

## Article

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# Antiherpetic activity of a flavonoid fraction from *Ocotea notata* leaves

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**Abstract:** This study describes the isolation of a flavonoid fraction from leaves of *Ocotea notata* (Nees & Mart.) Mez, Lauraceae, the identification of six major compounds (an A-type proanthocyanidin trimer [3], isoquercitrin [4], reynoutrin [5], miquelianin [6], quercitrin [7], afzelin [8]) and four minor compounds (catechin [1], epicatechin [2], quercetin [9], kaempferol [10]) present in the fraction and its activity against the Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). The 50% effective concentrations values (EC50) calculated from the dose-response curve and the selectivity indices (SI) against the virus were: EC50 35.8 µg/mL and SI 5.5 to HSV-1 and EC50 23.5 µg/mL and SI 8.5 to HSV-2. The flavonoid fraction was more active against HSV-2 than HSV-1. The mechanisms of antiviral action of the flavonoid fraction against the virus were also evaluated. The percentage inhibition (PI) obtained for HSV-2 was higher than 90% in the following assays: virucidal, pre-treatment of cells, treatment of cells after viral adsorption and treatment of cells after viral penetration. For HSV-1, the flavonoid fraction had no effect in pre-treatment of cells and showed 60% of inhibition in virucidal assay.

## Introduction

Herpesvirus is one of the most common human pathogen and the infections caused by its different virus types are widely distributed around the world (Brady & Bernstein, 2004; Hill et al., 2008). Among the Herpes simplex virus, the types 1 (HSV-1) and 2 (HSV-2) are the main human pathogens. The HSV-1 infection, also known as labial herpes, usually occurs by the contact with infected saliva during infancy. It causes diseases like gingivostomatitis, keratitis and encephalitis. On the other hand, HSV-2 infection, also known as genital herpes, is commonly associated with the beginning of the sexual activity in adolescence and it is responsible for genital diseases (Sacks et al., 2004; Mehnert & Candeias, 2005). Additionally, HSV infections may result from primary contact with the virus or reactivation of a latent infection (Hook et al., 1992). Several drugs are currently available for the treatment of HSV infections. Acyclovir is a potent drug and shows low cytotoxicity hence it is considered the first drug of choice (Kleymann, 2003; De Clercq,

2004). However, the emerging of resistant viral strains has been encouraging the search for new antiherpetic alternatives (Bacon et al., 2003; De Clercq, 2005; Fritz et al., 2007; Munding & Efferth, 2008). Different steps of the herpesvirus replication cycle can be chosen as targets for antiherpetic compounds. They could inactivate the virus particle or block its penetration at an early step of the infection, prevent the virus uncoating, inhibit the viral genome replication and others (Gomes et al., 2008).

Different compounds isolated from vegetal sources, including terpenes, phenolics, polyphenols and glycosides display antiherpes activities (Bourne et al., 1999; Benencia & Courreges, 2000; Chiang et al., 2002; Nohara, 2004; Kutluay et al., 2008). Over 4000 flavonoids have been identified to date in the plant kingdom. These compounds play an important role in plant protection against pathogens, UV-B radiation and in plant dispersion by providing attractive flower colors to pollinators. They are found in vegetables, fruits, seeds, flowers as well as in wine, tea and propolis (Harborne & Williams, 2000; Grotewold, 2006). The number of published papers about

antiviral activity of flavonoids had increased in the last two decades. The most cited flavonoid classes displaying antiherpes activity are flavonols and flavones (Hayashi et al., 1997; Amaral et al., 1999; Ma et al., 2001; Chiang et al., 2003; Lyu et al., 2005; Fritz et al., 2007; Gomes et al., 2008; Schnitzler et al., 2009; Martins et al., 2011).

*Ocotea notata* (Nees & Mart.) Mez, Lauraceae, is a medium-sized tree popularly known as “canela-branca”. It is widespread over the Brazilian Atlantic coast and grows mainly in sandy coastal plains, where it is used as timber-tree. The chemodiversity of *Ocotea* genus is well recognized and several alkaloids, terpenes, lignans and neolignans have been reported (Dias et al., 2003; Zanin & Lordello, 2007; Barbosa-Filho et al., 2008; Funasaki et al., 2009; Cuca et al., 2009). Nevertheless, only a small number of papers describe the presence of flavonoids in this genus (David et al., 1994; Garcez et al., 1995, 2005). The first published chemical study with *O. notata* was done by our research group in 2007 (Garrett et al., 2007) and this is the first report on the flavonoid content of this specie.

In the present study, we investigated the antiherpetic activity of a flavonoid fraction obtained from *O. notata* leaves, the viral inhibition mechanism and also the chemical composition of this fraction.

## Materials and methods

### Plant material

Aerial parts of *Ocotea notata* (Nees & Mart.) Mez, Lauraceae, were harvested during spring time (December 2007) in the region located between a periodically flooded forest and open *Clusia* scrub formation of the Restinga de Jurubatiba National Park sandy coastal plains, Rio de Janeiro state, Brazil. The specimen was identified by Dr. Marcelo G. Santos and a voucher sample (RFFP-10067) was deposited at the herbarium of the Faculdade de Formação de Professores-UERJ.

### General experimental procedures

All reagents used for the extraction and isolation process were analytical grade and reagents used for chromatography and mass spectrometry were hplc/spectrum grade. Acyclovir (Sigma) was used as standard compound in the experiments of cytotoxicity and antiviral screening. The flavonoids (+)-catechin and (-)-epicatechin (Fluka); isoquercitrin, quercitrin, quercetin and kaempferol (Aldrich); miquelianin (a gift from LPNBio-NPPN, UFRJ) were used as standards to identify the compounds present in the flavonoid fraction by co-injection in HPLC analyses.

Column chromatography was carried out on Sephadex LH-20 (25-100  $\mu$ m, Sigma). HPLC-DAD

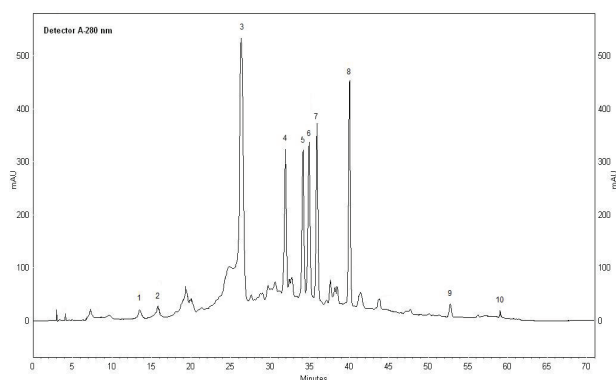
analysis was performed using a Waters Symmetry Shield reversed phase column (4.6 x 250 mm, 5  $\mu$ m particle size) with a gradient elution of acid water/acetonitrile (1:9; A) and acid water/acetonitrile (2:8; B) from 0 to 50% of B in 55 min. For preparative HPLC a Waters  $\mu$ -Bondapak column (7.8 x 300 mm, 10  $\mu$ m particle size) and UV detection at 280 nm were employed with a gradient elution of acid water (A) and acetonitrile (B) from 0 to 10% of B in 40 min. Mass spectra were recorded on a Micromass quadrupole-time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray ion source operating in the negative mode of ionization. Methanol-d<sub>4</sub> with TMS as internal standard was used for <sup>1</sup>H NMR (400 MHz) and <sup>13</sup>C NMR (100 MHz) analyses on a Varian MR-400. GC-MS analyses were recorded on a Shimadzu QP5000 GC-MS instrument (5% phenylmethyl silicone column, 30 m x 0.25 mm ID, 0.25  $\mu$ m film thickness; programmed column temperature from 110 to 290 °C, 5 °C min<sup>-1</sup>) (Borges et al., 2009).

### Extraction and isolation

Dried and powdered leaves of *O. notata* (400 g) were exhaustively extracted by Soxhlet, first with hexane and then with methanol. The methanol extract obtained was partitioned with ethyl acetate and concentrated using a rotary evaporator to provide the flavonoid fraction (3.2 g). This fraction (400 mg) was chromatographed on Sephadex column using ethanol and then methanol to afford 12 mg of the flavonoid quercetin (**9**), 24 mg of an A-type proanthocyanidin trimer (**3**) and a mixture of three flavonoids. This mixture was chromatographed in the same conditions described above to afford the 11 mg of isoquercitrin (**4**), 4 mg of reynoutrin (**5**) and 8 mg of quercitrin (**7**). In addition, 22 mg of the flavonoid afzelin (**8**) and 5 mg of kaempferol (**10**) were isolated by preparative HPLC. The flavonoids (+)-catechin (**1**), (-)-epicatechin (**2**) and miquelianin (**6**) were identified in the fraction by HPLC and MS analysis and were not isolated. Figure 1 shows the HPLC profile of the flavonoid fraction.

### Cells and viruses

Vero cells (African green monkey kidney cells) (Rio de Janeiro Cell Bank) were grown in Eagle's minimum essential medium (EagleMEM) (Cultilab) supplemented with 2 mM L-glutamine (Sigma), 50  $\mu$ g/mL garamicin, 2.5  $\mu$ g/mL fungizon (Gibco), 0.25 mM of sodium bicarbonate solution (Merck), 10 mM of HEPES (Sigma) plus 10% of heat-inactivated fetal bovine serum (FBS) (Cultilab) and maintained at 37 °C in atmosphere of 5% of CO<sub>2</sub>. Herpes simplex virus type 1 (HSV-1) was isolated from a typical lip lesion and Herpes simplex virus type 2 (HSV-2) from a typical genital lesion in the Virology Department of the Federal University of Rio de Janeiro (UFRJ), Brazil.



**Figure 1.** Reversed phase liquid chromatography profile of the flavonoid fraction (column 4.6 x 250 mm, 5  $\mu$ m;  $\lambda$  280 nm). Compounds: (+)-catechin (1), (-)-epicatechin (2), A-type proanthocyanidin trimer (3), isoquercitrin (4), reynoutrin (5), miquelianin (6), quercitrin (7), afzelin (8), quercetin (9) and kaempferol (10).

Viruses were typed by polymerase chain reaction (PCR) using specific primers for identification (Markoulatos et al., 2001).

#### Cytotoxicity assay

The cytotoxicity assay was performed prior to antiviral tests by incubating triplicate Vero cell monolayers cultivated in 96-well microplates with two-fold serial dilutions (3.1 to 200  $\mu$ g/mL) of the flavonoid fraction and acyclovir for 48 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. The morphological alterations of the treated cells were observed in an inverted optical microscope and the maximum non-toxic concentrations (MNTC) were determined (Walker et al., 1971). Cellular viability was evaluated by the neutral red dye-uptake method (Borenfreund & Puerner, 1985). 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

The antiviral activity of flavonoid fraction and acyclovir was evaluated by the titer reduction. The virus titers were calculated using the Reed and Muench statistical method (Reed & Muench, 1938) and expressed as 50% tissue culture infective dose (TCID<sub>50</sub>) per mL. Vero cell monolayers were treated with the flavonoid fraction and acyclovir at the MNTC and 100 TCID<sub>50</sub>/mL of HSV-1 or HSV-2 suspensions were added to treated and untreated cell cultures and incubated at 37 °C for 48 h in a 5% CO<sub>2</sub> atmosphere. After incubation, the supernatant was collected and virus titers in treated and untreated cells were determined. The antiviral activity was expressed as percentage inhibition (PI)

(Nishimura et al., 1977) using antilogarithmic TCID<sub>50</sub> 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, and the 50% effective concentration (EC<sub>50</sub>) was defined as the concentration required for 50% protection against virus-induced cytopathic effects. The selectivity index (SI) was determined as the ratio of CC<sub>50</sub> to EC<sub>50</sub>. The experiment was performed in triplicate and three times repeated.

#### Mechanism of action studies

##### Virucidal assay

HSV-1 and HSV-2 suspension (100  $\mu$ L) (10<sup>5</sup> TCID<sub>50</sub>/mL) were added to 900  $\mu$ L of the flavonoid fraction at the MNTC and MEM-Eagle without serum (virus control), according to Chen et al. (1988) and incubated at 37 °C for 2 h. After incubating, the virus titers were calculated in treated and untreated viral suspensions using the Reed and Muench statistical method (Reed & Muench, 1938) and the activity was expressed as PI.

##### Pre-treatment of cells

The flavonoid fraction was added to Vero cell monolayers before infection (pre-treatment) in order to evaluate their effect on cell receptors. Vero cell monolayers were pre-treated with the flavonoid fraction for 1 h at 4 °C. After this time, the cells were washed three times with MEM-Eagle for removing sample, and the treated and untreated cells were inoculated with 100 TCID<sub>50</sub>/mL of HSV-1 or HSV-2. After incubating the cells at 37 °C for 48 h, the supernatant was collected and virus titers in treated and untreated cells were determined and the activity was expressed as PI.

##### Treatment after viral adsorption

Vero cell monolayers were inoculated with 100 TCID<sub>50</sub>/mL of HSV-1 or HSV-2 and incubated for 1 h at 4 °C. After this period, the monolayers were washed with culture medium and the flavonoid fraction (at the MNTC) was added. The cultures were immediately shifted to 37 °C to allow the penetration of the particles into the cells for another hour. After incubation, the monolayer was washed, MEM-Eagle was added, and the cultures incubated at 37 °C for 48 h. After incubating, the supernatant was collected and virus titers in treated and untreated cells were determined and the activity was expressed as PI.

##### Treatment after viral penetration

Vero cell monolayers were inoculated with 100 TCID<sub>50</sub>/mL of HSV-1 or HSV-2 and incubated at 37 °C

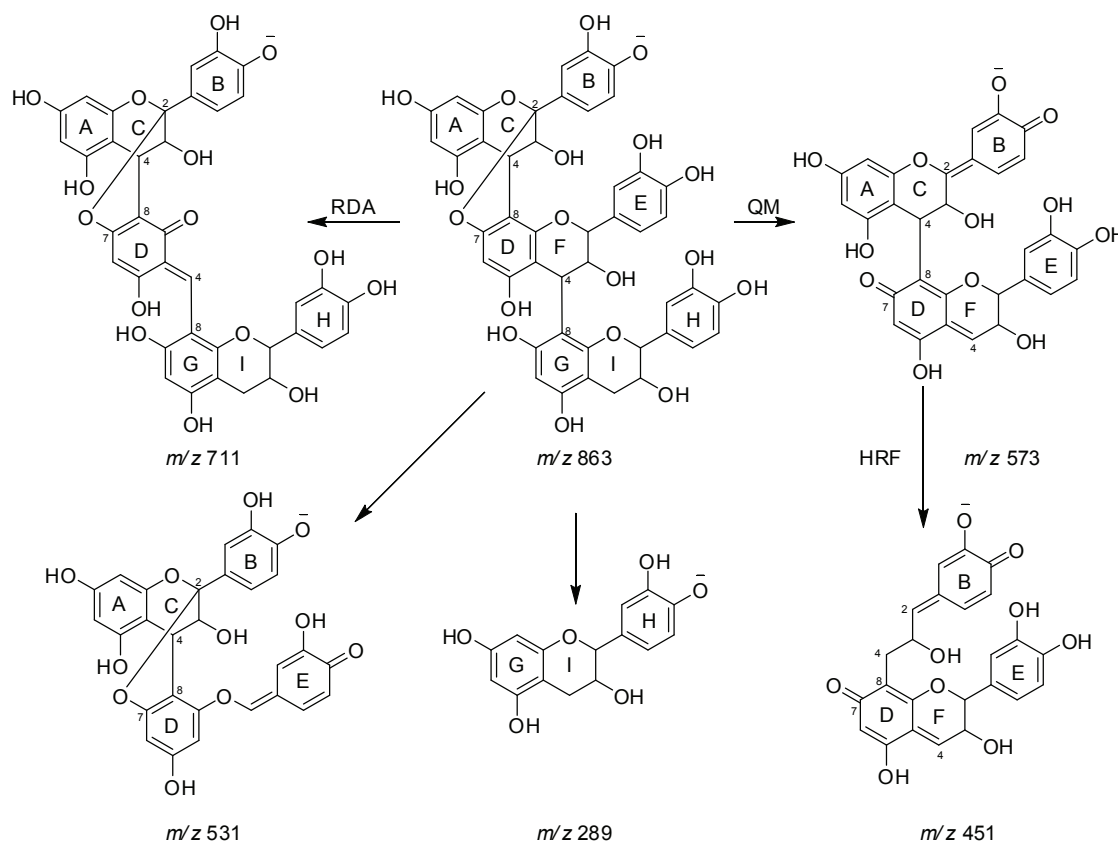
for 2 h. The cells were washed and the flavonoid fraction at concentration of 100  $\mu\text{g/mL}$  or MEM-Eagle (control) was added and the cultures incubated at 37 °C for 16 h. After incubation, the cells were washed to remove the flavonoid fraction before releasing viral particles. Then, MEM-Eagle was added and the cultures incubated for 32 h at 37 °C. After incubating, the supernatant was collected and virus titers in treated and untreated cells were determined and the activity was expressed as PI.

## Results and Discussion

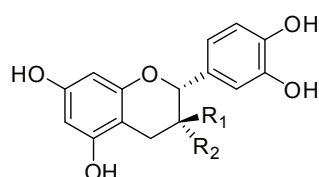
### Flavonoid fraction

Different methods were used to identify the compounds present in the flavonoid fraction. Compounds **1**, **2** and **6** were not isolated. They were directly identified in the flavonoid fraction by HPLC co-injection with standards and through the negative ions observed in the direct infusion ESI-MS of the flavonoid fraction. Quasi-molecular ions at  $m/z$  289 (compounds 1 and 2) and  $m/z$  477 (compound **6**; MS/MS 477, 301) were observed in the negative ESI mass spectra of the flavonoid fraction (Charrouf et al., 2007; Cavaliere et al., 2008). Compound **3** was isolated from the flavonoid fraction and showed an ion

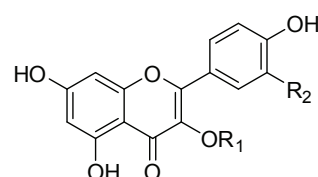
at  $m/z$  863. The MS/MS study of **3** revealed product ions at  $m/z$  711, 573, 531, 451, 411 and 289. Scheme 1 shows a chemical structure proposed for the compound **3** and the suggested fragmentation pathway. The  $m/z$  ion 711 is in agreement with a neutral loss of 152 Da from the A-type proanthocyanidin trimer rings E and F by a retro-Diels-Alder (RDA) fission. The loss of a lower *epi* (catechin) unit (290 Da) from **3** by a quinone methide (QM) fission generates the  $m/z$  ion 573, which corresponds to the upper and middle units of the proanthocyanidin with an A-type ether interflavan linkage. The  $m/z$  ion 451 is produced through the A ring loss from  $m/z$  573 by heterocyclic ring (HRF) fission. The presence of the (*epi*) catechin units is confirmed by the  $m/z$  ion 289 (Foo et al., 2000; Zhang et al., 2003; He et al., 2007, Li & Deinzer, 2007, 2008). A-type proanthocyanidins molecular ions displays two mass units lower regarding the B-types thus providing evidence for the presence of the additional ether linkage between C2 and C7 (Li & Deinzer, 2007, 2008). These considerations allowed the proposal of a A-type proanthocyanidin trimer (*epi*)catechin-A-(*epi*)catechin-(*epi*)catechin structure for compound **3**. Additional work is needed to elucidate the structure of this compound. Compounds **4**, **5** and **7-10** were isolated from the flavonoid fraction. The co-injection with standards on HPLC (compounds 4, 7, 9 and 10), the



**Scheme 1.** A chemical structure proposed for the ion at  $m/z$  863 (compound **3**) and the suggested fragmentation pathway by negative ESI-MS.



**1** R<sub>1</sub>=OH; R<sub>2</sub>=H  
**2** R<sub>1</sub>=H; R<sub>2</sub>=OH



**4** R<sub>1</sub>=glucose; R<sub>2</sub>=OH  
**5** R<sub>1</sub>=xylose; R<sub>2</sub>=OH  
**6** R<sub>1</sub>=glucuronic acid; R<sub>2</sub>=OH  
**7** R<sub>1</sub>=rhamnose; R<sub>2</sub>=OH  
**8** R<sub>1</sub>=rhamnose; R<sub>2</sub>=H  
**9** R<sub>1</sub>=H; R<sub>2</sub>=OH  
**10** R<sub>1</sub>=R<sub>2</sub>=H

negative ESI mass spectra at  $m/z$  463 (**4**; MS/MS 463, 301),  $m/z$  433 (**5**; MS/MS 433, 301),  $m/z$  447 (**7**; MS/MS 477, 301),  $m/z$  431 (**8**; MS/MS 431, 285),  $m/z$  301 **9**,  $m/z$  285 **10** and the comparison of <sup>1</sup>H NMR spectra of **4**, **5** and **7** (Lu & Foo, 1997; Lee et al., 2004) and <sup>1</sup>H NMR, COSY and HSQC spectra of **8** with literature (Min et al., 2003; Gohar et al., 2009) allowed the identification of these compounds in the flavonoid fraction. Furthermore, the flavonoid glycosides were acid hydrolyzed and the resulting monosaccharides were reduced and acetylated (Borges et al., 2009). The alditol acetates of glucose, xylose and rhamnose were identified by direct comparison with authentic samples using GC-MS.

#### Cytotoxicity

Changes in the cellular morphology were observed when 100 and 200 µg/mL of the flavonoid fraction were used (MNTC 50 µg/mL), although more than 50% of cells remained viable at the highest concentration used (CC50 > 200 mg/mL) (Table 1).

#### Antiviral activity

The antiviral experiments were performed with the flavonoid fraction at concentration of 50 µg/mL and acyclovir at 200 µg/mL. The EC50 values calculated from the dose-response curve (Figure 2) and the SI values were: EC50 35.8 µg/mL and SI > 5.5 to HSV-1 and EC50 23.5 µg/mL and SI > 8.5 to HSV-2. Acyclovir presented EC50 0.8 µg/mL and SI > 250 to HSV-1 and EC50 1.38 µg/mL and SI > 144.9 to HSV-2 (Table 1).

The flavonoid fraction showed antiherpes activity against both HSV-1 and HSV-2. Moreover, this fraction was more active against HSV-2 than HSV-1. All the compounds identified in the fraction had already demonstrated antiviral properties in literature. For instance, afzelin, quercitrin and kaempferol showed anti-HSV-1 activity (Almeida et al., 1998; Lyu et al., 2005) whereas quercetin and isoquercitrin showed activity against both HSV-1 and HSV-2 (Chiang et al., 2003; Gomes et al., 2008). A medicinal preparation from the plant extract *Rhododendron ungerinii* containing the flavonoids quercetin, isoquercitrin, quercitrin, (+)-catechin, (-)-epicatechin and others received the approval from the Georgia government to treat HSV-1 disorders in the oral cavity (Kemertelidze et al., 2007).

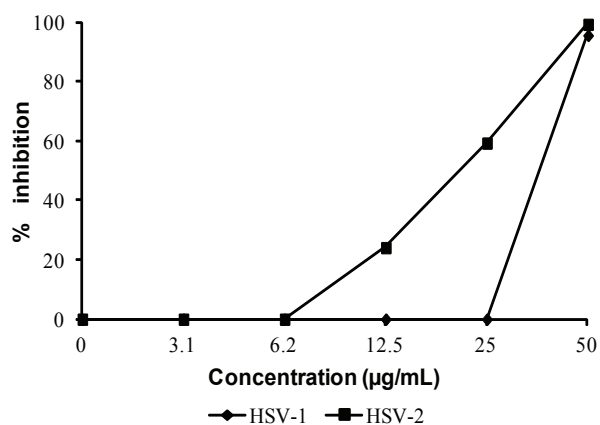
The mechanisms of antiviral action of the flavonoid fraction against the herpesvirus types 1 and 2 were evaluated and the inhibition of different steps of the virus replication cycle was observed (Figure 3). The PIs obtained for HSV-2 were higher than 90% in all performed experiments. Differently, for HSV-1, the flavonoid fraction had no effect in pre-treatment of cells and showed 60% of inhibition in virucidal assay.

Despite the drug acyclovir be the first choice for treating the HSV infections, new antiviral agents exhibiting different mechanisms of action are urgently needed. The flavonoid fraction had its major compounds and some minor compounds identified, showed different viral inhibition mechanisms and low toxicity and thus could be used as complement in the HSV-1 and HSV-2 infections treatment.

**Table 1.** Cytotoxicity and inhibitory effect of the flavonoid fraction from *Ocotea notata* leaves and acyclovir against Herpes simplex virus types 1 and 2.

Fraction and compound	MNTC (µg/mL)	CC50 (µg/mL)	EC50		SI	
			HSV-1	HSV-2	HSV-1	HSV-2
Flavonoid fraction	50	> 200	35.8	23.5	>5,5	> 8.5
Acyclovir*	≥200	> 200	0.8	1.38	> 250	> 144.9

MNTC: maximum non-toxic concentration; CC50: 50% cytotoxic concentration; HSV-1: Herpes simplex virus type 1, HSV-2: Herpes simplex virus type 2, PI: percentage of inhibition, EC50: 50% effective concentration, SI: selectivity index, \*Standard compound.



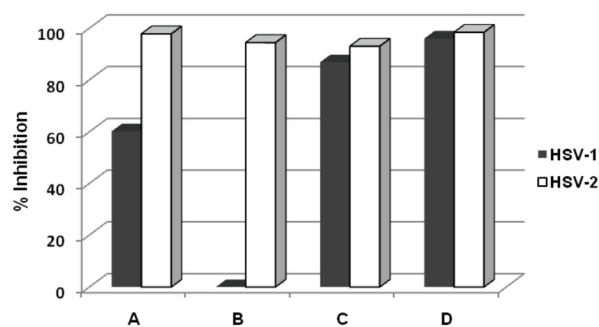
**Figure 2.** Dose-response curve of the flavonoid fraction from *Ocotea notata* leaves against Herpes simplex virus types 1 and 2. The dose-response curve was established starting from the MNTC. 100 TCID<sub>50</sub>/mL of virus suspensions were inoculated in the presence of several concentrations of the flavonoid fraction. After 48 h of incubation at 37 °C the supernatant was collected and virus titers in treated and untreated cells were calculated using the Reed and Muench statistical method (Reed & Muench, 1938) and the activity was expressed as PI.

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**Figure 3.** Mechanisms of antiviral action for the flavonoid fraction obtained from *Ocotea notata* leaves against Herpes simplex virus types 1 and 2. A. *Virucidal assay*: virus particles were treated with flavonoid fraction. The viral suspensions were incubated at 37 °C for 2 h. After incubating, the virus titers were calculated in treated and untreated viral suspensions and the activity was expressed as PI. B. *Pre-treatment of cells assay*: the flavonoid fraction was added to Vero cells after 1 h pre-infection. C. *Treatment after viral adsorption*: the flavonoid fraction was added to Vero cells after 1 h post-infection. D. *Treatment after viral penetration*: the flavonoid fraction was added to Vero cells 2 h post-infection. After 48 h of incubation at 37 °C, the supernatants were collected and virus titers in treated and untreated cells were calculated using the Reed and Muench statistical method (Reed & Muench, 1938) and the antiviral activities were expressed as PI.

of *in vitro* and *in vivo* activity against herpes simplex virus type 2. *Antiviral Res* 42: 219-226.

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