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Antiviral activity of the crude *n*-hexane extract from leaves of *Piper lepturum* var. *angustifolium* (C.DC.) Yunck. (Piperaceae)

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***Piper lepturum* var. *angustifolium* (C.DC.) Yunck. belongs to the family Piperaceae which is widely found from North to South of Brazil. Crude *n*-hexane extract from leaves of *P. lepturum* var. *angustifolium* was assayed to anti-herpes simplex viruses (HSV) activity against HSV-1 and HSV-2 in cell cultures, incubated at 37°C for 48 h in a 5% CO_2 atmosphere. Chemical profile of the extract was performed in a Shimadzu high performance liquid chromatography-diode array detector-ultraviolet (HPLC-DAD-UV), using a RP-18 column and mobile phase in gradient of acetonitrile and acid deionized water (pH = 3.0), at a flow rate of 1.0 ml/min. The HPLC analyses showed signals characteristics of non-polar compounds, mainly distributed between 50 and 70 min. UV spectra of the three main compounds (50.3% of the mixture) suggested isomers, with only one λmax at 235 nm. The crude *n*-hexanic extract showed 94.4% inhibition to HSV-1 with ED_{50} value of 5.2 µg/ml and selective index superior to 38.4. Considering HSV-2, the extract promoted 92.7% of inhibition with ED_{50} value of 1.1 µg/ml and selectivity index (SI) superior to 181.8. These results point out this species of Piperaceae as a source of active compounds for the treatment of infections caused by herpes simplex virus.**

Key words: Antiviral activity, herpes simplex virus type 1, herpes simplex virus type 2, *Piper lepturum* var. *angustifolium*, Piperaceae, high performance liquid chromatography-diode array detector-ultraviolet (HPLC-DAD-UV).

INTRODUCTION

Chemical studies with Piperaceae species have revealed several novel compounds from the secondary metabolism such as amides, terpenes, flavonoids and neolignans/lignans that are distributed in all plant organs, notably in the leaves (Parmar et al., 1997; Pessini et al., 2005; Alves et al., 2008; Mesquita et al., 2011). A broad spectrum of biological activities associated with these compounds has been proven, such as antitumor, antifungal, antimicrobial, trypanocidal and leishmanicidal (Andrade et al., 2005; Pessini et al., 2005: Nakamura et

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al., 2006; Regasini et al., 2009; Marques et al., 2011). According to studies published on *Piper* species in Brazil, it is interesting to note *Piper regnelli* C.DC., used in traditional medicine, whose fractions from the hexane extract showed activity against *Paracoccidioides brasiliensis*, a pathogen fungal for humans (Johann et al., 2010). *Piper aduncum* L., another species also used in folk medicine, showed antimicrobial activity in their extracts and volatile components (Braga et al., 2007; Lara Junior et al., 2012).

Although studies with different species of this family show satisfactory results, the knowledge of its antiviral activity is still scarce. Herpes simplex viruses (HSV) infections are among the most common diseases of humans, with a worldwide distribution (Aymard, 2002; Lorette et al., 2006; Fatahzadeh and Schwartz, 2007; Beydoun et al., 2010; Pereira et al., 2012). In spite of the availability of the effective antiviral agent acyclovir, a nucleoside analog, to treat these infections, resistant strains have already been isolated, most of them from immunocompromised patients (Safrin et al., 1991; Bergaoui et al., 2012; Chono et al., 2013). In recent years, there has been an increasing interest for application of natural products as anti-infective and concerns about the safety of synthetic compounds have encouraged more detailed studies of natural resources (Schuhmacher et al., 2003; Khan et al., 2005; Astani et al., 2009; Nolkemper et al., 2010). Since many species of the family Piperaceae have shown a broad spectrum of biological activities (Pessini et al., 2005; Johann et al., 2009; Silva et al., 2007; Santos et al., 2010), it was suggestive to investigate the assessment of antiviral activity of *Piper lepturum* against HSV-1 and HSV-2.

MATERIALS AND METHODS

Plant

Leaves of *P. lepturum* var. *angustifolium* (C.DC.) Yunck. were collected in the Tijuca Forest, which is located in the city of Rio de Janeiro, Brazil (22°56'57''S and 43°17'58''W), with an altitude of up to 917 m in the hollow. The taxon was identified by researcher Dr. Elsie Franklin Guimarães and a sample was deposited in the herbarium of the Botanical Garden Research Institute of Rio de Janeiro (RB 501328).

Extraction

P. lepturum var. *angustifolium* dried leaves (2 kg) were submitted to maceration with a total of 25 L of *n*-hexane, during 10 days. The crude *n*-hexane extract was filtered and the solvent was evaporated under reduced pressure yielding 26.4 g. The crude *n*-hexane extract was dark green.

HPLC apparatus and analyses

Analyses were done in a Shimadzu equipped with system controller SCL-10Avp, pump LC-10Advp, mixer FCV-10AL, degasser DGU-14A, column oven CTO-10AS, and diode array detector-ultraviolet (DAD-UV) detector SPD-M10A. The chromatograms were set in a PC-computer equipped with Shimadzu Class-VP workstation.

Samples were solubilizated with dichloromethane to a final concentration of 20 mg/ml and then filtrated in 0.45 μm Millipore membrane durapore PVDF filter. In all run experiments, 20 μl of the dichloromethane solution were injected. The mobile phase employed was composed by acetonitrile (A) and acid deionized water (pH 3.0 with glacial acetic acid) (B). Gradient programing started with 5% (A)/95% (B) and then 95% (A)/5% (B) in 80 min. Equilibration time after run was 10 min. A Merck Lichrospher 100 RP-18 column (250 mm × 4 mm × 5 μm) was used and equipped with a guard column Merck Lichrospher 100 RP-18 (4 mm × 5 μm). Flow rate was 1.0 ml/min and UV monitoring was done at λ220, 240, 275, and 340 nm.

Biological assays

Antiviral activity: Cells and virus

Vero cells (African green monkey kidney) were grown in Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 2.5 μ/ml fungizone and 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C in 5% CO_2 atmosphere. Herpes simplex virus type 1 and type 2 strains were isolated from typical oral and genital lesions, respectively, at the Virology Department of Universidade Federal do Rio de Janeiro (UFRJ), Brazil. The isolates were typed by polymerase chain reaction (PCR) using specific primers to identify HSV-1 and HSV-2 (Markoulatos et al., 2001) and propagated in a Vero cell. The titers were assessed by the cytopathic end-point assay and were expressed as 50% tissue culture infective dose ($TCID_{50}$) per milliliter (Reed and Muench, 1938). The virus suspensions were stored at -70°C until use.

Cytotoxicity assay

Crude *n*-hexane extract from *P. lepturum* var. *angustifolium* leaves was dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared in water at 400 μg/ml and sterilized by filtration using a 0.22 μm 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 compounds for 48 h at 37°C in 5% CO_2 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 (Borenfreund and Puerner, 1985). The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration which caused a 50% reduction in the number of viable cells.

Antiviral activity assay

Vero cell monolayers were treated with crude *n*-hexane extract from *P. lepturum* var. *angustifolium* leaves at the MNTC and 100 $TCID_{50}$/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_2 atmosphere. After incubation, the supernatants were collected and virus titers in treated and untreated cells were determined. The antiviral activity was expressed as the percentage inhibition (PI) using antilogarithmic $TCID_{50}$ values as follows: PI = [1 - (antilogarithmic test value/antilogarithmic control value)] × 100. The dose-response curve was established starting from the MNTC, and the 50% effective dose (ED_{50}) was defined as the concentration required achieving 50% protection against virus-induced cytopathic

Table 1. Cytotoxicity and antiviral activity of *n*-hexane extract from leaves of *P. lepturum* var. *angustifolium*.

Compound	MNTC (µg/ml)	CC_{50} (µg/ml)	HSV-1			HSV-2		
			PI	ED_{50} (µg/ml)	SI	PI	ED_{50} (µg/ml)	SI
Extract	50	>200	94.4	5.2	>38.4	92.7	1.10	>181.8
Acyclovir	200	> 200	99.0	0.8	> 250	99.0	1.38	> 145.0

MNTC, Maximum non-toxic concentration; CC_{50}, 50% cytotoxic concentration; PI, percentage of inhibition; ED_{50}, 50% effective dose; SI, seletivity index; HSV-1, Herpes simplex virus type 1; HSV-2, Herpes simplex virus type 2.

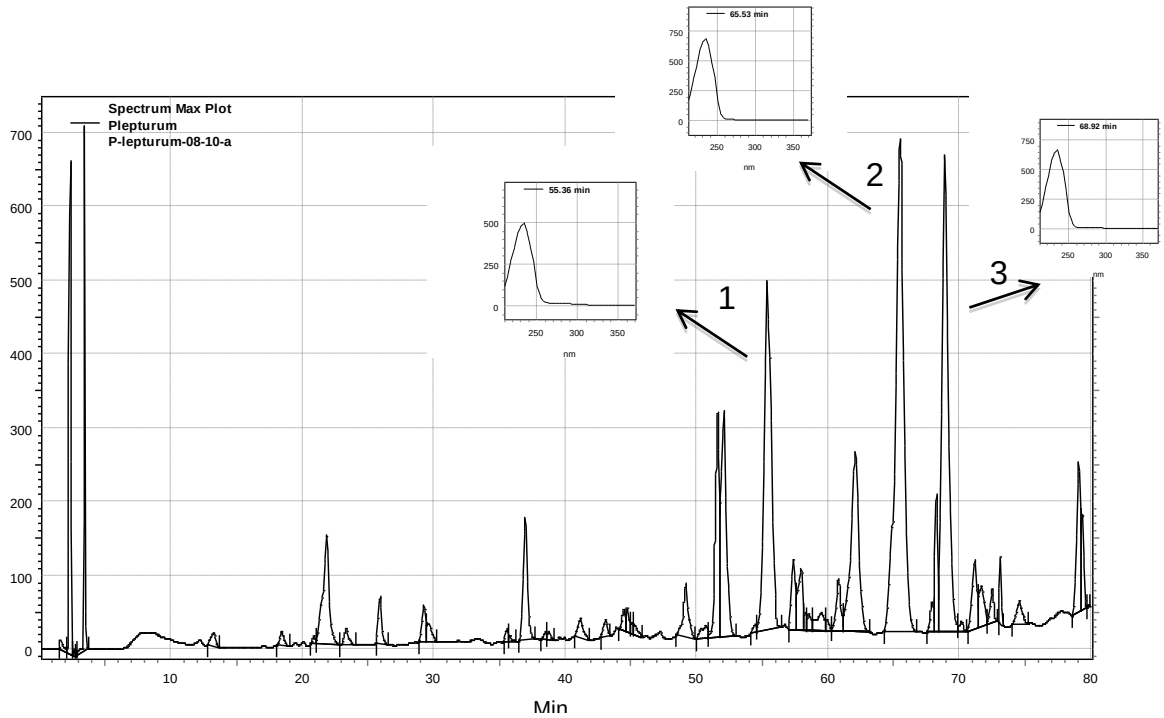

Figure 1. Chromatogram of the crude *n*-hexane extract from leaves of *P. lepturum* var. *angustifolium*.

effects. The selectivity index (SI) was determined as the ratio of CC_{50} to ED_{50}. Acyclovir (Sigma) was used as positive control.

RESULTS AND DISCUSSION

Chemical profile

The HPLC analyses showed signals from 20 to 70 min (Figure 1). In the used gradient programming, the polar compounds eluted until 10 min, medium polar between 10 and 40 min and non-polar after 40 min. Major signals were distributed between 50 and 70 min, suggesting the crude *n*-hexane extract is composed mainly by non-polar compounds. In fact, the three main compounds (assigned as 1, 2 and 3) represent about 50.3% of the mixture. UV spectra of the three main compounds (Figure 1) suggested isomers, with only one λmax at 235 nm. The observed UV pattern for the main three compounds suggested the presence of a simple chromophore in the structure (transitions π – π* and n - π*), such as conjugated double bounds (dienes) and carbonyl conjugated with a double bound (α,β-unsaturated keto compounds). The organic functions aldehydes, ketones and carboxyl acids and amides can be conjugated with double bounds and give λmax at 235 to 240 nm. These groups are very common in terpenes and fatty acids or fatty acid esters present in many plant extracts (Mabry et al., 1970; Silverstein et al., 2005; Larsen et al., 2008).

Antiviral activity

The anti-herpes simplex viruses activity of the crude *n*-hexane extract from leaves of *P. lepturum* var. *angustifolium* are shown in Table 1. The tested sample showed a potent antiviral activity against HSV- 1 and HSV- 2.

Although the CC_{50} was superior to 200 µg/ml, but was used in the concentration of 50 µg/ml due to morphological changes observed when the cells were exposed to higher concentrations. These alterations could affect the reading of the cytopathic effect caused by viruses.

The crude *n*-hexane extract showed 94.4 and 92.7% inhibitory activities to HSV-1 and HSV-2, respectively. In spite of a greater percentage of inhibition to HSV-1, the sample was more effective for HSV-2, since the ED_{50} was 1.1 µg/ml to HSV-2 and 5.4 µg/ml to HSV-1, thus showing a selectivity index, approximately, five times superior. In fact, the activity of the *n*-hexane extract against HSV-2 was very similar to the acyclovir activity (Table 1), a clinical drug used to treat anti-herpes viruses.

According to literature, many species of Piperaceae have shown antiviral activity. Lohézic Dévéhat-Le et al. (2002) demonstrated the antiviral activity against poliovirus for the methanol extract of *P. aduncum* L. collected in Indonesia. Aqueous and methanol extracts from fruits of *Piper cubeba* showed activity against Hapatitis C virus (Hussein et al., 2000). The hydroalcoholic extract from leaves of *P. regnelli* var. *pallescens* showed activity against Bovine herpes virus type 1 (BHV-1) and poliovirus (Bertol et al., 2012). According to the authors, this activity may be related to components in *n*-hexane, chloroform and chloroform/ethyl acetate fractions. Best results were obtained with chloroform/ethyl acetate fraction 9:1. However, it is not a common antiviral activity against HSV of Piperaceae species as it has been demonstrated in studies with *Piper lanceafolium* (Lopez et al., 2001), *Piper methysticum* (Locher et al., 1995) and *P. aduncum* (Lohézic Dévéhat-Le et al., 2002). By these means, the results achieved in this study points to *P. lepturum* var. *angustifolium* leaves *n*-hexane extract as an important source of natural compounds for the treatment of herpes virus.

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