAE and SBF treatment. The $IC_{50}$ values obtained with the simultaneous treatment were lower than the ones obtained with the postinfection treatment, even if the incubation time of the extract and its isolated compound is significantly shortened in the assay. The following step was to verify the effects of SEAE and SBF in the specific early stages of viral infection. Our results showed that HSV attachment, postattachment and penetration were inhibited by the extract and its isolated compound, at different levels, as can be verified by the SI values.

Moreover, a direct inactivation of viral particles by the extract and its isolated compound was observed. The virucidal activity was also assessed for another biflavone, the ginkgetin (Hayashi *et al.* 1992). Thus, it could be suggested that SEAE and SBE will be active when applied topically, as they are able to inactivate viral particles directly. This approach has a great appeal in the therapy of sexually transmitted diseases, including HSV infection (Ekblad *et al.* 2010).

To explore the effects of SEAE and SBF on the host cells, a pretreatment assay was conducted. The results suggest that the extract and isolated compound can protect cells against HSV-2 infection at relatively low concentrations. This effect corroborates the potential clinical application of SEAE and SBF as topical prophylactic/therapeutic agents. Intriguingly, HSV-1 infection was not impaired by the pretreatment of cells with both samples. Additional experiments including mutants strains will be performed in the future. This will be done to assess the influence of glycoproteins in the adsorption because we assume that the differences between HSV-1 and HSV-2 detected in this study are related to glycoproteins.

As previously mentioned, the $\alpha$ genes regulate viral replication; the $\beta$ genes are responsible for synthesize and packaging DNA, and the $\gamma$ genes synthesize the structural components of the virions. By Western blotting assays, a significant reduction of ($\beta$) UL42 and ($\gamma$) gD protein expression was detected, which were more affected by SEAE than by SBF treatment. Therefore, SEAE and SBF could interfere with the synthesis of DNA and structural components of the virions. Furthermore, SBF was able to inhibit the ($\gamma$) gD and ($\beta$) UL42 protein expression in a concentration-dependent manner. Concerning the ($\alpha$) ICP27, a minor inhibition was observed for SEAE and

SBF. These results suggest that, at the molecular level, the extract and isolated compound might interfere more intensively with expression of early and late proteins, in comparison with immediate early proteins.

The potential synergistic effects between SEAE or SBF and ACV were tested at different concentrations. Only SBF showed nearly additive effects with ACV against HSV-1 and HSV-2, since the obtained CI values ranged between 0·90 and 1·10 (Chou 2006).

The inflammatory response plays a crucial role in HSV infections. Chemokines are essential for the innate and adaptive immune responses to the viruses, but the outcomes of these responses may be deleterious to the host and affect, for example, the preservation of the visual axis, in the case of ocular HSV-1 infection (Carr and Tomanek 2006). Thereby, the selective control of chemokine expression during viral infection may be beneficial to the host. The flavonoids isolated from *S. pseudoquina* promoted a concentration-dependent inhibition of MCP-1 release by LPS-stimulated THP-1 cells, when assayed at concentrations below 100 $\mu$mol $l^{-1}$. It is noteworthy that 3MQ and SBF induced a selective MCP-1 inhibition, whereas they showed no effects or increased the levels of TNF-$\alpha$ and IL-1$\beta$ in LPS-stimulated cells, depending on the assayed concentrations. In the same way, Lin *et al.* (2012) investigated the effects of $\beta$-carotene in the production of pro-inflammatory mediators by RAW264.7 cells infected with pseudorabies virus. The treatment with $\beta$-carotene bellow 100 $\mu$mol $l^{-1}$ diminished significantly the production of NO, IL-1$\beta$, IL-6, TNF-$\alpha$ and MCP-1, with clear selective inhibitory effects on IL-1$\beta$ and IL-6.

The combination of antiviral therapy with anti-inflammatory agents seems to be a beneficial strategy for controlling HSV infection, as demonstrated by Boivin *et al.* (2013). Such authors reported a significant increase in the survival rate of mice infected with HSV-1 treated with a combination of valacyclovir and an anti-TNF-$\alpha$ antibody, in comparison to antiviral therapy alone. A better understanding of the role of pro-inflammatory cytokines in viral infection, and the outcomes of their inhibition, may impact positively on the development of new anti-HSV drugs. Therefore, natural products sharing antiviral and anti-inflammatory potential are especially desirable, as reported here for 3MQ and SBF.

In conclusion, our findings suggest that SEAE and SBF interfere with various steps of HSV replication cycle, mainly adsorption, postadsorption and penetration, as well as with $\beta$ and $\gamma$ viral protein expression. Besides, both flavonoids inhibit selectively the pro-inflammatory chemokine MCP-1, a feature that may be beneficial for the development of new antiherpes agents. These results corroborate the fact that natural compounds still could be regarded as a promising source of new drugs, especially as an alternative for the treatment of herpes simplex. In all experiments, the biflavone SBF was more active than the extract. In fact, the HPLC analysis (supplementary material) showed that SBF is the major compound detected in the standardized extract (189·51 $\mu$g $ml^{-1}$ of SBF *versus* 105·8 $\mu$g $ml^{-1}$ of 3MQ). Therefore, SBF could be regarded as the main bioactive compound of SEAE, although other active constituents cannot be ruled out at the moment. Note that, these are preliminary results obtained *in vitro*, and *in vivo* assays have to be performed to verify a successful future applicability.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## References

Adebowale, A., Naidoo, Y., Lamb, J. and Nicholas, A. (2014) Comparative foliar epidermal micromorphology of Southern African *Strychnos* L. (Loganiaceae): taxonomic, ecological and cytological considerations. *Plant Syst Evol* **300**, 127–138.

Andrighetti-Frohner, C.R., Sincero, T.C., da Silva, A.C., Savi, L.A., Gaido, C.M., Bettega, J.M., Mancini, M., de Almeida, M.T. *et al.* (2005) Antiviral evaluation of plants from Brazilian Atlantic Tropical Forest. *Fitoterapia* **76**, 374–378.

Argenta, D.F., Silva, I.T., Bassani, V.L., Koester, L.S., Teixeira, H.F. and Simões, C.M. (2015) Antiherpes evaluation of soybean isoflavonoids. *Arch Virol* **160**, 2335–2342.

Bertol, J.W., Rigotto, C., de Padua, R.M., Kreis, W., Barardi, C.R., Braga, F.C. and Simões, C.M. (2011) Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of *Digitalis lanata*. *Antiviral Res* **92**, 73–80.

Bettega, J.M., Teixeira, H., Bassani, V.L., Barardi, C.R. and Simões, C.M. (2004) Evaluation of the antiherpetic activity of standardized extracts of *Achyrocline satureioides*. *Phytother Res* **18**, 819–823.

Boivin, N., Menasria, R., Piret, J., Rivest, S. and Boivin, G. (2013) The combination of valacyclovir with an anti-TNF alpha antibody increases survival rate compared to antiviral therapy alone in a murine model of herpes simplex virus encephalitis. *Antiviral Res* **100**, 649–653.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Burleson, F.G., Chambers, T.M. and Wiedbrauk, D.L. (1992) *Virology: A Laboratory Manual*, p. 250. San Diego, CA: Academic.

Caon, T., Kaiser, S., Feltrin, C., de Carvalho, A., Sincero, T.C., Ortega, G.G. and Simões, C.M. (2014) Antimutagenic and antiherpetic activities of different preparations from *Uncaria tomentosa* (cat's claw). *Food Chem Toxicol* **66**, 30–35.

Cardozo, F.T., Camelini, C.M., Mascarello, A., Rossi, M.J., Nunes, R.J., Barardi, C.R., de Mendonca, M.M. and Simões, C.M. (2011) Antiherpetic activity of a sulfated polysaccharide from *Agaricus brasiliensis* mycelia. *Antiviral Res* **92**, 108–114.

Carr, D.J. and Tomanek, L. (2006) Herpes simplex virus and the chemokines that mediate the inflammation. *Curr Top Microbiol Immunol* **303**, 47–65.

Chou, T.C. (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* **58**, 621–681.

Da Rosa Guimaraes, T., Quiroz, C.G., Borges, C.R., de Oliveira, S.Q., de Almeida, M.T., Bianco, E.M., Moritz, M.I., Carraro, J.L. *et al.* (2013) Anti HSV-1 activity of halistanol sulfate and halistanol sulfate C isolated from Brazilian marine sponge *Petromica citrina* (Demospongiae). *Mar Drugs* **11**, 4176–4192.

Da Silva, M.A., Rafacho, B.P., Hiruma-Lima, C.A., da Rocha, L.R., dos Santos, L.C., Sannomiya, M., Souza-Brito, A.R. and Vilegas, W. (2005) Evaluation of *Strychnos pseudoquina* A. St. Hil. leaves extract on gastrointestinal activity in mice. *Chem Pharm Bull (Tokyo)* **53**, 881–885.

De Saint-Hilaire, A. (2009) *Plantas usuais dos brasileiros*. pp. 11–16. Belo Horizonte: Código Comunicação.

Ekblad, M., Adamiak, B., Bergstrom, T., Johnstone, K.D., Karoli, T., Liu, L., Ferro, V. and Trybala, E. (2010) A highly lipophilic sulfated tetrasaccharide glycoside related to muparfostat (PI-88) exhibits virucidal activity against herpes simplex virus. *Antiviral Res* **86**, 196–203.

Ferrari, M., Fornasiero, M.C. and Isetta, A.M. (1990) MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. *J Immunol Methods* **131**, 165–172.

Freitas, A.M., Almeida, M.T., Andrighetti-Frohner, C.R., Cardozo, F.T., Barardi, C.R., Farias, M.R. and Simões, C.M. (2009) Antiviral activity-guided fractionation from *Araucaria angustifolia* leaves extract. *J Ethnopharmacol* **126**, 512–517.

Gusman, G.S., Campana, P.R., Castro, L.C., Castilho, R.O., Teixeira, M.M. and Braga, F.C. (2015) Evaluation of the effects of some Brazilian medicinal plants on the production of TNF- alpha and CCL2 by THP-1 Cells. *Evid Based Complement Alternat Med* **2015**, 497123.

Hayashi, K., Hayashi, T. and Morita, N. (1992) Mechanism of action of the antiherpesvirus biflavone ginkgetin. *Antimicrob Agents Chemother* **36**, 1890–1893.

Honorio-Franca, A.C., Marins, C.M., Boldrini, F. and Franca, E.L. (2008) Evaluation of hypoglicemic activity and healing of extract from amongst bark of "Quina do Cerrado" (*Strychnos pseudoquina* A. St. Hil.). *Acta Cir Bras* **23**, 504–510.

Hu, J.M. and Hsiung, G.D. (1989) Evaluation of new antiviral agents: I. In vitro perspectives. *Antiviral Res* **11**, 217–232.

Kukhanova, M.K., Korovina, A.N. and Kochetkov, S.N. (2014) Human herpes simplex virus: life cycle and development of inhibitors. *Biochemistry* **79**, 1635–1652.

Lage, P.S., de Andrade, P.H., Lopes, A.S., Fumagalli, M.A., Valadares, D.G., Duarte, M.C., Lage, D.P., Costa, L.E. *et al.* (2013) *Strychnos pseudoquina* and its purified compounds present an effective in vitro antileishmanial activity. *Evid Based Complement Alternat Med* **2013**, 304354.

Lin, H.W., Chang, T.J., Yang, D.J., Chen, Y.C., Wang, M. and Chang, Y.Y. (2012) Regulation of virus-induced inflammatory response by beta-carotene in RAW264.7 cells. *Food Chem* **134**, 2169–2175.

Lorenzi, H. and Matos, F.J. (2008) *Plantas Medicinais no Brasil - Nativas e Exóticas*. pp. 347. Nova Odessa - SP: Instituto Pantarum.

Luckemeyer, D.D., Muller, V.D., Moritz, M.I., Stoco, P.H., Schenkel, E.P., Barardi, C.R., Reginatto, F.H. and Simões, C.M. (2012) Effects of *Ilex paraguariensis* A. St. Hil. (yerba mate) on herpes simplex virus types 1 and 2 replication. *Phytother Res* **26**, 535–540.

Monache, F.D., Aldo, P.T. and Marini-Bettolo, G.B. (1969) The occurrence of nor-dihydrotoxiferine in *Strychnos pseudoquina* A. St. Hil. *Tetrahedron Lett* **25**, 2009–2010.

Newman, D.J. and Cragg, G.M. (2016) Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod* **79**, 629–661.

Nicoletti, M., Goulart, M.O., de Lima, R.A., Goulart, A.E., Delle Monache, F. and Marini Bettolo, G.B. (1984) Flavonoids and alkaloids from *Strychnos pseudoquina*. *J Nat Prod* **47**, 953–957.

Philippe, G., Angenot, L., Tits, M. and Frederich, M. (2004) About the toxicity of some *Strychnos* species and their alkaloids. *Toxicon* **44**, 405–416.

Reynolds, S.J. and Quinn, T.C. (2005) Developments in STD/HIV interactions: the intertwining epidemics of HIV and HSV-2. *Infect Dis Clin North Am* **19**, 415–425.

Roizman, B., Knipe, D.M. and Whitley, R.J. (2013) Herpes simplex viruses. In *Fields Virology* ed. Knipe, D.M., Howley, P.M., Cohen, J.I., Griffin, D.E., Lamb, R.A., Martin, M.A., Racaniello, V.R. and Roizman, B. pp. 1823–1897. Philadelphia: Lippincott Willians & Wilkins.

Santos, F.V., Colus, I.M., Silva, M.A., Vilegas, W. and Varanda, E.A. (2006) Assessment of DNA damage by extracts and fractions of *Strychnos pseudoquina*, a Brazilian medicinal plant with antiulcerogenic activity. *Food Chem Toxicol* **44**, 1585–1589.

Silva, I.T., Costa, G.M., Stoco, P.H., Schenkel, E.P., Reginatto, F.H. and Simões, C.M. (2010) In vitro antiherpes effects of a C-glycosylflavonoid-enriched fraction of *Cecropia glaziovii* Sneth. *Lett Appl Microbiol* **51**, 143–148.

Thongphasuk, P., Suttisri, R., Bavovada, R. and Verpoorte, R. (2003) Alkaloids and a pimarane diterpenoid from *Strychnos vanprukii*. *Phytochemistry* **64**, 897–901.

Vichai, V. and Kirtikara, K. (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* **1**, 1112–1116.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** HPLC analysis of the standardized ethyl acetate extract (SEAE) prepared with stem barks of *Strychnos pseudoquina* A. St. Hil.