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Antiviral activity of fractions from leaves of *Piper regnelli* var. pallescens

Jéssica W. Bertol,[2] Patricia R. Santos,[1] Juliana Rodrigues,[1] Diógenes A. G. Cortez,[1] Benedito P. D. Filho,[1,2] Celso V. Nakamura,[1,2] Tania Ueda-Nakamura*[,1,2]

[1]Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Brazil,
[2]Departamento de Farmácia, Universidade Estadual de Maringá, Brazil.

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Abstract: This study investigated the antiviral potential of fractions and eupomatenoid-5 obtained from *Piper regnelli* (Miq.) C. DC., Piperaceae, leaves against bovine herpesvirus-1 (BHV-1) and poliovirus. VERO cell monolayers in 96-well cell culture plates were infected with BHV-1 or poliovirus and incubated in the presence and absence of samples for 48 h. The cells were then fixed and stained with sulforhodamine B, and the virus-induced cytopathic effect was measured in a 96-well plate reader at 530 nm. Cytotoxicity was assessed by incubating the cell monolayers with samples for 72 h. The hexane, chloroform, chloroform/ethyl acetate (19:1), and chloroform/ethyl acetate (9:1) fractions showed activity against BHV-1. The chloroform, chloroform/ethyl acetate (19:1), chloroform/ethyl acetate (9:1), chloroform/ethyl acetate (1:1), and ethyl acetate fractions were active against poliovirus. The chloroform/ethyl acetate (9:1) fraction presented the best selectivity index for both viruses. The present study reports the antiviral activity of the extract and fractions of *P. regnelli* leaves.

Introduction

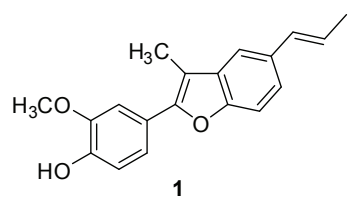
The Piperaceae family comprises a great number of species with medicinal uses and economic and commercial importance. It is used for essential oil production and spices and in the pharmaceutical and insecticide industries.

Piper regnelli var. *pallescens* (C. DC.) Yunck., popularly known in Brazil as "pariparoba," is an herbaceous plant found in tropical and subtropical regions of the world (Cronquist, 1981). Its leaves and roots are used in folk medicine in the form of crude extracts, infusions, and poultices for the treatment of wounds, swelling, and skin irritations (Yuncker, 1972). The extract and eupomatenoid-5, the neolignan isolated from the leaves of *P. regnelli*, have antimicrobial activity, including antibacterial (Pessini et al., 2003), antifungal (Koroishi et al., 2008), antileishmanial (Vendrametto et al., 2010), and trypanocidal (Luize et al., 2005) effects.

Bovine herpesvirus type 1 (BHV-1) is a member of the Herpesvirales order, Herpesviridae family, Alphaherpesvirinae subfamily, and Varicellovirus genus (Davison et al., 2009). BHV-1 is an important cattle pathogen that causes economic losses worldwide. BHV-1 is mainly implicated in respiratory infection (*e.g.*, infectious bovine rhinotracheitis), genital disease (*e.g.*, vulvovaginitis/pustular balanoposthitis), and abortion (Kahrs, 2001). This virus may be transmitted via respiratory and venereal routes and by artificial insemination with virus-contaminated semen, whereby samples of a single ejaculate may be inseminated into many females (Afshar & Eaglesome, 1990).

Poliovirus is a member of the *Enterovirus* genus and Picornaviridae family (Melnick, 1996). It is the etiological agent of poliomyelitis (Landsteiner & Popper, 1909), a disease that has been brought under control in most countries. Picornaviruses encompass numerous important human pathogens, including the enteroviruses polio, Coxsackie A and B, and echo and rhinoviruses, but no single antipicornavirus agent has been approved for clinical use (De Clerq, 2012). Although well studied, poliovirus remains one of the most appropriate models for the study of viral replication (Mueller et al., 2005).

Our previous studies found that the hydroalcoholic extract of *P. regnelli* leaves exhibited antiviral activity against BHV-1 but not against poliovirus. In the present study, we investigated the antiviral activity of fractions and eupomatenoid-5 (**1**) obtained from the leaves of *Piper regnelli* var. *pallescens* against BHV-1 and poliovirus.



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Materials and Methods

Cell culture and viruses

African green monkey kidney (VERO) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and 50 µg/mL gentamicin in a humidified 5% (v/v) CO_2 incubator at 37 °C. BHV-1 and vaccinal poliovirus were provided by Dr. Rosa Elisa Linhares, Microbiology Department, State University of Londrina. The viruses were titrated by inoculating the cells with 10-fold dilutions using the endpoint dilution technique (De Clercq, 1982).

Plant material

Piper regnelli var. *pallescens* (C. DC.) Yunck., Piperaceae, leaves were collected from the Profa. Irenice Silva Garden of Medicinal Plants, State University of Maringá. The plant material was identified by Marília Borgo, Botanical Department, Federal University of the Paraná, and a voucher specimen (no. HUM 8392) was deposited in the Herbarium of the State University of Maringá, Paraná, Brazil. The hydroalcoholic extract (crude extract), fractions, and pure compound eupomatenoid-5 (**1**) were obtained as described by Vendrametto et al. (2010). Briefly, the extract was obtained by extraction with ethanol/water (9:1, v/v) and evaporation under vacuum at 40 °C. This extract (275.0 g) was lyophilized, and the residue was partitioned with ethyl acetate. The organic layer was evaporated to yield the ethyl acetate extract (25.0 g). This extract was then subjected to vacuum column chromatography silica gel and eluted with hexane (2.8 g), chloroform (11.1 g), chloroform/ethyl acetate (19:1, 1.1 g), chloroform/ethyl acetate (9:1, 0.4 g), chloroform/ethyl acetate (1:1, 0.9 g), ethyl acetate (0.3 g), acetone (0.5 g), methanol (1.7 g), and methanol/water 9:1 (0.2 g), providing the corresponding fractions. The chloroform fraction was separated by column chromatography on silica gel 60 to yield eupomatenoid-5. The samples were stored at -20 °C until use. All of the compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium prior to use. The final concentration of DMSO did not exceed the minimum noncytotoxic concentration of 1% (v/v).

Antiviral assays

Confluent VERO cells in 96-well cell culture plates were washed with phosphate-buffered saline (PBS), infected with 25 µL/$TCID_{80}$/well of BHV-1 or poliovirus, and incubated at 37 °C in humidified 5% (v/v) CO_2 for 1 h. After incubation, 75 µL/well of culture medium was added, followed by 100 µL/well of culture medium that contained different concentrations of the fractions or eupomatenoid-5 (1).

The cells were incubated for 48 h. Afterward, the medium was removed, and the cells were washed with 100 µL/well of PBS. The monolayer was then fixed with 50 µL/well of 10% trichloroacetic acid for 1 h at 4 °C and subsequently washed four times with deionized water. The microplates were left to dry at room temperature for at least 1 h and then stained with 50 µL/well of 0.4% sulforhodamine B (SRB) in 1% acetic acid for 30 min. The microplates were then washed four times with 1% acetic acid. Bound SRB was solubilized with 150 µL/well of 10 mM buffered Tris-base solution, and the plates were left on a plate shaker for at least 15 min. Absorbance was read in a 96-well plate reader at 530 nm (Bio-Tek, Power XS). The virus-induced cytopathic effect (CPE) is expressed as a percentage of the optical density compared with the parallel virus control and cell control. The concentration that reduced 50% of the CPE compared with the virus control was estimated from the plots of the data and defined as the EC_{50} value (Papazisis et al., 1997).

Cytotoxicity assay

The cytotoxicity assay was performed as previously described, with some modifications (Skehan et al., 1990). Confluent VERO cell monolayers in 96-well cell culture plates had the medium replaced with 100 µL of serum-free medium. Subsequently, 100 µL/well of culture medium that contained different concentrations of fractions or eupomatenoid-5 was added. The plates were incubated at 37 °C in humidified 5% (v/v) CO_2. After 72 h, the plates were fixed and stained, and the optical density was determined as described above. The toxic concentration for 50% of the cells (CC50) is expressed as a percentage of the optical density of the control.

Results and Discussion

The fractions, and eupomatenoid-5 (**1**) obtained from the crude extract of *Piper regnelli* var. *pallescens* (C. DC.) Yunck., Piperaceae, leaves were evaluated against BHV-1 and poliovirus. Cytotoxicity was also tested. The results are summarized in Table 1.

Some *Piper* species have exhibited antiviral activity, such as the effects of the methanolic and aqueous extracts of *P. cubeba* fruits against hepatitis C virus (Hussein et al., 2000) and effects of the crude extract of *P. aduncun* leaves and flowers against poliovirus (Lohézic-Le Dévéhat et al., 2002). The methanolic extract of *P. lanceafolium* against Herpes simplex virus type 1 (HSV-1) and poliovirus (Lopez et al., 2001) and extracts of different parts of *P. methysticum* against HSV-1

(Locher et al., 1995) showed no antiviral activity.

As mentioned above, the hydroalcoholic extract (*i.e.*, crude extract) from the leaves of *Piper regnelli* had antiviral activity against BHV-1, with an EC50 6.0 µg/mL, but no antiviral activity against poliovirus, with an EC50 >100 µg/mL. The results of the antiviral activity of the fractions and eupomatenoid-5 obtained from this extract are shown in Table 1.

The anti-BHV-1 activity of the hydroalcoholic extract can be attributed to the components present in the hexane, chloroform, chloroform/ethyl acetate (19:1), and chloroform/ethyl acetate (9:1) fractions, with EC50 values that ranged from 26.2 to 3.6 µg/mL. The chloroform, chloroform/ethyl acetate (19:1), chloroform/ethyl acetate (9:1), chloroform/ethyl acetate (1:1), and ethyl acetate fractions were shown to be effective against poliovirus, although the crude extract had no effect. This issue should be assessed carefully in future investigations. The best activity against BHV-1 and poliovirus was obtained with the chloroform/ethyl acetate (9:1) fraction, with selectivity indices of 6 and 5 respectively. These results appear to show that this fraction contains the largest amount of the active substance and consequently is the fraction that exhibited the best antiviral activity against both tested viruses.

The antimicrobial activity of eupomatenoid-5 isolated from *Piper regnelli* (Pessini et al., 2003) was not observed against either BHV-1 or poliovirus, indicating that this compound has no antiviral activity.

Many active compounds have been isolated from this plant species, including amides, alkaloids, lignans, and neolignans, which have central nervous system depressant, antipyretic, analgesic, antiinflammatory, insecticidal, larvicidal, molluscicidal, anti-platelet-activating factor (PAF), and antimicrobial effects (Parmar et al., 1997; Santos et al., 2001; Sengupta & Ray, 1987). A phytochemical study of *Piper regnelli* isolated phenylpropanoids and dihydrobenzofuran neolignans. Previous studies indicated the presence of (+)-conocarpan as a major compound, in addition to eupomatenoid-6, eupomatenoid-5, and eupomatenoid-3 (Koroishi et al., 2008; Luize et al., 2006; Pessini et al., 2003).

Benzofuran neolignans are a subclass of neolignans with various biological activities, including anti-PAF, antifungal, antibacterial, insecticidal, trypanocidal, and leishmanial effects (Sartorelli et al., 2001). Many 2-arylbenzofuran derivatives, whose skeleton is present in the lignans isolated from *P. regnelli*, exhibit antiviral activity (Charlton, 1998; Craigo et al., 2000; Leung et al., 2000). Therefore, these compounds may be responsible by the antiviral activity of *P. regnelli*. The isolated eupomatenoid-5 (**1**) was also analyzed against BHV-1 and poliovirus. The chloroform fraction was chosen because of the greater amount of material provided by chromatography.

New vaccines have been developed against BHV-1 to ensure effective protection for calves. Rispoval 4 has presented good results under experimental conditions (Salt et al., 2007). Additionally, alternative therapeutics for the treatment of infection in calves have been interesting, especially in the presence of low serum neutralizing antibody titers.

The possibility of new outbreaks in the future and lack of specific and effective drugs encourage the resumption of the search for antipoliovirus compounds (Collett et al., 2008). The possibility that these compounds are also effective against other viruses of the Enterovirus genus cannot be ignored. The members of the Enterovirus genus cause a wide array of illnesses, such as meningitis, myocarditis, encephalitis, and respiratory diseases (Rotbart et al., 1998).

The antiviral potential of *Piper regnelli* was

Table 1. Antiviral activity and cytotoxicity of fractions and pure compound obtained from leaves of *Piper regnelli.*

Fractions/ pure compound	Bovine herpes virus-1			Poliovirus	
	CC50 (µg/mL)[a]	EC50 (µg/mL)[b]	Selectivity index[c]	EC50 (µg/mL)[b]	Selectivity index[c]
hexane	45.0±1.2	10.6±4.2	4	>50.0	<1
chloroform	69.8±5.0	26.2±0.4	3	31.1±1.5	2
chloroform/ethyl acetate (19:1)	8.2±0.3	3.6±0.9	2	8.2±1.6	1
chloroform/ethyl acetate (9:1)	31.1±7.5	5.6±0.7	6	6.7±1.4	5
chloroform/ethyl acetate (1:1)	28.9±4.4	>50.0	<1	15.5±12.0	2
ethyl acetate	53.9±3.9	>50.0	<1	10.9±1.3	5
acetone	89.0±9.9	>50.0	<2	>50.0	<2
methanol	261.9±6.7	>50.0	<3	>50.0	<3
methanol/water (9:1)	364.3±23.6	>50.0	<7	>50.0	<7
eupomathenoid-5	52.2±3.0	>50.0	<1	>50.0	<1

Results represent the mean±standard deviation of three independent experiments. [a]CC50 was the concentration that showed 50% cytotoxic effects in VERO cells; [b]EC50 was the concentration that inhibited 50% of virus replication in VERO cells; [c]The selective index (SI) was calculated as CC50/EC50.

demonstrated in the present study. Many fractions obtained from *P. regnelli* leaves exhibited antiviral activity against BHV-1 and poliovirus, demonstrating the potential antiviral activity of *Piper regnelli* var. *pallescens*.

Although eupomatenoid-5 has been shown to have trypanocidal (Pelizzaro-Rocha et al., 2011), antibacterial (Pessini et al., 2003), antifungal (Koroishi et al., 2008), and antileishmanial (Vendrametto et al., 2010) effects, it was not active against both viruses.

Future purifications and studies are needed to determine the substance or substances that are responsible for the antiviral activity and mechanism of action of these compounds.

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***Correspondence**

Tânia Ueda-Nakamura
Department of Basic Health Sciences, State University of Maringá
Colombo Avenue 5790, 87020-900 Maringá, PR, Brazil
tunakamura@uem.br
Tel. + 55 44 3011 5013
Fax: + 55 44 3011 5050