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## Full Length Research Paper

## ***In vitro* antiviral activity of Brazilian Cerrado plant extracts against animal and human herpesviruses**

**Marina A. Padilla<sup>1,2</sup>, Isabela C. Simoni<sup>1\*</sup>, Verônica Moreira H. Hoe<sup>1</sup>, Maria Judite B. Fernandes<sup>1</sup>, Clarice W. Arns<sup>2</sup>, Juliana R. Brito<sup>3</sup> and João Henrique G. Lago<sup>4</sup>**

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The Brazilian savanna known as “Cerrado” is very rich in medicinal plants that are used by the local population for treatment of several illnesses. The herpesvirus is a serious problem worldwide, and affects both animal and human health. This work aimed to study the antiviral activity of eight extracts from plants natives of “Cerrado” region against human (HSV-1), equine (EqHV-1) and swine herpesviruses (SuHV-1). The results showed that all plant extracts: *Banisteriopsis variabilis*, *Byrsonima intermedia*, *Campomanesia xanthocarpa*, *Erythroxylum deciduum*, *Lacistema hasslerianum*, *Ocotea pulchella*, *Stryphodendron adstringens* and *Xylopia aromatica* presented antiviral activity against at least one herpesvirus. Furthermore, it was observed a direct anti-herpes effect of extracts from *B. variabilis* and *B. intermedia* in non-toxic concentrations against all herpesviruses. *B. intermedia* crude aqueous extract showed the most promising results with selective index values of the  $41.76 \pm 0.04$ ;  $4.12 \pm 0.1$  and  $193.97 \pm 0.09$  respectively against HSV-1, EqHV-1 and SuHV-1. Due to this, *B. intermedia* extract was also analyzed by HPLC/MS allowing for the identification of gallic acid and quercetin as main compounds.

**Key words:** Brazilian savanna, Cerrado, *Byrsonima intermedia*, antiviral, herpesvirus, gallic acid, quercetin.

### INTRODUCTION

The herpes viruses are a serious worldwide problem that can affect the health of both animals and human beings. The herpes simplex virus (HSV-1) is pathogenic to humans (Wyler et al., 2017), whereas equine (EqHV-1) and swine herpesviruses (SuHV-1), are responsible for causing serious diseases in horses and pigs respectively,

resulting in large economic losses (Wernike et al., 2013; Gulati et al., 2016). These viruses belong to the subfamily Alphaherpesvirinae, characterized by a rapid lytic viral cycle and the establishment of neuronal latency, which can be reactivated (Riaz et al., 2017). The emerging of resistant virus strains to available drugs against human

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herpesvirus is a growing problem, particularly in immunocompromised patients (Vadlapudi et al., 2013). More recently, outbreaks of equine herpesvirus have had an impact on the equine industry, and stimulated interest in antiherpetic interventions (Vissani et al., 2016). The SuHV-1, also known as pseudorabies virus, is the causative agent of Aujeszky's disease affecting other mammals besides pigs, is still present in wild boar and there are no antiviral drugs approved for veterinary use (Zouharova et al., 2016). Therefore, it is necessary to find new alternative drugs against these diseases that affect both humans and animals (Martinez et al., 2015; Nocchi et al., 2016). Medicinal plants are natural resources, leading to valuable herbal products often used to treat a large number of various diseases in traditional medicine (Katiyar et al., 2012; Wachtel-Galor and Benzie, 2011).

The South American flora represents one of the world's richest sources of material with pharmacological activity, and Brazil has the biggest vegetal diversity (Dutra et al., 2016). One of them is the "Cerrado" (Brazilian Savannah) covering more than 2 million km<sup>2</sup> and land representing 25% of the area of the country (Sawyer, 2016). This vegetation consists of more than 6,000 vegetal species, having many compounds such as saponins, tannins, steroids, and others (Bessa et al., 2013), with biological activities including antifungal, antibacterial, antiprotozoal and antiviral ones (Alves et al., 2000; Mesquita et al., 2005, 2007; Silva Junior et al., 2009; Brandão et al., 2010).

Many plant extracts have been described for antiviral activity against herpesviruses *in vitro* such as *Conocephalum conicum*, *Polypodium glycyrrhiza* and the water-soluble extract from the narcissus bulb *Flos verbasci*, showed anti-HSV activity. The aqueous extracts of *Helichrysum aureonitens* (Asteraceae) shoots inhibited HSV-1 (Chattopadhyay and Khan, 2008). Compounds in green tea extract could also inhibit HSV-1 and black tea extract enriched with theaflavins has the potential to prevent the spread of HSV-1 (Cantatore et al., 2013). Moreover, *Lithraea molleoides*, *Sebastiania brasiliensis* and *Sebastiania klotzschiana* used against infectious diseases showed antiherpetic activity with no cytotoxicity, as did the aqueous extract of *Beta vulgaris* (Betancour-Galvis et al., 1999). The aqueous extracts from *B. vulgaris* and *S. brasiliensis* also exhibited antiviral activity against swine herpesvirus (Koseki et al., 1990; Simoni et al., 2014) and *Cecropia pachystachya*, *Melochia villosa* and *P. acuminatum* presented the most relevant results against bovine and swine herpesviruses (Simoni et al., 2014).

Simoni et al. (2007) reported on the *in vitro* research for potential antiviral properties of the 16 species collected from the Brazilian Cerrado against bovine herpesvirus (BoHV-1), infectious bursal disease virus (IBDV) and avian reovirus. They found 8 plant extracts active against BoHV-1 including *Byrsonima intermedia*, *Banisteriopsis variabilis*, *Stryphodendron adstringens* and *Xylopia*

*aromatica*.

Species of *Banisteriopsis* are described as having antioxidant and antibacterial properties, while also inhibiting the activity of monoamine oxidases. *Banisteriopsis caapi* is the most studied species of this genus because it is used by some religious groups in Brazil as an ingredient of the drink locally known as *ayahuasca*. Chemical compounds identified in this plant species include alkaloids, harmine and harmaline (Wang et al., 2010; Santos et al., 2017). *B. intermedia* belonging to the Malpighiaceae family popularly known as "murici" has been used in folk medicine to treat fever, as diuretic, for skin infections and ulcers. *B. intermedia* is described mainly as being antimicrobial and as anti-inflammatory (Moreira et al., 2011; Sannomiya et al., 2007). *S. adstringens* is one of the most widely used medicinal species by the Brazilian population. The stem bark is usually used to treat leucorrhea, gonorrhoea, vulvo-vaginal candidiasis, gastritis, sore throat, diarrhea and bleeding (Costa et al., 2010). Extracts from the leaves present trypanocidal activity associated with the chemical compound tannin (Ishida et al., 2009). Extracts from *X. aromatica* have been described for antibacterial, antifungal, insecticidal, antimicrobial, and antiparasitic activities. Many compounds in this genus have been identified, but the triterpenoids are present in higher concentrations (Stashenko et al., 2004; Costa et al., 2013).

The aim of this work was continue the search with these same eight positive plant extracts to evaluate the antiviral activity against equine, swine and human herpesviruses together with additional studies of antiviral and virucidal potential. Additionally, the phytochemical profile of the extract from *B. intermedia*, which displayed higher antiviral activity, was performed by HPLC/MS analysis.

## MATERIALS AND METHODS

### Plant materials

The "Cerrado" plant species *B. variabilis* B. Gates, *B. intermedia* A. Juss., *Campomanesia xanthocarpa* O. Berg, *Erythroxylum deciduum* A. St.-Hil., *Lacistema hasslerianum* Chodat, *Ocotea pulchella* (Nees) Mez, *S. adstringens* (Mart.) Coville, and *X. aromatica* (Lam.) Mart were collected in Mogi-Guaçu (22°18'S and 47°20'W), São Paulo State, Brazil. The specimens were authenticated by a taxonomist in the Herbarium of the Instituto de Botânica, São Paulo, Dr. Eduardo Luis Martins Catharino.

### Preparation of crude extracts

Crude aqueous extracts were prepared by grinding dried leaves with de-ionized distilled water (10%, w/v) in a mixer and maintenance at 4°C overnight. The aqueous extracts were filtered on Whatman-1 filter paper and freeze-dried in Flex-dry MP FTS systems. Lyophilized extracts were dissolved in equal parts of sterile de-ionized distilled water and Eagle minimum essential medium (MEM 2x) at a final concentration of 10,000, 4,000 or 2,000

µg/mL. The extracts were centrifuged at 2,500 g/10 min in a Hettich Zentrifugen model Rotina R and sterilized by filtration (0.22 µm filter).

#### HPLC/MS analysis

Crude aqueous extract from *B. intermedia* was filtered on a Sep-Pak column using MeOH as eluent. Samples containing 1 ml of the crude extract was analyzed by HPLC using a Luna C-18 (Phenomenex) column (5 mm, 250 X 4 mm), with a gradient from MeOH:H<sub>2</sub>O:HCCOH 1% 75:25 (0 min) to MeOH 100% (30 min), flow rate 1.0 ml/min and detection at 254 nm. HPLC/MS (negative form) were acquired on a Bruker micrOTOF-QII coupled to an Apollo ion source set as follows: dry temperature at 180°C and voltage at 4.5 kV. The mass/charge ratios were detected in scan (*m/z* 100–1200 Da) and product ion scan (*m/z* 50–1200 Da) modes, using the same chromatographic method described above.

#### Cell cultures and viruses

The equine herpesvirus 1 (EHV-1), strain A4/72 (Moreira et al., 1998) and the herpes simplex virus 1 (HSV-1), strain KOS (Silva et al., 2010) were propagated in Vero (African Green monkey – ATCC CCL 81). The swine herpesvirus 1 (SuHV-1) strain NP (Nova Prata) (Fonseca et al., 2010) was propagated in MDBK (Mardin and Darby Bovine Kidney – ATCC CCL 22). All cells were grown in MEM with 10% fetal bovine serum (FBS) (Simoni et al, 2007).

#### Cytotoxicity assay

The assays were performed using 96- well microtiter plates with 30,000 cell/well. After 24 h of incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, each cell type was exposed to decreasing concentrations of plant extract in triplicate. Any cell morphology alteration was observed at light microscopy during the next 3 days to determine the maximum non-cytotoxic concentration (MNCC). Monolayers of cells incubated only with MEM were used as a control.

#### Antiviral assay

For the assays, MDBK or Vero cells were prepared in 96-well microplates at density of  $3 \times 10^4$  cells per well and incubated for 24 h at 37°C with 5% CO<sub>2</sub>. After 24 h of incubation, the medium was poured off and 100 µL of extracts at dilutions corresponding at MNCC were added. The cells were incubated for 1 h and after this period, 50 µL of logarithmic dilutions of viruses were inoculated for 96 h. MDBK cells were infected with SuHV-1 and Vero cells were infected with EHV-1 or HSV-1. Controls consisted of untreated infected (virus titer), treated non-infected (extract control), untreated non-infected (cell control) cells. In addition, to verify if the antiviral activity of the extract remained below its MNCC, a dose-response experiment was carried out using it at range of 250 until 1.9 µg/mL.

#### Direct anti-herpes assay

The assay was described previously by Simoni et al. (1996). Briefly, 100 µL of each extract at MNCC was mixed with 100 µL of logarithmic dilutions of each virus, incubated at 37°C in 5% CO<sub>2</sub> for 1 h. Then, 50 µL of mixture was inoculated at the monolayers in each well of a 96-well plate. In addition, to verify if the direct anti-herpes effect of the extract remained below its MNCC, a dose-response experiment was carried out using it at range of 250 until

1.9 µg/mL.

#### MTT assay

The MTT assay was performed to confirm qualitatively the MNCC and the antiviral activity of the extracts that presented PI% more than 97%. Cells in a 96-well microplate were incubated with 100 µL/well of increasing concentrations of crude extracts in quadruplicate with at least 5 different concentrations. Thereafter, 100 TCID of virus was added. After incubation for 72 h, the medium was removed and added on cell monolayer 50 µL of MTT (1 mg/mL) to each well. The microplate was then incubated at 37°C for 4 h. The supernatant was then removed from each well without disturbing the cell clusters containing formazan crystals. To solubilize the formazan crystal, 100 µL of SDS was added and the microplate was incubated again overnight. The absorbance of the wells was read in a computer-controlled microplate reader (Spectra Max Plus 384) at 540 nm wavelengths. The 50% cytotoxic concentration (CC<sub>50</sub>) of the test compound was defined as the concentration that reduced the absorbance of mock-infected cells by 50% of that of the control. The 50% antiviral effective concentration (IC<sub>50</sub>) was expressed as the concentration that achieved 50% protection of virus- infected cells from the HSV-1, EHV-1 and SuHV-1 induced destruction (Takeuchi et al., 1991).

#### Data and statistical analysis

The antiviral and virucidal activities were based on reduction of viral titers using CPE criteria. Values were expressed as titer (TCID 50 µL) (Reed and Muench, 1938) and viral inhibition index (VII) calculated as the difference of virus titer between treated and untreated infected control cultures. The VII was considered positive when  $\geq 1.5$  (Barros et al., 2012).

The 50% cytotoxic (CC<sub>50</sub>) and 50% inhibition (IC<sub>50</sub>) concentrations were calculated from concentration-effect curves obtained from nonlinear regression analysis of concentration-effect curves by the GraphPad Prism 5 Demo program. The results were obtained from triplicate independent assays. The percentage of cytotoxicity was calculated as  $[(A - B)/A] \times 100$ , where A and B are the OD<sub>540</sub> of untreated and of treated cells, respectively. The percentages of protection were calculated as  $[(A - B) \times 100/(C - B)]$ , where A, B and C indicate the absorbance of the extracts/fractions, virus and cell controls, respectively. Each obtained EC<sub>50</sub> value was defined as the effective concentration that reduced the absorbance of infected cells to 50% when compared with cell and virus controls. The selectivity index (SI) was determined by the ratio of CC<sub>50</sub> to EC<sub>50</sub> and expressed as mean  $\pm$  s.e.m. The statistically different effects of tested extracts on the inhibition of virus replication were compared with the control group using the Student's t-test with  $p \leq 0.05$  for significant result.

## RESULTS

### Cytotoxicity

The MNCCs of the extracts were determined in two different cell lines, MDBK and Vero and the concentrations used in bioassays are presented in Table 1. In Vero cells, the CC<sub>50</sub> was lower than that one observed in MDBK cells. *X. aromatica* presented the lowest cytotoxic to Vero and MDBK cells with MNCC of 1250 and 625 µg/mL, respectively. The extract from *O. pulchella* was the most cytotoxic for both cell lines with



**Table 1.** Antiviral activity of extracts at MNCC and CC<sub>50</sub> against HSV-1, EHV-1 and SuHV-1.

Plant extract	MNCC <sup>a</sup> Vero	CC <sub>50</sub> <sup>b</sup>	HSV-1 <sup>c</sup>	EHV-1	MNCC MDBK	CC <sub>50</sub>	SuHV-1
<i>Banisteriopsis variabilis</i>	625	435.8	2.25	3.75	312	>2,000	3.24
<i>Byrsonima intermedia</i>	31.2	141.1	3.25	4.87	250	782.1	5.45
<i>Campomanesia xanthocarpa</i>	62.5	n.t.	0	0.62	250	1,299	2.0
<i>Erythroxylum deciduum</i>	125	n.t.	1.0	0.26	31.2	n.t.	1.16
<i>Lacistema hasslerianum</i>	62.5	n.t.	0.5	0.26	62.5	821	2.0
<i>Ocotea pulchella</i>	31.2	n.t.	0	0.26	31.2	290.9	1.76
<i>Stryphodendron adstringens</i>	625	n.t.	2.75	1.0	62.5	>2,000	2.0
<i>Xylopiya aromatica</i>	1250	203.1	1.5	1.76	625	>2000	3.24

a: MNCC: Maximum non-cytotoxic concentration; HSV-1: Human Herpesvirus type 1; SuHV-1: Swine Herpesvirus type 1; EHV-1: Equid Herpesvirus type 1; b: 50% cytotoxic concentration (µg/mL); c: VII: Viral inhibition index; n.t.: CC<sub>50</sub> not tested.

**Table 2.** Direct anti-herpes effect of extracts at MNCC against herpes simplex virus, equine herpesvirus and swine herpesvirus in cell cultures.

Plant extract	VII <sup>a</sup>	VII	VII
	HSV-1 <sup>b</sup>	EHV-1 <sup>c</sup>	SuHV-1 <sup>d</sup>
<i>Banisteriopsis variabilis</i>	3.0	3.67	3.34
<i>Byrsonima intermedia</i>	4.5	4.0	5.22
<i>Stryphodendron adstringens</i>	n.t.	n.t.	0.75
<i>Xylopiya aromatica</i>	1.5	0.84	1.67

a: VII: Viral inhibition index; b: Human Herpesvirus type 1; c: Equid Herpesvirus type 1; d: Swine Herpesvirus type 1; n.t.: Not tested. MNCC: Maximum non-cytotoxic concentration.

MNCC of 31.2 µg/mL. The other extracts showed MNCC ranging between these values for both cells.

for SuHV-1 and HSV-1, while the extracts from *S. adstringens* had action only on HSV-1.

### Antiviral activity

Results are presented in Table 1. The extracts from *B. variabilis*, *B. intermedia*, *S. adstringens* and *X. aromatica* presented VII greater than or equal to 1.5 for HSV-1. The extracts of *B. variabilis*, *B. intermedia* and *X. aromatica* were positive for EHV-1 while the extracts *B. variabilis*, *B. intermedia*, *C. xanthocarpa*, *L. hasslerianum*, *O. pulchella*, *S. adstringens* and *X. aromatica* were effective for SuHV-1.

### Direct anti-herpes effect

Assays to study the direct anti-herpes effect were done only with the extracts that were effective for at least two of the viruses tested in the antiviral assays. These results are presented in Table 2.

The extracts of *B. variabilis* and *B. intermedia*, presented an inhibition against the three viruses with VII greater or equal to 3.0. *X. aromatica* was only effective

### Additional studies with *Byrsonima intermedia*

*B. intermedia* was selected for additional studies and to characterize its antiviral properties because it showed the highest values of VII against all herpesviruses.

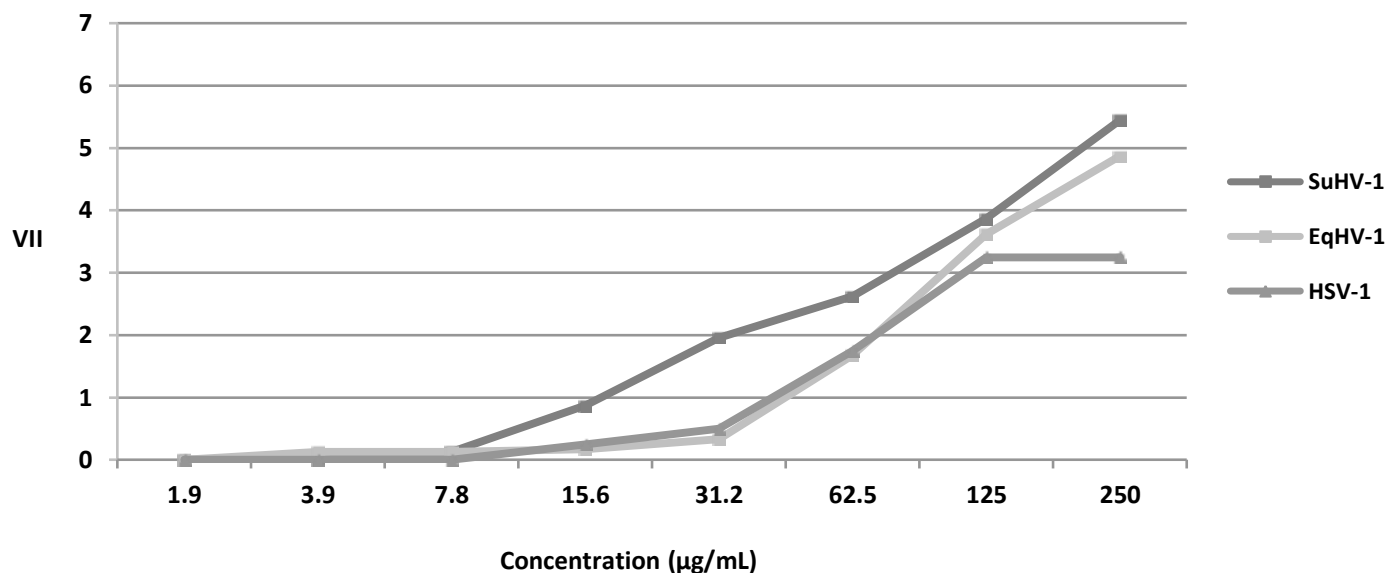
Table 3 shows the results of the cytotoxic, antiviral activities and selectivity index (SI) obtained from *B. intermedia* extracts. The MNCC of *B. intermedia* extracts that did not cause alterations in the morphology of the cells visible under the optical microscope (Table 2) were lower than that obtained with CC<sub>50</sub> of 141.1 for Vero cells and 782.1 for MDBK cells. *B. intermedia* presented SI values of 41.76 ± 0.04; 4.12 ± 0.1 and 193.97 ± 0.09 respectively against HSV-1, EHV-1 and SuHV-1.

Figure 1 shows the antiviral activity of extract from *B. intermedia* at 8 set concentrations against the three viruses showing that treatment with *B. intermedia* extract resulted in reduced viral titers in dose-response curves. At 15.6 µg/mL, the initial reduction occurred firstly with SuHV-1 and then with EHV-1 and HSV-1 at 62.5 µg/mL. At 250 µg/mL, the greater VII was also obtained with

**Table 3.** Selectivity index, cytotoxic and antiviral activities from *B. intermedia* aqueous extract against swine herpesvirus, equine herpesvirus and herpes simplex virus by the MTT assay.

Plant extract	Vero	HSV-1	SI <sup>c</sup>	EqHV-1	SI	MDBK	SuHV-1	
	CC <sub>50</sub> <sup>a</sup>	IC <sub>50</sub> <sup>b</sup>		IC <sub>50</sub>		CC <sub>50</sub>		IC <sub>50</sub>
<i>Byrsonima intermedia</i>	141.1	3.37	41.76 ± 0.04	34.24	4.12 ± 0.1	782.1	4.03	193.97 ± 0.09

a: 50% cytotoxic concentration (µg/mL); b: 50% inhibitory concentration (µg/mL); c: SI - Selectivity Index - ratio of CC<sub>50</sub> to IC<sub>50</sub>. SuHV-1: Swine Herpesvirus type 1; EHV-1: Equid Herpesvirus type 1; HSV-1: Human Herpesvirus type 1.

**Figure 1.** Antiviral activity of different concentration of extract from *B. intermedia* against Swine Herpesvirus type 1 (SuHV-1); Equine Herpesvirus type 1 (EHV-1); Human Herpesvirus type 1 (HSV-1); VII: Viral Inhibition Index.

SuHV-1 followed by EHV-1 and HSV.

Likewise, Figure 2 shows the direct anti-herpes effect against SuHV-1, EHV-1 and HSV-1 showing that treatment of virions with this various concentrations of extracts for one hour resulted in reduced viral titers. The extracts were active at the concentrations range 250 to 31.2 µg/mL. At 3.9 µg/mL, the reduction occurred firstly with SuHV-1 and then with EHV-1 and HSV-1 at 7.8 µg/mL. At 250 µg/mL, the greater VII was obtained for SuHV-1 and EHV-1 followed by HSV.

#### Phytochemical profile of *Byrsonima intermedia*

As follow-up, the *B. intermedia* extract was subjected in the current study to HPLC/MS analysis (Figure 3). The peak at  $R_t = 10.2$  min corresponds to a  $[M - H]^-$  quasi-molecular ion peak at  $m/z$  169, suggesting the occurrence of gallic acid (A). The second peak, detected at  $R_t = 13.6$  min corresponds to a  $[M - H]^-$  quasi-molecular ion peak at  $m/z$  301, suggesting the presence of flavonoid quercetin (B).

## DISCUSSION

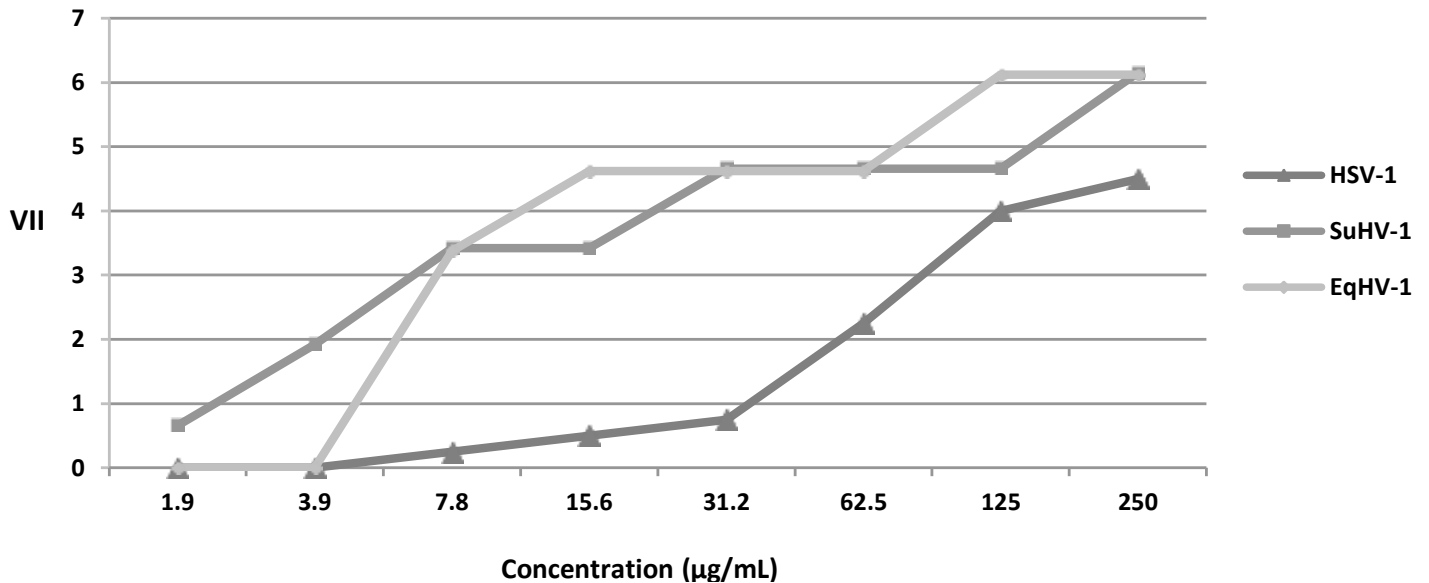
### Cytotoxicity

The cytotoxic assay at the first stage of screening is very important to determine the maximum concentration of the aqueous extract that did not induce changes in cell morphology. This step is critical because the virus is an intracellular parasite and uses the cell machinery to replicate, so it must be ensured that the virus have the ideal conditions for their growth (Cos et al., 2006).

The study of cytotoxicity in different cell lines is important because one of the inherent drawbacks of *in vitro* antiviral testing is the environmental sensitivity of animal cells in culture, although the *in vitro* methodology is faster and less costly (McCutcheon et al., 1995).

### Antiviral activity

Brazilian Cerrado plants may lead to the study of a broad source of new natural compounds; however, most are



**Figure 2.** Direct anti-herpes effect of different concentrations of extract from *B. intermedia* against Swine Herpesvirus type 1 (SuHV-1); Equine Herpesvirus type 1 (EHV-1); Human Herpesvirus type 1 (HSV-1); VII: Viral Inhibition Index.

being developed for use as antibiotics more than antiviral drugs. This is because the viruses present certain characteristics among others of replicating within the cell and persisting in the host. Their combat must be more rational by the choice of species of plants that have already been used by the population to treat the diseases.

Many Brazilian Cerrado species have already been described as having antiviral activity. Chattopadhyay et al. (2015) showed that *Byrsonima verbascifolia* had strong anti-HSV activities, whereas Maldini et al. (2011) found that *B. crassifolia* used as anti-inflammatory has been described as having compounds that also display antiviral activity against HSV-1 and HIV.

In the present study, eight extracts reduced at least one of the viral titers, as compared to untreated control cells. *B. variabilis*, *B. intermedia* and *X. aromatica* species were active against all these herpesviruses. Furthermore, a strong inhibition was obtained from *B. variabilis* and *B. intermedia*. *S. adstringens* extract also showed an effective inhibition against SuHV-1 and HSV-1. Extract from *X. aromatica* presented the highest values of VII against SuHV-1. Antiviral activity was also observed for extracts from *C. xanthocarpa* and *O. pulchella* only against SuHV-1.

Frias et al. (2012) isolated from methanolic extract of *B. variabilis*, the flavonoids quercetin, rutin, and apigenin. Quercetin is found in a wide variety of plants and studies showed that it could reduce infectivity of target cells and replication against herpesviruses. The results of our work indicated that the extracts of *B. variabilis* showed inhibitory effect against the three herpesviruses and probably this inhibitory effect is due to presence of

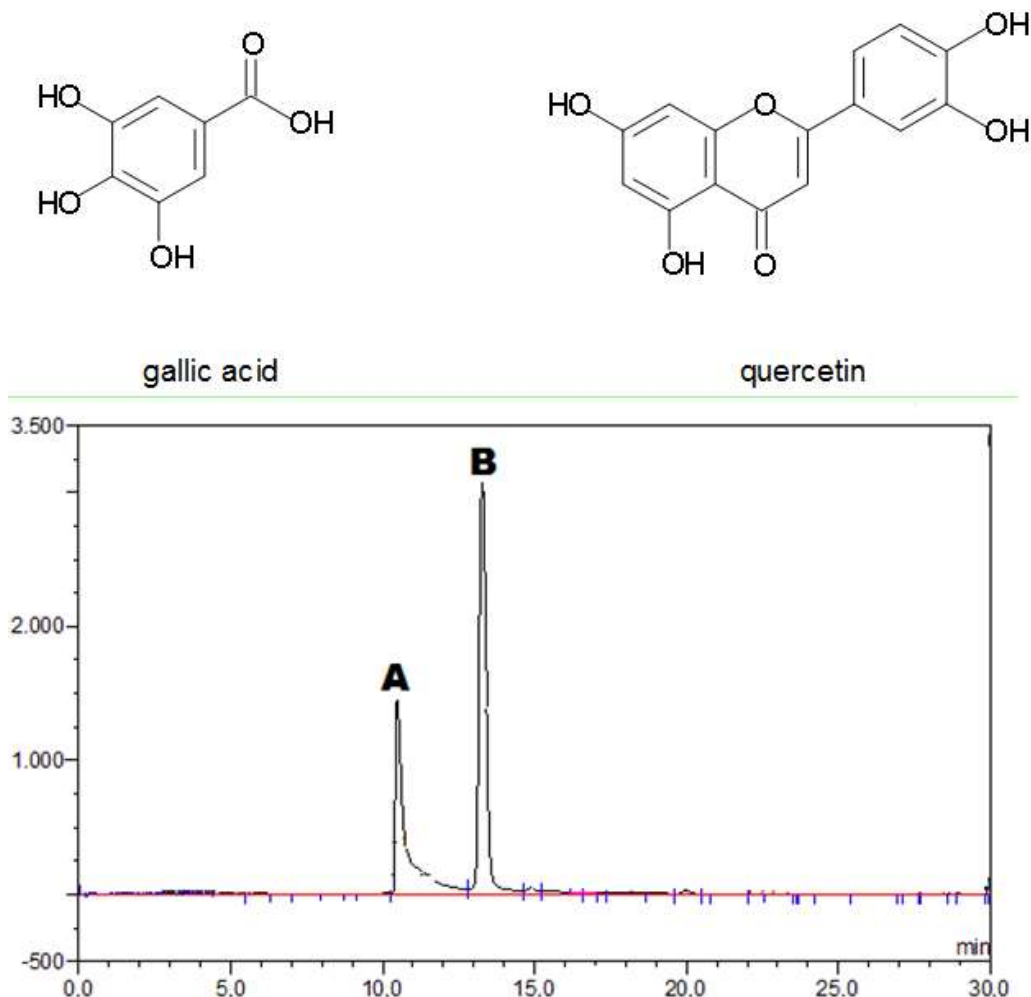
these flavonoids.

Felipe et al. (2006) described the inhibition BoHV-1 replication of aqueous extracts from *S. adstringens* stem bark. Simoni et al. (2007) showed that the leaves presented moderate activity against BoHV-1. In the present work, leaves extracts from *S. adstringens* also presented moderate activity against EHV-1 but strong inhibition against HSV-1 and SuHV-1.

Antiviral drugs have been reported for herpetic infections for both human and animal herpesviruses. Although the regular use of vaccines helps in improvement of animal herds and avoids viral shedding, treatment of herpesvirus infections is not completely prevented by immunization (Gulati et al., 2016; Maxwell, 2017). Thus, antiviral agents can be useful not only as preventives but also as therapeutic in herpesvirus infections which leads to a great interest in discovering new effective and safe drugs. The results of this present work suggest the importance of searching for new compounds for veterinary and human medicine in Brazilian Cerrado plants because they have shown potential for developing new antivirals.

Several plant extracts with promising results have been described for their antiviral potential against diverse herpes viruses. For example, *Cardamine angulata*, *Conocephalum conicum* and *Polypodium glycyrrhiza* showed activity against bovine herpesvirus and HSV-1 (McCutcheon et al., 1995). Aqueous extract from *Guettarda angelica* seeds exhibited strong inhibition for three animal herpesviruses including bovine, swine and equine herpesviruses 1 (Barros et al., 2012).

The present results were promising especially for *B. intermedia* and indicating that materials derived from their



**Figure 3.** HPLC analysis of crude aqueous extract from *B. intermedia* leaves and identified compounds gallic acid (A) and quercetin (B).

leaves exhibited antiviral activity and selectivity toward three herpesviruses HSV-1, EqHV-1 and SuHV-1. In 2007, Simoni et al. (2007) described the antiviral activity of the *B. intermedia* against bovine herpesvirus (BoHV-1) and against avian reovirus indicating a broad spectrum of action of this plant species.

#### Direct anti-herpes effect

To verify if the mechanism of action of extracts is also due to inhibition on the viruses in extracellular conditions, the selected extracts showing antiviral activity were studied.

Many plants may exhibit different mechanisms of viral inhibition acting on more than one target during viral replication. The *Opuntia streptacantha* inhibited virus replication and inactivated extracellular virus, such as HSV, equine herpes virus, pseudorabies virus (Jassim

and Naji, 2003). *Bidens pilosa* extract was effective against HSV-1 and HSV-2 with potent virucidal activity (Vadlapudi et al., 2013). Virucidal activity has also been described in crude hydroethanolic extract from the stem bark of *S. terebinthifolia* and was effective against HVS-1 in the attachment and penetration stages (Nocchi et al., 2016). Moreover, hot water extract of *B. pilosa* showed potent inhibitory activity against HSV-1 and HSV-2 in Vero cells. The authors suggest multiple targets of activity because this extract showed virucidal activity to block the binding of virus to host cells and viral cell penetration. The *B. pilosa* extract also inhibits virus adsorption to cells and affects some intracellular steps of viral replication (Nakama et al., 2012). These findings are in agreement with those observed in this study.

#### Additional studies with *Byrsonima intermedia*

The extracts from *B. intermedia* were tested in their

maximum non-toxic concentrations in which no alteration of normal cell morphology was observed, and the CC<sub>50</sub> for both MDBK and Vero cells was obtained. Therefore, the extract showed no toxicity even when used at high concentrations in the reduction of viral titers assay and direct anti-herpes assay. Further, *B. intermedia* extracts showed anti-herpes activity in a concentration-dependent manner in both assays, suggesting that this effect may have occurred either acting directly on the viral particle or in other stages of the replicative cycle.

Additional studies should be done to study the mechanism of action of extracts of *B. intermedia* against those herpesviruses indicating at which stage the extract has activity against viruses.

### Phytochemical profile

Using dereplication procedures by HPLC/MS, the occurrence of gallic acid and quercetin in the active extracts from *B. intermedia* was identified. As previously reported, these compounds were detected in the MeOH leaves extract from *B. intermedia* which displayed mutagenic activity (Sannomiya et al., 2007).

Gallic acid is a phenolic compound present in many plants, fruits and vegetables. Studies have demonstrated that gallic acid exhibits a strong inhibitory effect against several viruses such as the herpes simplex virus, hepatitis C virus, HIV and enterovirus 71. Therefore, the potential activity observed by GA suggests that this activity is due to the hydrophobic interaction between the functional group, (hydroxyl) and virion components of enveloped herpesvirus. In addition, gallic acid showed an inhibitory effect in a time-dependent manner, notably similar to the current study (Govea-Salas et al., 2016). Quercetin, a flavonoid found in several plant species, including fruits and edible vegetables, displayed several biological potentials (Sharma et al., 2018), including antiviral against HSV-1 (Garrett et al., 2012). Additionally, Choi et al. (2009) described that quercetin-3-rhamnoside from *Houttynia cordata* extract inhibit influenza A replication in the initial stage of virus infection by indirect interaction with virus particles.

New studies are needed to bring the active substances present in *B. intermedia* extract and to identify in detail the ways in which they act. The other extracts that can be considered promising must also pass through the processes of fractionation and isolation of substances in order to verify the potential of this activity. All of them here have shown their potential to lead to the development of an alternative therapy for the treatment of such important infections worldwide.

### Conclusion

This study has demonstrated a broad antiviral activity of Brazilian Cerrado plants and indicated that they can be

effective potential candidates for the development of new strategies to treat viral infections. *B. intermedia* was chosen for further research as well as gallic acid and quercetin all necessary to elucidate the mechanism of action of the extract or compounds both on herpesviruses replication and on direct antiviral effect.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

## Pharmaceutical topical gel containing proanthocyanidin polymers-rich fraction from *Stryphnodendron adstringens*

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The stem bark of *Stryphnodendron adstringens*, popularly known in Brazil as "barbatimão", has many biological activities, including antifungal activity. Considering the increasing interest of using "barbatimão" extract in the treatment of vaginal candidiasis, the aim of this study was to propose a pharmaceutical topical gel containing a proanthocyanidin polymers-rich fraction for use as a pharmacological agent in vaginal gels, as well as to evaluate the validation parameters for the determination of phenolic compounds. UV/Vis spectrophotometry was used as a quantitative method for quality control of topical gel and a proanthocyanidin polymers-rich fraction was used in this work. The proposed gel seems suitable for use in vaginal infections, and the analytical method was linear, specific, precise, accurate, reproducible and robust. This methodology complies with analytical application demands and it is easily performed in work routine.

**Key words:** Barbatimão, polyphenols, *Stryphnodendron adstringens*, UV/Vis spectrophotometry, vaginal gel, validation method.

### INTRODUCTION

The genus *Stryphnodendron* belongs to the *Leguminosae* family, which has about 48 species, all rich in tannin and native to the savannah in tropical and subtropical climates. Among them is *Stryphnodendron adstringens* (Mart.) Coville. The content of tannins in the bark of *S. adstringens* reaches a minimum of 8% according to the Brazilian Pharmacopeia (Albuquerque et al., 2007; ANVISA, 2010).

The stem bark of *S. adstringens*, popularly known in Brazil as "barbatimão", is employed in popular culture as an anti-inflammatory, antibacterial and antiulcer treatment (Agra et al., 2008; Albuquerque et al., 2007). The use of *S. adstringens* is well documented in the literature as demonstrating anti-inflammatory, antibacterial, healing, antiviral, antiulcer, antitrypanosomal, antileishmanial and antifungal activities (Audi et al., 2004; Felipe et al., 2006;

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Glehn and Rodrigues, 2012; Hernandez et al., 2010; Herzog-soares and Alves, 2002; Ishida et al., 2009; Lanchoti Fiori et al., 2013; Luize et al., 2005; Morey et al., 2016; Pinto et al., 2015). In addition, in Brazil there is a commercialized pharmaceutical ointment named Fitoscar<sup>®</sup> containing *S. adstringens* extract for wound-healing. Phytochemical studies performed with *S. adstringens* showed that it is composed of proanthocyanidin polymers, mainly several flavan-3-ols derivatives, such as prodelphinidins and prorobinetinidins (Lopes et al., 2008; Mello et al., 1999, 1996).

Additionally, other species from the genus *Stryphnodendron* have shown promise for the treatment of some pathologies. *S. polyphyllum* showed potential molluscicidal activity (Bezerra et al., 2002), *S. obovatum* showed potential antileishmanial activity (Ribeiro et al., 2015) and both showed good activity for wound healing, antibacterial and antioxidant potential (Lopes et al., 2005). *S. rotundifolium* has been ethnopharmacologically very important and used for inflammatory and infectious diseases, gastroprotection and pain complaints (Oliveira et al., 2014); its laboratorial evaluation has shown several activities for the extract of its leaves, such as antiulcer (Awaad et al., 2013; Lopes et al., 2008), extract of bark showed a leishmanicidal and trypanocidal activity and gastroprotection (Oliveira et al., 2018; Vandesmet et al., 2017). The effect of plants from the genus *Stryphnodendron* may be related with some substances of the class of tannins.

Vulvovaginal candidiasis is one of the most frequent mycoses seen in daily practice in gynecology. This fungus affects approximately 70% of women and ranks second among the causes of vaginitis. Despite its clinical importance, no ideal medication for this disease exists. Various imidazole derivatives have achieved higher cure rates than polyenics (85-90%), but with similar side effects. Besides, reports have shown the incident of strains with decreased sensitivity or resistant to some antifungal agents (Fidel, 2007; Odds et al., 2003; Ostrosky-Zeichner, 2008; Sanglard, 2016).

Considering the increasing interest in vaginal candidiasis treatment, we propose a pharmaceutical topical gel for vaginal use containing a proanthocyanidin polymers-rich fraction from *S. adstringens* (F2). Previous reports demonstrated the antifungal activity of F2 against planktonic cells (in suspension) from vaginal isolates of *Candida* spp., and F2 also altered some virulence factors of *C. albicans* as well as led to alterations in budding and cell wall morphology (Ishida et al., 2006). Treatment with F2 reduced biofilm metabolic activity (in sessile and in dispersed cells) during biofilm formation, and in mature biofilms it reduced biofilm biomass during biofilm formation and led to the appearance of dumbbell-shaped blastoconidia and blastoconidia clusters in biofilms (Freitas et al., 2018; Luiz et al., 2015). In addition, toxicological studies using rodents also reported low side effects after oral treatment with F2 (Costa et al., 2013,

2010).

Several methods were already shown for quantitative analyses of polyphenols in *S. adstringens* and its herbal preparations (ANVISA, 2010; Isler et al., 2010; Nascimento et al., 2013). Currently, there is a tendency to prefer HPLC methods for quality control, probably assuming that they are reproducible, can identify and quantify single substances and provide a suitable chromatographic fingerprint. But, the current HPLC methods for herbal drugs with polyphenols are limited to detection of, besides gallic acid, only monomers, such as gallocatechin, epigallocatechin, and epigallocatechin gallate, or dimers whose identification and quantity are difficult due to the absence of reference substances. The UV/Vis spectrophotometric method by colorimetric reaction using Folin-Ciocalteu reagent is a classical method used to determine the content of polyphenols in herbal drugs, including for *S. adstringens* (ANVISA, 2010; Schofield et al., 2001). This method is not limited to some substances but measures the concentration, which is directly proportional to the total phenolic hydroxyl groups, therewith including polymers of high molecular weight (Schofield et al., 2001). Chemical characterization of F2 and its subfractions by mass spectrometry ES-MS and <sup>13</sup>C NMR spectroscopy showed the presence of proanthocyanidin polymers (a hexameric compound), composed of prodelphinidin and prorobinetinidin units and gallic acid residues, with an average molecular weight of 2,114 (Ishida et al., 2009, 2006).

In this context, the aim of this study was to validate a method of quality control for quantitative determination of phenolic compounds in a proanthocyanidin polymers-rich fraction (F2) from the stem bark of *S. adstringens*, as well as in a topical vaginal gel (TG), by spectrophotometric method in the ultraviolet region.

## MATERIALS AND METHODS

### Plant material

Stem bark was collected in São Jerônimo da Serra, Paraná, Brazil (S23° 43' 7.8", W50° 45' 23.5"; altitude 926 m), in March 2008. A voucher herbarium specimen was deposited under number HUM 14321 at the Universidade Estadual de Maringá, and was identified by Prof. Dr. Cássia Mônica Sakuragui, Universidade Federal do Rio de Janeiro. Stem bark was dried at room temperature and then pulverized (Tigre ASN-5).

### Preparation of extracts

The crude extract was obtained by turbo-extraction (Skymssen) of 1,000 g of bark with 70% acetone in water for 15 min and temperature under 40°C. The organic solvent was eliminated using a rotavapor under reduced pressure, and the residue was lyophilized to yield a crude extract (F1; 300 g). Next, the F1 (50 g) was suspended in water (500 mL) and partitioned with ethyl acetate (500 mL; 1:1) to obtain a proanthocyanidin polymers-rich fraction (lyophilized water fraction; F2; 35 g).

### Preparation of pharmaceutical topical gel and quality control

The composition of the TG is given in Table 1. The gel was prepared by dispersing the gel-forming material in sterile distilled water. Methylparaben was added as a preservative, and sodium carbonate was added as a neutralizer with gentle agitation to avoid the inclusion of air, until the gel acquired the intended consistency and transparency. Afterwards, F2 was incorporated at 0.2% with mixing until the desired homogenate was obtained. The pH value was measured. The TG was transferred into polyethylene tubes to a total amount of 50 g under laminar flow conditions.

### Centrifugation test

A sample of 5 g of TG was submitted to centrifugation under the following conditions:  $25 \pm 2^\circ\text{C}$ , 3,000 rpm and 30 min. Afterwards, the sample was immediately evaluated for the presence of any instability signals.

### Preliminary stability study

Tubes containing TG were stored in climate chambers under the following conditions:  $30^\circ\text{C}/75\%$  relative humidity (RH) and  $40^\circ\text{C}/75\%$  RH for 3 months. We evaluated possible changes regarding the organoleptic characteristics, pH value, total polyphenols content and antifungal potential.

### Antifungal potential by determination of minimum inhibitory concentration (MIC)

Strains of *Candida albicans* (ATCC 10230) were provided by the Oswaldo Cruz Institute (Rio de Janeiro–RJ, Brazil) and stored in water suspension at room temperature. MIC determination was performed as described in document M27-A3. Briefly, serial dilutions of TG in RPMI 1640 medium without sodium bicarbonate (Sigma Chemical Co., MO, USA) buffered with 0.165 M MOPS (Sigma Chemical Co., MO, USA) were made in 96-well microtiter trays, to obtain equivalent concentrations of F2 from 10 to 5,000  $\mu\text{g}/\text{mL}$ . A suspension of *C. albicans* of  $1.5 \times 10^6$  cfu/mL was prepared, diluted 1:1000 and 100  $\mu\text{L}$  was dispensed into each well containing 100  $\mu\text{L}$  of medium to obtain a final concentration of  $0.5\text{--}2.5 \times 10^3$  cfu/mL. The microtiter trays were incubated at  $35^\circ\text{C}$  for 48 h in a humidity chamber. The MIC values were considered to be the lowest concentration that visibly inhibited *Candida* spp. growth. The F2 was used as positive control and was prepared under the same conditions as TG. The experiment was performed in triplicate in different days.

### Total polyphenols content of the proanthocyanidin polymers-rich fraction of *S. adstringens* (F2) and topical gel containing this fraction (TG)

All dilution operations were protected from light.

**Stock solution:** 15 mg of F2 was diluted with 25 mL of water in a volumetric flask; 5 mL of this solution was transferred to another 25 mL volumetric flask and diluted with water (F2 stock solution); 1.5 g of TG was diluted with 25 mL of water in a volumetric flask (TG stock solution).

**Total polyphenols (TP) solution:** The stock solution of F2 or TG (2 mL) was transferred to a 25 mL volumetric flask and mixed with 1 mL of 2 N Folin-Ciocalteu reagent plus 10 mL of water and

volumetrically diluted to 25 mL with a 10.75% w/v solution of anhydrous sodium carbonate. After 30 min, the absorbance was measured at 760 nm using water as the compensation liquid and a quartz cell (1 cm path length) in a UV/Vis spectrophotometer (Shimadzu PC-1650).

**Calibration curve:** In three replicates, 50.0 mg of pyrogallol was dissolved in water immediately before use and diluted to 25 mL. Aliquots of 1.0, 1.75, 2.5, 3.25 or 4.0 mL were diluted to 25 mL in water, and aliquots of 5 mL from these solutions were diluted to 25 mL (stock solution). Each stock solution (2 mL) was prepared according to the procedure described above for TP solution and the absorbance was measured under the same conditions. The final concentrations were 1.28, 2.24, 3.20, 4.16 and 5.12  $\mu\text{g}/\text{mL}$  of pyrogallol and the specific absorptivity of pyrogallol was determined from the linear equation curve (concentration vs absorbance).

The percentage of total polyphenols expressed as pyrogallol in F2 and TG was calculated, respectively, by the equations:

$$TP_{F2} = \frac{1562.5.A}{A^{1\%}.m}$$

$$TP_{TG} = \frac{312.5.A}{A^{1\%}.m}$$

Where A = absorbance of the sample for TP solution;  $A^{1\%}$  = specific absorptivity of pyrogallol; m = mass of the sample examined, in grams; 1562.5 and 312.5 = dilution factors for the samples F2 and TG, respectively.

### Analytical method validation

For validation of the analytical method, the guidelines established by the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005) and by Brazilian regulation RE n° 899/2003 of the National Health Surveillance Agency (ANVISA, 2003) were employed.

### Linearity

To establish linearity of the proposed method, five stock solutions were prepared in three replicates from 5.0, 10.0, 15.0, 20.0 or 25.0 mg of F2. These stock solutions were used to prepare the TP solutions at the concentrations: 3.2, 6.4, 9.6 and 12.8  $\mu\text{g}/\text{mL}$ , and their absorbance was measured. TG was prepared with five different concentrations of F2: 0.1, 0.15, 0.2, 0.3 and 0.35%. Then, 1.5 g of each gel, in three replicates, was used to prepare the stock solutions and TP solution for determining the absorbance.

### Specificity

Specificity was determined by adding 1 mL of pyrogallol solution (1.0 mg/mL) to each stock solution of F2 described in the linearity test or 1 mL of pyrogallol solution (0.2 mg/mL) to each stock solution of TG described in the linearity test. The method is considered specific if the slopes of the linear equation in tests for linearity and specificity are equal or very similar. Additionally, TG prepared in three replicate samples with placebo (gel base) to confirm no interference of other compounds from formulation in absorbance at 760 nm.

### Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were

**Table 1.** Composition of topical gel.

Gel base	
Ingredient	Quantity
Carbopol-940®	1.0%
Methylparaben	0.2%
Sodium carbonate	q.s.
Purified water	100 g

determined from the curves of linearity of F2 and TG. The LOD was established by using the expression  $3\sigma/S$  and LOQ by expression  $10\sigma/S$ , where  $\sigma$  is the standard deviation of the response and S is the slope of the linear equation.

### Precision

Precision was evaluated on two levels: repeatability (intra-day) and intermediate (inter-day). The repeatability was assessed using six samples of 15.0 mg of F2 or 1.5 g of 0.2% TG, and intermediate level was considered as variation between days of at least 2 days, in six replicates. A coefficient of variation over 10% and a significant difference between days were considered unacceptable for the complex matrix.

### Accuracy

The accuracy of the method was established based on the recovery tests and different concentration levels were evaluated: lower concentration (LC), intermediate concentration (IC) and higher concentration (HC). There was addition of 1, 2 or 3 mL of pyrogallol solution (1.0 mg/mL) to the F2 stock solution, in three replicates, or addition of 1, 2 or 3 mL of F2 aqueous solution (0.72 mg/mL) to TG stock solution, in three replicates. Theoretical absorbance was calculated for the sum of the absorbance from samples in the repeatability test and expected absorbance of pyrogallol by calibration curve in each level or expected absorbance of F2 by linearity curve. The measured value was compared with the theoretical value. The accuracy was assessed as the recovery percentage and the method was considered accurate if the recovery percentages were between 85 and 115%.

### Robustness

Robustness was demonstrated for F2 by analyzing the influence of natural light (operations without light protection), and changing of anhydrous sodium carbonate solutions to 7.50% and 14.06% (w/v); it was evaluated in three replicates. For TG, the absorbance of samples were evaluated at different times (25 and 35 min) and wavelengths (755 and 765 nm), in three replicates.

### Statistical analysis

Data were analyzed with Statistica® 8.0 program (Copyright StatSoft, Inc., 1984-2007) by one-way analysis of variance (ANOVA) followed by Tukey's test, as well as by T-test for two groups, considering  $P < 0.05$  as significant. The data were expressed as mean  $\pm$  standard deviation [relative standard deviations (%)]. Linear correlation tests and residual analyses were

performed by simple linear regression, considering  $R^2$  equal or higher than 0.99, and the residual sum of squares was evaluated.

## RESULTS AND DISCUSSION

Among the several pharmaceutical forms of topical application for the vaginal canal, topical gel is promising because it can adhere to the vaginal surface for a reasonable time and releases drug faster than cream or ointment if there is no interaction between drug and polymers. Besides, hydrogels, such as gels from Carbopol, showed special advances due to their elastic consistency, which reduces the friction between the pharmaceutical form and physiological tissue (Johal et al., 2016).

The physical stability of gels indicates the absence of chemical integration between the polymer and drugs. The centrifugation test is considered as a screen test for the development of gels, since instability signals indicate increased particle mobility and the need of a new formulation (Dantas et al., 2016). According to our evaluation, TG remained translucent and without precipitates or lumps after centrifugation. Other polymers (sodium carboxymethyl cellulose (Na CMC), hydroxyethyl cellulose – Natrosol and hydroxypropylmethylcellulose – (HPMC) were tested for formation of gel and incorporation of F2. Natrosol and HPMC were incompatible, showing precipitates/lumps. The gel from Na CMC showed viable characteristics and could be a potential polymer for this formulation. Both Carbopol 940 and Na CMC are anionic polymers and there is the probability that there might be repulsion between these polymers and the proanthocyanidins, which have many hydroxyl groups that reduce the possibility of involving the active substance by this formulation (Tatavarti et al., 2004).

The preliminary stability study is important in development phases to anticipate possible problems with stability, therewith saving time in an advanced phase as opposed to industrial development. The organoleptic characteristics assist in evaluating the acceptance parameters of the product by consumers, as well as permit realization of physical stability problems such as lumps, precipitation and turbidity (Chang et al., 2013). According to our observation, TG showed no change in

physical parameters and maintained homogeneity in both storage conditions. However, there was a slight darkening and odor change at 40 °C/75% RH, as well as reduced consistency and shine. Besides, the pH value was 6 at the initial time and was constant after storage in both conditions. The female genital tract can show pH from slightly basic to moderately acidic, and it is important that a topical formulation maintain its pH near physiological values to prevent discomfort in patients. Besides, the change of pH in a pharmaceutical formulation can show some degradation reactions, compounds interactions, microbial contamination, viscosity alteration and others (Cook and Brown, 2018). The TP content in TG was smaller than 5% during the storage time in both conditions. These data comply with the one acceptable for assays in herbal preparations, but special attention should be given to this parameter in future studies. Other factors, besides the formulation, could affect the stability of chemical marker, for example the package material and its isolation. Finally, the MIC of TG was equivalent to 31.25 µg/mL of F2; the same value was determined for positive control (pure F2) and was constant during the study. The determination of microbiological potential in an antimicrobial preparation shows that the chosen formula will not negatively affect the efficacy of pharmaceutical agents by compounds interactions or use of wrong preserving agents.

The data on pharmacotechnical development and from the preliminary stability study showed the formulation was suitable for using the proposed gel in treatment of vaginal infections.

The analytical conditions for UV/Vis spectrophotometric method were evaluated as described by Blainski et al. (2013) and Bueno et al. (2012), and were confirmed with the use of pyrogallol, for 30 min and 760 nm, respectively; reference substance, time reaction and wavelength, as described in material and methods.

The calibration equation was  $y = 0.141x + 0.0044$  ( $n = 5$ ,  $R^2 = 0.996$ ) for pyrogallol. Based on statistical analysis of results of the calibration curve of pyrogallol, the points fall near the line, showing a normal distribution for the samples and observing that the residues are distributed randomly around the mean zero. The method satisfies the conditions statistics, showing that the linear model does not show error for lack of fit (sum of pure error bigger than error for lack of fit). Furthermore, variance analysis showed that the regression is significant and lower values of parameters like standard error (SE) of slope and intercept indicated high precision of the proposed methods. Goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated  $F$ -values (Table 2).

Spectrophotometric methods for natural products are normally performed using the linearity test for the sample in order to posteriorly analyze the specificity of the method, as well as the LOD and LOQ. In this case, the specificity was evaluated by the absence of matrix

effects, considering that natural products are complex matrices in which it is not possible to obtain a pure analyte. The absence of matrix effects is verified if the curves of linearity and specificity are parallel, in other words, if both curves show the same or very similar slopes (Blainski et al., 2013; Bueno et al., 2012; Ribani et al., 2004). The data obtained in the linearity and specificity tests for F2 and TG appear in Table 2 and are represented in Figure 1. The linear regression analysis of all four curves showed satisfactory results for the statistical conditions and were similar to the discussion of the calibration curve. The specificity of the method for F2 and TG was confirmed because the slopes of linear equation were, respectively, equal ( $= 0.046$ , for linearity and specificity) and very similar ( $= 1.846$  for linearity;  $1.821$  for specificity; difference smaller than 1.4%).

Additionally, the determination of TP in TG verified there was no interference from formulation compositions. The placebo absorbance [ $0.0250 \pm 0.0006$  (2.28%)] was below the limit of detection, probably due to noise of equipment.

Based on the data from linear regression of the linearity test for F2, the LOD and LOQ were 0.702 and 2.341 µg/mL in TP solution, respectively. These concentrations are equivalent to the absorbance of 0.075 and 0.150 uA, respectively. In the same way, the LOD and LOQ for TG were 0.02 and 0.06% of F2 in TG, respectively. These concentrations are equivalent to the absorbance of 0.076 and 0.147 uA, respectively. It is important to consider that the experimental determinations may be affected by several factors, such as equipment noise, human manipulation and laboratorial conditions. Besides, according to the law of Lambert-Beer there is no proportionality between concentration and absorbance after a certain concentration (Vogel, 2002). Then, despite the LOQ results it is recommended that the range between 0.2 and 0.8 uA be used in spectrophotometric analyses.

The repeatability and intermediate precision for F2 shows  $0.494 \pm 0.018$  [3.6%] and  $0.500 \pm 0.011$  [2.1%], respectively, and there was no significant difference between them ( $t_{1,10} = -0.74$ ,  $P = 0.26$ ). For the TG, the repeatability and intermediate precision shows  $0.440 \pm 0.010$  [2.3%] and  $0.464 \pm 0.007$  [1.6%], respectively, and also no significant difference between them ( $t_{1,10} = -5.28$ ,  $P = 0.47$ ). Thus, the proposed methods for F2 and TG have precision for determination of TP.

The results for the accuracy test (Table 3) showed a total recovery of 98.4 and 85.8% for F2 and TG, respectively, with all levels (LC, IC and HC) between 85 to 115%. These results indicate that the method of TP determination has good accuracy for F2 and acceptable accuracy for TG.

For robustness test, the method was insensitive to tested changes for F2 and TG. For F2, there was no significant difference by changing of anhydrous sodium carbonate solution (7.5% and 14.06%, respectively,

**Table 2.** Statistical data for the regression equations of the calibration curve for pyrogallol, linearity test and specificity test for F2 and TG.

Regression analysis	Calibration curve of pyrogallol		Linearity test of F2		Linearity test of TG		Specificity test of F2		Specificity test of TG	
Slope (SE)	0.1407 (0.0023)		0.0457 (0.0010)		1.8460 (0.0426)		0.0455 (0.0009)		1.8208 (0.0285)	
Intercept (SE)	0.0044 (0.0078)		0.0428 (0.0107)		0.0451 (0.0102)		0.1471 (0.0094)		0.0915 (0.0068)	
Regression coefficient (R <sup>2</sup> )	0.9964		0.9932		0.9926		0.9948		0.9966	
Calculated F-value (critical F-value)	3.30 (3.71)		0.29 (3.71)		0.28 (3.71)		0.29 (3.71)		0.29 (3.71)	
Sum of pure error	0.0990		0.0020		0.0005		0.0013		0.0003	
Lack of fit error	0.0097		0.0021		0.0003		0.0018		0.0010	
Analysis of variance	F <sub>1,13</sub> = 3901.2, P < 0.001		F <sub>1,13</sub> = 2053.0, P < 0.001		F <sub>1,13</sub> = 1878.2, P < 0.001		F <sub>1,13</sub> = 2669.5, P < 0.001		F <sub>1,13</sub> = 12,265.7, P < 0.001	
95% CL slope	0.1359; 0.1456		0.0435; 0.0479		1.7540; 1.9381		0.0436; 0.0474		1.7592; 1.8823	
95% CL intercept	-0.0125; 0.0213		0.0197; 0.0659		0.0231; 0.0670		0.1268; 0.1673		0.0768; 0.1062	
	<b>SS</b>	<b>MS</b>	<b>SS</b>	<b>MS</b>	<b>SS</b>	<b>MS</b>	<b>SS</b>	<b>MS</b>	<b>SS</b>	<b>MS</b>
Regression	0.5476	0.5476	0.6409	0.6409	0.4396	0.4396	0.6371	0.6371	0.4277	0.4277
Residual	0.0018	0.0001	0.0040	0.0003	0.0030	0.0002	0.0031	0.0002	0.0014	0.0001
Total	0.5494		0.6450		0.4426		0.6402		0.4290	

SE, Standard error; SS, Sum of squares; MS, Mean square; CL, Confidence limits.

**Table 3.** Accuracy results determined for recovery test in F2 and TG.

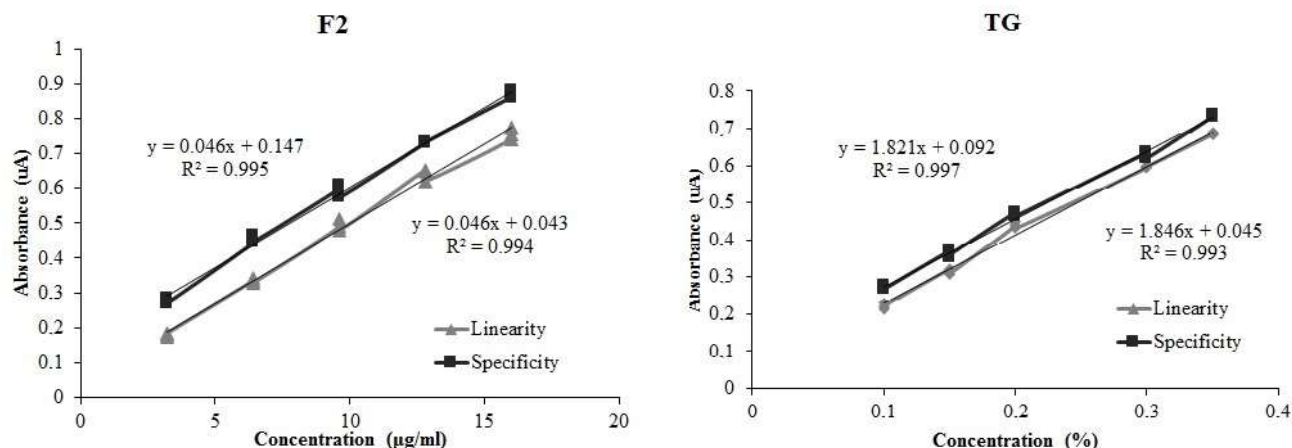
Parameter	F2			TG		
	Theory absorbance (uA)	Obtained absorbance (uA) $(\bar{x} \pm dp)$ [CV%]	Recovery (%)	Theory absorbance (uA)	Obtained Absorbance (uA) $(\bar{x} \pm dp)$ [CV%]	Recovery (%)
LC	0.588	0.593 ± 0.020 [2.9]	100.9	0.586	0.503 ± 0.007 [1.3]	85.8
IC	0.679	0.673 ± 0.010 [1.7]	99.1	0.689	0.596 ± 0.009 [1.6]	86.5
HC	0.769	0.733 ± 0.020 [3.1]	95.3	0.793	0.676 ± 0.004 [0.5]	85.2
<b>Total recovery</b>			<b>98.4</b>			<b>85.8</b>

 $\bar{x}$ , Mean; sd, Standard deviation; RSD, Relative standard deviations; LC, Lower concentration; IC, Intermediate concentration; HC, Higher concentration.

0.510 ± 0.003 [0.57%] and 0.498 ± 0.003 [0.65%]), as well as by dilution operations without light protection (0.513 ± 0.001 [1.95%]) by

statistical analysis (F<sub>3,11</sub> = 2.8, P = 0.09). For TG, there was no significant difference in the amount of absorbance at different times (25 and 35 min,

respectively, 0.440 ± 0.01 [2.28%] and 0.440 ± 0.01 [2.19%]), or different wavelengths (755 and 765 nm, respectively, 0.422 ± 0.010 [2.83%] and



**Figure 1.** Curve of specificity test compared with curve of linearity test for absorbance of F2 and TG obtained by reaction with Folin-Ciocalteu reagent. (Reproduction size: full page width, 27 cm).

$0.424 \pm 0.010$  [2.91%]) by statistical analysis ( $F_{3,8} = 2.5$ ,  $P = 0.14$ ). This demonstrated the robustness of the method under the evaluated conditions.

Considering the proposed methods for F2 and TG, we could determine the content of TP relative to pyrogallol in both samples. The specific absorptivity is the absorbance of a substance in solution at 1% (w/v; 10,000  $\mu\text{g/mL}$ ). The specific absorptivity of pyrogallol was calculated using the linear equation ( $y = 0.141x + 0.0044$ ); a value of 1,407.3 was obtained. Therewith, the TP content is 36.600% in F2 and 0.067% in TG, which complies with the expected concentration for a TG with 0.2% of F2.

## Conclusion

The UV/Vis spectrophotometric method described was successfully validated as an appropriate approach for the determination of total polyphenols relative to pyrogallol in a proanthocyanidin polymers-rich fraction of *S. adstringens* (F2), as well as in topical gel (TG). Furthermore, the proposed TG is an appropriate formulation and may be considered as a potential pharmaceutical agent in the treatment of vaginal candidiasis.

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## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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