

**IN VITRO ANTI-HSV-2 ACTIVITY OF ISOQUERCETIN FROM *HYPTIS*
FASCICULATA BENTH.**

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ABSTRACT

Ethyl acetate extract from leaves and flowers of *Hyptis fasciculata* Benth. (Lamiaceae) and the isolated flavonoid isoquercetin showed inhibitory activity against herpes simplex virus type 2 in vitro, with EC₅₀ values of 2 µg/ml and 1.4 µg/ml, respectively, at non cytotoxic concentrations. The selective index of ethyl acetate extract and isoquercetin was higher than 100 and 142.8, respectively. Antiviral activity of the extract and isoquercetin was found to occur at the early stage of virus replication. Furthermore, ethyl acetate extract and isoquercetin were able to directly inactivate the virus particles (virucidal action). These results suggest that *H. fasciculata* ethyl acetate extract and the isolated flavonoid isoquercetin have the potential to inhibit the infection caused by HSV-2.

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) usually causes genital infection and has been responsible for serious and persistent disease, particularly in immunocompromised patients. The drug of choice for treating this disease is acyclovir, but due to the emergence of acyclovir resistant herpes simplex viruses and the cost of the traditional treatment, new strategies are being developed as alternative therapy.

Screening of plant extracts for anti-herpetic effect has been performed by our group (Miranda et al. 1997, Wigg 1997, Almeida et al. 1998, Amaral et al. 1999, Gonçalves et al. 2001, Esquenazi et al. 2002, Miranda et al. 2002, Gonçalves et al. 2005), as part of a project to study the antiviral activity of plants cultivated in Brazil.

The genus *Hyptis* belongs to the family Lamiaceae and has approximately 400 species distributed from the South of the United States of America all the way to Argentina (Falcão et

al. 2003). This genus includes species with great economic and ethnopharmacological importance. Some of them are used in traditional medicine and have already been shown to contain bio-active molecules in studies carried out in Brazil (Falcão et al. 2003); Mexico (Pereda-Miranda et al., 1993); India (Mukherjee et al. 1984); China (Lee et al. 1988); Ecuador and Thailand (Almtorp et al. 1991); Jamaica (Porter et al. 1995); the North of Nigeria (Onayade et al. 1990), and other parts of Africa (Porter et al. 1995).

In this study, we examined the anti-HSV-2 activity of an ethyl acetate extract of *Hyptis fasciculata* leaves and flowers and the isoquercetin flavonoid isolated thereof.

MATERIALS AND METHODS

Cells and virus.

Vero cells (African green monkey kidney) were grown in Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50µg/ml gentamicin, 2.5µg/mL fungizone and 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37⁰C in a 5% CO₂ atmosphere. HSV-2 was isolated from typical genital lesions, at the Virology Department, Federal University of Rio de Janeiro (UFRJ), Brazil. The isolate was typed by polymerase chain reaction (PCR) using specific primers to identify HSV-2 (Markoulatos et al. 2001) and propagated in Vero cells. The titer (10^{6.5} TCID₅₀/ml) was assessed by the cytopathic end-point assay and was expressed as 50% tissue culture infective dose (TCID₅₀) per ml. The virus was stored at -70⁰C until use.

Plant extract and flavonoid.

Leaves and flowers of *H. fasciculata* were collected at Campo Bom City, Rio Grande do Sul State, Brazil. Their authenticity was confirmed by Dr. Sérgio Augusto de Loreto Bordignon (Universidade Federal do Rio Grande do Sul) using morphological and anatomical

techniques. A voucher specimen of the plant is deposited at the Herbarium of the Department of Botany, Universidade Federal do Rio Grande do Sul, Brazil, under the number 537. Leaves and flowers of *H. fasciculata* were dried at room temperature before grinding. Extraction of the ground plant material was obtained by maceration in ethanol. The ethanol extract was concentrated under reduced pressure, suspended in water and then submitted to a liquid-liquid partition procedure with different solvents in order to achieve different new extracts of increasing polarities. The ethyl acetate (EtOAc) extract obtained in this way was fractionated over a silica gel column starting with dichloromethane as mobile phase. This was followed by increasing concentrations of methanol in the solvent until pure methanol (MeOH) has been attained. The fractions obtained in this chromatographic procedure were analyzed by thin-layer chromatography (TLC) and grouped according to their chromatographic profile. Fractions 73 and 74, eluted with EtOAc: MeOH (4:1) yielded isoquercetin in large amounts.

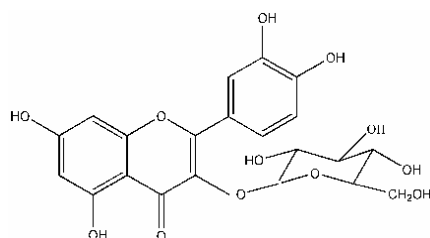


Figure 1. Chemical structure of isoquercetin isolated from *Hyptis fasciculata*. Isoquercetin was isolated from the ethyl acetate fraction of the ethanolic extract from *H. fasciculata* leaves and flowers. After several chromatographic procedures the flavonoid was isolated and purified, and the structure was established by NMR techniques.

Purification of these fractions led to the pure compound (Figure 1) that had the structure elucidated by different techniques of nuclear magnetic resonance (NMR): ^1H and ^{13}C . The extraction of isoquercetin from the ethyl acetate extract yields 1.88%.

Standard compound.

The flavonoid rutin (Merck & Co., USA) and the anti-HSV drug acyclovir (Sigma Chemical Company, USA) were used as standard compounds.

Cytotoxicity assay.

The ethyl acetate extract and isoquercetin of *H. fasciculata* leaves and flowers, and rutin were suspended in DMSO. Stock solutions were prepared in water at 400µg/ml and sterilized by filtration using a 0.22µm Millipore membrane filter. The cytotoxicity assay was performed by incubating triplicate Vero cell monolayers cultivated in 96-well microplates with two-fold serial dilutions of extract and compounds for 48 h at 37°C in a 5% CO₂ atmosphere. The morphological alterations of the treated cells were observed in an inverted optical microscope (Leitz) and the maximum non-toxic concentrations (MNTC) were determined (Walker et al. 1971). Cellular viability was further evaluated by the neutral red dye-uptake method (Neyndorff et al. 1990). The 50% cytotoxic concentration (CC₅₀) was defined as the compound concentration which caused a 50% reduction in the number of viable cells.

Antiviral activity assay.

Vero cell monolayers cultivated in 96-well microtiter plates were treated with the extract and compounds at the MNTC. Immediately after treatment, logarithmical dilutions of HSV-2 suspension were added to treated and untreated cell cultures. After 48 h incubation at 37°C in a 5% CO₂ atmosphere, the virus titers in treated and untreated cells were determined. The antiviral activity was expressed as the percentage inhibition (PI) (Nishimura et al. 1977) using antilogarithmic TCID₅₀ values as follows: $PI = [1 - (\text{antilogarithmic test value}/\text{antilogarithmic control value})] \times 100$. The dose-response curve was established starting from the MNTC. The 50% effective concentration (EC₅₀) was defined as the concentration required for 50% protection against virus-induced cytopathic effects and was calculated from

the dose-response curve. The selective index (SI) was determined as the ratio of CC₅₀ to EC₅₀. The results show a representative result of an experiment performed in triplicate and three times repeated.

Mechanism of action studies.

Virucidal assay.

100µl of the HSV-2 suspension were added to either 900µl of ethyl acetate extract or isoquercetin, at the MNTC, or MEM-Eagle without serum (control), according to Chen et al. (1988). The samples were incubated at 37°C for 2h, diluted, and then inoculated in Vero cell monolayers. After 48 h incubation the residual titers of the treated and untreated virus were determined and expressed as PI.

Cellular receptor assay.

Ethyl acetate extract and isoquercetin were added to Vero cell monolayers before infection (pretreatment) in order to evaluate the effect of the compounds on cell receptors. Vero cell monolayers were pretreated with ethyl acetate extract and isoquercetin for 1 h at 4°C. After being washed three times with MEM- Eagle, the treated and untreated cells were inoculated with logarithmical dilutions of HSV-2 and incubated at 37°C for 48 h. The virus titres in treated and untreated cells were determined and the activity was expressed as PI.

Penetration assay.

Vero cell monolayers were inoculated with logarithmical dilutions of HSV-2 and incubated for 1 h at 4°C. After this period, the monolayers were washed with culture medium and then 100µl of ethyl acetate extract or isoquercetin at the MNTC was added. The cultures were immediately incubated at 37°C to allow the penetration of the particles into the cells for another hour. After incubation, the monolayers were washed, MEM- Eagle was added, and the cultures were incubated at 37°C for 48h. The virus titers in the control (absence of ethyl

acetate extract or isoquercetin), and in the test were determined, and the activity was expressed as PI.

Intracellular assay.

Vero cell monolayers were inoculated with logarithmical dilutions of HSV-2 and incubated at 37°C for 2h. The cells were washed and 100µl of ethyl acetate extract or isoquercetin at the MNTC or MEM-Eagle (control) was added and the cultures incubated at 37°C for 16 h. After incubation, the cells were washed to remove the extract and the flavonoid before releasing of viral particles. Then, MEM-Eagle was added and the cultures incubated for 32 h at 37°C. The virus titers in the control and test cells were determined, and the activity was expressed as PI.

RESULTS

Cytotoxicity.

Experiments to evaluate the cytotoxic concentration of the extract and compounds were performed before the antiviral activity assay. The ethyl acetate extract and rutin changed the cellular morphology at the concentration of 50µg/ml, while no alteration was observed when the cells were exposed to isoquercetin at concentrations as high as 200µg/ml. The cellular viability was not affected by the addition of the extract and compounds.

Antiviral activity.

The ethyl acetate extract, isoquercetin and the standards rutin and acyclovir showed inhibitory effects at different concentrations (Table 1). The 50% effective concentrations (EC₅₀) calculated from the dose-response curve were 2; 1.4; 11.3 and 3.9 µg/ml, respectively. The SI of the ethyl acetate extract and isoquercetin was higher than 100 and 142.8, respectively.

Table 1. Antiviral activity of ethyl acetate extract and isoquercetin from *H. fasciculata* leaves and flowering

Material	Concentration (µg/ml)								
	0.7	1.5	3.1	6.2	12.5	25	50	100	200
EtOAc extract	0 ^a	0	82.2	90.0	94.4	96.8	^b	^b	^b
Isoquercetin	0	60.2	87.0	87.4	87.4	87.4	91.5	96.0	99.9
Rutin ^c	0	0	0	0	60.2	87.4	^b	^b	^b
Acyclovir ^c	0	0	0	90	96.8	96.8	99	99	99.9

Virus titer = 10^{6.5} TCID₅₀/ml

^a Percentage inhibition; ^b Cytotoxic concentration; ^c Standard compound

Mechanisms of antiviral action.

To determine the mechanism of action of the ethyl acetate extract and isoquercetin against HSV-2 infection, experiments were performed to evaluate their inhibitory effect at different stages of virus infection of cells. Figure 2 shows that the samples presented virucidal activity with 99.9% inhibition. Besides, it was observed that addition of the extract and isoquercetin to the cells before virus inoculation presented inhibition of viral infection by 82.2% and 90% respectively. Considering the penetration step, 92% and 99.6% inhibition were observed. No inhibition occurred when the extract and isoquercetin were added after penetration of the virus (intracellular effect).

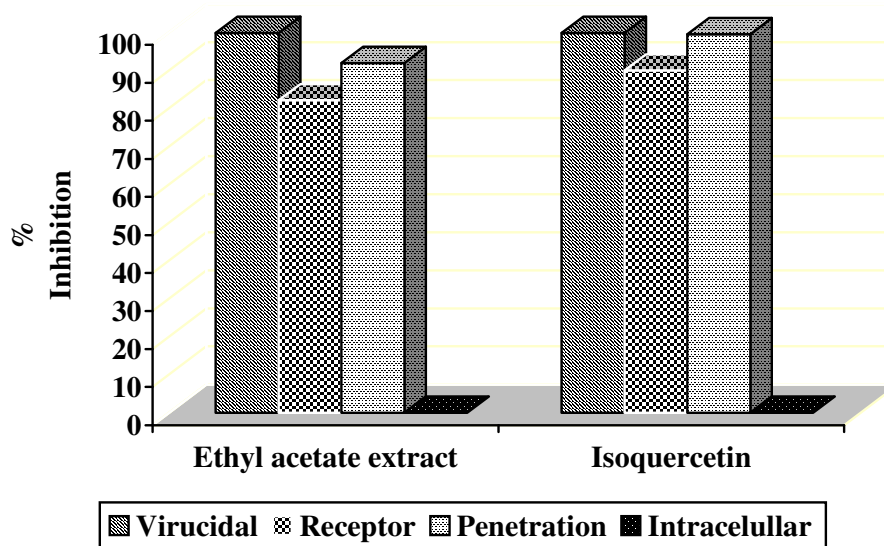


Figure 2: Mechanism of action of ethyl acetate extract and isoquercetin from *H. fasciculata* leaves and flowers. Virucidal effect - virus particles were treated with ethyl acetate extract and isoquercetin, at concentrations of 25 µg/ml and 200 µg/ml, respectively. The samples were incubated at 37°C for 2h, diluted, and then inoculated onto Vero cell monolayers. Receptor - extract and flavonoid were added to Vero cells at 1h pre-infection. Penetration - extract and flavonoid were added to Vero cells after 1h post-infection. Intracellular - extract and flavonoid were added to Vero cells 2h post-infection. After 48h of incubation at 37°C the residual titers of the treated and untreated virus were determined and expressed as percentage inhibition (PI).

DISCUSSION

The development of viral resistance towards antiviral agents increases the need for new effective compounds against viral infection. Medicinal plants have a variety of chemical molecules which present antiviral activity. Among them, the alkaloids (Szlavik et al. 2004), terpenes (Pavlova et al. 2003), tannins (Cheng et al. 2002, Notka et al. 2004), coumarins (Barnard et al. 2002), stilbenes (Docherty et al. 2004), cinnamic acid esters (Galabov et al. 1998) and flavonoids (Gonçalves et al. 2001, Chiang et al. 2003, Bettega et al. 2004, Sokmen et al. 2005) have already demonstrated their inhibitory effect on different viruses. Regarding the anti-HSV-2 activity, some flavonoids have demonstrated antiviral effect (Chiang et al. 2003) but to our knowledge there is no mention in the literature about the anti-HSV-2 activity

of isoquercetin. In this study, we demonstrate, for the first time, the ability of this flavonoid to inhibit the multiplication of HSV-2.

Ethyl acetate extract and isoquercetin showed virucidal action, demonstrating that one of their modes of action is the capacity of interacting with HSV-2 particles. The other possible mechanism is the ability of the extract and isoquercetin to react with cellular receptors, blocking virus attachment. Inhibitory activity was also observed when the extract and isoquercetin were added to the cell culture immediately after adsorption (penetration), whereas no intracellular effect was detected.

The step of virus penetration is an attractive target for therapy because the virus can be blocked at an early stage of infection. Our results show that ethyl acetate extract and isoquercetin, besides presenting virucidal activity, were also capable of inhibiting the early stages of the infection at non cytotoxic concentrations. These results suggest that *H. fasciculata* extract and its isoquercetin flavonoid have the potential to inhibit the infection caused by HSV-2.

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