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Action of extracts obtained with organic solvents from *Minthostachys verticillata* (Griseb.) Epling on viability of *Herpes simplex* Type 1 virus (HSV-1)

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ABSTRACT

Introduction: It has demonstrated "in vitro" antimicrobial activity exerted by extracts from *Minthostachys verticillata* (peperina). **Objective:** Investigate antiviral action of organic extracts of peperina against *Herpes simplex* virus. **Experimental:** Vero cells and *Herpes simplex* virus type 1 strain KOS were used. Aerial vegetal parts of plant were submitted to sequential extraction with n-hexane, chloroform and methanol for 48 h at room temperature. Extracts n-hexanic (N-HE), chloroformic (CE) and methanolic (ME) were obtained. They were added on cell monolayers, and incubated for 48 h at 37°C. Cell viability was determinated by Neutral Red uptake (NRU) after treatment of cells with different concentrations of extracts (10-1000 µg/ml). The same method was performed to evaluate antiviral action exerted by N-HE (100-250 µg/ml), CE (100-500 µg/ml) and ME (range 80-200 µg/ml). **Results and discussion:** The three extracts showed dose-dependent cytotoxic activity on Vero cells with CC_{50} values: 502 µg/ml for CE, 244 µg/ml for N-HE and 192 µg/ml for ME. The replication of *Herpes simplex* virus type 1 "in vitro" was inhibited by each extract at non cytotoxic concentrations. The extracts showed selectivity of action because all concentrations tested inhibited more than 60% of viral production. The results are promising for future natural therapy antiherpetic.

Keywords: *Minthostachys verticillata*, cytotoxicity, antiviral activity, organic extracts, *Herpes simplex* virus, Neutral Red uptake assay.

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Introduction:

Herpes simplex virus type 1 (HSV-1) and *Herpes simplex* virus type 2 (HSV-2) are human pathogens. Genital herpes is an important sexually transmitted disease, commonly caused by HSV-2 and occasionally by HSV-1.

Infections caused by HSV-1 are very common and a high percentage of the adult population has antibodies against the virus, (Khan *et al*., 2005). Many strains of HSV are resistant to available drugs therefore, new antiviral agents with different mechanisms of action are urgently required.

Minthostachys verticillata (Griseb.) Epling, (Labiatae) commonly referred to as "peperina", has a wide geographical distribution and is known for its ethno-medicinal properties. Its infusion is widely utilized to treat diarrhea and vomiting. It is also used as a digestive, sedative, anti-spasmodic, stimulant and bronchial-dilating agent (Núñez and Cantero, 2000). Previous studies have shown antimicrobial and immunomodulatory properties "in vitro" for this specie, (Elstein *et al*., 2005; Escobar *et al*., 2005; Cariddi *et al*., 2009). Background on the ability to inhibit in vitro *Herpes suis* virus type 1 (Zanon *et al*., 1999), stimulated this study, whose aim was to investigate the antiviral action of extracts obtained from peperina with organic solvents against human *Herpes simplex* virus type 1.

Experimental

Cell culture and virus

VERO cells line (clone 76) (*Cercopithecus aethiops* kidney) were propagated in Eagle's minimal essential medium (EMEM – Gibco, USA) supplemented with 10% fetal calf serum (Natocor, Argentina), glutamine (30 µg/mL) and gentamicin sulfate (200 µg/mL) (all from Sigma-Aldrich, Italy) at pH 7.2 (growth medium, GM). Cell cultures were maintained at 37°C under a humidified 5% CO_2 atmosphere. This substrate was used to evaluate cellular cytotoxicity and antiviral activity exerted by plant extracts. The virus used was *Herpes simplex* virus type 1 strain KOS. Viral stocks were stored at -70ºC.

Plant material

The specie studied *M. verticillata* was collected in Alpa Corral (Córdoba) in April 2007 and was identified by professionals Botany Section of Universidad Nacional de Río Cuarto. A voucher specimen has been deposited under number RCV-1955 at the Herbarium, (Río Cuarto Vasculares).

Preparation of extracts

Aerial vegetal parts dry and finely ground were submitted to sequential extraction with n-hexane, chloroform and methanol for 48 h at room temperature.

After filtration the liquid extracts were concentrated to dryness in rotary evaporator to obtain the extracts: n-hexanic (N-HE), chloroformic (CE) and methanolic (ME). Each vegetal extract was dissolved in dimethyl sulfoxide (DMSO) to achieve an initial concentration of 100 mg/ml and were stored at -20°C.

Determination of 50% cytotoxic concentration (CC_{50}) by Neutral Red Uptake assay (NRU)

Cell viability was determined by Neutral Red Uptake test (NRU), (Seth *et al*., 2004). Different concentrations of extracts were obtained by dissolution in Maintenance Medium (MM): (MEM + 2% FCS). They were tested in a range from 10 to 500 µg/mL for ME and from 100 to 1000 µg/mL for CE and N-HE. Cell monolayers grown in 48-well culture plates (Cellstar, Greiner Bio-One, Germany) were incubated for 48 h at 37ºC with different concentrations of extracts, for triplicate. Then, medium was removed and 500 µL of NR solution (30 µg/mL en MM) was added to each well. The plates were incubated once more for 3 h at 37ºC to promote the uptake of the dye by cells. Subsequently, the supernatant was removed. The monolayers were washed with PBS, and 500 µL/well of extraction solution (H_2O: acetic acid: ethanol) (49:1:50) was incorporated. After gently shaking the plates, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, Elx 800) at 540 nm.

Monolayers incubated only with MM were used as control. The CC_{50} were calculated from concentration-effect curves after non linear regression analysis (Boltzman sigmoidal Origin 6.0). The results represent the mean ± standard error of the mean values of three different experiments.

Determination of antiviral activity by Neutral Red Uptake assay

The "in vitro" action of plant extracts on replicative capacity of *Herpes Simplex* virus type 1 was evaluated according to the protocol described by Watanabe *et al*., 1994, with modifications.

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Briefly, the test was performed using Vero cell monolayers preformed in 48 well-culture plates, which were infected with 100 PFU/well of virus. The system was incubated for 1 h at 4°C and after viral adsorption, different non-cytotoxic concentrations of each extract: N-HE (100-250 µg/ml), CE (100-500 µg/ml) and ME (80-200 µg/ml) were added to the cells. Controls of virus, cells and extracts were included in all assays.

The plates were incubated for 48 h at 37ºC. The same NRU method used to evaluate cell viability was followed.

The optical densities (OD) of treated and control cells were read in a spectrophotometer at 540 nm. Antiviral activity (AA) was determined by the following formula:

$$\% AA = \frac{OD\ (_{virus\ +\ compound}) - OD\ (_{virus})}{OD\ (_{CC\ +\ compound}) - OD\ (_{virus})} \times 100$$

These values allowed create a graph of concentration of extract *versus* viral inhibition.

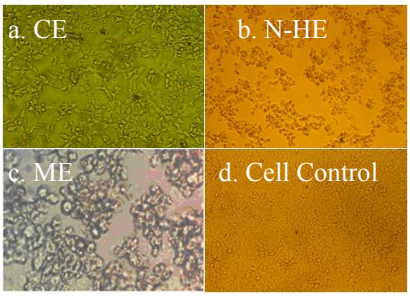
Results and discussion

Determination CC_{50} by Neutral Red Uptake assay (NRU)

Figure 1 shows the correlation between the percentages of cell viability in terms of concentrations of the extracts used: CE, N-HE and ME, obtained from *M. verticillata***.** The three extracts showed dose-dependent cytotoxic activity on Vero cells with values of CC_{50} of 502 µg/mL for CE, 244 µg/mL for N-HE and 192 µg/mL for ME, which allowed to establish the following increasing order of toxicity: CE> N-HE> ME.

At 48 h after treatment with extracts and before the addition of RN, cell monolayers were observed under light microscope. It was possible to detect some structural changes in those cell monolayers treated with high concentrations of extracts respect to cellular control that did not show any change, **(Photographs 1)**.

Monolayers treated with CE exhibited holes formation with retraction of cells even attached. Treatment with N-HE generated round cells grouped and refractile, intracytoplasmic granulations, in addition to cell detachment. The ME induced rounding and refraction of the cells with thickening of the membranes.



Photographs 1: Morphological alterations of monolayers of Vero cells, induced by different extracts obtained from *M. verticillata*, 20X. a. CE: Chloroformic extract; b. N-HE: N-hexanic extracts; c. ME: Methanolic extract; d. Cell control.

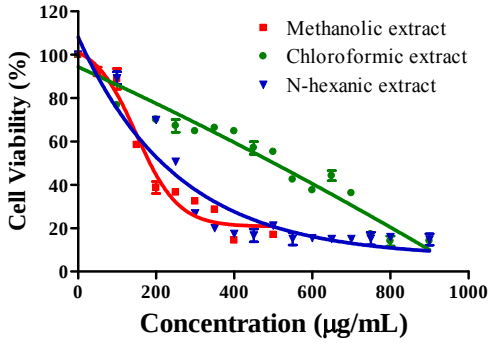


Figure 1: Curves of cytotoxicity of organic extracts to *Minthostachys verticillata*, determined by Neutral Red Uptake.

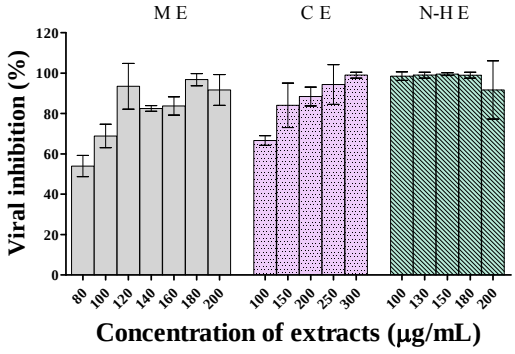


Figure 2: Percentages of viral inhibition in terms of non-cytotoxic concentrations of extracts: methanolic (ME), chloroformic (CE) and n-hexanic (N-HE).

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Determination of antiviral activity by Neutral Red Uptake assay

All three extracts of the specie *M. verticillata* inhibited the replication of *Herpes simplex* virus type 1 in Vero cells at non cytotoxic concentrations. The viral inhibition was independent of the concentrations tested for each extract and therefore it was not possible to calculate the value of 50% inhibitory concentration (IC_{50}) for each of them **(Figure 2).** According to these results it was not possible to calculate the selectivity index (CC_{50}/IC_{50}) for each extract.

However, the antiviral action is selective because the virus was inhibited in more than 60% with all non-toxic concentrations of the extracts. This confirms the importance of further studies to elucidate active molecules responsible present in each extract obtained from *M. verticillata.* The results suggest that peperina extracts could be used as chemotherapeutic antiherpetic, even ignoring its chemical composition.

Conclusion:

Our findings show that extracts of *Minthostachys verticillata* at concentrations tested exerted selective antiviral action against *Herpes Simplex* virus type 1.

Acknowledgments

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Note: Part of this study was presented at the 'II Reunión de Biotecnología aplicada a plantas medicinales y aromáticas' (Second Biotechnology Meeting on Medicinal and Aromatic Plants), Córdoba, Argentina, 2009.

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