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Cordia americana: Evaluation of *in vitro* anti-herpes simplex virus activity and *in vivo* toxicity of leaf extracts

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Abstract

Herpes simplex virus (HSV) type 1 and type 2 are responsible for causing infections whose symptoms can vary from subclinical to severe manifestations. *Cordia americana* is a plant used by traditional communities for the treatment of wounds and diarrhoea, as well as infections like flu and syphilis. Scientific evidence has shown that, among other biological activities, the plant possesses antiviral properties; however, the evaluation of the *in vivo* toxicity of preparations of this plant is still lacking. This study assessed the *in vitro* anti-HSV-1 and anti-HSV-2 activity of a crude extract (CE) obtained from the leaves of *C. americana*, as well as its aqueous (FAq) and ethyl-acetate fractions (FAc). In addition, the *in vivo* toxicity of the FAq was assessed. The sulforhodamine B method was performed to determine the antiviral activity and the *in vivo* toxicity was evaluated according to Brazilian federal regulations. The CE, FAq, and FAc demonstrated antiviral activity against HSV-1 *in vitro*, presenting EC_{50} values of 7.0±1.4, 1.5±0.35, and 7.5±3.8, respectively. The FAq also had activity against HSV-2 with an EC_{50} of 11.8±1.02. The toxicological study of FAq in animals showed that it had very low toxicity. No death occurred during acute or subchronic experiments, where up to 5000 mg/kg and 150 mg/kg FAq were tested respectively; and there were no signs of toxicity in the subchronic test. The results of this study, in conjunction with further studies, pave the way for a potential topical treatment for skin and mucosal diseases, such as HSV-1 and HSV-2 infections.

Keywords: anti-HSV activity; *Cordia americana*; *in vivo* toxicity.
Abbreviations: AST_ aspartate aminotransferase, CC_{50}_ Cytotoxic concentration 50%, CE_ Crude Extract, CGen_ Genetic Heritage Management Council, DMEM_ Dulbecco's Modified Eagle Medium, EC_{50}_ Effective concentration 50%, FAc_ Acetate Fraction, FAq_ Aqueous Fraction, FCS_ Fetal Calf Serum, HIV_ Human Immunodeficiency Virus, HSV-1_ Herpes Simplex Virus Type 1, LC-ESI-MS_ Liquid Chromatography – Electrospray Ionization –Mass Spectrometry, LD_{50}_ Lethal Dose 50%, SI_ Selectivity Index, WBC_ White Blood Cells.

Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are double-stranded DNA viruses, responsible for causing recurrent infection in adults and children as well as neonatal infection. The symptoms can vary from infections with a severe outcome, such as encephalitis and keratitis, to mild or subclinical manifestations, such as tingling, itching or burning sensations (Kleinschmidt-DeMasters et al., 2020; Tomasini, 2020). HSV infections affect more than 80% of people worldwide and persist throughout the lifetime of the host. Modified nucleosides or their prodrugs, such as acyclovir, are currently used for the treatment, but the side effects often cause an issue and the emergence of resistant strains is becoming problematic. Thus, these concerns are motivation for researchers to seek new antiviral treatments (Vilarreal, 2003; Kukhanova et al., 2014; Kopp et al., 2014).

Plants have been used medicinally since the beginning of human history. In Brazil, which has a great abundance of biodiversity, indigenous people passed along knowledge of the

beneficial uses of medicinal plants to slaves and Europeans, further propagating the use of these plants (Simões et al., 1998).However, it is a common misconception that plants used to treat illnesses or ailments are all harmless and non-toxic. In fact, many medicinal plants can exhibit toxicity, for example *Abrus precatorius* used as an aphrodisiac in Indian folk medicine and *Larrea tridentata* for arthritis by American indigenous cause gastrointestinal symptoms and nephrotoxicity, respectively. These plants are well-known by the population that uses them, as well as having been well-studied, and thus are used carefully. The greatest risk, however, is the widespread use of plants without prior safety assessments and subsequent recommendations regarding their use to the public (George, 2011; Farzaei et al., 2020).

Cordia americana (L.) Gottschling & J. S. Mill. was previously classified as *Patagonula americana* L. and other names (The Plant List, 2013). It is a tree that is native to the southern and southeastern states of Brazil. This species belongs to the Boraginaceae family and is locally known as 'guajuvira' (Gottschling and Miller, 2006). The wood is typically used as firewood as well as for construction, and household and agriculture equipment. In addition the traditional communities have reported that this plant has medicinal effects. The population of the indigenous reserve of Rio das Cobras, Paraná, Brazil uses the bark and leaves to treat flu and wounds (Moura-Costa et al., 2012). Other traditional groups in South America also use it for the treatment of skin and mouth wounds like oral aphthae and gingivitis, diarrhoea, and even against syphilis (Oza and Kulkarni, 2017; Rovedder et al., 2016; Scarpa and Rosso, 2018; Simões et al., 1998). Biological activities have been reported for *C. americana* including anti-inflammatory, antiulcer, and wound healing (Bolson et al., 2015; Moura-Costa et al., 2012; Rovedder et al., 2016). The main compound that has been isolated from this species is rosmarinic acid, which has been shown to have antioxidant, anti-inflammatory, and antiviral effects against HSV-1 and human immunodeficiency virus (HIV) (Geller, 2010; Tewtrakul et al., 2003). Previously, a 50% hydroalcoholic extract of the leaves of *C. americana* was reported to have anti-HSV-1 activity (Moura-Costa et al., 2012). Although this plant is commonly used by the indigenous population of the Rio das Cobras reserve, Paraná, Brazil, no study has properly investigated the safety of the potential medicinal use of this plant. The present study evaluated the antiviral activity of an aqueous fraction (FAq) against HSV-1 and HSV-2; as well as an ethyl-acetate fraction (FAc) and the crude extract (CE) of *C. americana* leaves against HSV-1. Using tests of both acute and subchronic toxicity the safe use of the FAq was evaluated *in vivo*.

Results

LC-ESI-MS/MS analysis

The chemical composition presented in the FAq and FAc from the leaves of *C. americana* were determined by comparing the MS/MS ion fragments with the literature. The identified compounds with their respective molecular weights are listed in Table 1.

Antiviral activity and cytotoxicity

The CE, FAq, and FAc of *C. americana* leaves presented activity against HSV-1, with a statistical difference for all groups in terms of the number of viable cells when compared to the infected cells without the presence of these treatments. Between the fractions, the FAq was significantly more effective in terms of inhibiting viral activity than the CE and FAc, thus the antiviral activity of FAq was also assessed against HSV-2. The FAq was also effective against HSV-2 but not to the same degree as its anti-HSV-1 activity (Table 2). Acyclovir, the common HSV treatment drug, was used as a positive control, which had EC_{50} values against HSV-1 and HSV-2 of 0.35 and 0.24 μg/ml, respectively.

The cytotoxicity of the fractions was also evaluated against the host cells. The cytotoxic range was very close between the CE and the two fractions, which had no statistical difference (Table 2).

Acute toxicity

Clinical signs of toxicity (e.g., changes in locomotion, respiratory difficulties, piloerection, diarrhoea, drooling, alterations in muscle tone, hypnosis, hyperexcitability of the central nervous system, convulsions and abdominal writhing) were not observed in any of the mouse groups given any concentration of FAq. Furthermore, no deaths occurred during 14-day course of the experiment meaning the LD_{50} could not be determined. The use of FAq did not cause any weight variation in the mice during the experiment as compared with the control group. The macroscopic observation of the organs after 14 days did not reveal any significant morphological changes that were caused by the extract, even up to highest concentration (5000 mg/kg) test. There were also no significant differences in relative organ weight as compared with the control group (Figure 1).

Repeated-dose oral toxicity (subchronic toxicity)

Weight gain progressed normally in both the control group of rats and the groups receiving different concentrations, up to 150 mg/kg daily doses, of FAq. No differences were observed in the weight gain between the sex of the rats for each group. For most of the analysed organs there were no significant differences in the relative weight. The spleens of male rats and the lungs of female rats presented significant differences in relative weight, both in the group that received a dose of 15 mg/kg FAq, but this increase in relative organ weight was not dose-dependent. The macroscopic observation of these and the other organs did not present any signs of morphological or haemorrhagic changes that could be caused by the FAq. Histopathological examination showed that the organs in the control and treated groups had the same characteristics, with no pathological features. The biochemical analysis of the whole blood showed that 75 and 150 mg/kg FAq decreased AST in males, 75 mg/kg FAq decreased uric acid in males, and 150 mg/kg FAq decreased albumin in females. The other parameters evaluated were not significantly different from the control group. There were also no significant differences in the haematological parameters in male or female rats receiving repeated-dose oral administration of FAq compared with the control group.

Table 1. Characterization of compounds detected in both FAq and FAc fractions from the leaves of *Cordia americana* by LC-ESI-MS/MS in positive $[M+H]^+$ and negative mode $[M-H]^-$.

Compound	*m/z* $[M+H]^+$	*m/z* $[M-H]^-$	MS^2	Reference
Caffeic acid	181		163, 145, 135	Wu et al., 2009
Quercetin	303		274, 257, 229, 165, 153, 149, 137	Tsimogiannis et al., 2007
Rosmarinic acid		359	197, 179, 161, 135	Wang et al., 2013
Quercetin-3-O-Rhamnoside (Quercitrin)	449		303	de Brito et al., 2007
Quercetin-3-O-glucoside (Isoquercitrin) or Quercetin-3-O-galactoside (Hyperoside)	465		303	de Brito et al., 2007 Lin et al., 2000 Wang and Sporns, 2000
Quercetin-3-O-Rutinoside (Rutin)	611		465, 303	Ma et al., 2000

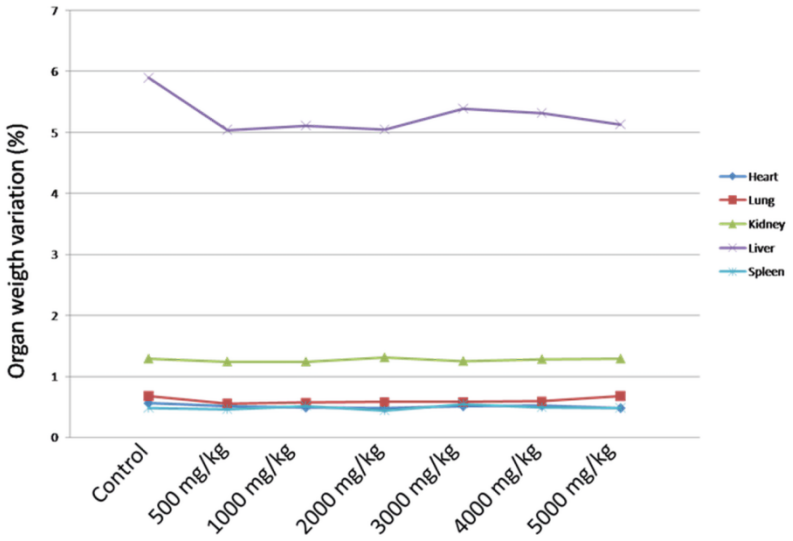

Figure 1. Effect of FAq of *Cordia americana* on the relative weight of the main vital organs of Swiss mice in acute toxicity tests.

Table 2. Antiviral activity and toxicity of the crude 50% hydroalcoholic extract and fractions from *Cordia americana*.

		HSV-1		HSV-2	
Extract	CC_{50} (mg/ml)	EC_{50} (mg/ml)	SI	EC_{50} (mg/ml)	SI
CE	233 ± 12	7.0 ± 1.4 [a]	33	-	-
FAq	233 ± 50	1.5 ± 0.35 [a, b]	155	11.8 ±1.02	20
FAc	250 ± 30	7.5 ± 3.8 [a]	33	-	-

CC_{50}: Cytotoxic concentration 50%; EC_{50}: Effective concentration 50%; SI: Selectivity Index. $P < 0.05$ statistic test ANOVA followed by the Dunnett test. [a] Statistically different when compared to values found in the viral action, without using the extract on the cells.
[b] Statistically different activity when compared to fractions CE and FAc.

Discussion

Cordia americana is a medicinal plant that is used for wound healing by indigenous people of the Rio das Cobras reserve in Paraná, Brazil. The decoction or maceration of the leaves and bark has been reported to be used for its anti-inflammatory, antiulcer, and wound healing effects and for the treatment of malignant diseases, diseases of the skin, eye, ear, nose, and oropharynx, as well as gastrointestinal, endocrine, cardiovascular, and central nervous system disorders (Bolson et al., 2015; Geller, 2010; Oza and Kulkarni, 2017). As reported previously, crude extracts of *C. americana* leaves have shown activity against HSV-1 (Moura-Costa et al., 2012). In the present study, the CE, an aqueous (FAq), and an ethyl-acetate (FAc) fraction demonstrated activity against HSV-1. As the FAq had the best anti-HSV-1 activity, it was also tested against HSV-2, showing that the FAq is effective both HSV types.

Tannins from the leaves, quinones and phenolic aldehydes from the heartwood, and coumarin and tannins from the bark of *C. americana* have previously been identified (Geller, 2010;

Simões, 1998). In the present study, LC-ESI-MS/MS suggested the presence of phenolic compounds in the FAq and FAc. In positive ion mode $[M+H]^+$, caffeic acid (*m/z* 181), quercetin (*m/z* 303), quercitrin (*m/z* 449), and rutin (*m/z* 611) were identified. In negative ion mode $[M-H]^-$, rosmarinic acid (*m/z* 359) was identified. The analyses also showed the presence of *m/z* 465 in positive ion mode, a compound that could be either isoquercitrin or hyperoside, as both have the same mass and fragment ions (de Brito et al., 2007, Lin et al., 2000; Wang and Sporns, 2000). Geller et al. (2010) previously reported the presence of rosmarinic acid, caffeic acid, quercitrin, and rutin in *C. americana* leaves. Quercetin and isoquercitrin were also identified in other *Cordia* species, *C. sinensis* and *C. verbenaceae* (Al-Musayeib, 2011; Santi et al., 2014). As far as we know, hyperoside has not been described in the genus *Cordia* before. Rosmarinic acid, a caffeic acid dimer, is a characteristic constituent of the Boraginaceae family and was identified as the major constituent in *C. americana* leaves (Geller et al., 2010). Caffeic acid, quercetin, quercitrin, and isoquercitrin have all been reported to have anti-HSV-1 and anti HSV-2 activity (Chen et al., 2011; Hung et al., 2015; Ikeda et al., 2011; Lyu and Rhim, 2005; Prinsloo and Vervoort, 2018). Rosmarinic acid is also reported to be effective against HSV-1 (Astani et al., 2012), whilst rutin has shown moderate activity against HSV-1 (Boligon et al., 2013). According to Fritz et al. (2007), hyperoside itself does not have antiviral activity, but Amaral et al. (1999) described combinatorial antiviral activity of this compound when in the presence of other flavonoids.

In addition to the antiviral activity that has been described in the literature, all of the phenolic compounds that were isolated from *C. americana* have demonstrated anti-inflammatory activity (Chao et al., 2009; Guardia et al., 2000; Melzig et al., 2001; Ozbilgin et al., 2015; Swarup et al., 2007). Interestingly, *C. americana* is able to fight against HSV-1 and HSV-2 infection by both exerting effects on the causal agent and minimizing the inflammatory response that is caused by the virus.

Based on the greater activity the FAq exhibited against HSV-1, its safe use was assessed in animal models. *In vivo* evaluations of mice and rats administered with different concentrations of FAq revealed no clinical signs of toxicity. Furthermore, no deaths occurred during the course of the evaluations. Thus the use of FAq in animals may be considered nontoxic according to Loomis and Hayes (1996), who stated that chemical substances with an LD_{50} of 5000-15000 mg/kg can be considered practically nontoxic. The administration of the FAq also did not cause any observable difference in animal behaviour. The differences in relative organ weights were not statistically significant and macroscopic observations of the organs did not reveal any morphological changes. According to Hilaly et al. (2004), weight changes during treatment may indicate adverse effects. As no significant changes were observed in animal weight or behaviour in the acute or subchronic toxicity tests compared with the control groups, this suggests that the FAq does not impact the normal growth of the animals, thus indicating a lack of toxicity.

The macroscopic observation of the organs in the acute single-dose toxicity test and the histopathological findings in the subchronic repeated-dose test did not reveal any pathological features in any of the examined organs, which also suggests that FAq is nontoxic.

Biochemical analysis of the whole blood of rats in the repeated-dose oral toxicity test indicated that the FAq induced changes in AST, uric acid, and albumin. However, these results do not suggest toxicity because decreases in these parameters are not necessarily clinically relevant (Burtis et al., 2005). Finally, no haematological changes were caused by the repeated-dose oral administration of the FAq, thus providing additional evidence that *Cordia americana* extracts can be considered nontoxic.

Materials and methods

Plant material

Leaves from *Cordia americana* (L.) Gottschling & J. S. Mill. were collected in Nova Laranjeiras, Paraná, Brazil, in December 2008. This study was authorized by the Genetic Heritage Management Council (CGen; no. 68/2011), and a voucher specimen was deposited in the West of Paraná State University Herbarium, Cascavel, Paraná, Brazil (UNOP#5300).

Preparation of the extract and fractions

The leaves were dried at room temperature and then pulverized in a hammer mill (Tigre ASN5). The crude extract (CE) was obtained from the pulverized leaves using an Ultra-Turrax apparatus (UTC115KT, Ika Works) at 1500 x *g* with 50% ethanol in water for 15 min. The organic solvent was eliminated by rotavapor (Büchi R-114) under reduced pressure and lyophilized to yield 195 g of CE. Next, 100 g of the CE was resuspended in water (1000 mL) and partitioned with ethyl acetate at a 1:1 ratio. Both fractions were concentrated in vacuum and lyophilized to obtain the aqueous fraction (FAq; 79 g) and ethyl-acetate fraction (FAc; 21 g).

LC-ESI-MS/MS analyses

High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was performed using a 1525 µ HPLC instrument linked to a Micromass Quattro *micro*™ API benchtop triple quadrupole mass spectrometer (both from Waters, Milford, MA, USA). The electrospray ionization (ESI) source was operated in positive and negative ion detection modes. All of the data were acquired and processed using MassLynx 4.0 software (Waters, Milford, MA, USA). The following MS source parameters were used for ion detection: capillary voltage (2.5 kV), extraction cone (2 V), desolvation temperature (450ºC), cone voltage (25 V), source temperature (130ºC), cone gas flow (50 L/h), and desolvation gas flow (900 L/h), with argon as the collision gas (collision energy, 15-30 eV). Chromatographic separation of the diluted samples (1 mg/mL in water:acetonitrile 1:1, v/v) was performed using a reverse-phase column (3.5 µm Symmetry C18 column; 75 x 4.6 mm, Waters, Milford, MA, USA) that was maintained at room temperature. The mobile phase consisted of the following sequences of linear gradients and isocratic flows of solvent A (water with 0.1% formic acid, v/v) and solvent B (acetonitrile with 0.1% formic acid, v/v): 0-1 min (5% B), 1-3.4 min (12% B), 3.4-8 min (20% B, maintained for 1 min), 9-15 min (31% B), 15-20 min (41% B), 20-30 min (95% B, maintained for 2 min), and finally 95-5% B for 2 min. The flow rate was 0.5 mL/min, and the sample injection volume was 10 µL. The MS/MS identification of the ion fragments was compared with the literature.

Antiviral activity

Viability of cells infected with HSV-1 and HSV-2 was assessed by a colorimetric method using sulforhodamine B (Sigma-Aldrich). VERO cells, an African green monkey kidney epithelial cell line, were grown in 96-well plates using Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal calf serum (FCS) and 50 μg/mL gentamycin. The cells were incubated at 37°C with 5% CO_2 for 24 h and after the formation of a confluent monolayer, the cells were infected with a viral suspension and tested with different concentrations of the extracts (0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 mg/mL). The cells were incubated at 37°C with 5% CO_2 for 72 h. A cell control (cells without viral particles or extracts) and virus control (infected cells without the addition of extracts) were included. The tests were performed three times in triplicate (Skehan et al., 1990). Acyclovir (Sigma, St. Louis) was solubilized in phosphate-buffered saline (PBS) to give a stock solution of 1 mg/mL and used as a positive control drug. Sulforhodamine B was added at a concentration of 0,4% and read on a microplate reader at 510 nm

Cytotoxicity assay

VERO cells were grown in DMEM plus 10% FCS and gentamycin, distributed in a 96-well microplate, and incubated until a confluent monolayer formed. Different concentrations of the extracts were added to the wells in triplicate and incubated at 37°C with 5% CO_2 for 72 h. The cell viability was revealed by the sulforhodamine B colorimetric method. Control cells without the addition of extracts were also included. The tests were performed in three independent experiments (Skehan et al., 1990).

Toxicity studies

The *in vivo* toxicity studies were performed according to Brazilian federal regulations "Guide for Conducting Pre-Clinical Toxicity Studies on Herbal Medicines" from the National Health Surveillance Agency (Brasil, 2004), and approved by the Ethical Committee of the State University of Maringá (Protocol n° 57/2011).

Animals

Adult Swiss mice (weighing 39.5 ± 6.8 g) and Wistar rats (weighing 159.8 ± 16.0 g), both male and female, were used in this study and housed in groups of six per cage (Swiss mice) or five per cage (Wistar rats) with food and water available *ad libitum*. The animals were maintained under a 12 h/12 h light/dark cycle and controlled temperature (22°C ± 1°C).

Acute toxicity

Swiss mice were divided into seven groups with 12 animals each (six male and six female). They were treated orally by gavage with a single dose of 500, 1000, 2000, 3000, 4000, and 5000 mg/kg FAq per group. The negative control group was treated with distilled water. General behaviour and the number of surviving animals for each treatment were evaluated at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 24 h, and then daily up to 14 days. Clinical signs of toxicity and mortality (LD_{50}) were evaluated. The animals were weighed daily to observe variations in body mass. At the end of the 14-day period, all of the animals were sacrificed. The organs were removed, weighed, and macroscopically evaluated for abnormalities.

Repeated-dose oral toxicity (subchronic study)

Wistar rats were divided into four groups with 20 animals each (10 males and 10 females).Three groups received different doses of the FAq (15, 75, and 150 mg/kg) that was resuspended in water and administered orally by gavage daily for 30 days. The negative control group received oral gavage of distilled water only.

Behaviour was observed daily. Body weight was recorded weekly. At the end of the 30-day period, the animals were sacrificed. Blood was collected for biochemical and haematological analyses, and the organs were removed, weighed, and macroscopically and histopathologically examined.

Biochemical analyses of whole blood were performed to determine glucose, triglycerides, total cholesterol, total protein, albumin, creatinine, uric acid, blood urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, and bilirubin. Haematological analyses were performed using whole blood to evaluate erythrocyte count, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, platelet count, white blood cell (WBC) count, and differential WBC count.

The organs (lung, heart, kidneys, liver, and spleen) of all of the animals were examined macroscopically for signs of abnormalities. The organs were weighed, fixed in Bouin's solution, and preserved in 70% ethanol. Tissue slides were prepared and stained with haematoxylin and eosin for microscopic examination.

Statistical analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Dunnett test and T test. The histopathology results were analysed using Fisher's exact test. Differences were considered significant at $p \leq 0.05$.

Conclusion

The present study provided valuable information regarding the antiviral activity and toxicity profile of the aqueous fraction (FAq) of a crude extract (CE) obtained from the leaves of C*ordia americana*. The FAq presented better antiviral activity than the CE. The FAq was also considered to have low toxicity when tested *in vivo*, as even at the highest dose tested in an acute toxicity test did not result in death. The FAq also did not affect the normal growth of the animals in either the acute or subchronic experiments. In the repeated-dose oral toxicity experiment, there were no relevant changes in haematological, biochemical, or histopathological parameters in animals that received the FAq. These toxicity studies suggest that the FAq of the CE from *Cordia americana* leaves may be considered safe for treatment of lesions caused by HSV-1 or HSV-2 and serves to contribute to the development of a new medicine in the topical treatment of the skin and mucosal diseases caused by these viruses.

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Conflict of interest

The authors declare that there are no conflicts of interest**.**

Author´s contributions

Moura-Costa,GF conducted the extracts preparation, antiviral and cytotoxicity assays, performed the in vivo toxicity tests and draft the manuscript; Panizzon, GP and Dias Filho BP conducted the CG-MS and helped to draft the manuscript; Mello JCP were involved in the extracts preparation, CG-MS and helped to draft the manuscript; Oliveira, TZ conducted the in vivo toxicity; Costa, MA conducted the in vivo toxicity, performed the statistical analysis and helped to draft the manuscript; Nakamura, CV and Kaneshima, EM were involved in the toxicity tests and helped to draft the manuscript; Ueda-Nakamura, T idealized the study and acted as principal investigator throughout its execution. All authors read and approved the final manuscript, conducted and analysed the experimental assays and took part in the design of the study.

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