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Article

Antiviral Activity of Red Propolis Against Herpes Simplex Virus-1

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Brazilian Red Propolis is a resinous material produced by *Apis mellifera* bees through the collection of the exudates of *Dalbergia ecastaphyllum*, rich in flavonoids, and *Symphonia globulifera* and *Clusia* species, which are rich in polyprenylated benzophenones. More than 200 compounds, including flavonoids and polyprenylated benzophenones have been found in Brazilian red propolis. The objective of the present study was to evaluate the chemical composition and antiviral activity of an ethanolic extract of red propolis from Alagoas, Brazil, against Herpes Simplex Virus (HSV-1). In the HPLC-PDA-ESI-MS/MS analysis were detected flavanones, isoflavones, chalcones, pterocarpans and polyprenylated benzophenones. The measurement of antiviral activity of red propolis extract was performed by DNA quantification through quantitative real-time PCR assay and negative staining Electron Microscopy. The pretreatment, post-treatment, and virucide assays using ethanolic extract of red propolis with concentration of 8, 12, 24, 48, or 96 μg/mL, indicated an inhibition of the viral binding and viral entry into cells as well as the replication of HSV-1. In the electron microscopy imaging was observed the disruption of the viral membrane in the HSV-1 treated with red propolis, when compared with HSV-1 that was treated with phosphate buffered saline alone, indicating that ethanolic extract from red propolis can act directly on the viral envelope, through lipid membrane degradation and/or directly blocking the enriched proteins on the viral surface.

Keywords: Antiviral activity. Red propolis. *Apis mellifera*. Herpes Simplex Virus-1. Flavonoids. Polyprenylated benzophenones.

INTRODUCTION

Apiculture with the management of *Apis mellifera* bees, order Hymenoptera, Apoidea family, known for producing honey, bee pollen, propolis, beeswax, royal jelly, and bee venom, started at least four thousand years ago. Apitherapy dates back 9,000 years and is the science that uses bee products to prevent or treat disease (Ghosh *et al*., 2022; Rocha *et al*., 2022).

Propolis is used by bees to maintain the internal temperature, repair honeycombs, embalm dead insects and protect the hive against the invasion of microorganisms (Tran *et al*., 2020). The resin source used in the production of propolis is collected from the leaves and flower buds of numerous tree species, which is chewed by the honeybee and are added salivary enzymes and wax (Salatino, Salatino, Negri, 2021). The chemical composition and pharmacological activity of propolis can vary widely from region to region, according to its botanical and geographical origins, collection season, climatic zones, and bee species (Zulhendri *et al*., 2021).

Although brown, green, and red propolis show differences in their chemical composition, justified by

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the biodiversity of Brazil, all these types of propolis exhibited antimicrobial activities (Salatino, Salatino, Negri, 2021; Salatino, 2022). Flavanones, isoflavanones, chalcones and pterocarpans were detected in red propolis from Mexico and Cuba (Freires *et al.*, 2016), brazilian red propolis (Righi *et al.*, 2011) and propolis from the Midwest region of Brazil (Costa Jr. *et al.*, 2020). The presence of polyprenylated benzophenones in Brazilian red propolis from Amazon region was attributed to *Clusia* ssp as the resin source (Ishida *et al.*, 2011), while *Clusia scrobiculata*, *Clusia minor* and *Clusia major* as the resin source for red propolis from Venezuela and *Clusia rosea* for propolis from Cuba (Boeing *et al.*, 2021). Saturated and unsaturated aromatic hydrocarbons, ketones, alcohols, ethers, and terpenes were detected in hexane extracts from red propolis (De Carvalho *et al.*, 2020).

The red propolis produced on the littoral of the northeastern states, mainly Maceió, Alagoas State, exhibited isoflavonoids, chalcones and pterocarpans as main constituents and *Dalbergia ecastaphyllum* (Leguminosae, Faboideae) as resin source (de Carvalho *et al.*, 2020; dos Santos *et al.*, 2021; Vieira de Morais *et al.*, 2021; Barreto *et al.*, 2022; Salatino, Salatino, Negri, 2021; Silva *et al.*, 2022). On the other hand, the presence of polyprenylated benzophenones in red propolis indicated *Symphonia globulifera* and *Clusia* species as resin source (Ccana-Ccapatinta *et al.*, 2020). *Symphonia globulifera* occur in the Atlantic Forest (Salatino, Salatino, Negri, 2021). The State of Alagoas, Brazil, became recognized as a red propolis producer with the designation of origin (IG 201101) obtained in July 2012 by the National Institute of Industrial Property (Giesbrecht *et al.*, 2016, Silva *et al.*, 2020; dos Santos *et al.*, 2021).

Propolis extracts exhibited activity against *Herpes simplex* virus (Coelho *et al.*, 2015; Rocha *et al.*, 2022; Bankova *et al.*, 2014), antimicrobial activity (Wieczorek *et al.*, 2022), preclinical efficacy against adenoviruses, influenza viruses, respiratory tract viruses (Zulhendri *et al.*, 2021; Salatino, 2022), human immunodeficiency virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Yosri *et al.*, 2021; Ghosh *et al.*, 2022; Sobrinho *et al.*, 2022; Dilokthornsakul *et al.*, 2022) and antifungal activity (Wolska, Antosik, 2023).

Red propolis exhibited antifungal, antiviral (Silva-Beltrán *et al.*, 2020; Silva *et al.*, 2020), antimicrobial activity against several microorganisms, including Gram-positive and Gram-negative bacteria (Barreto *et al.*, 2022) and anticancer activity against ovarian cancer (Justino *et al.*, 2023). Brazilian red propolis was more effective against Enterovirus surrogates than green propolis (Silva-Beltrán *et al.*, 2020), exhibited anti-Helicobacter pylori activity (Mendonça *et al.*, 2020) and can be used in the treatment against multidrug-resistant bacterial infections (Xavier *et al.*, 2023). Neovestitol and vestitol, isoflavonoids found in red propolis inhibited Chikungunya virus infection (Silva *et al.*, 2022). A new variety of red propolis, Brazilian Amazon red propolis with high concentration of isoflavones and pterocarpans exhibited leishmanicidal activity against *Leishmania amazonenses* (Dutra *et al.*, 2023).

The *Herpes simplex* virus of the Herpesviridae family is a neurotropic virus that has two distinct serotypes, human herpesvirus 1 and 2, which are viral pathogens closely related, however, they contain sufficient differences to enable type identification (Hayashi *et al.*, 2019). The lesions caused by *Herpes simplex* virus (HSV-1) in skin or mucous membranes are highly infectious, often painful, burning, or pruritic, and tend to recur in most patients. HSV-1 possesses an outer envelope that consists of a lipid bilayer with viral glycoproteins, is composed of linear double-stranded DNA and possesses a replicative cycle, which produce lytic infections, establishing latent infection in host cells (Hayashi *et al.*, 2019). This virus causes a wide spectrum of diseases, from primary or recurrent mucosal infections, such as, keratoconjunctivitis, visceral infection in immunocompromised patients, neonatal infection, herpetic encephalitis, among others (Hayashi *et al.*, 2019; Garber, Barnard, Pickrell, 2021). Numerous vaccines were investigated in clinical trials, however, there is no licensed vaccine available for the prevention of HSV-1 infection (Garber, Barnard, Pickrell, 2021).

HSV-1 infection occurs involving the relocalization of cellular proteins and the ordered assembly of replication compartments, which are large globular domains within the nucleus of infected cells, in which occur gene expression, DNA replication, cleavage and packaging.

The antiviral drugs acyclovir, valacyclovir, famciclovir, cidofovir, and foscarnet are commonly used to targeting the Viral DNA and RNA polymerases, which are very important enzymes that synthesize the genetic materials of the virus survival (van de Sand *et al*., 2021). The treatment with acyclovir, which acts as a highly selective inhibitor of HSV-1 DNA replication, can accelerate the healing of lesions caused by HSV. The continuous use of acyclovir therapy is often prescribed for people with frequent recurrences (Poole, James, 2018), however, it can cause a wide array of side effects, including renal failure, hepatitis, and anaphylaxis (Poole, James, 2018). Beside this, the effectiveness of the treatments with antiviral drugs has decreased, due to the resistance of the HSV-1 (van de Sand *et al*., 2021; Garber, Barnard, Pickrell, 2021).

Propolis have exhibited many antiviral effects in assays carried out in cell infections *in vitro* (Salatino, 2022) and may be useful for treating active HSV-1 lesions or preventing recurrences. In this study was evaluated the chemical profile of an ethanolic extract of red propolis from Alagoas by HPLC-PDA-ESI-MS/MS analysis and its antiviral activity against HSV-1, through viral DNA quantification experiments and electron microscopy experiments.

MATERIAL AND METHODS

Ethanolic extract from red propolis

All materials and solvents were of analytical reagent grade. Red propolis produced by *Apis mellifera* was collected in May 2015, in an apiary located in Maceio, state of Alagoas, in northeastern Brazil and was provided by a beekeeper. The present study has access permission to the components of plant genetic heritage registered in the SisGen Platform (Registration A710EE), in accordance with the Brazilian Biodiversity Law (13.123/2015). Extraction with ethanol is suitable to obtain dewaxed propolis extracts, rich in polyphenolic compounds. The propolis sample was cut into small pieces with a blender. Ethanolic extract was prepared using 30 g of propolis dissolved in 150 mL of ethanol (99%) Sigma-Aldrich, through the maceration extraction-method at room temperature for 6 days. The solvent exchange, adding more 150 mL of ethanol (99%) Sigma-Aldrich was performed every 48 h, with daily stirring in the maceration process. The extraction process was performed in the dark, due to the photosensitivity of the extract. After maceration, the ethanolic extract obtained every 48 h was kept in the refrigerator at 4 ^{o}C for cooling to form a wax layer overnight. The ethanolic extracts was completed with other extracts collected ever 48 h, filtered using cotton and Whatman No. 4 filter paper and kept in the refrigerator at 4 ^{o}C for cooling to form a wax layer overnight. Thus, all the ethanolic extract collected each 48 h was filtered using cotton and Whatman No. 4 filter paper and kept in the refrigerator for cooling to form a wax layer overnight. This process was repeated three times until the waxy layer was completely removed. After this, it was again filtered using cotton and Whatman No. 4 filter paper, evaporated off in a rotary evaporator at a temperature of 60 °C to constant weight and stored at – 20°C in amber glass tubes to guarantee the preservation of bioactive compounds. The yield result was calculated based on the initial amount of propolis.

HPLC-PDA-ESI-MS/MS analysis of ethanolic extract from red propolis

Dry red propolis extract (10 mg/3 mL) was dissolved in H_2O:MeOH (1:1 v:v) and filtered with 0.45 μm polytetrafluoroethylene membrane, prior to injection of 28.0 μL into the HPLC system. The HPLC-PAD-ESI-MS/MS analysis was conducted on DADSPD-M10AVP Shimadzu system equipped with a photodiode array detector coupled to Amazon Speed ETD, Bruker Daltonics, which consisted of two LC-20AD Shimadzu pumps, SPD-20A photodiode array detector, CTO-20A column oven and SIL 20AC autoinjector (Shimadzu Corporation Kyoto, Japan). The detector, a quadrupole ion trap equipped with atmospheric pressure ionization source through electrospray ionization interface, was operated in the full scan MS/MS mode. All the operations, acquisition and data analysis were controlled by CBM-20A software. The wavelength range of the PDA detector was 210-500 nm. The mobile phase was composed by eluent A (0.1% aq. formic acid) and eluent B (methanol) at the constant flow rate 1.0 mL/min and constant temperature of the oven at 35 °C. The following elution program, based on concentrations

of the B solvent, was used: 0 min, 20%; 10 min, 40%; 20 min, 60%; 30 min, 80%; 40 min, 100%; 50 min, 20%. Helium was used as the collision gas, and nitrogen as the nebulizing gas, respectively. Nebulization was aided with a coaxial nitrogen sheath gas provided at pressure of 27 psi. Desolvation was facilitated using a counter current nitrogen flow set at a flux of 7.0 L/min. The ionization conditions were, electrospray voltage of the ion source -38 V, a capillary voltage 4500 V and a capillary temperature of 325 °C. Constituents were separated using a reversed-phase Phenomenex Luna C-18 (250 mm × 4.6 mm, 5 μm) connected to a guard column. The full scan mass covered the range from *m/z* 100 up to *m/z* 1000. Collision induced dissociation spectra were performed in the ion trap using helium as collision gas. The data dependent MS/MS events were performed on the most intense ions detected in full scan MS. The collision energy was set with voltage ramping cycles from 0.5 up to 1.3 V. The constituents were characterized by ultraviolet and mass spectral data (MS) and the proposed structures are based in MS data reported in literature (Table I), together on-line chemical databases Scifinder (http://www.scifinder.cas.org), Reaxys (http://www.reaxys.com), RIKEN-Respect (http://metadb.riken.jp/db), and HMDB (www.hmdb.ca).

Cell Viability – MTT Assay

Cells of Vero lineages (African green monkey kidney cells, ATCC CCL 81) were grown at 37 °C in 25 cm^2 plastic cell culture flasks containing 10 ml of Dulbecco's Minimum Eagle Essential medium (DMEM) supplemented with 10% inactive fetal bovine serum (Invitrogen/Life Carlsbad, CA, USA) and 20 mM *L*-glutamine (Invitrogen, USA). Viable cells were determined by [4,5-dimethylthiazol 2-yl]-2,5 diphenyl tetrazolium bromide (MTT) method, based on the principle that active mitochondria cleave the MTT tetrazolium ring, which is reduced to insoluble formazan and used as a measure of cell viability to identify non-cytotoxic concentrations of propolis, as reported by Coelho *et al.* (2015). Vero cells were cultured in 96-well plates in DMEM supplemented with 5% of fetal bovine serum (Invitrogen/Life Carlsbad, CA, EUA). The plates were incubated for 24 h at 37°C. After this period, the plate was washed twice with sterile phosphate buffered saline (PBS) at 37°C; 100 μl of MTT solution (0.5 mg/mL) was added to all wells and the plate was incubated for 2 h at 37°C. After 48 h, ethanolic extract from red propolis with concentrations ranging from 2 μg/mL to 156 μg/mL was added to the wells culture medium. After 24 h of exposure, the supernatant was discarded and MTT at concentration of 500 μg/mL was added to the growth medium for 4 h. After this, the culture medium was removed, 100 mL of DMSO was added and the plates were shaken for 30 minutes and read on a spectrophotometer at 570 nm. The percentage of viable cells in each sample was calculated from the absorbance value obtained by subtracting the average of the controls by the average of the blanks, the latter value being considered as 100% viable cells.

Determination of the Virus Infectious Dose

The most common method for determining viral titer is 50% tissue culture infectious dose (TCD) by using microscope observation of cytopathic effect (CPE). Quantitative real-time PCR (qRT-PCR) method is accepted as the best and most validated quantification method for evaluation of antiviral activity. Determination of the virus infectious dose was carried out using the methodology reported by Coelho *et al.* (2015, 2018) and was evaluated by 50% tissue culture infectious dose ($TCID_{50}$) assays. Herpes simplex virus strain (McIntyre) stock virus (HSV-1) was quantified by medium tissue culture infections with 0.01 MOI (multiplicity of infection). The confluent monolayers of Vero cells were inoculated with 100 μL diluted virus in quadruplicates. After 1 hour of adsorption at 25° C, each well received 200 μL of DMEM medium with 2% FBS. Uninfected cultures were also prepared and treated identically as controls. Plate cultures were observed for cytopathic effect (CPE), a set of cell changes or alterations resulting from a viral infection, daily during, 7 days, when the test was concluded. Virus titers were determined by monitoring the CPE and the endpoint dilution were determined as the highest dilution of virus able to induce CPE in 50% of cells. All titers were given as log 10 $TCID_{50}$ per 0.1 mL of virus.

Antiviral Effect of red propolis on Infected Cells

The antiviral effect was evaluated according to the method described by Coelho *et al.* (2015). Vero cells were grown to approximately 90 % confluency in 96-well plates in DMEM supplemented with 2 mM L-glutamine and 10 % of FBS. Plates were incubated at 37 ºC in a humidified 5% CO_2 atmosphere. After Vero cells to reached confluency, they were infected with HSV-1 at a concentration of 10^{-8} and monitored for cytopathic effects for 3 days. Three antiviral tests were carried out. Ethanolic extract from red propolis (8, 12, 24, 48 or 96 μg/mL) was added to the Vero cells at 3 h prior to virus infections and 1 h after virus infection. The evaluation of virucidal activity was carried out with the HSV-1 at the concentrations described above incubated with 24, 48 or 96 μg/mL of ethanolic extract of red propolis for 1 h at 4 °C, being then placed in 96-well plates containing Vero cells. After this time of contact, unabsorbed virions were then aspirated, and the wells were washed with DMEM to remove the virus that did not penetrate the cells, the medium was replaced, and the culture maintained at 37 °C and 5% CO_2. The antiviral screenings were independently repeated, three times. As a negative control, was used the DMEM medium + 10% DMSO was used. Samples containing the HSV-1 were analyzed by real-time polymerase chain reaction (qPCR).

Quantitative Real-Time PCR Assay

Quantitative real-time PCR (qPCR) is used for the quantification of viral nucleic acids. In qPCR, the viral load is measured as the copy number per cell or percentage of total DNA by using a standard curve, generated using a dilution series of a DNA template, which is commonly generated from plasmid DNA or DNA oligonucleotides (Coelho *et al.*, 2015). Genomic DNAs including viral DNAs were isolated from the harvested cells using the MagNA Pure extractor (Roche, Basel, Switzerland) according to the manufacturer's instructions. The forward primer sequence for HSV-1 is 5′-TGGGACACATGCCTTCTTGG while the reverse sequence is 5′-ACCCTTAGTCAGACTCTGTTACTTACCC with amplicon size of 147 bp. For the Real-Time PCR, the 20 μL reaction mixture were added 12 μL of the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 μM of each primer, 6.5 μL H_2O, and 5 μL of cDNA. The assay was performed using the ABI 7300 Real-Time PCR Systems (Applied Biosystems, Foster City, USA) under the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 20 s, and 72 °C for 40 s. Standard curves were prepared by qPCR using serial dilution of the known copies number of purified amplification products for HSV-1. The copy number of the samples was calculated from the standard curves. Percentage of reduction was defined as [copy number of infected − copy number of treated]/copy number of infected] × 100. All experiments were made in triplicate.

Direct Electron Microscopy (DEM)

Vero cells infected with herpes virus at concentration of 10^{-8} and treated with virucide concentrations of ethanolic extract from red propolis (24, 48 or 96 μg/mL) for 1 hour at 4 °C were resuspended in 50 mL of phosphate buffered saline (PBS) (Coelho *et al.*, 2015) at pH 7.2. One drop of the suspension was put on an EM grid and submitted to a negative staining technique with 2% potassium phosphotungstate at pH 6.4. The viruses were documented in a JEM-1011 (JEOL, Japan) electron microscope.

Statistical Analysis

Statistical analysis was performed using Exstat software. For analyses of the cell viability data and the antiviral activity, the Student's T test was used with the *p*-value corrected by the Bonferroni– Sidak method.

RESULTS AND DISCUSSION

HPLC-PDA-ESI-MS/MS analysis of ethanolic extract from red propolis

The extraction yield was 3.75 g of dry extract per 30.00 g of red propolis sample. Reversed-phase HPLC method are very useful for the characterization and quality control of different types of propolis extracts

(Vecchi, dos Santos, Bruschi, 2023). Data of UV/VIS and mass spectra relevant for dereplication of constituents **1-16** found in ethanolic extract from red propolis are shown in Table I. The chromatography profile of ethanolic extract from red propolis is shown in Figure 1.

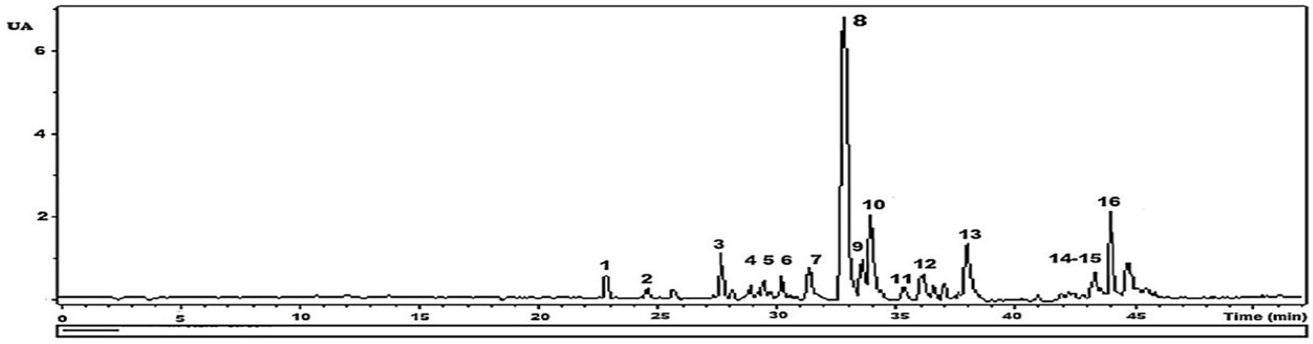

FIGURE 1 - Chromatography profile of ethanolic extract from red propolis. For peak assignments, see Table I.

The proposed structures for flavanones **1-3**; isoflavones **4-5** and **7**; chalcones **6**, **8** and **9**, pterocarpans **11-12**, isoflavans **10** and **13**, and polyprenylated benzophenones **14-16** were based on UV/VIS and MS data reported in literature (Table I), together the comparison using the MS/MS information found on-line in chemical databases.

TABLE I – HPLC-PAD-ESI-MS/MS data and proposed structure for compounds detected in red propolis produced by *Apis mellifera* from Maceio, state of Alagoas, Brazil

Comp. no	RT Min	UV $\lambda_{max - nm}$	HPLC-PAD-ESI-MS/MS *m/z* (% base peak) $[M - H]^-$	Proposed structure	References
1	22.8	289, 313sh	$[M - H]^-$ - 331 MS/MS – 313 (60), 177 (100)	3,3',5-trihydroxy-4',7-dimethoxy flavanone	Yen *et al*., 2012, Vieira de Moraes *et al*., 2021
2	24.5	289, 313sh	$[M - H]^-$ - 301 MS/MS – 283 (100), 177 (50)	3,3',5-trihydroxy-4'-methoxy flavanone	Yen *et al*., 2012, Vieira de Moraes *et al*., 2021
3	27.6	280, 314sh	$[M - H]^-$ - 255 MS/MS - 135	liquiritigenin – (4',7-dihydroxy flavanone)	Silva *et al*., 2020
4	28.6	249, 291sh	$[M - H]^-$ - 313	5,7-dihydroxy-3',4'-dimethoxy isoflavone	Vieira de Moraes *et al*., 2021
5	29.5	254	$[M - H]^-$ - 283	calycosin – (3'-hydroxy formononetin)	Vieira de Moraes *et al*., 2021, Silva *et al*., 2020, Bankova *et al*., 2019
6	30.2	300sh, 370	$[M - H]^-$ - 271 MS/MS - 151	2',3',4, 6'-tetrahydroxy chalcone	Ferreira *et al*., 2017

TABLE I – HPLC-PAD-ESI-MS/MS data and proposed structure for compounds detected in red propolis produced by *Apis mellifera* from Maceio, state of Alagoas, Brazil

Comp. no	RT Min	UV $\lambda_{max - nm}$	HPLC-PAD-ESI-MS/MS *m/z* (% base peak) $[M - H]^-$	Proposed structure	References
7	31.3	265	$[M - H]^-$ - 283	biochanin A – (5,7-dihydroxy-4'-methoxy isoflavone)	Silva *et al.*, 2020
8	32.8	300sh, 365	$[M - H]^-$ - 271 MS/MS – 135 (50), 109 (100)	2',3,4,4'-tetrahydroxy chalcone	Righi *et al.*, 2011
9	33.5	300sh, 370	$[M - H]^-$ - 255 MS/MS - 135	isoliquiritigenin – (4,2',4'-trihydroxy chalcone)	Vieira de Morais *et al.*, 2021 Silva *et al.*, 2020
10	33.9	290	$[M - H]^-$ - 271	vestitol – (2',7-dihydroxy-4'-methoxy isoflavan)	Silva *et al.*, 2020
11	35.3	290	$[M - H]^-$ - 255	demethyl medicarpin	Vieira de Morais *et al.*, 2021
12	36.5	290	$[M - H]^-$ - 283	homopterocarpin – (3,9-dimethoxy pterocarpan)	Silva et al., 2020 Vieira de Morais *et al.*, 2021
13	38.0	290	$[M - H]^-$ - 285 MS/MS – 149 (50), 123 (100)	7,3'-dihydroxy-4'-methoxy-8-methylflavan	Silva et al., 2020, Vieira de Moraes *et al.*, 2021

The proposed structure for flavonoids detected in ethanolic extract from red propolis were based on Retro–Diels–Alder (RDA) reactions, which play a key role in the identification of flavonoids and their derivatives (Zhang *et al.*, 2013, Zhao *et al.*, 2020). The Collision induced Dissociation (CID) of flavanones produce fragment ions resulting from RDA fragmentation, which can be more abundant than the loss of other radical ions, CH_3, CO, OH, or H_2O (Zhang *et al.*, 2013; Zhao *et al.*, 2020). The mass spectra of compounds **1** and **2** exhibited deprotonated ions of *m/z* 331 and *m/z* 301 and fragment ions of *m/z* 313 (60) and *m/z* 283 (100), respectively, corresponding to the loss of water. The B ring RDA fragment ion $[^{1,4}B - H]^-$ at *m/z* 177 $[C_{10}O_3H_9]^-$ observed in mass spectra of compound **1** and **2**, was attributed to fragmentation pathway that produce RDA cleavage from 1,4-position of the C-ring, as reported for polymethoxylated flavanones (Zhang *et al.*, 2013; Zhao *et al.*, 2020). Compounds **1** and **2** were assigned as 3',5,7-trihydroxy-4',6-dimethoxyflavanone **1** and hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone) **2**, respectively. The identification of hesperetin **2** was carried out through comparison of its mass spectrum with the mass spectra database MSBNK-RIKEN Respect – in PS078002 and PS078003.

The flavanone **3** and chalcone **9** exhibited similar mass spectra and were differentiated by their UV/VIS spectra. Liquiritigenin **3** (λ_{max} - 280 nm) and isoliquiritigenin **9** (λ_{max} - 370 nm) exhibited deprotonated ions $[M - H]^-$ of *m/z* 255 and yielded RDA $[^{1,3}A - H]^-$ fragment ion at *m/z* 135 $[C_7O_3H_3]^-$, as base peak (Vieira de Morais *et al.*, 2021; Silva *et al.*, 2020; Zhao *et al.*, 2020). 5,7-Dihydroxy-3',4'-dimethoxy isoflavone **4** exhibited deprotonated ion of *m/z* 313. Compounds **5** and **7** exhibited the same deprotonated ion of *m/z* 283 and based on retention time and UV/VIS maximum absorption approximately

at 260 nm (isoflavones) were assigned as calycosin **5** and biochanin A **7**, respectively (Vieira de Moraes *et al.*, 2021; Silva *et al.*, 2020).

Compounds **6**, **8** and **10** exhibited the same deprotonated ions of *m/z* 271. Compound **10** exhibited UV/VIS maximum absorption at 290 nm and was assigned as vestitol (2',7-dihydroxy-4'-methoxyisoflavan) (Righi *et al.*, 2011). The chalcones **6** and **8**, exhibited UV/VIS maximum absorption at 365 nm (band I, ring B, catechol absorption), respectively, attributed to the presence of OH in 4-position of the B-ring (Minsat *et al.*, 2021). 4,2',4',6'-Tetrahydroxy chalcone **6** produced base peak at *m/z* 151 obtained by RDA fragmentation $[^{1,3}A - H]^-$ from the loss of hydroxy vinyl benzene moiety (120 Da), consistent with the presence of OH group at the 4-position of the B-ring (Minsat *et al.*, 2021). 3,4,2′,4′-Tetrahydroxy chalcone **8** exhibited A-ring RDA $[^{1,3}A - H]^-$ fragment ion at *m/z* 135 (50%) $(C_7O_3H_3)^-$ and base peak at *m/z* 109 $(C_6O_2H_5)^-$ (Righi *et al.*, 2011, Minsat *et al.*, 2021).

7,3'-Dihydroxy-4'-methoxy-8-methylflavan **13** exhibited deprotonated ion of *m/z* 285, the B-ring RDA fragment $[^{1,3}B - H]^-$ at *m/z* 149 (50%) $(C_9O_2H_9)^-$ and base peak at *m/z* 123 $(C_7O_2H_7)^-$. The pterocarpans, demethyl medicarpin **11** and 3,9-dimethoxypterocarpan **12** exhibited UV/VIS maximum absorption at 290 nm and deprotonated ions of *m/z* 255 and *m/z* 283, respectively (Silva *et al.*, 2020; Vieira de Morais *et al.*, 2021).

In the retention time of 43.3 min was observed a co-elution of polyprenylated benzophenones **14** and **15**, as shown in Figure 1 and Table I. The polyprenylated benzophenone **14** exhibited deprotonated ion of *m/z* 601 and was assigned as guttiferone F. The unknown polyprenylated benzophenone **15** exhibited deprotonated ion of *m/z* 715 and possess more one prenyl group that guttiferone F and E, as occur in guttiferones C, D and B (Silva *et al.*, 2020). The polyprenylated benzophenone **16**, detected in the retention time of 44.0 min, exhibited deprotonated ion of *m/z* 599 and was suggested as a dimethyl weddellianone B derivative (Porto *et al.*, 2000). The presence of guttiferone F and others guttiferones were reported in ethanolic extracts of red propolis from Alagoas (Silva *et al.*, 2020). The presence of flavonoids, chalcones, pterocarpans and polyprenylated benzophenones in red propolis from Alagoas are corroborated by other authors (de Carvalho *et al.*, 2020; dos Santos *et al.*, 2021; Vieira de Morais *et al.*, 2021; Silva *et al.*, 2020; Reis *et al.*, 2020). The proposed structure for compounds **1 – 16**, common markers of red propolis collected in northeastern Brazil, are shown in Figure 2.

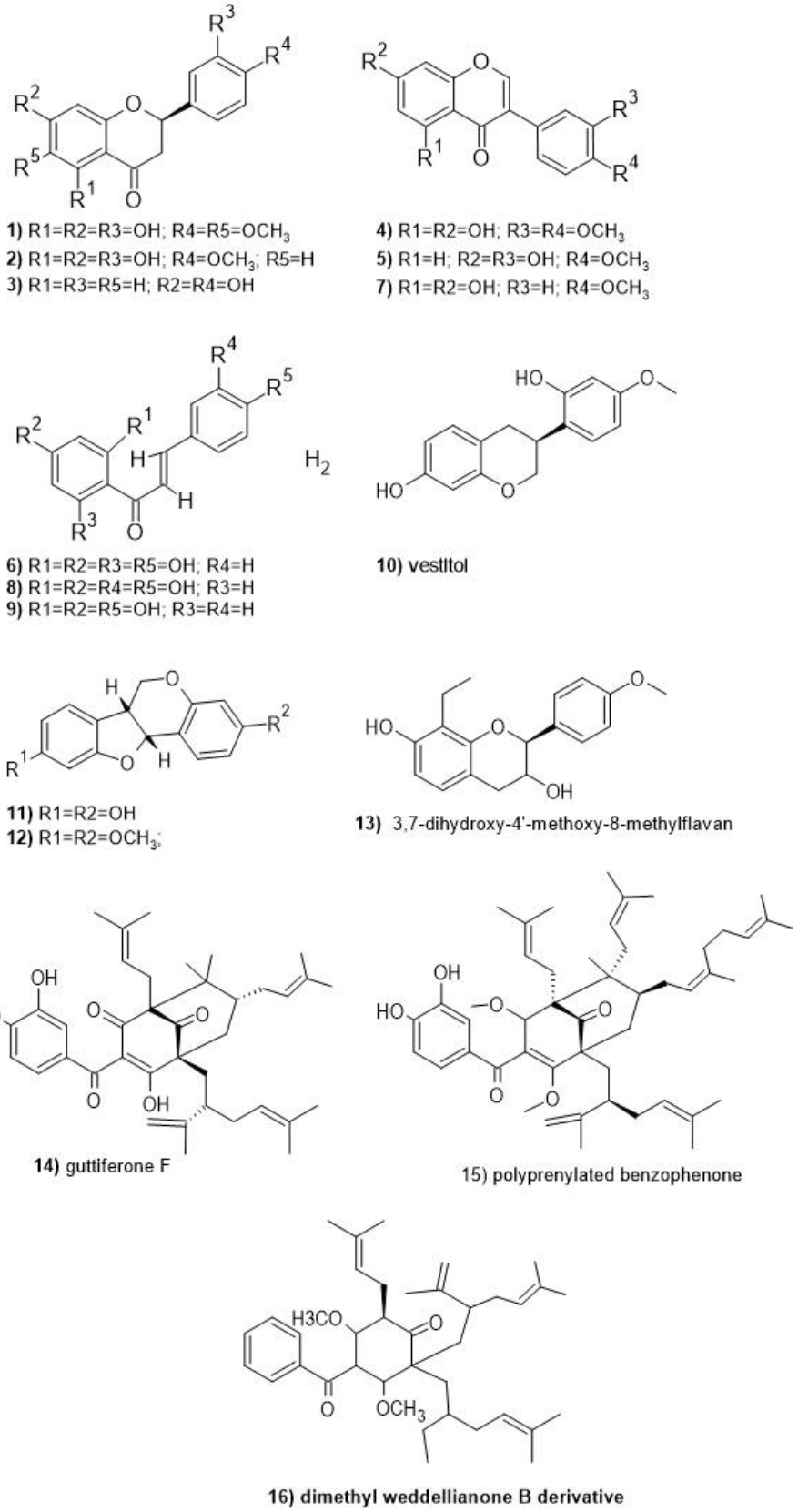

FIGURE 2 - The proposed structures for compounds **1-16** are based on MS data compared with data reported in literature.

Antiviral activity against herpes simplex virus-1

Vero Cells treated with ethanolic extracts from red propolis at concentration below 96 μg/mL did not present a reduction in cell viability (Figure 3) with CC50 value of 156 μg/mL. Therefore, 96 μg/mL was determined as the minimum cytotoxic concentration (MCC or maximum non-toxic dose). In a study carried by other authors, Brazilian red propolis was not toxic below the concentrations 750 μg/mL in a test of toxicity using *C. elegans* (Silva *et al*., 2022).

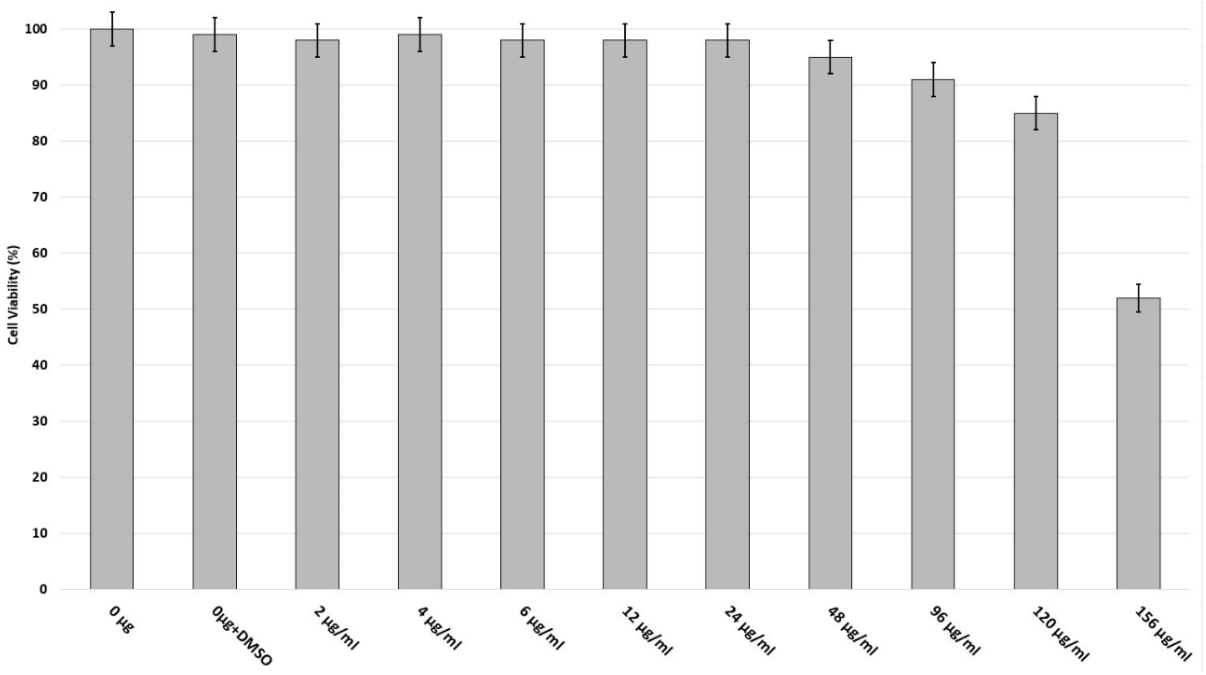

FIGURE 3 - Cell viability of Vero cells treated with different concentrations of ethanolic extracts from red propolis. The number represents the mean of three replicates.

Based on results obtained previously by our group to evaluate of the antiviral activity of propolis (Coelho *et al*., 2015, 2018), the antiviral activity of ethanolic extract from red propolis were carried out through three protocols, pre-treatment, posttreatment and virucide effect. The pre-treatment using concentrations of 8, 12, 24, 48, or 96 μg/mL were carried out at 3 h before infection, while the post treatment, using the same concentration, 1 h after infection. The results showed that these treatments were effective in reducing the level of infection. The virucide effect was evaluated at the concentrations of 24, 48 or 96 μg/mL. Cultures were observed daily for cytopathic effects, which was based on comparison with uninoculated cell cultures. The evaluation of cytopathic effect in Vero cell culture showed to be effective in inhibiting HSV-1 viral replication, and were not observed any cytopathological changes, such as aggregation, nuclear enlargement, or cell rounding, at red propolis concentrations of $\leq$ 96 μg/mL (data not shown), indicating inhibitory effect on the HSV-1 by reducing its CPE in infected Vero cells.

The quantification of viral DNA from HSV-1 were reduced in all conditions, and concentrations tested of ethanolic extract from red propolis (Figure 4), showing more than 85% of inhibition of viral replication with a concentration of 48 μg/mL. The pre-treatment was more effective than post-treatment against the HSV-1. The reduction of infection, when the ethanolic extract of red propolis was added to Vero cells 3 h before viral infection could indicate that the

constituents of extract acted on the cell surface or even intracellularly, impairing the viral cycle. The treatment conducted post-infection (post-treatment) also resulted in inhibitory effect of viral activity and could indicate that the constituents can pass through cell membranes and target the viral cycle's intracellular stages. Moreover, the compounds present in propolis extract could block viral attachment to cell receptors and consequent entrance in the host cell (Magnavacca *et al.*, 2022; Yosri *et al.*, 2021).

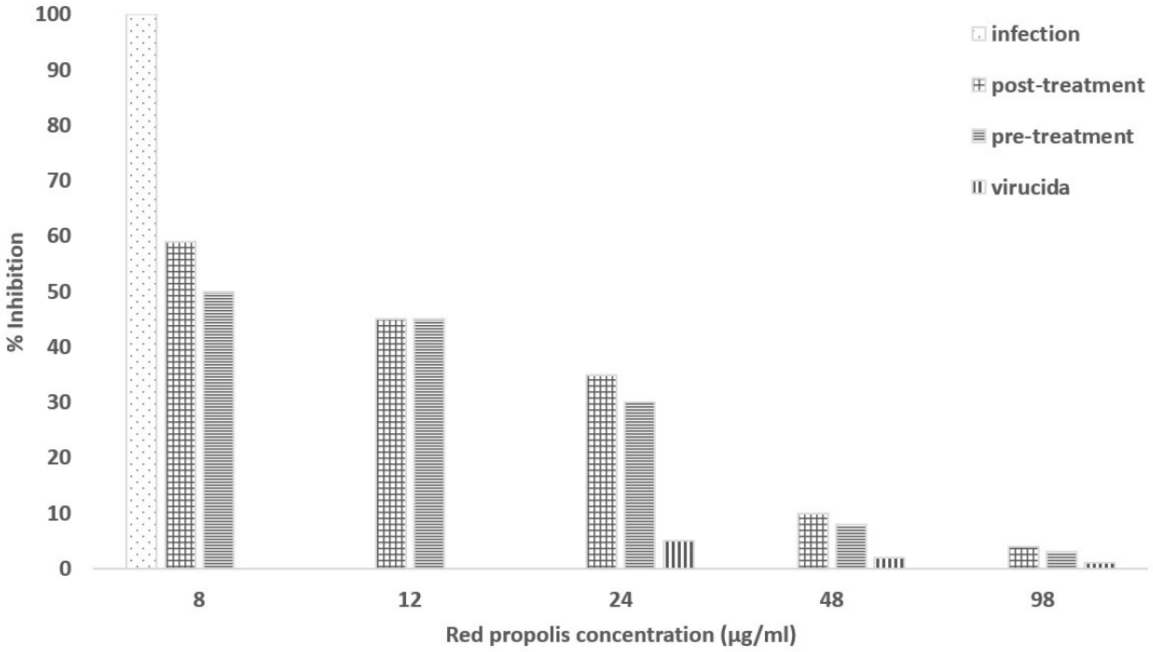

FIGURE 4 - Real-Time PCR (qPCR) of HSV-1 treated with ethanolic extracts from red propolis. Total DNA was performed at 3 hours before infection (pretreatment), 1 hour post infections (pos-treatment) and the virucide effect was evaluated at the concentrations 24, 48, or 96 μg/mL.

The images obtained through negative staining electron microscopy are shown in Figure 5. The virus particle or virion of HSV-1 on the surface of Vero cells treated with phosphate buffered saline (PBS) is shown in Figure 5A. For HSV-1 treated with ethanolic extract from red propolis (Figure 5B) were observed the lipid membrane degradation, i.e., the envelope integrity was disrupted on the surface of Vero cells, when compared with HSV-1 that was treated with PBS alone (Figure 5A). Thus, in the DEM was observed the rupture of the virus membrane, showing that the compounds could act directly on the viral envelope, through lipid membrane degradation and/or directly blocking the glycoproteins on the viral surface.

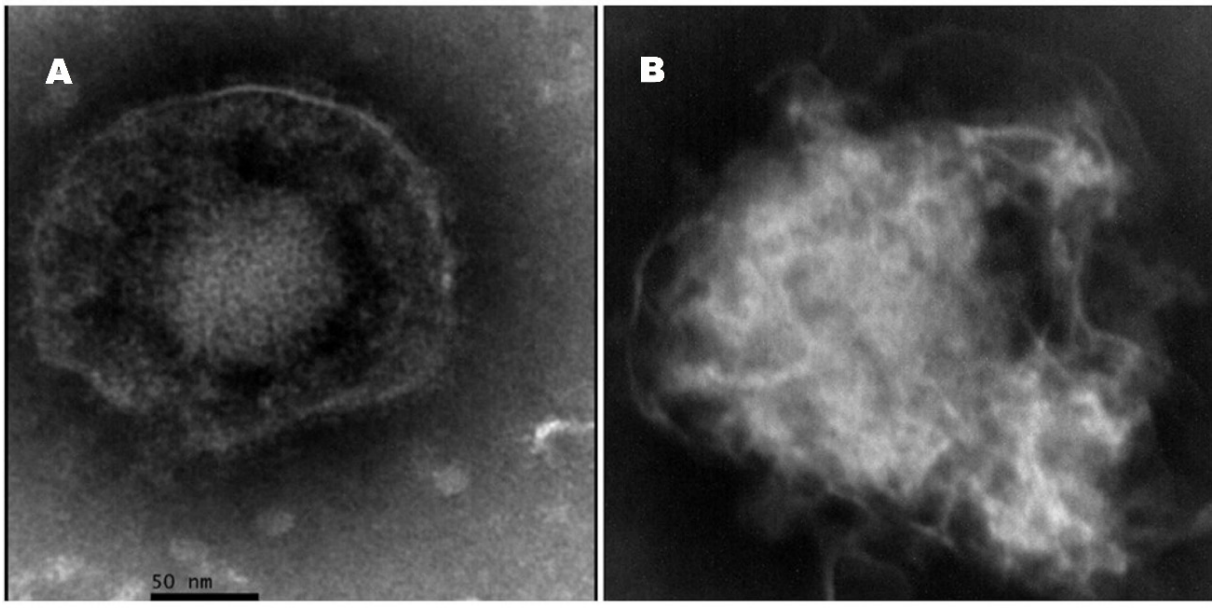

FIGURE 5 - The virus particle or virion of HSV-1 treated with phosphate buffered saline (PBS) (5A) and HSV-1 treated with red propolis (5B), where lipid membrane degradation is observed.

The first stage of HSV infections involves binding of the viral glycoproteins to a protein on the host cell surface (Montenegro-Landívar *et al*., 2021). The rupture of the virus membrane of HSV-1 treated with propolis was observed in this study and corroborated by other authors (Silva *et al*., 2020, Alkhalefa *et al*., 2022). The phenolic compounds present in extract of propolis can cause functional and structural damage to the bacterial cytoplasmic membrane or cell wall, interfering with glycoproteins of the viral envelope (Bankova *et al.,* 2014; Silva *et al*., 2020, Alkhalefa *et al*., 2022).

Antiviral agents can interrupt transcription or viral protein synthesis and replication of the viral genome, avoiding virus entry or absorption into the host cells, exhibiting an effect on cell receptors, and possibly also an effect at the viral adsorption stage (Demir *et al*., 2020). Acyclovir act as competitive inhibitors for nucleosides or nucleotides that are used by the viral DNA polymerases to transcribe the viral DNA chain, causing a highly selective inhibition of herpes virus DNA replication, however as described in introduction section, this antiviral drug possesses side effects (Poole, James, 2018). Propolis demonstrated antiviral activities on viral infectivity and replication, *in vitro* and *in vivo*, as well as the modulatory actions on cytokine production and immune cell activation as part of both innate and adaptive immune responses (Magnavacca *et al*., 2022; Silva-Beltrán *et al*., 2022; Zulhendri *et al*., 2021). As far as was known, propolis was not toxic below the concentrations 750 μg/mL and there are no reports about side effects of propolis (Silva *et al*., 2022, Alkhalefa *et al*., 2022). Other studies indicated that propolis can to disrupt the ability of the virus to invade the host cells by forming a physical barrier, can exerting their inhibitory action early in the viral infection cycle, mainly during virus absorption or host cell penetration inhibiting enzymes and proteins needed for invasion, through direct interaction with viral particles or can prevent infection by mimicking cellular proteins (Ripari *et al*., 2021; Magnavacca *et al*., 2022; Yosri *et al*., 2021, Montenegro-Landívar *et al*., 2021). The antifungal effect of Brazilian red propolis against the fungi *Paracoccidioides brasiliensis* was exerted through activation of neutrophils, preventing fungal dissemination, and controlling excessive inflammation process (Santos *et al*., 2021). Many authors reported the antiviral activity of propolis against *Vaccinia* virus, *Herpes Simplex* virus, *retroviruses* and *Influenza* virus, attributed to compounds present in propolis that exert its antiviral activity through their role in preventing

virus transmission to other cells, inhibiting virus propagation and causing structural damage to the bacterial cytoplasmic membrane or cell wall (Alkhalefa *et al*., 2022, Ripari *et al*., 2021; Magnavacca *et al*., 2022; Yosri *et al*., 2021, Montenegro-Landívar *et al*., 2021).

In this ethanolic extract of red propolis were found flavanones, pterocarpans, chalcones and polyprenylated benzophenones. Flavonoids are considered the main compounds responsible for the biological activity of propolis and exhibited potential antiviral activity against different DNA and RNA viruses, in different antiviral assays, *in vitro*, *in vivo* (mice model) and *in silico* (dos Santos *et al*., 2021; Silva *et al*. 2022, Cui *et al*., 2022). Chalcones exhibited antiviral activities on Middle East Respiratory Syndrome Coronavirus, Human Immunodeficiency, Influenza, Human Rhinovirus, Herpes Simplex, Dengue, Human Cytomegalovirus, Hepatitis B and C, Rift Valley Fever and Venezuelan Equine Encephalitis (Elkhalifa *et al*., 2021). Guttiferones belong to the polyprenylated benzophenone, a very restricted class of compounds, especially found in the Clusiaceae family and in the genus Garcinia and Symphonia. Studies indicate a low toxicity and a broad spectrum of pharmacological activities of these compounds, as anti-inflammatory, immunomodulatory, antioxidant, antitumor, antiparasitic, antiviral, and antimicrobial (Conceição *et al*., 2023).

Hesperetin (3',5,7-trihydroxy-4'-methoxy flavanone) showed great potential for binding with the molecular targets of SARS-CoV-2 (Sobrinho *et al*., 2022). In some studies, it was observed that the antiviral activity of individual substances isolated from propolis was not greater than of the total extract (Bankova *et al*., 2014, Demir *et al*., 2020). A mixture of different constituents presents in propolis extract is more effective, becoming possible that different antiviral mechanisms can occur, and this is not surprising if we take into consideration the high number of compounds that have been identified in propolis to date (de Carvalho *et al*., 2020; dos Santos *et al*., 2021; Vieira de Morais *et al*., 2021; Silva *et al*., 2020; Reis *et al*., 2020; Cui *et al*., 2022). The extracts contain many constituents with unknown and untested antiviral potential, and some of them could possess valuable activity against HSV-1.

CONCLUSION

The results found in the present study are in line with other reported studies about the chemical composition of red propolis and its antiviral activity. The ethanolic extract from red propolis showed more than 90% antiviral activity without affecting the cell viability. Results of cell viability and cell proliferation assays indicated that this extract was safe and not toxic to cultured Vero cells, based on their CC50 values. The results obtained by viral binding assay, antiviral assay, real-time PCR, and electron microscopy demonstrate that red propolis can be able to inhibit the production of infectious HSV-1 particles, which was significantly inhibited by pretreatment with ethanolic extract from red propolis prior to the infection, indicating that the compounds present in extract affected the virus before adsorption to cell surface. This extract inhibited the replication of a virus of great importance to public health; however, more studies are necessary to identify the mechanism of action and the main antiviral compounds.

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CONFLICTS OF INTEREST

No conflict of interest mentioned.

AUTHORS' CONTRIBUTIONS

GN, JMD-A, and ARTP contributed to the *Data curation*, *Formal analysis*, *Methodology*, original draft and revising the manuscript. CAF contributed to the *Formal analysis* and *Methodology*. FCPT and TFB contributed to English translation, *Formal analysis,* and *Investigation*. RZM contributed to the *Conceptualization, Methodology, Supervision, Validation,* original draft and revising the manuscript. All authors reviewed and approved the content of the manuscript.

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