In vitro antiviral activity of *Chamaecrista nictitans* (*Fabaceae*) against herpes simplex virus: Biological characterization of mechanisms of action

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Abstract: We have previously identified a crude extract of the plant *Chamaecrista nictitans* (*Fabaceae*) with antiviral activity against herpes simplex virus. The main objectives of this research were to identify the step of the replication cycle of herpes simplex inhibited by the extract, and to attempt to characterize the chemical characteristics of this extract. The crude extract from *Chamaecrista nictitans* (*Fabaceae*) was extracted with a mixture of dichloromethane/methanol, and further fractionated following a bioassay-guided protocol using a combination of preparative thin layer and column chromatography. Toxicity and bioassay experiments were carried out in monolayers of Vero cells. The antiviral activity of the extract was assessed by total inhibition of cytopathic effect after three-day incubation. The highest concentration of the extract which was not toxic to the cells was 200 μg/ml.

Western blot and immunofluorescence techniques were used to elucidate the antiviral mechanism of the extract by infecting Vero cells with the virus at different times and monitoring the synthesis of viral proteins. A 60 kDa protein was detected at 2 hr and 8 hr post-infection but no additional proteins were synthesized at later time intervals, and cytopathic effect was not observed after 24 hr. This result indicates that the extract acts at the intracellular level in order to inhibit late transcription. However, it does not inhibit transcription/translation of early viral proteins. These results were confirmed by immunofluorescence experiments. A strong fluorescent signal was observed in control cell monolayers at 24 hr post infection, accompanied with a clear cytopathic effect. In contrast, in the presence of acyclovir or the extract, cells showed very discrete immunofluorescence, characterized by a punctuated pattern, and no cytopathic effect was observed. Neutralization assays were performed using pre-incubation of virus with either specific herpes simplex-1 antiserum, 200 μg/ml of the extract or 20 μg/ml of acyclovir. After 1 hr incubation, cells were infected and monitored for cytopathic effect. Only the virus treated with acyclovir showed viral activity, while no cytopathic effect was induced by samples of virus incubated with the extract. It is concluded that the extract inhibits both the attachment of the virus to the cell and the secondary transcription of the virus within the cells. Chemical characterization of the extract showed the presence of tannins.


Key words: Chamaecrista, tannins, bioassay-guided-fractionation, herpes simplex virus.

Palabras clave: Chamaecrista, taninos, bioensayo, fraccionamiento guiado, virus herpes simplex.
Costa Rica possesses an extremely rich biodiversity. Twenty five percent of Costa Rican territory is protected by conservation policies aimed at preserving this extraordinary biodiversity. These areas contain about half a million species of wild plants, animals and microorganisms, representing a rich natural resource for drug discovery programs (Obando 2002), and have been scarcely tested.

Development of nontoxic antiviral agents by selective inhibition of virus replication within infected cells has been a challenging task. The difficulties are related to the use of similar substrate and enzyme systems for synthesis of both virus and cellular macromolecules. Thus, biophysical or biochemical interference with viral events in the infected cell often result in similar interference with cellular function. Thus, although some specificity is occasionally achieved, inhibition of virus replication often results in significant overall cellular toxicity. An interesting proposal for specific inhibition of virus replication is to selectively inhibit enzyme targets that are uniquely present in virus infected cells, but absent in normal host cells (Hovi 1988, Bean 1992).

Antiviral drugs against herpes simplex virus (HSV) have proven to be selective and specific inhibitors for viral replication and are clinically effective (Bean 1992, Crumpacker 2001). The value of therapy has had a major impact on altering the spectrum of human disease and has implications for long range control of HSV infections (Whitley and Gnann 1992). Acyclovir guanine is a guanosine analog with an acyclic side chain at the 9 position. It is a specific inhibitor of HSV-1, HSV-2 and Varicella Zoster virus replication, with little toxicity for host cells (Schaeffer 1978). It requires a virus encoded thymidine kinase (TK) for efficient intracellular activation, which accounts, in part, for its selectivity. However, resistance to this antiviral drug has already been reported (Crumpacker et al. 1982, Bean 1992).

The aim of this study was to search for new natural product scaffolds with potential antiviral activity. A selection of 50 different plant species and their parts from Costa Rican biodiversity, mainly from the families Euphorbiaceae, Fab/Caesalpinaceae and Rubiaceae, were evaluated. From a preliminary study, 6.5% of extracts were chosen for further evaluation for their anti-herpes simplex activity (G. Tamayo et al. unpublished results).

One of the most promising extracts found among the 12 final extracts selected, was the organic extract from Chamaecrista nictitans (Fig. 1) belonging to the Fabaceae family, Caesalpinaceae subfamily. To our knowledge, C. nictitans has never been investigated for its chemical composition, nor for its biological activity. This study describes its biological activity against HSV and a preliminary characterization of its antiviral effect and its chemical composition.

MATERIALS AND METHODS

Collection of plant material: Plant material used in this research was collected from 1997 to 2000 in different locations of Costa Rica. Voucher specimens are preserved in the collection of the National Biodiversity Institute (INBio) under the register numbers of SS354 (1997, collection in Nandayure), AR5429 (collection in Guápiles), LA1995 (collection in Rincón River, Osa). Permits for collection were obtained from the Ministry of the Environment and Energy. Plant material collected consisted
of aerial parts mostly separated from roots. Original voucher specimen of an initial collection in 1991 of this plant material is kept under Prof. Luis Poveda custody, at the Herbarium of the National University.

**Preparation of extracts:** All collected material was dried using an oven at 50°C, grounded and extracted with a 1:1 mixture of methylene chloride for 6 hr and re-extracted with the same mixture overnight. The two extracts were combined and evaporated by vacuum. A sample ranging from 1 mg to 100 mg was further prepared for biological activity determination.

**Separation protocol:** A bioassay-guided fractionation strategy was used to concentrate the activity on active components. Organic extracts were partitioned as follows (Fig. 2): 19 grams from the crude extract were dissolved in 90% aqueous methanol. The resulting hydroalcoholic solution was extracted three times with 150 ml of hexane. All hexane fractions were combined and evaporated to dryness and labeled “PH” for hexane partition (14% of total mass recovered). The remaining methanolic aqueous solution was evaporated to yield a fraction labeled as “Maq”, diluted with water to 200 ml and extracted with three portions of 125 ml methylene chloride. All methylene chloride fractions were combined and evaporated to dryness and labeled “PD” for dichloromethane partition (14% of total mass recovered). The aqueous fraction was subsequently extracted with three portions of 125 ml of n-butanol. All n-butanol fractions were combined and evaporated to dryness and labeled “PB” for butanol partition (22% of total mass recovered). The aqueous residue was freeze-dried and labeled “RA” for aqueous residue (50% of total mass recovered). “PD” and “PB” were selected for further separation. A fraction of 2.89 g of PD was submitted to vacuum liquid chromatography with silica gel 60F (Merck), initiating with hexane and then using increments in polarity of hexane: ethyl acetate mixtures up to 100%.

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**Fig. 2. Scheme of the fractionation procedure.** The Crude Extract was partitioned by solvent affinity and the obtained fractions screened for anti HSV-1 activity. Positive fractions were further sub-fractionated by chromatography. Positive sub-fractions were partitioned and analyzed by Nuclear Magnetic Resonance.
ethyl acetate. The final elution was done with 
ethyl acetate: methanol 1:1 mixture, methanol 
100%. Following this procedure, 35 fractions 
were obtained, which after thin layer chroma-
tography (TLC) control, were grouped into 8 
fractions numbered 1 (32.8 mg), 2 (7.8 mg), 3 
(25.1 mg), 4 (13.4 mg), 5 (188.3 mg), 6 (56.4 
mg), 7 (1270 mg) and 8 (20.1 mg). 2.89 g of 
PB were re-suspended in water and separated 
with Diaion HP-20 (Mitsubishi), de-salted and 
eluted with water : methanol 1:1, methanol 
and acetone. A total of 18 fractions were col-
lected and grouped into 6 fractions numbered 
as 1 (1050 mg), 2 (89.3 mg), 3 (2300 mg), 4 
(76.3 mg), 5 (53.8 mg) and 6 (9.3 mg) after 
TLC control.

Qualitative analysis of tannins: The pre-
viously described procedure used to separate 
tannins of different degree of polymerization 
was used (Sun et al. 1998) with the following 
modifications: one glass cartridge, filled with 
2 g of LiChroprep RP-18 (Merck), was pre-
conditioned with deionised water, methanol 
and water at pH 7. In the second elution step, 
methylene chloride was used instead of diethyl 
ether. All sub fractions were eluted on TLC 
using toluene:acetone:acetic acid 3:3:2. 100 
mg of tannin were used as standard for com-
parative purposes with the active fraction.

Structure characterization: NMR mea-
surements were performed to some of the 
active fractions. Vainillin and sulphuric acid 
(VAS) reagent was used for TLC fingerprints 
and visualization.

Toxicity of the extract: Ten milligrams of 
dry extract were diluted in 0.2 ml of dimeth-
ysulfoxide (DMSO) and 0.8 ml of sterile 
phosphate buffer solution (PBS) to attain an 
initial concentration of 10 mg/ml. Two fold 
dilutions were performed in minimal essential 
medium (MEM) complemented with 2% fetal 
calf serum, sodium bicarbonate and penicill-
in/streptomycin. Confluent Vero cell mono-
layers (ATCC-CCL-81) were overlayed with 
the different dilutions of the extract and were 
incubated at 37°C with 5% CO₂ during three 
days. Toxicity was determined by observation 
of the morphology of the cells in comparison 
with the cell control without the extract. After 
three days of incubation, neutral red was added 
for confirmation of cell viability.

Bioassay: The F strain of herpes simplex 
virus (ATCC-VR-733) was used at 100 ID₅₀, 
and viral dilutions were performed using PBS 
as diluent. Vero cell monolayers in 96 well 
microplates were inoculated with 100 μl of 
the virus and were adsorbed at 37°C. After 
one hour, the inoculums were removed and the 
monolayers were overlayed with the extract 
diluted 1/50 in the maintenance medium and 
were incubated at 37°C with 5% CO₂ for three 
days. Inhibition of the typical herpes simplex 
cytopathic effect (CPE) was recorded as the 
inhibitory effect of the extract. The inhibitory 
potency was tested performing two fold dilu-
tions, starting from the 1/50 dilution.

Effect of the extract and acyclovir 
on the virus: To compare the effect of the 
extract or acyclovir on the virus, 500 μl of 
concentrated F strain was mixed separately 
with an equal amount of acyclovir at 20 
μg/ml or extract at 400 μg/ml. Two neutral-
izing units (NU) of human specific antise-
rum against HSV-1 and maintenance medium 
were used as controls. They were incubated 
for 1 hr at 37°C, after which 5 ml of PBS 
were added and were ultracentrifuged for 1 
hr at 30 000 rpm (121.000 g) in a Beckman 
ultracentrifuge using a SW 50.1 rotor. The 
pellets were resuspended in PBS and ultra-
centrifuged once more to eliminate the anti-
viral compounds and antiserum. The pellets 
were then resuspended in 500 μl of mainte-
nance medium. The virus was diluted to 100 
TCID₅₀, filtered through a Millipore 0.22 μm 
filter and inoculated in Vero cell monolayers 
which were overlayed with and without extract 
or acyclovir in the medium.

Effect of the extract on the cells: Vero 
cell monolayers were treated with 200 μl of 
extract in maintenance medium or acyclovir at 
a 10 μg/ml for 1 and 24 hr at 37°C. The mono-
layers were then washed 5 times with PBS and 
were infected with 100 ID₅₀ of the F strain of 
HSV. They were incubated with and without 
extract or acyclovir in the medium.
Western blot: Confluent Vero cells grown on 24-well plates were infected with 100 ID_{50} of the F strain of HSV-1 in the presence of 200 μg /ml of the extract under study or acyclovir at 20 μg /ml. Cells were incubated with the virus for different times as indicated in figure legends and the viral cycle stopped by lysis of the cells in Laemmli sample buffer (Laemmli 1970). Protein concentration in cell lysates was determined and 30 μg of each were loaded on a 12.5% SDS-PAGE gel, corresponding to different stages in the viral replication cycle. Separated proteins were blotted on nitrocellulose membrane and probed with a human immune anti-HSV-1 serum diluted 1/1000 and further incubated with a goat anti-human peroxidase conjugate (Sigma, USA). The immune complexes were detected by chemiluminescence reaction (Boehringer, Germany).

Immunofluorescence: Confluent Vero cells grown on 13 mm coverslips were infected with 100 ID_{50} HSV-1 for 6 and 24 hr in the presence of 200 μg /ml of the extract or 20 μg/ml acyclovir. Cells were then fixed in methanol and processed for immunofluorescence using a commercial diagnostic kit (BioRad, Pathfinder). Cover slips were mounted in glycerol and photographed using aca 400 film.

RESULTS

Cell toxicity was determined by observation of the cell morphology and confirmed by staining of the monolayer with neutral red. The minimal dilution of the extract not toxic to the cells was 1/50 which was equivalent to 200 μg/ml. To determine the direct effect of extract or acyclovir on the virion, equal amounts of the antiviral compounds were incubated with HSV for one hr at 37ºC and a specific antiserum against the virus was used as a control. As shown in Table 1, the extract had an inhibitory effect when preincubated with the virus and this effect was maintained even when the extract was not present in the culture medium during the replication cycle. Acyclovir had no effect on the virion in pre-incubation experiments since this antiviral drug has to be present during the replication cycle in the culture medium to maintain its inhibitory effect. Treatment of virus with antiserum neutralized the virus, as evidenced by the lack of CPE.

To determine if the extract and acyclovir had some effect on the cells, they were treated for 1 or 24 hr with the antiviral compounds. No inhibitory effect was detected, indicating that both acyclovir and extract must be present in the maintenance medium during the replication cycle to have an inhibitory effect on the virus.

In order to understand the effect of the extract on the viral cycle at the molecular level, HSV protein synthesis was monitored by Western blot using a specific immune serum against HSV. A 60 kDa protein was detected at 2 hr post infection (Fig. 3). The intensity of this band increased steadily while other viral proteins appeared. The expression of HSV proteins was evident after 24 hr of infection correlating with the appearance of its characteristic cytopathic effect (Fig. 4a, b). When the same experiment was repeated in the presence of the extract, expression of the 60 kDa band was similar to the controls, however, the rest of the protein bands were weak or did not appear at all. The intensity of the 60 kDa band ceased to increase at 8 hr, and at 24 hr its intensity had diminished considerably, although it was still present. No CPE was observed in the cultures treated with the extract. In agreement
with these findings, cells infected for 24 hr in the presence of extract were processed by immunofluorescence specific for HSV-1. The cells that did not show any cytopathic effect presented a very discrete and punctuated immunofluorescence indicating, again, that at least the synthesis of some viral proteins took place but further translation was inhibited (Fig. 4c-f). In order to compare the activity of the extract to other known substances with anti HSV effect, acyclovir was used as a control under the same experimental conditions. When cells were infected with HSV-1 in the presence of acyclovir, the same electrophoretic pattern was observed as with the extract, i.e., the expression of a viral 60 kDa protein started at 2 hr, increased up to 8 hr and decreased at 24 hr (Fig. 3). No other viral proteins were observed in the gel. The immunofluorescence in the presence of acyclovir followed the same pattern as when the extract was included in the culture medium (Fig. 4c-f).

The activity profile of C. nictitans extract is shown in tables 2, 3 and 4. The activity found suggests a real antiviral action since a dose-dependent effect was observed. The activity was followed to polar components, initially in the methanolic aqueous fraction and then to the dichloromethane and butanol partitions. The fraction #31 originated from a vacuum liquid chromatography performed on PD, was chosen for further evaluation of its biological activity profile. Attempts to chemically characterize this fraction are currently underway, although a preliminary fingerprint on NMR showed that it consists of a complex mixture of tannins. To demonstrate this, a TLC analysis was conducted with a tannin control, treated with the procedure described in the experimental section. Fig. 5 shows the result of this qualitative test.
analysis is based on a separation on a reverse phase, first by eliminating phenolic acids with water, then eluting with ethyl acetate and concentrating oligomers in this fraction, and finally eluting polymers (F3) using methanol. The oligomers are re-eluted to obtain F1 and F2. These fractions (F1, F2 and F3) were eluted together on a thin layer chromatogram with fraction # 31, evidencing the tannin nature and the complexity of this fraction.

Fig. 4. Morphology of cells infected with HSV 1. Vero monolayers infected with HSV-1 for 24 hr were processed for light microscopy (a, b) or immunofluorescence (c-f). Control cells were non infected (c), positive cells were infected with HSV-1 (d) and infected with HSV-1 in the presence of the extract (e) or acyclovir (f).
These experiments demonstrated that inhibition by the plant extract and acyclovir takes place at the level of late protein synthesis since a 60 kDa protein is detected at 2 to 8 hr p.i., but late proteins are not synthesized and CPE is not observed. From these results, it is possible to conclude several aspects related to the antiviral effect of the extract. First, its antiviral action is exerted intracellularly. This conclusion is supported by the fact that even if no CPE is detected, the synthesis of at least one viral protein takes place. For this event to occur, the virus has to be adsorbed to cells even in the presence of the extract, implying that the first steps of the viral cycle were not inhibited in these experimental conditions. Since only one viral protein was detected in the presence of the extract, we suggest that the effect of the extract takes place by inhibiting the production of this specific protein.
either at the level of transcription or translation of late proteins. This pattern correlates well with the known antiviral effect exerted by acyclovir, which blocks the activity of thymidine kinase (Schaeffer et al. 1978). Since the extract behaved similarly to acyclovir, it could be speculated that the active compound in the extract would have a similar mechanism of action as acyclovir. Alternatively, the possibility remains that both acyclovir and the extract inhibit at the same point in the viral cycle but the molecular target may be different. At present, it is not possible to discern between these two alternatives.

A difference in the mechanism of action between the extract and acyclovir was detected, since the former also exerts a direct action on the viral particle, inhibiting the adsorption of the virus to the cell receptor after one hour of incubation. This suggests that during a primary infection or reactivation, the virus can enter the cells and undergo its primary cycle of replication, but the progeny can then be inhibited by the extract in the second cycle of replication either by blocking the adsorption, by inhibiting its contact with the cell receptor or by blocking the secondary transcription or translation. The involvement of tannins in the anti-viral activity of various plant extracts has been well documented. In his review of 1996, Haslam cites the findings of Cadman, in which the anti-viral effect of a plant extract was ascribed to polyphenolic compounds, since they bind to the viral protein coat, thus inactivating the virion. More recently, De Bruyne et al. (1999) evaluated 15 tannins for biological activity, in order to find models for structure-activity relationships. They found that epicatechin-containing dimers exhibit more anti-HSV activity than other compounds assayed. This is consistent with the tannin profile and the degree of activity observed in the sample.

In conclusion, it was found that the extract of Chamaecrista nictitans studied inhibits HSV in two steps of its replication cycle, i.e., during adsorption and second transcription or translation. Fig. 6 shows the possible mode of action of the extract as compared to acyclovir.

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RESUMEN

Previoamente se había identificado un extracto crudo a partir de Chamaecrista nictitans (Fabaceae) con actividad antiviral contra el virus herpes simplex. Los objetivos de esta investigación fueron determinar el paso en el ciclo de replicación del herpes simplex inhibidos por el extracto y caracterizar la naturaleza química de dicho extracto. El extracto crudo de la planta se obtuvo con una mezcla de diclorometano/metanol y fraccionado, utilizando la guía de un bioensayo, mediante cromatografía preparativa de capa fina y cromatografía en columna. Los ensayos se llevaron a cabo en monolapas de células Vero. La actividad antiviral exhibida por el extracto se determinó mediante la inhibición total del efecto citopático después de tres días de incubación. La concentración máxima de la fracción positiva que no presentó citotoxicidad fue 200 µg/ml. Se utilizaron técnicas inmunoquímicas y de inmunofluorescencia para elucidar el mecanismo antiviral ejercido por el extracto; con este propósito, se infectaron células Vero con el virus herpes simplex y se determinó la producción de proteínas virales a diferentes tiempos después de la infección. Se detectó la producción de una proteína de aproximadamente 60 kDa a las 2 hr y 8 hr luego de la infección; sin embargo no se detectó producción de ningún tipo de proteína tardía, hecho correlacionado con la ausencia de efecto citopático a las 24 hr. Este resultado indica que el extracto actúa intra-celularmente siendo capaz de inhibir la transcripción secundaria. Sin embargo, el extracto permite la transcripción y traducción de proteínas tempranas. Estos resultados fueron confirmados mediante inmunofluorescencia. En células control no tratadas con el extracto, se observó una fuerte señal fluorescente acompañada de la aparición del efecto citopático característico 24 hr después de la infección con el virus herpes simplex. Por el contrario, células en presencia de acyclovir o del extracto desarrollaron un patrón muy discreto de inmunofluorescencia sin presencia de efecto citopático. Se realizaron adicionalmente ensayos de neutralización utilizando preincubaciones con un antisuero específico contra herpes simplex 1, 200 µg/ml del extracto o 20 µg/ml de acyclovir. Solamente el virus tratado con acyclovir fue capaz de producir un efecto citopático, mientras que el extracto inhibió el virus y no se detectó efecto citopático. Se concluye que el extracto inhibe la adherencia inicial del virus a las células y los eventos de transcripción secundaria del virus. La caracterización química del extracto demostró la presencia de taninos en el mismo.

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