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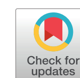
Original Article

Determination of phenolic profile by HPLC–ESI-MS/MS, antioxidant activity, *in vitro* cytotoxicity and anti-herpetic activity of propolis from the Brazilian native bee *Melipona quadrifasciata*



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A B S T R A C T

Propolis is a rich source of bioactive molecules made by bees by collecting an infinite variety of secondary metabolites from plants. This study determined the chromatographic and mass spectrometry profile along with the biological activity of propolis produced by the native Brazilian bee *Melipona quadrifasciata*. A hydroalcoholic crude extract was prepared and partitioned in solvents of different polarities, generating aqueous, dichloromethane, ethyl acetate, butanol and insoluble fractions. The phenolic and flavonoid content was also determined in crude aqueous and methanolic extracts as well. The antioxidant activity was determined by three different methods and correlated with the phenolic and flavonoid content. Extracts and fractions were tested against the herpes simplex virus type 1, and the cytotoxicity was evaluated in fibroblast L929 cells. The chromatographic and mass spectrometry analysis revealed the presence of catechin, epicatechin, aromadendrin, naringenin, pinocembrin and *p*-coumaric acid. The methanolic extract, ethyl acetate fraction and insoluble fraction possess higher phenolic and flavonoid content along with better antioxidant activities. The ethyl acetate and butanol fractions, along with the FM14 and FM45 subfractions demonstrated better viral inhibition, with 50% inhibitory concentrations of 90.5, 294, 188 and 58.5 $\mu g\,ml^{-1}$, respectively. Among these, the ones presenting the best selectivity index were the ethyl acetate and FM45 fractions, with a pronounced virucidal effect. The methanolic extract stood out in the antioxidant activities and showed no cytotoxicity bellow 125 $\mu g\,ml^{-1}$, exhibiting the most promising biological activity. Although this propolis seems to act in the early stages of herpetic infections, it is still difficult to correlate this activity with a single constituent of this complex mixture, suggesting a synergism in the extract components. To our knowledge, this is the first study of its kind with this propolis type.

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Introduction

Propolis is a resinous, complex and heterogeneous chemical substance produced by bees from exudates collected from various plants. For this reason it contains several secondary metabolites that depend on the local flora and generates different chemical types of propolis. Propolis is considered a rich source of bioactive molecules because it is very likely that even in areas never explored by scientists, that the bees have already found the best molecules with promising biological activities (Sforcin and Bankova, 2011; Aminimoghadamfarouj and Nematollahi, 2017). Because of this complexity, several therapeutic activities of propolis have been proven, including antibacterial, anti-tumor, anti-inflammatory, antioxidant and antiviral (Bankova et al., 2014a).

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Brazilian propolis are classified into thirteen different types, according to the predominance of the secondary metabolite class, which impart a characteristic coloration to each sample ranging between several shades of greenish brown to reddish (Toreti et al., 2013; Machado et al., 2016). The most common type in Brazil is the green propolis, which gained market preference due to its physicochemical characteristics, its pleasant smell and its color ranging from yellow-green to dark green (de Castro Ishida et al., 2011; Nunes and Guerreiro, 2012). Its main feature is the high content of cinnamic acid derivatives mainly containing prenylated groups, which may be present as esters, sesquiterpenes, diterpenes, pentacyclic triterpenes, as well as alcohols and aldehydes (Nunes and Guerreiro, 2012). Previously, the studied propolis was characterized by our group through HPLC–UV, ^{13}C NMR and ^{1}H NMR as a type of green propolis with nuances of yellow propolis. Its high content of phenolic compounds and flavonoids include catechin, epicatechin, aromadendrin, *p*-coumaric acid, naringenin, pinocembrin, quercetin, epigallocatechin, *p*-OH-benzoic acid, epigallocatechin gallate and coumaric acid (dos Santos et al., 2017).

One of the great challenges for medical application and better therapeutic use of propolis is the standardization of its minimum quality parameters. Thus, this study aimed to determine the phenolic profile of a hydroalcoholic crude extract (HCE) of propolis produced by *Melipona quadrifasciata* – a native Brazilian bee (not Africanized) – through HPLC–ESI-MS/MS, to evaluate the phenolic and flavonoid content obtained by using different solvents, and to examine its biological properties. The obtained extracts were then used to evaluate the antioxidant activity: reducing potential, inhibition of free radical DPPH and inhibition of lipid peroxidation. These results were correlated with phenolic and flavonoid content. It is important, when testing a new complex mixture, to also test its cytotoxicity. It is known that *in vitro* studies can minimize the use of animals and so far, only *in vivo* toxicity studies have been reported. For this test fibroblast cells (L929) were used to obtain a preliminary *in vitro* study of this propolis sample. Additionally, the antiviral activity against the herpes simplex-1 was measured. To the best of our knowledge, this is the first study of this kind with this propolis type.

Material and methods

Propolis extract preparation

The propolis sample from the native Brazilian bee species *Melipona quadrifasciata* was obtained in May of 2013 in the city of Blumenau, SC, Brazil at the university apiary (26°54′21.3″S 49°04′49.1″W). In order to obtain a hydroalcoholic crude extract (HCE), 284.3 g of propolis were pulverized and macerated in 70% ethanol (m/m), left in a dark chamber for 7 days at room temperature (20–25 °C), and then filtered under vacuum and dried in a rotary evaporator under reduced pressure. In order to increase the extract yield, this procedure was repeated three times on the same sample. HCE (50 g) were dissolved in 500 ml of water and submitted to liquid–liquid partitioning with 250 ml of different polarities solvents (dichloromethane, ethyl acetate and butanol). Five fractions were generated, namely, an aqueous fraction (AqF), a dichloromethane fraction (DiF), en ethyl acetate fraction (AcF), a butanol fraction (ButF), and an insoluble fraction (InF – solid residue after liquid–liquid partitioning). To obtain the aqueous crude extract (ACE), 100 g of the powdered original sample were added to 400 ml of ultrapure water, incubated in a water bath for 2 h at 60 °C, filtered and then lyophilized. To obtain a methanolic crude extract (MCE), the methodology described by Valencia et al. (2012) was used. Specifically, 100 g of the powdered original sample were macerated with 400 ml of methanol in a dark chamber for 3–4 days at room temperature (20–25 °C) with occasional stirring; the extract was then filtered and the solvent evaporated in a rotary evaporator at reduced pressure to complete dryness; finally the extract was washed three times with 200 ml of hexane in order to remove lipids.

Due to the better yield, an aliquot of 15 g of the dichloromethane fraction was subjected to silica gel column chromatography (17 × 6.5 cm column, silica gel 60–200 mesh Vetec®, Rio de Janeiro, Brazil), using as eluents hexane, ethyl acetate and methanol in an increasing polarity gradient, starting from 100% hexane to 100% ethyl acetate and finally 100% methanol. Fifty fractions of 125 ml each were collected and the solvent was recovered in a rotary evaporator. The fractions were analyzed by thin layer chromatography using hexane/ethyl acetate, pure chloroform and chloroform/methanol in several proportions with one drop of acetic acid as eluent. The plates were stained with sulfuric anisaldehyde and according to their chromatographic similarity; the fractions were combined resulting in twenty fractions. Of these fractions, only those with very distinct TLC profiles (FM6, FM9, FM14, FM24, FM34, FM45) were selected for the antiviral assays (Fig. 1).

Phenolic profile determination in the propolis HCE by HPLC–ESI-MS/MS

The HCE was analyzed by HPLC–ESI-MS/MS at the LabEC/INCT-Catalise laboratory (Graduate Program in Chemistry, Federal University of Santa Catarina, Florianopolis – UFSC). Analyses were conducted in an Agilent® 1200 (Agilent Technologies, Germany) liquid chromatograph system with a Phenomenex Synergi® 4μ Polar-RP 80A column (150 mm × 2 mm, particle size of 4 μm, stationary phase of ether-linked phenyl with polar end capping) at a temperature of 30 °C. The eluents were formed by mixing solvents A ($MeOH/H_2O$ 95:5, v/v) and B (H_2O/0.1% formic acid) as follows: 1st stage – 10% solvent A and 90% solvent B (isocratic mode) for 5 min; 2nd stage – linear gradient of solvents A and B (from 10 to 90% of A) for 2 min; 3rd stage – 90% solvent A and 10% B (isocratic mode) for 3 min; 4th stage – linear gradient of solvents A and B (from 90 to 10% of A) for 7 min with a flow rate of 250 μl min^{-1} of mobile phase. In all analyses, the injected sample volumes, prepared in 70% ethanol, was 5 μl. The liquid chromatograph was coupled to a mass spectrometry system consisting of a hybrid triple quadrupole/linear ion trap mass spectrometer Qtrap® 3200 (Applied Biosystems/MDS SCIEX, USA) with TurboIonSpray® as the ionization source in negative ionization mode with the following source parameters: ion spray interface at 400 °C; ion spray voltage of −4500 V; curtain gas, 10 psi; nebulizer gas, 45 psi; auxiliary gas, 45 psi; collision gas, medium. The Analyst® (version 1.5.1) software was used for recording and processing the data. Pairs of ions were monitored in Multiple Reaction Monitoring (MRM) mode. For compound identification, 45 standard phenolic and flavonoid compounds (4-aminobenzoic acid, salicylic acid, cinnamic acid, *p*-anisic acid, mandelic acid, vanillin, 4-hydroxymethylbenzoic acid, protocatechuic acid, umbelliferone, *p*-coumaric acid, methoxyphenylacetic acid, vanillic acid, gallic acid, 4-methylumbelliferone, coniferyl aldehyde, caffeic acid, syringaldehyde, scopoletin, ferulic acid, syringic acid, sinapaldehyde, sinapic acid, resveratrol, chrysin, pinocembrin, apigenin, galangin, naringenin, kaempferol, eriodictyol, aromadendrin, fustin, catechin, epicatechin, hispidulin, ellagic acid, quercetin, taxifolin, myricetin, carnosol, chlorogenic acid, rosmarinic acid, isoquercetin, naringenin and rutin) (Sigma®) were dissolved in methanol (1 mg l^{-1}) were analyzed in the same conditions as described above.

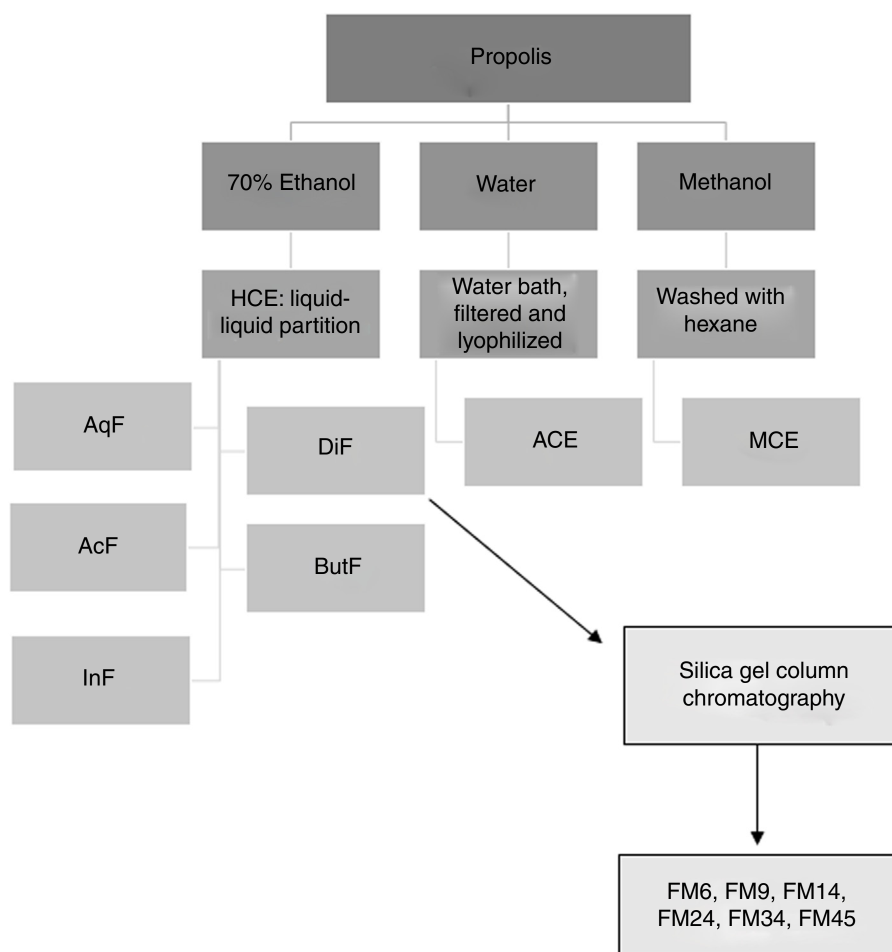

Fig. 1. Fractionation scheme of the *Melipona quadrifasciata* propolis raw extract.

Flavonoid and phenolic compound content

All samples were prepared in methanol solutions at a concentration of 8 mg ml^{-1}. The phenolic compound content was determined using the Folin–Ciocalteu based method described by Anagnostopoulou et al. (2006). The concentration of phenolic compounds in the samples was estimated by interpolation with a calibration curve ($y = 0.0064x - 0.1798$; $r^2 = 0.9935$) constructed with standard solutions of gallic acid (purity >98%, Sigma®) and expressed in milligrams of gallic acid equivalents per gram of dry extract (GA mg g^{-1}). Three replicates were performed for each test. The flavonoid content was determined by the method of Woisky and Salatino (1998). The flavonoid content in the samples was determined by interpolation with a calibration curve ($y = 0.0048x + 0.0121$, $r^2 = 0.997$) constructed with standard solutions of quercetin (purity >98%, Sigma®) and expressed in milligrams of quercetin equivalents per gram of the dry extract (quercetin mg g^{-1}). Three replicates were performed for each test.

Antioxidant activity evaluation

For the antioxidant testing, all samples were prepared in methanol solutions at a concentration of 8 mg ml^{-1}.

Reducing potential: This assay is based on the Price and Butler (1977) method, with adaptations proposed by Waterman and Mole (1994). The reducing potential was estimated by interpolation with a calibration curve ($y = 0.0026x + 0.1708$; $r^2 = 0.9994$) constructed with standard solutions of ascorbic acid (purity >98%, Vetec®), expressed in mg of ascorbic acid equivalent per 100 g of sample (mg 100 g AA^{-1}). Three replicates were performed.

Inhibition of the free radical DPPH: The assay is based on the method of Cavin et al. (1998). Serial dilutions were taken with the extracts and fractions in methanol, and then 2 ml of the DPPH solution was added to the tubes, measuring the absorbances at 517 nm for each sample at different concentrations. The values were plotted in a graph expressed as percentage (%) of absorbance increase as a function of the concentration of the test sample. The Microcal Origin® (version 5.0) software was used to determine the concentration required to reduce the radical DPPH by 50% (IC_{50}) in the solutions.

Inhibition of lipid peroxidation: Using the Mokbel and Hashinaga (2006) methodology, the inhibition of lipid peroxidation was evaluated with the β-carotene-linoleic acid model. An emulsion was prepared with 3 mg of β-carotene, 1 ml of chloroform, 45 mg of linoleic acid and 215 mg Tween-80 for testing. To the resulting mixture, 6 ml of distilled water were added while stirring to form an emulsion. This in turn was poured into a volumetric flask and filled to 100 ml with 0.01 M hydrogen peroxide. An emulsion aliquot (4 ml) was added to the tubes containing 0.2 ml of each sample. A positive control was performed using a butylated hydroxytoluene (BHT) solution. The negative control was performed with a mixture of 4 ml of the emulsion mentioned above and 0.2 ml of methanol. To calibrate the test, an emulsion prepared as described above but without the presence of β-carotene was used. The absorbance of

the solutions was determined at 470 nm at time zero and every 30 min up to 180 min. The antioxidant activity (potential inhibitor of lipid peroxidation in percentage), represented by AA, was calculated using the equation $AA = 100 \times [1 - (A0 - At)/(A00 - A0t)]$, where A0 = absorbance of the sample at time zero, At = sample absorbance after 180 min, A00 = absorbance of the negative control at time zero, and A0t = absorbance of the negative control after 180 min.

Cytotoxicity assay: Stock cells of the mouse fibroblasts L929 cell line were cultured at 37 °C and 5% CO_2 in a 96-well cell culture microplate. Each well was filled with 100 µl of culture media. In the first well of each row, 100 µl of propolis extracts at a concentration of 4 mg ml^{-1} previously diluted in dimethyl sulfoxide (DMSO) (a final DMSO concentration of less than 3% in the well) and culture medium was added. Then, a serial dilution of the propolis extracts for the other cavities was made. Finally, 100 µl of a suspension containing L929 cells was dispensed in order to obtain a concentration of 2×10^4 cells per cavity. The cells were precounted in a Neubauer chamber and cell viability was confirmed using the Trypan blue dye. A line of positive control (only cells) and blank (only culture medium) were carried out. Twenty-four hours after the incubation, the wells were washed three times with culture medium containing fetal bovine serum and subsequently 100 µl of a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma®) 5 mg ml^{-1} was added and the plates incubated for 4 h. After this period, the ability of cells to capture and reduce MTT to form formazan crystals was assessed. The crystals were then diluted with a solution of sodium dodecyl sulfate (SDS) 10% and *N*,*N*-dimethylformamide (DMF) 50%, and after 30 min of soft stirring the absorbance of the solutions were measured in a microplate UV spectrophotometer at 570 nm (Mosmann, 1983; Li et al., 2011). Eight replicates were performed for each sample. The results were expressed in percentage of cell viability relative to the control according to the following equation (Lu et al., 2014): Cell viability = $(SA - BA)/(CA - BA) \times 100$; where SA = sample absorbance, BA = blank absorbance, CA = cell growth control absorbance.

Statistical analysis

The statistical analyses were performed using the least significant difference test (LSD) and the Statistica® (version 7) software (Statsoft Inc.). *p* values of less than 0.05 were considered statistically significant at a 95% confidence level.

Antiviral assay

Cells and viruses: Vero cells (African green monkey kidney epithelial cells, ATCC CCL-81) were cultured in DMEM (*Invitrogen-Gibco, USA) supplemented with 10% fetal bovine serum (*), 2 mM glutamine (Sigma Chem Co., USA) and treated with 100 µg ml^{-1} streptomycin (Gibco BRL, USA), 100 IU ml^{-1} penicillin (Novafarma Ind. Farm., BR) and amphotericin B 2.5 µg ml^{-1} (Meizler Biopharma S/A, BR). The cell cultures were maintained at 37 °C with a 5% CO_2 tension until the cell monolayer was obtained. The HSV-1 (strain KOS) was provided by the LITDFC/DBS/UEM, Maringá, PR, Brazil. The viral stock was obtained by inoculation in Vero cells until the cytopathic effect was observed in about 90% of the cells and then aliquoted with 10% glycerol and kept at −20 °C.

Cytotoxicity over the viral cell culture substrate: The toxicity of the crude extract and propolis fractions on Vero cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Mosmann, 1983; Sieuwerts et al., 1995), with minor modifications. The cells were cultured in 96-well microplates (TPP, Switzerland) at 37 °C, 5% CO_2 for 24 h until 70% of the cell monolayer were formed. Several concentrations of the extracts (4–0.125 mg ml^{-1}) were added to the cells and incubated under the same conditions during 72 h. After that period, cell viability was assessed by adding 10 µl of the MTT reagent followed by further incubation for 3 h at 37 °C, 5% CO_2. Then, the MTT diluent (90 µl) was added under constant stirring for 15 min. The absorbance was read in a spectrophotometer at wavelengths of 570 and 690 nm and the percentage of cell viability (% VC) was calculated by the formula: $\% VC = (At_{570\,nm} - At_{690\,nm})/(Acc_{570\,nm} - Acc_{690\,nm}) \times 100$, where At and Acc refer to the absorbances of test substance and cell control, respectively. The cytotoxic was assessed and expressed as CC_{50} (concentration that reduced the absorbance of treated cells by 50% when compared to the cell control – untreated cells). The CC_{50} values were used for screening antiherpetic activity of the extracts.

Antiviral activity: The antiviral assays were based upon cell viability also using the MTT method as reported by Takeuchi et al. (1991), with minor modifications. Vero cells (10^5 cells ml^{-1}) were cultured in 96-well microplates for 24 h at 37 °C and 5% CO_2 until obtaining 70–80% confluence. The medium was removed and 100 µl of a mix containing HSV-1 (MOI of 1) plus different concentrations of extracts were simultaneously added to the cells. Cell (CC) and viral (VC) controls were performed by adding 100 µl of DMEM or viral suspension, respectively. The microplates were incubated for 72 h, under the same conditions of temperature and CO_2, with subsequent addition of the MTT reagent and its diluent. The percentage of viral inhibition was calculated by the formula: $\%VI = [(At_{570\,nm} - At_{690\,nm}) - (Avc_{570\,nm} - Avc_{690\,nm})/(Acc_{570\,nm} - Acc_{690\,nm}) - (Avc_{570\,nm} - Avc_{690\,nm})] \times 100$; where At – absorbance of virus-inoculated cells and treated with test substance; Avc – absorbance of VC; and Acc – absorbance of the CC. The IC_{50} 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 calculated from the CC_{50}/IC_{50} ratio and the compounds that presented values greater than 4 were investigated for virucidal and adsorption inhibition action (Lyu et al., 2005; Sidwell, 1986). The medicine Acyclovir (Zynvir – Novafarma Ind. Farmac., BR, 10 µg ml^{-1}) was used as positive control for HSV-1.

Determination of extracellular virucidal assay: The test was performed according to Faccin-Galhardi et al. (2012) with some modifications. Briefly, Vero cells (10^5 cells ml^{-1}) were grown in 24-well plates (TPP, Switzerland) by 48 h, at 37 °C, 5% CO_2 until confluence. The HSV suspension (10^4 UFP ml^{-1}) was preincubated (v/v) with varying concentrations of fractions (25, 50, 100 and 200 µg ml^{-1} for AcF and 50, 100, 200 and 290 µg ml^{-1} for FM45), at 37 °C for 1 h. A 10-fold dilution from suspensions were inoculated (100 µl) in monolayer Vero cells, with new incubation under 5% CO_2, at 37 °C for more 1 h, followed by a plaque reduction assay (PRA). For the PRA, the incubating solution was discarded and cell cultures were overlaid with nutrient agarose (DMEM 2×/1.8% agarose [v/v]) containing 25 mM $MgCl_2$ and incubated for 72 h at 37 °C. Cells were fixed with 10% formaldehyde PBS, pH 7.3, and stained with 0.5% crystal violet in 20% ethanol for counting the plaque forming units (PFU). The percentage of viral inhibition (%VI) was calculated by the following formula $\%VI = 1 - [(\text{PFU in treated cells}/\text{PFU in virus control})] \times 100$. The 50% inhibitory concentration (IC_{50}) was determined as the concentration of fractions capable of reducing the number of PFU in 50%.

Adsorption inhibition assay: The monolayer Vero cells were preincubated at 4 °C for 30 min, followed by viral infection (10^5 PFU ml^{-1}) and simultaneously submitted to varying concentrations of the fractions as described before. After 1 h of adsorption at 4 °C, the cells were washed with PBS and then PRA was performed (Lopes et al., 2017).

Statistical analysis: CC_{50} and IC_{50} values were obtained from linear regression analysis of concentration-effect curves. One-way

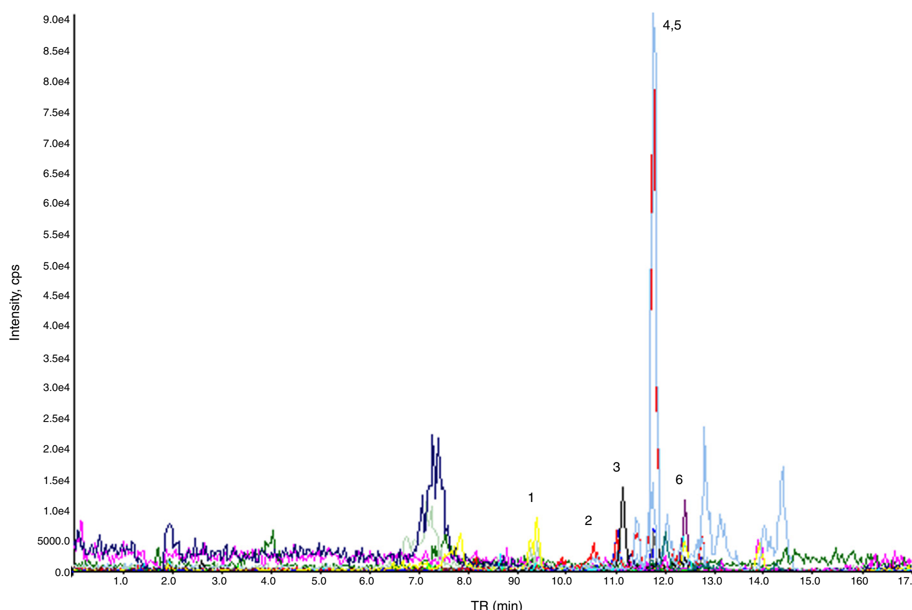

Fig. 2. HPLC–ESI-MS/MS chromatogram of phenolic and flavonoid compounds identified in the hydroalcoholic and aqueous extracts.

analysis of variance (ANOVA) was used to determine statistical differences followed by Tukey's multiple comparison. Statistical significance was set at $p < 0.05$.

Results and discussion

The HPLC/MS–MS technique is very useful to study high molecular weight compounds along with other nonvolatile and labile molecules. In this technique, liquid chromatography can be coupled to two mass spectrometers (MS–MS). When the ion of interest is isolated and compared to the other ions, which were generated during the induced fragmentation, the interference of the other ions contained in the matrix is minimized (Chiaradia et al., 2008). Thus, its sensitivity and reliability becomes sufficient to draw the profile of phenolic compounds and flavonoids of a sample.

The propolis HCE fingerprint (Fig. 2) was subjected to analysis by HPLC–ESI-MS/MS and comparing the molecular ion, obtained by MRM, to commercially available standards identified the compounds. Table 1 summarizes the six compounds identified in our propolis sample. This technique revealed the presence of the flavonoids catechin, epicatechin, aromadendrin, naringenin, pinocembrin and the phenolic *p*-coumaric acid.

Although the compounds found in this propolis sample are quite common and were reported in several types of propolis sourced from different parts of the world, it is important to highlight that each propolis chemical profile should always be determined. It should be kept in mind that each propolis type possesses singular features and, once its major components are determined, the particular propolis type can be directed to specific therapeutic indications, such as anti-inflammatory, antioxidant, and antibacterial, among others.

In addition to the inherent ability of flavonoids to block free radicals, some of them still have other specific activities. To illustrate, aromadendrin is a pharmaceutically important plant-based flavonoid with anti-inflammatory and anticancer activity (Malla et al., 2012). Pinocembrin is a flavanone commonly isolated from a range of sources including bee honey. Its pharmacological activities are quite versatile, presenting anti-tumor, antimicrobial and neuroprotective effects (Rasul et al., 2013).

Success in obtaining active compounds from the raw material depends on the solvent employed in the extraction process. Since the biologically active components of propolis are mainly polyphenolic compounds, aromatics and flavonoids (Park et al., 2015), the use of higher polarity solvents is a good way to achieve better yields, resulting in better antioxidant activities. Based upon this, three different extraction methods with different solvents were employed, and the results of the flavonoid and phenolic compound content obtained from each extraction are shown in Table 2.

Our results support the higher polarity solvent assertion, since the methanol extraction yielded phenolic and flavonoid compound levels significantly higher ($p < 0.05$) compared to water and 70% ethanol extraction. Propolis pretreatment employing non-polar solvents like hexane or enzyme lipase is also used by some authors who intend to remove lipids and waxes in order to achieve better yields of bioactive compounds and a significant increase in antioxidant activity (Park et al., 2015). This effect can be clearly observed in the MCE, which was washed with hexane and 5% (m/m) of lipids

Table 1
Phenolic and flavonoid compounds identified in the hydroalcoholic crude extract (HCE) of propolis from *Melipona quadrifasciata*.

Compound	RT (min)	Calculated mass (M)	Experimental mass $[M+H]^+$	MS/MS (*m/z*)
Aromadendrin (1)	9.39	288.25	287.00	125.00
p-Coumaric acid (2)	10.24	164.04	162.92	119.10
Naringenin (3)	11.02	272.25	270.98	151.00
Catechin (4)	8.57	290.26	289.04	109.00
Epicatechin (5)	8.57	290.26	289.04	109.00
Pinocembrin (6)	12.41	256.25	255.05	65.00

RT, retention time; MS/MS, Tanden mass spectometry

Table 2
Phenolic compound content (in milligrams of gallic acid equivalents per gram of extract) and flavonoid content (in milligrams of quercetin equivalents per gram of extract) in methanolic, aqueous and hydroalcoholic crude extracts, and fractions of hydroalcoholic propolis extract.

	Phenolic compounds(mg GA g^{-1})	Flavonoids(mg QUE g^{-1})
MCE	57.53 ± 0.2^{a}	8.48 ± 0.3^{a}
ACE	11.96 ± 1.5^{b}	3.67 ± 0.3^{b}
HCE	4.87 ± 0.2^{c}	3.99 ± 0.4^{b}
AqF	Not detected	0.78 ± 0.2
DiF	8.10 ± 0.3	2.20 ± 0.3
AcF	64.93 ± 0.6	4.23 ± 0.5

Among the crude extracts, the same letters in a given column indicate no significant difference ($p > 0.05$); 95% confidence level. MCE, methanolic crude extract; ACE, aqueous crude extract; HCE, hydroalcoholic crude extract; AqF, aqueous fraction; DiF, dichloromethane fraction; AcF, ethyl acetate fraction; ButF, butanol fraction; InF, insoluble fraction.

was removed, resulting in a higher concentration of the compounds of interest. Among the water and 70% ethanol extractions, the phenolic compound concentration was significantly higher ($p < 0.05$) in the first method and was not significantly different ($p > 0.05$) for the flavonoid content. When carrying out the fractionation of HCE, the ButF and AcF fractions stood out, demonstrating that polar solvents can concentrate flavonoids and phenolic compounds in the sample. The InF fraction also gathered a considerable amount of phenolics and flavonoids, but they seemed to be hydrolysable tannins, due to their low solubility in various solvents.

The antioxidant activity of the propolis sample was verified by three different methods: the reducing potential, *i.e.*, the ability of the molecule to donate electrons to a free radical; the DPPH assay, which evaluates the concentration of the extracts and fractions required to inhibit 50% (IC_{50}) of free radicals contained in the solution; and the lipid peroxidation assay, in which the sample's ability to inhibit the action of the free radicals over β-carotene is evaluated. The results are shown in Table 3.

The reducing potential was evaluated by the equivalent of ascorbic acid per 100 g of sample, which means that the action of the molecules in the sample at a concentration of 8 mg ml^{-1} can act in the same way as ascorbic acid molecules in preventing the spread of a free radical cascade.

The best results were achieved by the AcF fraction (127.83 ± 1.45 mg 100 g AA^{-1}), followed by the MCE (117.19 ± 2.77 mg 100 g AA^{-1}) and the InF fractions (111.93 ± 1.45 mg AA 100 g^{-1}), which showed higher activity compared to ascorbic acid. Many authors have found stronger antioxidant activity in propolis extracts than that seen with well-recognized antioxidants such as vitamins C and E. Therefore, our results are in agreement with these previous studies (Banskota et al., 2000).

In the DPPH assay, the MCE fraction demonstrated the best ability to capture free radicals in solution ($IC_{50\%}$ 151.37 ± 7.92 mg l^{-1}), followed by the AcF, InF and ButF fractions. For the other fractions, the values found exceeded the limits of sensitivity of this test (1 mg ml^{-1}). A key reaction factor between the DPPH radical and donor electrons is the steric accessibility of the radical. Small molecules have greater access to the radical site than large molecules (Alves et al., 2010). This may explain the lower performance of certain fractions such as the DiF, since this fraction contains non-polar molecules with higher molecular weight, such as terpenes. This is a particular feature of green propolis, which may not have the same steric accessibility.

In the lipid peroxidation assay with propolis crude extracts and fractions, all of them showed a statistically different activity ($p < 0.05$) compared to the positive control (BHT), but among the samples tested, the MCE, HCE and AcF fractions stood out. Between the MCE and AcF fractions there was no significant difference, as well as between the HCE and AcF fractions ($p > 0.05$). The AqF fraction showed a negative result since its performance was worse than that of the negative control, which is a reflection of the low values of phenolic compounds and flavonoids found in this fraction. The decrease of activity of the extracts and fractions *versus* time can be seen in Fig. 3.

The lipid peroxidation process is responsible mainly for the degradation of phospholipids of the cell membranes of different biological systems and of the fatty acids contained in food. The ability of propolis to inhibit the lipid peroxidation directly reflects the ability of the molecules contained in the samples to prevent the damage of excess free radicals formed during intense physical activity, for instance (Alves et al., 2010).

Currently, propolis has been widely used for manufacturing healthy drinks and foods, and to prevent inflammatory diseases, heart diseases, diabetes and even cancer, which are known to be linked to the imbalance between the production and neutralization of free radicals (Banskota et al., 2001; López et al., 2013).

In order to analyze the interference of the phenolic and flavonoid contents with the reducing potential, the sequestration potential of the free radical DPPH and the inhibition of lipid peroxidation were graphically correlated. The results can be viewed in Fig. 4.

Among the analyzed parameters, the phenolic compound content and the reducing potential of the samples ($R^2 = 0.895$) showed a good correlation, confirming that the samples with higher phenolic content can more effectively reduce the ferric ions to their ferrous state. For the other parameters, there was a low correlation with the flavonoid and phenolic compound content. This may be due to the fact that green propolis is mainly composed of terpene compounds. These compounds have a functional isoprene unit with π bonds that may also contribute to the antioxidant (González-Burgos and Gómez-Serranillos, 2012); sharing this task with phenolic and flavonoid compounds. According to Banskota et al. (2000), the antioxidant activity of aqueous and hydroalcoholic propolis extract is mainly due to the presence of caffeic

Table 3
Reducing potential test results (in milligrams of ascorbic acid equivalents per 100 grams of extract), IC_{50} DPPH inhibition assay, and lipid peroxidation assay of propolis crude extracts and fractions.

Fraction	Reducing potential(mg AA 100 g^{-1})	DPPH IC_{50}(μg ml^{-1})	Lipid peroxidation (%)
MCE	117.19 ± 2.8^{a}	151.37 ± 7.9^{a}	38.48 ± 0.6^{a}
ACE	33.81 ± 5.0^{b}	>1000^{b}	9.85 ± 1.0^{b}
HCE	38.08 ± 0.8^{b}	>1000^{b}	32.50 ± 4.0^{c}
AqF	16.29 ± 1.0	>1000^{b}	−10.00 ± 1.0^{d}
DiF	58.34 ± 2.7^{d}	>1000^{b}	28.37 ± 0.5^{e}
AcF	127.83 ± 1.4^{e}	311.47 ± 8.2^{c}	35.53 ± 0.2^{a,c}
ButF	49.62 ± 0.8^{f}	588.8 ± 0.0^{d}	14.57 ± 4.7^{g}
InF	111.93 ± 1.5^{g}	489.8 ± 0.0^{e}	22.91 ± 3.9^{h}

The same letters in a given column indicate no significant difference ($p > 0.05$); 95% confidence level. AA, ascorbic acid; DPPH, 2-2'-diphenyl-1-picryhydrazyl; IC_{50}, inhibitory concentration of 50%; MCE, methanolic crude extract; ACE, aqueous crude extract; HCE, hydroalcoholic crude extract; AqF, aqueous fraction; DiF, dichloromethane fraction; AcF, ethyl acetate fraction; ButF, butanol fraction; InF, insoluble fraction.

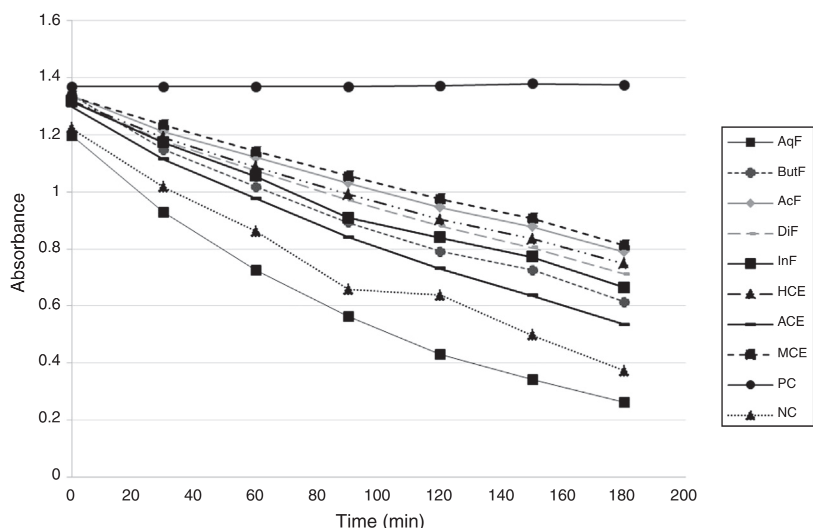

Fig. 3. Decrease of the lipid peroxidation inhibition of the propolis crude extracts and fractions as a function of time.

Fig. 4. Correlation between the phenolic and flavonoid content with the reducing potential, inhibition of DPPH and inhibition of lipid peroxidation of the samples along with their respective R^2.

and cinnamic acid derivatives, respectively, while the methanolic extracts mainly concentrate flavonoids and derivatives of cinnamic acid.

In the face of the diversity of molecules found in propolis, it is difficult to determine precisely which ones are mainly responsible for antioxidant activity. Therefore more studies are needed to confirm this correlation.

Cytotoxicity and biocompatibility tests simulate biological reactions to materials and molecules when they are placed in contact with cells and body tissues. The use of cell lines can predict whether

a molecule might be toxic to the body, since it is possible to measure how much the cell metabolism was affected by the tested material. Currently, in light of the establishment of ethics committees for the use of animals in research, cell culture is the main alternative model for the replacement of animal experiments (Alves and Guimarães, 2010). Although tests assessing cytotoxicity *in vitro* may not have an absolute correlation with *in vivo* tests, if a material induces a proven cytotoxic reaction in cell culture tests, it is very likely that it will develop toxicity when applied to living tissue (Martins et al., 2009).

We have used the cytotoxicity assay with mouse fibroblast L929 cell line, as they are cells that adhere to the microplate bottom, have easier growth and present a good correlation with human cells. The cells put in contact with different concentrations of extracts and fractions of propolis had their viability assessed after 24 h by reaction with the MTT reagent. The results were expressed in percentage of cell viability compared to cell growth control (CGC), and can be seen in Table 4.

When analyzing the data presented in Table 4, it becomes clear that the HCE, MCE, ACE, DiF and AcF fractions at 1000 mg l^{-1} were significantly different from the CGC ($p < 0.05$), demonstrating a marked cytotoxicity with a sharp decay of cell viability. An experimental detail can be observed between the first dilutions of the HCE and DiF fractions. The resin contained in these samples deposited on the plate bottom and seemed to have a cytotoxic effect at the concentrations of 500 and 250 mg ml^{-1}, but this effect was absent after the fourth sample dilution.

The HCE and AcF fractions showed some cytotoxicity, albeit slight, even at the lowest concentration tested (1.9 mg ml^{-1}). The ACE fraction presented significantly reduced cytotoxicity only in the concentration of 1.9 mg ml^{-1} and the DiF fraction, only at 3.9 mg ml^{-1}; no longer presented a significant difference when compared to the CGC.

The AqF fraction showed no cytotoxicity vis-à-vis the L929 cells, since even at the highest concentration tested it did not differ significantly from the positive control ($p > 0.05$). The MCE fraction showed cytotoxicity up to the concentration of 250 mg ml^{-1} and at the concentration of 125 mg ml^{-1}, it showed no significant difference from the CGC. The ButF fraction showed a significant difference from the positive control ($p < 0.05$), but curiously, this difference is toward a slight increase in cell proliferation. This difference ceased to be significant ($p > 0.05$) beyond a concentration of 125 mg ml^{-1}. These results lay the foundation for future investigations of the wound healing potential of this fraction.

Generally propolis can be considered safe in low doses, but allergic reactions may be common (Groot, 2013). Due to the wide range of propolis compositions found all over the world, it is necessary to investigate the toxicity of each sample individually as generalizations can lead to confusion. However, studies with the cytotoxic effect of propolis extracts usually involve the use of animals and previous research with *in vitro* models is scarce.

Comparing our results with the ones found in da Silva et al. (2015) with *in vivo* models, one can see that there is a considerable difference in toxic doses, as *in vivo* assays tolerate higher doses of propolis. On the other hand, if a given sample concentration induces toxicity; it can in turn be investigated for its anti-tumor potential. In this way, these studies are considered preliminary studies and further research with tumor cell lines should be undertaken.

Antiherpetic activity

We evaluated whether the propolis extract would or would not be cytotoxicity over the cell line used for virus culture (Vero cells). The substance with the highest cytotoxicity was FM09, with a CC_{50} of 88.4 μg ml^{-1}, followed by HCE (143.7 μg ml^{-1}), FM14 (225 μg ml^{-1}), DiF (272.5 μg ml^{-1}), FM45 (290 μg ml^{-1}), FM34 (300 μg ml^{-1}), ButF (350 μg ml^{-1}), FM06 (380 μg ml^{-1}), AcF (420 μg ml^{-1}) and FM24 (472.5 μg ml^{-1}). The least cytotoxic fraction was AqF with a CC_{50} of 1240 μg ml^{-1}. The ethanol control used in the test did not present cytotoxicity.

The anti-HSV-1 effect of all tested compounds was carried out using the MTT method, in the simultaneous treatment. The percentages of viral inhibition (%VI) of the crude propolis extract and their respective fractions against HSV-1, at CC_{50} values, are shown in Fig. 5. The best %VIs were those of AcF, ButF, FM14 and FM45 with 64.3%, 57.7%, 69% and 68.4% of inhibition, respectively. The other extracts weakly inhibited HSV infection, presenting %VI <50%.

For the fractions AcF and ButF along with compounds FM14 and FM45 it was possible to determine the IC_{50} values of 90.5 μg ml^{-1}, 294 μg ml^{-1}, 188 μg ml^{-1} and 58.5 μg ml^{-1} respectively. The FM14 compound and ButF fraction showed a low selectivity index of 1.2, while the AcF and FM45 samples presented a SI of 4.64 and 4.95, respectively (Table 5) and were evaluated for virucidal and inhibition of adsorption activities.

Both AcF and FM14 showed strong virucidal activity at a concentration of 200 μg ml^{-1}; inhibiting about 90% of the viral replication and showed dose-dependent inhibition (Fig. 6). For FM45, this activity was maintained up to 100 μg ml^{-1}, which was not observed for AcF. The fractions also showed activity in the viral adsorption test, but to a smaller degree compared to the virucidal activity. AcF presented a better activity, inhibiting 60.4% of viral replication, at 200 μg ml^{-1}. The other concentrations inhibited viral adsorption weakly, with %VI ranging from 20–37%. For FM45, the %VI ranged from 23–49%, in all concentrations tested. The IC_{50}/SI values for FM45 and AcF were respectively of 124 μg ml^{-1}/3.4 and 36 μg ml^{-1}/8 in the virucidal and 149 μg ml^{-1}/2.8 and 227.9 μg ml^{-1}/1.3 in the adsorption inhibition assays (Table 5). Comparing these values, determined in the different treatment protocols (simultaneous, virucidal and adsorption inhibition), showed that the virucidal activity for FM45 was strongly responsible for viral inhibition, presenting lower IC_{50} and higher SI.

The low toxicity found in the present work for AqF in both cell models evaluated (Vero and L929), corroborates the studies carried out by Noureddine et al. (2017) and dos Santos et al. (2017). However, the low biological activity coincides with the low levels of phenolic compounds and flavonoids found in this fraction, suggesting a strong relationship between these constituents and the evaluated activities. The HCE and its fractions showed similar CC_{50} values in both cell lines, except for AcF and BuF, which can be explained by the longer contact time with the cells in the Vero model (72 h) compared to L929 cells (24 h). The toxicity of the hydroalcoholic extract and the dichloromethane fraction obtained from the propolis in cultured cell lines has also been reported in other studies (Bonamigo et al., 2017; Peter et al., 2017; Teerasripreecha et al., 2012; Utispan et al., 2017).

The studied propolis was previously characterized as being a particular propolis type, with characteristics of both the green and yellow types (dos Santos et al., 2017), common in southern Brazil. This type of propolis is characterized by the presence of many terpene compounds, as well as phenolics and flavonoids, which, after extraction and purification, become significantly concentrated in polar fractions. Our results showed that the fractions with the highest anti-HSV activity (AcF and BuF) also contained higher amounts of flavonoids and phenolic compounds. Some flavonoids galangin, kaempferol, quercetin and flavone luteolin (Amoros et al., 1992), and also the minor constituents of propolis (3-methyl-but-2-enyl caffeate) (Amoros et al., 1994) have already been correlated with the antiviral activity of propolis against HSV-1. In addition,

Table 4
Cell viability of the L929 line exposed to the propolis crude extracts and fractions at different concentrations (mg ml^{-1}) expressed in percentage and ±standard deviation.

	1000	500	250	125	62.5	31.2	15.6	7.8	3.9	1.9
CGC	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
HCE	$8.13^{b} \pm 12.6$	$4.32^{b} \pm 17.7$	$1.82^{b} \pm 31.9$	$55.28^{b} \pm 10.9$	$84.65^{b,d} \pm 5.6$	$87.07^{b} \pm 3.8$	$92.84^{b} \pm 5.7$	$93.59^{b} \pm 6.3$	$90.88^{b} \pm 3.7$	$89.64^{b} \pm 5.6$
AqF	$98.35^{a} \pm 5.8$	$96.57^{a} \pm 7.3$	$102.46^{a,d} \pm 4.8$	$97.83^{a} \pm 11.0$	$101.4^{a} \pm 3.2$	$98.12^{a} \pm 4.1$	$103.72^{a} \pm 5.5$	$100.50^{a} \pm 4.2$	$102.27^{a} \pm 5.9$	$95.55^{a,b} \pm 8.4$
DiF	$6.72^{b} \pm 11.8$	$4.89^{b} \pm 15.1$	$4.50^{b} \pm 15.2$	$56.40^{b} \pm 10.9$	$94.10^{b,d} \pm 7.1$	$92.17^{b} \pm 6.8$	$91.84^{b} \pm 4.2$	$91.29^{b,c} \pm 7.0$	$93.77^{a,b} \pm 3.8$	$94.13^{a,b} \pm 5.2$
AcF	$46.19^{c} \pm 11.4$	$79.72^{c} \pm 10.3$	$72.32^{c} \pm 13.2$	$84.73^{c} \pm 16.5$	$73.18^{c} \pm 10.3$	$77.84^{c} \pm 9.5$	$77.13^{c} \pm 3.9$	$87.63^{b,c} \pm 7.9$	$89.92^{b} \pm 10.2$	$90.55^{b} \pm 9.0$
ButF	$108.10^{d} \pm 4.7$	$110.6^{d} \pm 8.0$	$106.9^{d} \pm 7.0$	$107.0^{a} \pm 5.0$	$104.60^{a} \pm 11.2$	$106.8^{d} \pm 5.1$	$102.7^{a} \pm 6.3$	$102.6^{a} \pm 6.1$	$98.3^{a} \pm 10.2$	$106.1^{a} \pm 7.6$
MCE	$11.72^{b} \pm 5.6$	$9.17^{b} \pm 18.4$	$5.93^{b} \pm 9.5$	$88.7^{a,c} \pm 4.7$	$89.00^{a,b,d} \pm 5.2$	$91.24^{a} \pm 6.7$	$89.74^{b} \pm 5.7$	$96.31^{a,b} \pm 7.6$	$101.69^{a,b} \pm 3.5$	$104.13^{a} \pm 7.3$
ACE	$86.06^{e} \pm 10.0$	$90.01^{e} \pm 5.5$	$86.33^{e} \pm 6.4$	$84.38^{c} \pm 6.3$	$86^{d} \pm 6.6$	87.65 ± 9.3	$86.75^{b} \pm 8.2$	$83.28^{c} \pm 8.7$	$88.59^{b} \pm 6.5$	$92.88^{a,b} \pm 5.9$

The same letters in a given column indicate no significant differences ($p > 0.05$); 95% confidence level. CGC, cell growth crontrol; HCE, hydroalcoholic crude extract; AqF, aqueous fraction; DiF, dichloromethane fraction; AcF, ethyl acetate fraction; ButF, butanol fraction; InF, insoluble fraction; MCE, methanolic crude extract; ACE, aqueous crude extract.

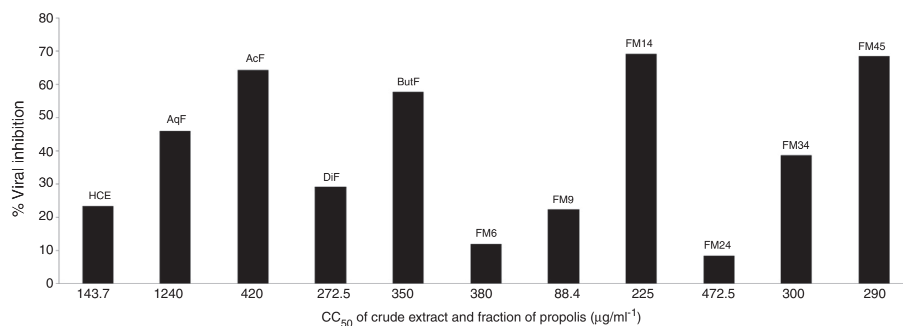

Fig. 5. Inhibition of herpes Simplex virus (HSV) by crude extract and fractions ($\mu g\,ml^{-1}$) of propolis produced by the *Melipona quadrifasciata* bee in Vero cells. Percentage of viral inhibition (%VI) was determined in comparison to controls. HCE, hydroalcoholic crude extract; AqF, aqueous fraction; DiF, dichloromethane fraction; AcF, ethyl acetate fraction; ButF, butanol fraction; FM06, FM09, FM14, FM24, FM34, FM45, fractions generated by DiF.

Table 5
Antiviral activity of the crude extract and fractions of propolis produced by the *Melipona quadrifasciata* bee against herpes simplex virus type 1, in Vero cells. The values were determined *via* different treatment protocols: simultaneous, virucidal and adsorption inhibition. AcF (ethyl acetate fraction), ButF (butanol fraction) and FM14 and FM45 (fractions generated by DiF).

		Inhibitions assays					
		Simultaneous		Virucidal		Adsorption inhibition	
	CC_{50} [a]	IC_{50} [b]	IS [c]	IC_{50}	IS	IC_{50}	IS
AcF	420	90.5	4.6	124	3.4	149	2.8
ButF	350	294	1.2	NT	NT	NT	NT
FM14	225	188	1.2	NT	NT	NT	NT
FM45	290	58.5	4.9	36	8	227.9	1.3

[a] Cytotoxic concentration of 50% ($\mu g\,ml^{-1}$).
[b] Inhibitory concentration of 50% ($\mu g\,ml^{-1}$).
[c] Selective Index (CC_{50}/IC_{50}).
NT, not tested.

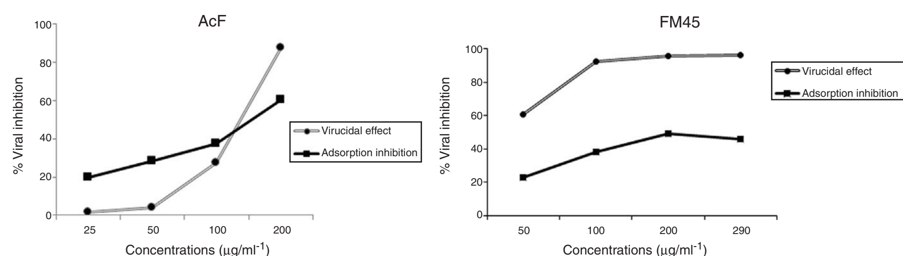

Fig. 6. Virucidal and inhibition of adsorption activities of the AcF (ethyl acetate) fraction and FM45 compound obtained from propolis produced by the *Melipona quadrifasciata*, bee in the HSV-1 model.

triterpenes, such as moronic acid, isolated from Brazilian propolis demonstrated potential activity against HIV (Ito et al., 2001).

When testing the crude extract and fractions of propolis against the HSV-1, some samples showed significant results. FM45, obtained from the purification of DiF, was identified as a catechin derivative (compounds 4 and 5 in Fig. 2), while AcF, extracted from HCE with ethyl acetate, has a greater amount of phenolic compounds and flavonoids. Thus, as reported by other authors (Amoros et al., 1992; Marcucci, 1995), it is difficult to correlate only one class of compounds with the antiviral activity. A synergism occurring between these molecules seems to be the most likely explanation.

Corroborating our virucidal results, Bankova et al. (2014b) also demonstrated a pronounced virucidal effect of a propolis extract against HSV-1 and HSV-2. These authors suggest that the action of propolis occurs in the structure of the viral envelope or by modifying structural components necessary for adsorption or entry of the virus into the cell. In the study by Peter et al. (2017), significant virucidal activity was also reported in which three extracts of propolis were effective against bovine herpesvirus type 1 and bovine viral diarrhea virus.

Conclusion

In conclusion, it was shown that the studied propolis possesses significant antioxidant activity, which is directly dependent on the method used for extraction. It was observed that not all the antioxidant activity is related to phenolic compounds and flavonoids. Thus, more studies are needed to determine whether the terpene compounds influence the performance of these activities.

The highest concentration of flavonoids and phenolics occurs in the extraction with methanol. The MCE also showed good antioxidant activity and had low cytotoxicity at doses beginning with 125 $mg\,ml^{-1}$, demonstrating effectiveness when employing a polar solvent and subsequently removing interfering resins.

It was also shown that Brazilian native bee propolis and its purified fractions have an important antiviral effect against an HSV-1 strain. This study suggests that its action is mainly due to interference with the surface structures of the virus, with cell receptors or both, in the early stages of viral infection. However, further studies are needed to clarify the mechanism of anti-HSV-1 activity. It is still difficult to correlate the antiviral activity of a single constituent

in a complex mixture such as propolis, so it is suggested that the synergism between them contributes to the optimization of this activity.

Author contributions

SH performed the propolis extraction and fractioning, the chemical and antioxidant analysis, and the manuscript draft and revision writing; AG supervised the propolis extraction and fractioning process; LF, DRZ, CN and REL performed the antiviral assays; HSF performed the cytotoxicity assay; MR contributed to the propolis fractioning process and its chemical characterization; DS and GM contributed to the chemical characterization of the propolis samples; CMMC was responsible for the overall design of the work and supervision; all authors participated in the draft of the manuscript, analysis of the results and revision of the final version.

Conflicts of interest

The authors declare no conflicts of interest.

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