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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 diclorometane/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. Rev. Biol. Trop. 52(3): 807-816. Epub 2004 Dic 15.

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

Palabras clave: Chamaecrista, taninos, bioensayo, fraccionamiento guiado, virus herpes simplex.

Before the introduction of modern western medicine in our cultures, people depended only on traditional medicine to treat and cure diseases. In recent years, traditional medicine has received growing attention from the scientific community as an alternative in the development of new therapeutic alternatives. Additionally, the development of resistance by microorganisms to the existing drugs is a major problem that urges new combined approaches in the search of new antimicrobial agents (Bean 1992).

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

Fig. 1. *Chamaecrista nictitans.* (Source: INBIO).

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%

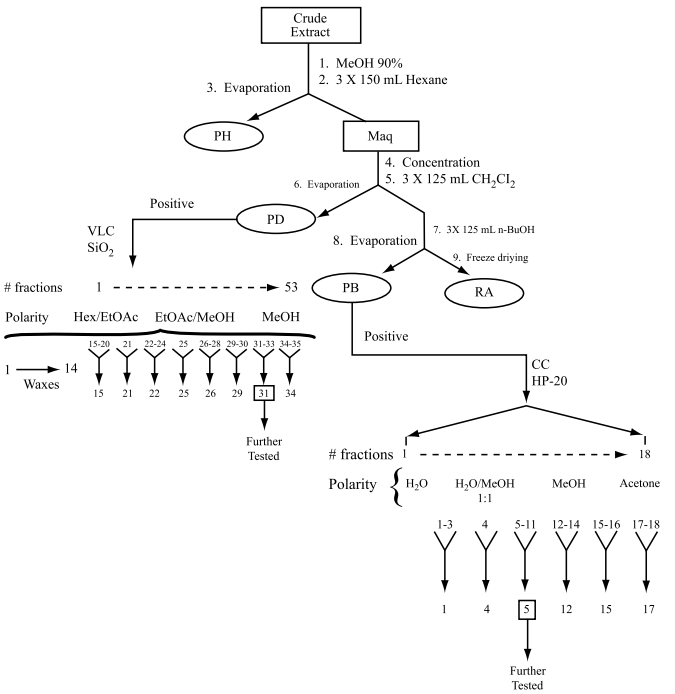

Fig. 2. **Scheme of the fractionation procedure**. The Crude Extract was partitioned by solvent affinity and the obtained fractions screened for anti HSV-1 activity. Positive fractions were further sub-fractionated by chromatography. Positive sub-fractions were partitioned and analyzed by Nuclear Magnetic 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 chromatography (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 collected 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 previously 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 preconditioned 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 comparative purposes with the active fraction.

Structure characterization: NMR measurements 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 dimethylsulfoxide (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 penicillin/streptomycin. Confluent Vero cell monolayers (ATCC-CCL-81) were overlayed with the different dilutions of the extract and were incubated at 37ºC with 5% CO_2 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 $ID_{50\%.}$ 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 37ºC. 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 37ºC with 5% CO_2 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 dilutions, 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 neutralizing units (NU) of human specific antiserum against HSV-1 and maintenance medium were used as controls. They were incubated for 1 hr at 37ºC, 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 ultracentrifuged once more to eliminate the antiviral compounds and antiserum. The pellets were then resuspended in 500 µl of maintenance medium. The virus was diluted to 100 $TCID_{50}$, filtered through a Millipore 0.22 µm filter and inoculated in Vero cell monolayers which were overlaid 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 37ºC. The monolayers were then washed 5 times with PBS and were infected with 100 ID_{50} 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 immunofluorescense using a commercial diagnostic kit (BioRad, Pathfinder). Cover slips were mounted in glicerol and photographed using asa 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.

TABLE 1
Effect of antiviral compounds on the virion

Treatment (1 hr)	Cytopathic effect
Extract	Inhibition
Acyclovir	No Inhibition
Antiserum	Inhibition
Control	No inhibition

Virions from herpes simplex were pre incubated for 1 hour with extract (200 μg/ml), acyclovir (20 μg/ml), anti HSV-1 antiserum and PBS as control. Virions were washed three times by ultracentrifugation and subsequently added onto Vero cell monolayers and the appearance of a cytopathic effect was monitored.

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

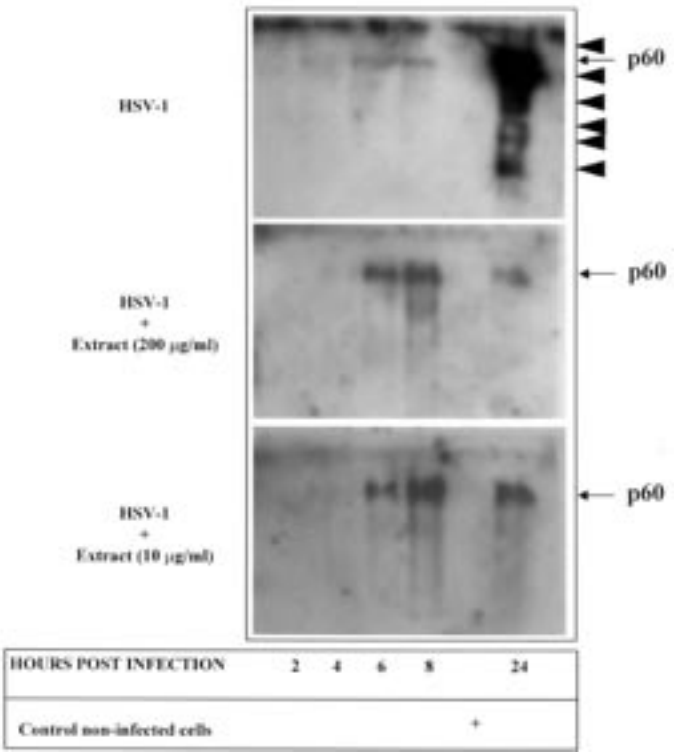

Fig. 3. **Effect of the subfraction on HSV 1 protein replication.** Proteins from cells infected with HSV-1 in the presence of the extract or acyclovir were separated by 12.5% SDS PAGE, transferred to nitrocellulose and probed with anti HSV-1 antibodies to follow the viral cycle. Control cells were infected only with HSV-1 without any antiviral substance.

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. The

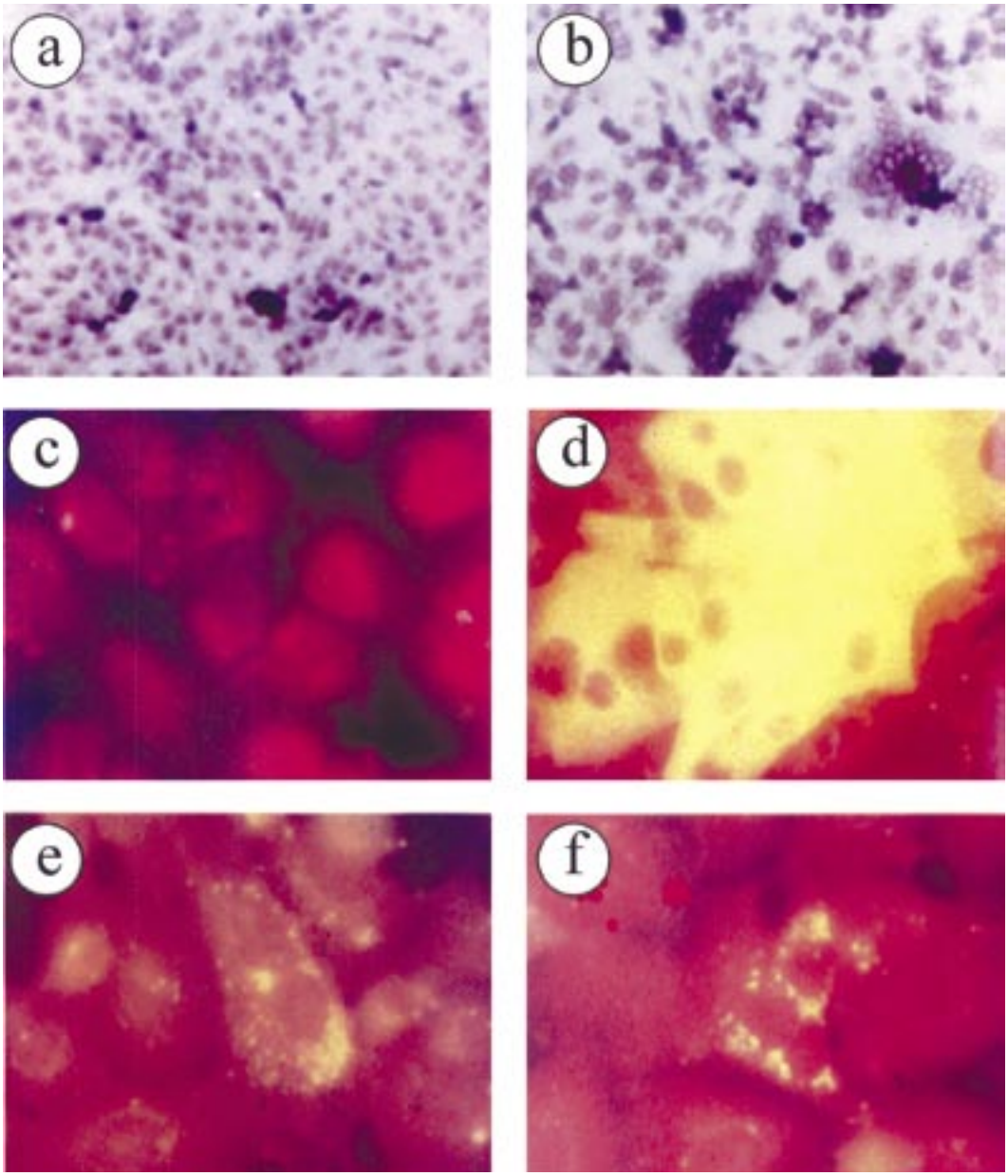

Fig. 4. **Morphology of cells infected with HSV 1.** Vero monolayers infected with HSV-1 for 24 hr were processed for light microscopy (a, b) or immunofluorescence (c-f). Control cells were non infected (c), positive cells were infected with HSV-1 (d) and infected with HSV-1 in the presence of the extract (e) or acyclovir (f).

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.

TABLE 2
Antiviral activity of various partitions and concentrations of extracts of C. nictitans

Extract or Partition	µg/ml	Cytopathic effect
Crude Extract	168.75	Positive
Crude Extract	675.00	Positive
Maq	20.70	Negative
Maq	82.81	Partial
Maq	41.41	Partial
PD	51.56	Positive
PB	19.14	Negative
PB	76.56	Positive
RA	593.57	Negative
RA	2375.00	Negative

Positive: 100% CPE inhibition during 3 days incubation, Partial: 50% CPE inhibition during 3 days incubation, Negative: No inhibition. PD: dichloromethane partition, RA: aqueous residue, PB: butanol partition, Maq: methanol aqueous.

TABLE 3
Antiviral activity of VLC fractions of dichloromethane partition (PD)

Fractions of PD	µg/Ml	Cytopathic effect
15	23.44	Negative
21	23.05	Negative
22	81.25	Negative
29	19.14	Negative
31	9.18	Negative
31	36.72	Positive
34	2.64	Negative
34	10.55	Partial

Positive: 100% CPE inhibition during 3 days incubation, Partial: 50% CPE inhibition during 3 days incubation, Negative: No inhibition.

TABLE 4
Antiviral activity of CC-HP20 fractions of butanol partition (PB)

Fractions of PB	µg /ml	Cytopathic effect
1	159.38	Negative
4	168.75	Partial
5	73.44	Positive
12	9.18	Negative
15	19.92	Negative
17	62.50	Negative

Positive: 100% CPE inhibition during 3 days incubation; Partial: 50% CPE inhibition during 3 days incubation; Negative: No inhibition.

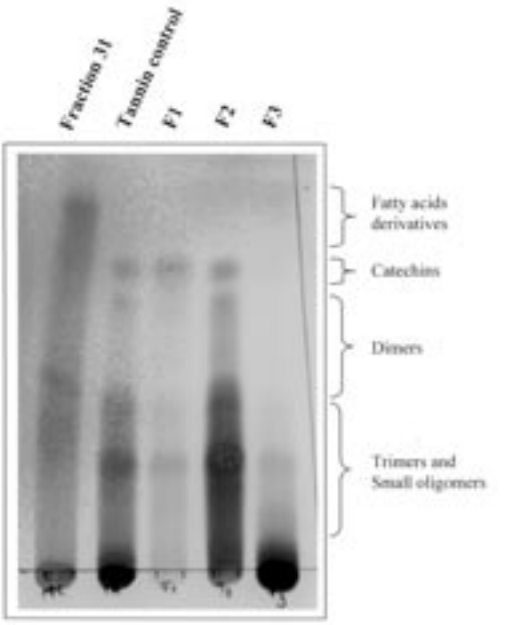

Fig. 5. **Determination of tannins.** Bioactive fraction 31 was further separated into fractions F1, F2 and F3 as indicated in materials and methods. The samples were eluted by thin layer chromatography using toluene:acetone:acetic acid (3:3:2). 100 mg of a typical tannin was used for comparison purposes.

DISCUSSION

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

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 adscribed 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.

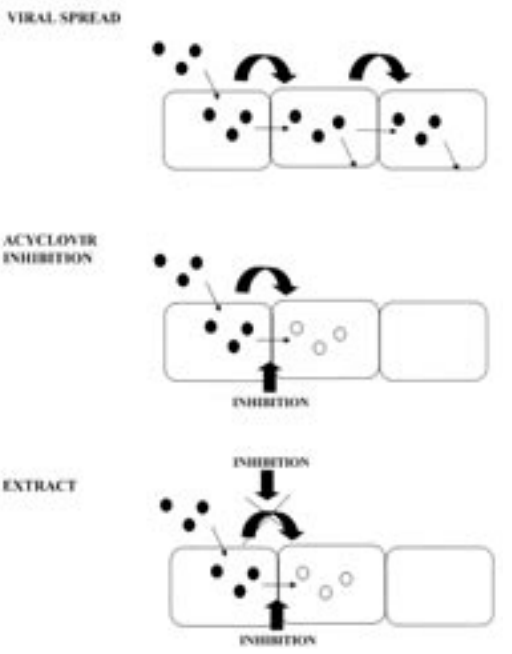

Fig. 6. Mechanism of action of antivirals.

From a therapeutic point of view, our findings suggest that this extract has a relevant therapeutic potential, since it will inhibit the second cycle of replication of the virus, thus decreasing the severity of the lesion and the time of recurrency.

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RESUMEN

Previamente 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 monocapas de células Vero. La actividad antiviral exhibida por el extracto fue determinada 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 intracelularmente 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.

REFERENCES

Bean, B. 1992. Antiviral Therapy: Current Concepts and Practices. Clin. Microbiol. Rev. 5: 146-182.

Crumpacker, C.S., LE. Schnipper, S.I. Marlowe, P.N. Kowalsky, B.J. Hershey & M.J. Levin. 1982. Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N. Engl. J. Med. 306: 343-346.

Crumpacker, C. 2001. Antiviral Therapy, pp. 393-434. *In* D.M. Knipe & P.M. Howley (eds.). Fields Virology. Lippincott Williams & Wilkings. Philadelphia, Baltimore, New York.

De Bruyne, T., L. Pieters, M. Witvrouw, E. De Clercq, D. Vanden Berghe & A.J. Vlietinck. 1999. Biological Evaluation of Proanthocyanidin Dimers and Related Polyphenols. J. Nat. Prod. 62: 954-958.

Haslam, E. 1996. Natural Polyphenols (Vegetable Tannins) as Drugs: Possible Modes of Action. J. Nat. Prod. 59: 205-215.

Hovi, T. 1988. Successful selective inhibitors of viruses, pp. 1-21. *In* H.J. Field (ed.). Antiviral Agents: the development and assessment of antiviral chemotherapy. CRC, Boca Raton, Florida.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680-685.

Obando, V. 2002. Biodiversidad en Costa Rica: estado del conocimiento y gestión. INBio, Heredia. p. 81.

Schaeffer, M.J., L. Beauchamp, P. de Miranda, G.B. Ellion, D.J. Bauer & P. Collins. 1978 . 9-(2-hydroxy ethoxymethyl)guanine activity against viruses of the herpes group. Nature 272: 583-585.

Sun, B., C. Leandro, J.M. Ricardo da Silvia & I. Spranger. 1998. Separation of grape and wine proanthocyanidines according to their degree of polymerization. J. Agric. Food. Chem. 46: 1390-1396.

Whitley, R.J. & J. Gnann. 1992. Acyclovir. A decade later. N. Engl. J. Med. 327: 782-789.