Revista Brasileira de Farmacognosia 29 (2019) 309–318

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Chemical characterization, antioxidant and anti-HIV activities of a Brazilian propolis from Ceará state

Caroline Cristina Fernandes da Silva [a], Antonio Salatino [a,*], Lucimar Barbosa da Motta [b], Giuseppina Negri [a,b], Maria Luiza Faria Salatino [a]

[a] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil
[b] Universidade Paulista, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 28 September 2018
Accepted 4 April 2019
Available online 14 May 2019

Keywords:
Anti-HIV
Antioxidant
Brazilian propolis
Flavonoids
Botanical source
HIV-1 reverse transcriptase

ABSTRACT

Propolis (bee glue) a product of *Apis mellifera* L. is a resinous mixture containing chiefly beeswax and resin harvested by bees from plant leaves, buds and exudates. Extracts of a propolis sample from Salitre, a municipality of Ceará state (northeast Brazil) were obtained with solvents of increasing polarity (hexane, chloroform, ethyl acetate and methanol). A chemical profile was carried out by GC–EI-MS and HPLC–DAD–ESI-MS/MS. Lupenone, lupeol, octanoic acid tetracosyl ester and octanoic acid hexacosyl ester were identified by GC–EI-MS. Antioxidant activity was evaluated by the DPPH and β-carotene discoloring methods, and anti-HIV activity by the *in vitro* inhibition of HIV-1 reverse transcriptase. The ethyl acetate extract exhibited the highest antioxidant and anti-HIV activity and was fractioned by column chromatography using silica gel and seven different eluents. The active fractions were submitted to semi preparative HPLC and the following compounds were isolated: caffeic acid, *p*-coumaric acid, diprenylcinnamic acid, quercetin, naringenin, isorhamnetin, quercetin-3-*O*-diglucoside,4,2′,4′-trihydroxy-2-methoxychalcone, gossypetin-3,3′,4′,7-tetramethyl ether, myricetin-3,7,3′-trimethyl ether and 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone. The ethyl acetate extract and its fractions F5-F7, as well as quercetin, isorhamnetin, myricetin-3,7,3′-trimethyl ether and *p*-coumaric acid exhibited high antioxidant activity on both DPPH and β-carotene antioxidant methods. Isorhamnetin exhibited moderate inhibitory effect against HIV-1 reverse transcriptase (56.99 ± 3.91%), followed by naringenin (44.22 ± 1.71%), quercetin (43.41 ± 4.56%) and diprenylcinnamic acid (41.59 ± 2.59%). These results agree with previous authors who reported anti-HIV activity of flavonoids.

Introduction

Propolis (bee glue) is a resinous mixture produced by *Apis mellifera* L. It contains bee secretions (including beeswax) and resins, which bees collect from various botanical sources, such as buds, leaves, and exudates. Other propolis constituents are pollen, sugars, amino acids and minerals. Propolis has been shown to exert many biological activities (wound-healing, anti-inflammatory, gastroprotective, hepatoprotective, immunomodulatory, antineoplastic, antidiabetic, among many others) (Hashemi, 2016; Berretta et al., 2017). Honey bees, independently of the geographical zone, can locate appropriate propolis plant resin sources for production a propolis possessing biological activity (Bittencourt et al., 2015; Machado et al., 2016; Berretta et al., 2017; Kocot et al., 2018). Propolis chemical composition varies substantially, according to geography and local flora (Bertelli et al., 2012; Petelinc et al., 2013; Toreti et al., 2013). Propolis types are characterized by geography, chemical profile and source plants (Berretta et al., 2017; Kocot et al., 2018).

Propolis from distinct regions exhibited characteristic chemical profiles and source plants (Berretta et al., 2017; Kocot et al., 2018). In temperate zones (Europe, nontropic regions of Asia, North America, and continental Australia), the propolis known as poplar type propolis is originated mainly from bud exudates of *Populus nigra*, Salicaceae, and possess flavonoids with no B-ring oxygenation, phenolic acids and their esters as main constituents (Ristivojević et al., 2015; Al-Ani et al., 2018). However, in Russia a distinct propolis type is produced, derived from birch (*Betula verrucosa*, Betulaceae) containing flavones and flavonols different from those of poplar propolis (Miguel and Antunes, 2011; Martinotti and Ranzato, 2015). Mediterranean or Italian cypress propolis is originated from the resin of *Cupressus sempervirens*, Cupressaceae.

* Corresponding author.
E-mail: asalatin@ib.usp.br (A. Salatino).

https://doi.org/10.1016/j.bjp.2019.04.001

This type of propolis is produced in countries such as Greece, Sicily, Malta, Cyprus, Croatia, and Algeria, and contains chiefly diterpenes (Popova et al., 2010, 2011). At least three plant species provide resin for Omani propolis: *Azadirachta indica*, Meliaceae, *Acacia* spp. (probably *A. nilotica*, Fabaceae, and *Mangifera indica*, Anacardiaceae. Omani propolis contains C_5-prenyl flavanones as main constituents (Popova et al., 2013). Tropical propolis from Cuba and Venezuela originates from resin exuded by flowers of *Clusia* spp., Clusiaceae, and contains benzophenones as main constituents (Cuesta-Rubio et al., 2002). The propolis from tropical Pacific Ocean islands (Taiwan, Okinawa, and Indonesia) originated from *Macaranga tanarius*, Euphorbiaceae, is characterized by *C*-geranyl flavanones (Trusheva et al., 2011; Kumazawa et al., 2014). Bolivian propolis from the valleys (Cochabamba, Chuquisaca and Tarija) is rich in prenylated phenylpropanoids, while samples from La Paz and Santa Cruz exhibited cycloartane and pentacyclic triterpenes as main constituents (Nina et al., 2016).

In Brazil, several types of propolis have been characterized, each deriving from a distinct resin source (Berretta et al., 2017). Among Brazilian propolis, the green and red types have been the most frequently studied among Brazilian propolis (Huang et al., 2014). Green propolis from southeast Brazil is derived from young tissues of *Baccharis dracunculifolia*, Asteraceae, and contains mainly prenylated phenylpropanoids, caffeoylquinic acids and diterpenes (Salatino et al., 2005; Fernandes-Silva et al., 2013; Righi et al., 2013). Red propolis, produced in the littoral of Brazilian northeast, is originated from *Dalbergiae castaphyllum*, Fabaceae, and possesses chalcones and isoflavones as main constituents (Righi et al., 2011; Mendonça et al., 2015; Rufatto et al., 2017). Several other types of Brazilian propolis have been characterized. A green propolis produced in the northeast of the country contains chiefly flavonoids and is derived from *Mimosa tenuiflora*, Fabaceae (Ferreira et al., 2017). A dark-colored propolis from the Amazon is originated from *Clusia* spp., Clusiaceae, and contains prenylated benzophenones (Castro Ishida et al., 2011). Some propolis types have been characterized chemically, but still not botanically. Thus, there are two propolis types in Piauí state, one containing cycloartane triterpenoids (Silva et al., 2005) and another containing flavanones and glycosyl flavones (Righi et al., 2013); a yellow propolis from Mato Grosso State (central west, Brazil) possesses triterpenes (Machado et al., 2016) and a propolis type from Pirenópolis (Goiás State, central west Brazil) contains predominantly prenylated flavonoids and triterpenes (Righi et al., 2013). Brown propolis from Campo Grande, Mato Grosso do Suland Paraná contain, mainly, prenylated phenylpropanoic acids, flavonoids and chlorogenic acids (Fernandes et al., 2015; Andrade et al., 2017; de Oliveira Dembogurski et al., 2018). On the other hand, the brown propolis from Bahia State, possesses saturated hydrocarbons, methyl cinnamate, sitosterol cinnamate and anani xanthones main constituents (Santos et al., 2017).

In developing countries, acquired immunodeficiency syndrome (AIDS) is still a great cause of death. Human immunodeficiency virus-1 (HIV-1) leads to AIDS *via* destruction of T-cells after invasion and replication inside the host cells. Most conventional drugs used as anti-HIV (anti-human immunodeficiency virus) are nucleoside-based. Their use has serious limitations because of adverse effects, resistance and toxicity. They undergo first pass metabolism, leading to inactivation and generation of toxic metabolites (Singh et al., 2010; Pasetto et al., 2014; Saravanan et al., 2015). Natural products have been used as lead compounds aiming to obtain effective drugs against many ailments, including AIDS. Several flavonoids have been shown to be active against HIV (Pasetto et al., 2014). Although flavonoids are common constituents of several propolis types, not much has been investigated about propolis antiretroviral activity. Among the methods used to test anti-HIV activity, the anti-reverse transcriptase method has been widely used for bioassay guided fractionation and determination of IC_{50} of isolated compounds (Reutrakul et al., 2007; Zhang et al., 2017).

The objective of the present study is to determine the chemical composition of a propolis produced in the municipality of Salitre (state of Ceará, northeast Brazil). In addition, it is intended to isolate constituents of this propolis with antioxidant and anti-HIV activity, aiming to detect constituents accounting for the activity of the extracts.

Material and methods

Propolis sample and extraction

The propolis sample was collected in the municipality of Salitre (7°16′56″ S, 40°27′21″ W), Ceará state (northeast Brazil). Approximately 50 g of propolis was submitted to extraction in Sohxlet for 6 h, using 500 ml of solvents with increasing polarity (hexane, chloroform, ethyl acetate and methanol). The extracts were concentrated under reduced pressure, re-suspended in chloroform (hexane and chloroform extracts) or methanol (ethyl acetate and methanol extracts) and filtered.

Characterization of constituents by GC–EI-MS

Analyses of 1 μl of chloroform and methanol solutions at 1 mg/ml were carried out by GC–MS in split mode, using a 6850 Agilent gas chromatograph operating at the split mode and equipped with a capillary column DB-5HT (30 m × 0.32 mm, 0.25 μm) coupled to an injector using a pulsed split ratio 1:10. The chromatograph was coupled to a 5975C VL MSD Agilent mass spectrometer operating with electron impact ionization at 70 eV. The temperature of both injector and detector was 300 °C. Helium was used as carrier gas at 1.5 cm^3 min flow rate. Oven temperatures ranged from 100 to 300 °C at 10 °C/min, finishing with a 15 min isothermal period. The mass spectrometer was set to detect in the *m/z* range 50–650 in the scan mode using a source temperature of 250 °C, a quadrupole temperature of 110 °C and a filament emission current of 35 μA. Characterization of constituents was achieved by matching query spectra to spectra present in a reference library. Thus, the fragmentation pattern of the EI mass spectra of constituents was compared *via* spectrum matching with the reference mass spectra in the library NIST 08 and Wiley-275 (Hewlett Packard, Wiley/NBS, Koo et al., 2013), and a list of most probable identities was produced based on the best mass spectrum matching score. Comparison with data reported in literature also was used for characterization, as well as comparison with authentic standard of lupeol. Relative amounts of constituents were assumed to be proportional to the areas under the corresponding chromatogram peaks.

Characterization of constituents by HPLC–DAD and HPLC–DAD–ESI-MS/MS

RP HPLC–DAD–ESI-MS/MS analyses of ethyl acetate extract and its fractions were conducted using a DADSPD-M10AVP Shimadzu system equipped a photodiode array detector coupled to Esquire 3000 Plus mass spectrometer, Bruker Daltonics, which consisted of two LC-20AD pumps, SPD-20A diode array detector, CTO-20A column oven and SIL 20AC autoinjector (Shimadzu Corporation Kyoto, Japan). All the operations, acquisitions and data analyses were controlled by the Shimadzu CBM-20A controller. Separations were carried out using a C18 RP Luna Phenomenex reverse phase column (4.6 × 250 mm i.d., 5 μm), protected with a security guard cartridge (Gemini C18, 4.0 × 2.0 mm i.d.). The mass spectrometer was a quadrupole ion trap with atmospheric pressure ionization source through electrospray ionization interface (ESI) operating in

the scan MS mode from *m/z* 100 to 1500. The ethyl acetate extract and its fractions (2 mg/ml) were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane filters, and a 10 μl aliquot was injected into the chromatograph. The column temperature was maintained at 40 °C and the flow rate was 1 ml/min. The DAD acquisition wavelength was set in the range of 240–400 nm and chromatograms were recorded at 250, 300 and 352 nm. After passing through the flow cell of the DAD, the column eluate was split, and a flow of 100 μl/min was diverted to the mass spectrometer. The mobile phase was composed by eluent A (0.1% aqueous acetic acid) and eluent B (methanol). 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, 95%; 50 min, 95%. Helium was used as the collision, and nitrogen as the nebulizing gas, respectively. Nebulization was aided with a coaxial nitrogen sheath gas provided at pressure of 27 psi. Mass spectra were acquired in both negative and positive modes with ion spray voltage at 3.0 kV, capillary temperature at 300 °C, capillary voltage at 45 V and drying gas flow 6 l/min. Collision induced dissociation spectra were obtained in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.5 to 1.3 V. The constituents were characterized by ultraviolet and mass spectral data (MS). The MS data were compared with mass spectral databases Phenol-Explorer (www.phenol-explorer.eu), ChemSpider (http://www.chemspider.com), Metlin (http://metlin.scripps.edu) and HMDB (www.hmdb.ca), besides MS data reported in literature (see Table 2). Quercetin, naringenin, isorhamnetin, caffeic acid and *p*-coumaric acid from Sigma-Aldrich were used as standards using the same chromatographic conditions.

Bioguided isolation of active constituents

The extracts with higher antioxidant activity (evaluated according to section *Antioxidant activity*; see below) were fractionated by column chromatography using silica gel and the following solvents: F1: hexane; F2: hexane:chloroform (50:50); F3: chloroform; F4: chloroform:ethyl acetate (50:50); F5: ethyl acetate; F6: ethyl acetate:methanol (50:50) and F7: methanol. Fractions that exhibited higher antioxidant and anti-HIV activities were submitted to semi-preparative HPLC to isolate the main active constituents. The isolation was performed with a reverse phase Zorbax C18 RP-18 column (Hewlett Packard; 9.6 × 250 mm, 5 μm) and mobile phase composed by eluent A (0.1% aq. acetic acid) and eluent B (methanol) at 2.5 ml/min constant flow rate and 40 °C constant temperature. The following elution program was used, based on the concentration of B: 0 min: 20%; 10 min: 40%; 20 min: 60%; 30 min: 80%; 40 min: 90%; 50 min: 95%. The antioxidant and anti-HIV activities of isolated compounds were evaluated and those obtained in high contents were analyzed by ^{1}H NMR and ^{13}C NMR.

NMR spectra of isolated compounds

NMR spectra of isolated compounds were recorded in MeOD with a Bruker Advance III (300 MHz). Chemical shifts are given in ppm and were referenced to the solvent (MeOD) signal at 3.31 ppm for ^{1}H and 49.3 ppm for ^{13}C.

Antioxidant activity

Antioxidant activities were measured by the DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging (Moreno et al., 2000) and the β-carotene discoloring methods (Koleva et al., 2002), with minor modifications. For the DPPH method, samples at 10, 15 and 30 μg/ml were maintained in the dark for 30 min. The absorbance was measured in a Synergy™ Neo2 Multi-Mode Microplate Reader at 517 nm. The percentage of free radical scavenging was calculated by the following formula:

$$\%\,\text{Free radical scavenging} = \frac{\text{DPPH absorbance} - \text{sample absorbance}}{\text{DPPH absorbance}} \times 100$$

Quercetin in methanol solutions (5–30 μg/ml) was used as reference control and a solution of DDPH in methanol as negative control. The antioxidant activity of the samples was calculated according to the regression equation $y = 13.043x$ ($R^2 = 0.96$), obtained with the quercetin solutions. The results of free radical scavenging capacity are given as mg of quercetin equivalent per g of propolis (mg QE/g). Tests for all samples were run in triplicates.

For the β-carotene discoloring method, a reactive solution containing 200 μg of β-carotene, 25 μl of linoleic acid and 200 μl of Tween 40 was solubilized in 50 ml of distilled water saturated with oxygen. The absorbance of this solution was set to lie between 0.7 and 0.8 at 470 nm (Righi et al., 2013). For the oxidation reaction, 1 ml of the reactive solution was added to 120 μl of methanol solutions of the samples at the 120 μg/ml concentration. The solutions were left standing in the dark for 2 h. Then, at every 30 min, the absorbance of the solutions was read at 470 nm, using a Synergy™ Neo2 Multi-Mode Microplate. The results are expressed as percent of inhibition, comparing the decrease in absorbance of the samples with the decrease of the absorbance of the control (reactive solution + methanol), according to the following formula:

$$\%\,\text{Antioxidant activity} = 1 - \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

Quercetin in methanol solutions (20–120 μg/ml) was used as reference control. The antioxidant activity of the samples was calculated according to the regression equation $y = 102.14x + 13.92$ ($R^2 = 0.92$), obtained with the quercetin solutions. Results of antioxidant activities are given as mg of quercetin equivalent per g of propolis (mg QE/g). All samples were tested in triplicates.

Anti-HIV activity

Among the methods used to test antiviral activity against HIV, the quantitative determination of retroviral reverse transcriptase activity is used for bioassay guided fractionation and determination of IC_{50} of isolated compounds (Reutrakul et al., 2007; Zhang et al., 2017). The colorimetric enzyme immunoassay for the quantitative determination of retroviral reverse transcriptase activity was evaluated with recombinant HIV-1 enzyme, using a non-radioactive HIV-1 RT colorimetric ELISA kit (Roche Applied Sciences, Mannheim, Germany), Roche®, following the general recommendations of the manufacturer. Samples were diluted in 10% dimethyl sulfoxide (DMSO) to a final concentration of 200 μg/ml. Aliquots of 20 μl of the samples were applied on 96 well plates. Foscarnet was used as positive control at 10 μg/ml. Controls used were control A (without sample and without reverse transcriptase) and control B (without sample, but with reverse transcriptase). The absorbance was read on a Thermo Scientific™ Multiskan™ FC Microplate Photometer at 405 nm. The percentage of inhibition was calculated by the following formula (Meragelman et al., 2001; Ono et al., 1990):

$$\%\,\text{Inhibition} = 1 - \frac{\text{sample absorbance} - \text{control A absorbance}}{\text{control B absorbance} - \text{control A absorbance}} \times 100$$

Results

Characterization of isolated constituents by NMR

Naringenin (**9**): ^{1}H NMR (300 MHz, MeOD, TMS), δ H (ppm) *J* (Hz) 5.43 (1H, dd, *J* = 13, 2.8 Hz, H-2), 3.26 (1H, dd, *J* = 13, 16.5 Hz, H-3a), 2.65 (1H, dd, *J* = 16.5, 2.8 Hz, H-3b), 5.93 (1H, d, *J* = 2.1, H-6), 5.94 (1H, d, *J* = 2.1, H-8), 6.85 (2H, d, *J* = 7.8 Hz, H-3′, 5′), 7.35 (2H, d, *J* = 7.8 Hz, H-2′, 6′). ^{1}H NMR chemical shifts are in accordance with literature data reported by Lemos da Silva et al. (2015).

4,2′,4′-Trihydroxy-2-methoxychalcone (**12**): ^{1}H NMR (300 MHz, MeOD, TMS), δ H (ppm) *J* (Hz) 7.68 (1H, d, *J* = 14 Hz, H-β), 7.66 (1H, d, *J* = 14 Hz, H-α), 7.63 (1H, d, *J* = 6.5 Hz, H-6′), 7.62 (1H, d, *J* = 7 Hz, H-6), 7.09 (1H, dd, J = 3, 6.5 Hz, H-5′), 7.07 (1H, dd, J = 2.8, 7 Hz, H-5), 6.6 (1H,d, J = 3 Hz, H-3′), 6.7 (1H,d, J = 2.8 Hz, H-3), 3.96 (3H, s). The identification was carried out through comparison with ^{1}H NMR and MS data reported by Khamsan et al. (2012) and Kajiyama et al. (1992).

Myricetin-3,3′,7,-trimethyl ether (**14**): ^{1}H NMR (300 MHz, MeOD, TMS) δ 7.56 (2H, s, H-2′, 6′), 6.52 (1H, d, *J* = 2.0 Hz, H-8), 6.40 (1H, d, *J* = 2.0 Hz, H-6), 3.96 (3H, s, OMe), 3.94 (3H, s, OMe), 3.83 (3H, s, OMe). ^{13}C NMR (150 MHz, CD3OD) δ 180.8 (C-4), 163.3 (C-7), 160.8 (C-5), 157.4 (C-9), 154.1 (C-2), 152.0 (C-3′, 5′), 140.0 (C-3), 122.3 (C-4′), 116.4 (C-1′), 112.5 (C-2′, 6′), 104.7 (C-10), 92.2 (C-8, 6), 61.2 (OMe), 60.7 (OMe), 57.1 (OMe). ^{1}H NMR and ^{13}C NMR chemical shifts are in accordance with literature data reported by Kranjc et al. (2016).

5-Hydroxy-3,6,7,8,4′-pentamethoxyflavone (**15**): ^{1}H NMR (400 MHz, MeOD) δ 8.13 (2H, d, *J* = 8.8 Hz, H-2′,6′), 7.10 (2H, d, *J* = 8.8, H-3′,5′), 3.97 (3H, s, OMe), 4.10 (3H, s, OMe), 3.96 (3H, s, OMe), 3.90 (3H, s, OMe), 3.87 (3H, s, OMe). The ^{1}H NMR chemical shifts are in accordance with literature data reported by Paula et al. (2002) and Zou et al. (2010).

Characterization of constituents by GC–EI-MS

Fig. 1 represents the chromatogram of the GC analyses of the hexane extract. The main constituents detected in hexane and chloroform extracts are listed in Table 1. They were characterized based on mass spectra (Koo et al., 2013) comparisons with the library NIST 08 and Wiley-275, with similarity percentage higher than 95% and NIST Match Factor – 850. Two main constituents were detected and characterized in both extracts, namely, octanoic acid tetracosyl ester (**3**) and octanoic acid hexacosyl ester (**4**), with molecular ions (M^+) at *m/z* 480 ($C_{32}H_{64}O_2$) and *m/z* 508 ($C_{34}H_{68}O_2$), respectively. The base peak at *m/z* 143 corresponds to the protonated acid moiety ($(C_8H_{15}O_2)^+$) (Palacios et al., 2015). Saturated straight-chain ester contains product ions derived from the fatty acid moiety of the ester ($[R_1COO]^+$) (Tada et al., 2014). The triterpenoid lupenone **1** exhibited molecular ion M^+ at *m/z* 424 (30) and a base peak at *m/z* 205 (100), agreeing with data of Xu et al. (2018), while lupeol (**2**) had M^+ at *m/z* 426 (**1**) and base peak at *m/z* 189 (100) (Table 1), agreeing with data of Pereira Beserra et al. (2018).

Characterization of constituents by RP HPLC–DAD–ESI-MS/MS

Fig. 2 represents the chromatogram obtained by RP HPLC–DAD–ESI-MS/MS analysis of the ethyl acetate extract. Compounds characterized are listed in Table 2, including retention times, $[M+H]^+$ and $[M-H]^-$ ions corresponding to protonated and deprotonated molecules, respectively, and relevant MS/MS ions. Caffeic acid (**5**), *p*-coumaric acid (**6**), quercetin (**8**), naringenin (**9**) and isorhamnetin (**10**) were identified by comparison with authentic standards (Sigma-Aldrich). Naringenin (**9**) was identified also by ^{1}H NMR spectrum and comparison with data reported by Lemos da Silva et al. (2015). Isorhamnetin (**10**) exhibited

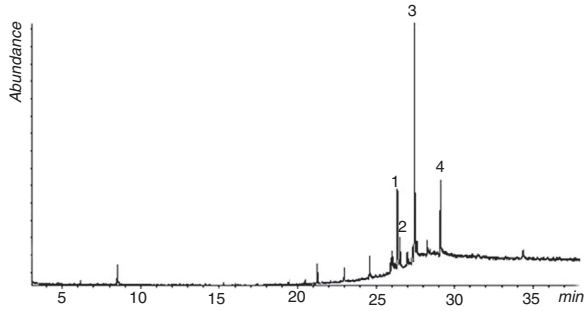


Fig. 1. Chromatogram obtained by GC–EI-MS analysis of the hexane extract of a propolis sample from the municipality of Salitre (state of Ceará, northeast Brazil). Digits on the chromatograms peaks correspond to compounds characterized and listed in Table 1.

UV/vis maximum absorption at 270, 295 sh, and 360 nm, and also $[M+H]^+$at *m/z* 317 (Table 2). The mass spectrum of deprotonated molecule at $[M-H]^-$ at *m/z* 315 exhibited base peak at *m/z* 300, produced by a stable radical anion $[M-H-15]^{\bullet-}$ (Engels et al., 2012; Cao et al., 2009).

Compound **7**, tentatively characterized as diprenylcinnamic acid, exhibited UV/vis maximum absorption at 310 nm, typical of phenylpropanoids, and $[M+H]^+$ at *m/z* 285. The base peak at *m/z* 147 is produced by the loss of two prenyl groups (138 Da) (Righi et al., 2013; Taddeo et al., 2016). Compound **11** exhibited UV/vis maximum absorption at 260 and 360 nm, characteristic of flavonols, as well as $[M-H]^-$ at *m/z* 625. Its base peak was *m/z* 301 (characteristic of quercetin) and the observed losses of 324 Da are consistent with *O*-glycoside (hexoside) fragmentation (Karar and Kuhnert, 2015; Kumar et al., 2017). Compound **11** was characterized as quercetin-3-*O*-diglucoside. UV/vis maximum absorption of chalcones lies in the range 340–390 nm. The maximum absorption of compound **12** was 370 nm. Its MS spectrum provided a deprotonated molecule at *m/z* 285 and an adduct $[2M-H]^-$ ion at *m/z* 570 (Table 2 and Fig. 3). The ESI MS under positive mode provided a dominant product ion at *m/z* 257, corresponding to the loss of 30 Da and a neutral loss of CH_2O (methanal). According to George et al. (2009) ESI methods protonate chalcones at the carbonyl oxygen, producing species that undergo Nazarov type cyclizations, capable of catalyzing proton migrations in the gas phase. Substituent such as OCH_3 at the 2-position can be sufficiently basic to assist in proton transport. The ^{1}H NMR spectrum from **12** exhibited a pair of doublet *trans* protons at δ 7.68 (1H, d, *J* = 14.0 Hz) and 7.66 (1H, d, *J* = 14.0 Hz). The signals at δ 7.62 (1H, d, *J* = 7 Hz, H-6), 7.07 (1H, dd, *J* = 2.8, 7 Hz, H-5) and δ 6.7 (1H,d, *J* = 2.8 Hz, H-3) are consistent with substitution at 2 and 4 position of the B ring. The signals at 7.63 (1H, d, *J* = 6.5 Hz, H-6′), 7.09 (1H, dd, *J* = 3, 6.5 Hz, H-5′) and at δ 6.6 (1H,d, *J* = 3 Hz, H-3′) is consistent with A ring possessing hydroxyl groups at 2′ and 4′ positions. The methoxyl group appeared at δ 3.96 (3H,s, OCH_3). Based on ^{1}H NMR and MS data reported by Kajiyama et al. (1992), George et al. (2009), Khamsan et al. (2012), Li et al. (2017), and Sun et al. (2017), compound **12** was assigned as 4,2′,4′-trihydroxy-2-methoxy chalcone (2-methoxy isoliquiritigenin).

Compound **13**, tentatively identified as gossypetin-3,3′,4′,7-tetramethyl ether, exhibited UV maximum absorption at 260–360 nm, a sodium adduct at *m/z* 397 and $[M+H]^+$ at *m/z* 375. The characterization was based on the mass spectral database HMDB (www.hmdb.ca) and MS data reported by Ali Khan et al. (2018) and Kranjc et al. (2016). Compound **14**, assigned asmyricetin-3,3′,7-trimethyl ether, exhibited UV maximum absorption at 260–355 nm, $[M-H]^-$ at *m/z* 359 and $[M+H]^+$ at *m/z* 361, respectively (Table 2 and Fig. 3). The MS/MS experiments at *m/z* 359 produced a fragment ion at *m/z* 344 arising from the loss of the methyl radical (Hussein et al., 2003;

Table 1
Compounds characterized by GC–EI-MS analyses from the *n*-hexane and chloroform extracts of a propolis sample from Salitre (state of Ceará, northeast Brazil).

Comp. number	RT (min)	Proposed characterization	MS data (M^+) and fragment ions (*m/z*)[a]
1	26.48	Lupenone	424 (30) 205 (100), 189 (60), 218 (50), 245 (40), 313 (40), 409 (30), 109 (90)
2	26.65	Lupeol	426 (1), 218 (50), 207 (80), 189 (100), 121 (80), 95 (100)
3	27.58	Octanoic acid tetracosyl ester	480 (1), 207 (13), 143 (100), 125 (51), 95 (16)
4	29.62	Octanoic acid hexacosyl ester	508 (1), 207 (13), 143 (100), 125 (51), 95 (16)

GC, gas chromatography; EI, electron impact; MS, mass spectrum data; RT, retention time; NIST, National Institute of Standards and Technology.
[a] NIST Match Factor: 850.

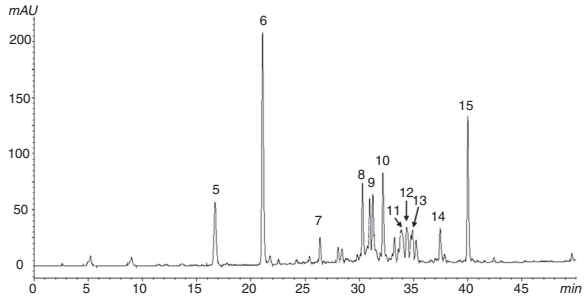


Fig. 2. Chromatogram obtained by CLAE–DAD-MS/MS analysis of the ethyl acetate extract of a propolis sample from the municipality of Salitre (state of Ceará, northeast Brazil). Digits on the chromatogram peaks correspond to compounds characterized and listed in Table 2.

Kranjc et al., 2016). Methoxylated flavonoid structures lead to stable radical anions $[M-H-15]^{\bullet-}$ (Engels et al., 2012). In positive ionization mode fragment ions at *m/z* 345 and *m/z* 328 were observed, corresponding to the sequential losses of methyl group and water. The ^{1}H NMR spectrum exhibited three distinct methoxy proton resonances at δ 3.96, 3.94 and 3.83 ppm. In the aromatic region, the spectrum exhibited a singlet at δ 7.56 ppm corresponding to the two protons (H-2′ and H-6′) and two meta coupled proton doublets (J = 2 Hz) at δ 6.52 and 6.40 ppm, corresponding to theH-6 and H-8 protons of chromone moiety. In ^{13}C NMR spectrum, the most upfield signals at δ 61.2, 60.7 and 57.1 were assigned to the carbons of the methoxy groups. Etherification at position 3, led to the downfield shift of C-3 to δ 140.6 ppm, as reported by Kranjc et al. (2016).

Compound **15**, assigned as 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone, provided UV/vis spectrum with maximum absorption at 255, 267 sh and 350 nm, $[M+H]^+$ at *m/z* 389 and sodium adduct at *m/z* 411. The mass spectrum was compared with data reported by Zhang et al. (2011). The ^{1}H NMR spectrum showed signals at δ 8.13 (2H, d, J = 8.8 Hz, H-2′, 6′) and δ 7.10 (2H, d, J = 8.8, H-3′,5′) corresponding to the protons of ring B and methoxy groups at δ 3.97 (3H, s), 4.10 (3H, s), 3.96 (3H, s), 3.90 (3H, s), 3.87 (3H, s), which is in accordance with data reported by Paula et al. (2002) and Zou et al. (2010).

Antioxidant activities

The antioxidant activity of the extracts carried out with the DPPH method varied widely, for example5.32 ± 1.08 (hexane extract) and 240.20 ± 4.90 mg EQ g^{-1} (ethyl acetate extract); chloroform and methanol extracts showed activities of 30.46 ± 5.82 and 102.94 ± 2.95 mg EQ g^{-1}, respectively. On the other hand, with the β-carotene method, the antioxidant activities of extracts ranged from 4.4 ± 0.31 (hexane extract) to 37.95 ± 1.3 mg EQ g^{-1} (ethyl acetate extract). Chloroform and methanol extracts showed activities of 12.76 ± 0.16 and 17.76 ± 1.22 mg EQ g^{-1}, respectively. Ethyl acetate extract exhibited greater antioxidant activity with both evaluated methods, thus, bioassay-guided fractionation was

Table 2
Constituents from the ethyl acetate extract of a propolis sample from Salitre (state of Ceará, northeast Brazil), characterized by HPLC–DAD–ESI-MS/MS analysis.

Comp. number	RT (min)	UV_{max} (nm)	MS, negative mode (*m/z*)[a]	MS, positive mode (*m/z*)[a]	Proposed characterization	Means used for characterization
5	16.2	300, 330	$[M-H]^-$ 179	$[M+H]^+$ 181	Caffeic acid	Standard
6	21.1	310	$[M-H]^-$ 163	$[M+H]^+$ 165	*p*-Coumaric acid	Standard
7	25.8	310	ND	$[M+Na]^+$ 307 $[M+H]^+$ 285 MS/MS 147	Diprenyl cinnamic acid	Tadeo et al. (2016) and Righi et al. (2013)
8	30.3	256, 300 sh, 360	$[M-H]^+$ 301	$[M+H]^+$ 303	Quercetin	Standard
9	30.9	290, 337 sh	$[M-H]^-$ 271 MS/MS 253, 225	$[M+H]^+$ 273 MS/MS 153, 147	Naringenin	Standard; ^{1}H NMR analysis, Lemos da Silva et al. (2015)
10	32.1	270, 295 sh, 360	$[M-H]^-$ 315 MS/MS 300	$[M+H]^+$ 317	Isorhamnetin	Standard; Engels et al. (2012) and Cao et al. (2009)
11	34.2	260, 360	$[M-H]^-$ 625 MS/MS 301	ND	Quercetin 3-*O*-diglucoside	Karar and Kuhnert (2015) and Kumar et al. (2017)
12	34.3	260 sh, 370	$[M-H]^-$ 285	$[M+Na]^+$ 309 $[M+H]^+$ 287 MS/MS 257, 147, 137	4,2′,4′-Trihydroxy-2-methoxychalcone	^{1}H NMR analysis, Li et al. (2017)
13	36.9	260, 360	ND	$[M+Na]^+$ 397 $[M+H]^+$ 375	Gossypetin-3,3′,4′,7-tetramethyl ether	MS database HMDB, Ali Khan et al. (2018) and Kranjc et al. (2016)
14	37.4	255, 265 sh, 352	$[M-H]^-$ 359 MS/MS 344	$[M+H]^+$ 361 MS/MS 345, 328	Myricetin-3,7,3′-trimethyl ether	NMR (^{1}H and ^{13}C) analyses, Engels et al. (2012) and Hussein et al. (2003)
15	39.9	255, 267 sh, 350	ND	$[M+Na]^+$ 411 $[M+H]^+$ 389	5-Hydroxy-3,6,7,8,4′-pentamethoxyflavone	^{1}H NMR analysis, Zhang et al. (2011), Paula et al. (2002) and Zou et al. (2010)

HPLC, High Performance Liquid Chromatography; DAD, diodo array detecter; ESI, electrospray ionization; MS, mass spectra data; RT, retention time; UV, ultraviolet spectrum; ND, not detected.
[a] ND: not determined.

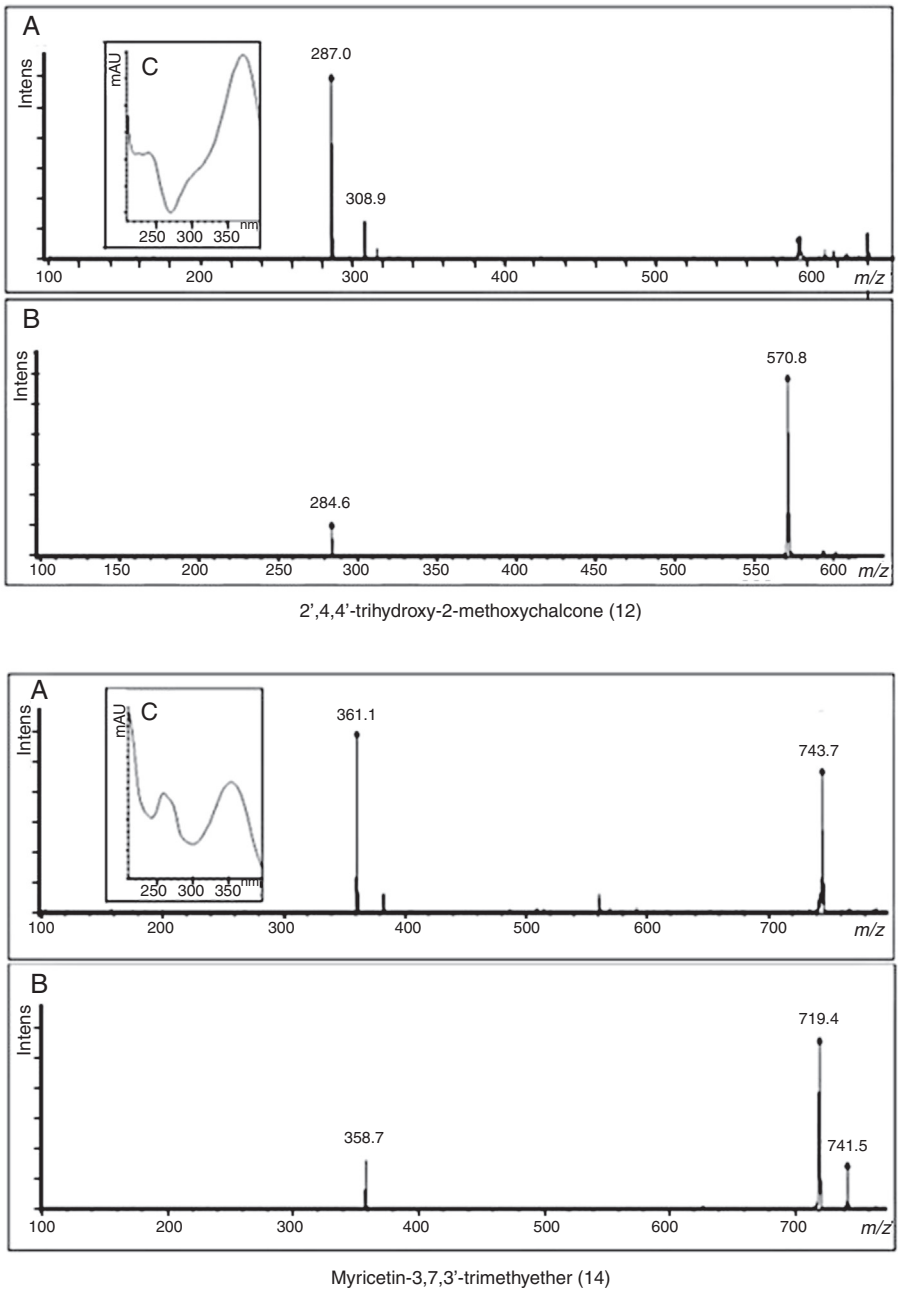


Fig. 3. UV/vis spectroscopy and MS spectrometry of flavonoids isolated from the ethyl acetate extract of a propolis sample from the municipality of Salitre (state of Ceará, northeast Brazil: 2′,4,4′-trihydroxy-2-methoxychalcone (**12**) and myricetin-3,7,3′-trimethy ether (**14**), both isolated from the ethyl acetate extract of propolis sample from the municipality of Salitre (state of Ceará, northeast Brazil). **A**: MS, positive mode; **B**: MS, negative mode; **C**: UV/vis absorption spectrum.

achieved by column chromatography for isolation of its constituents.

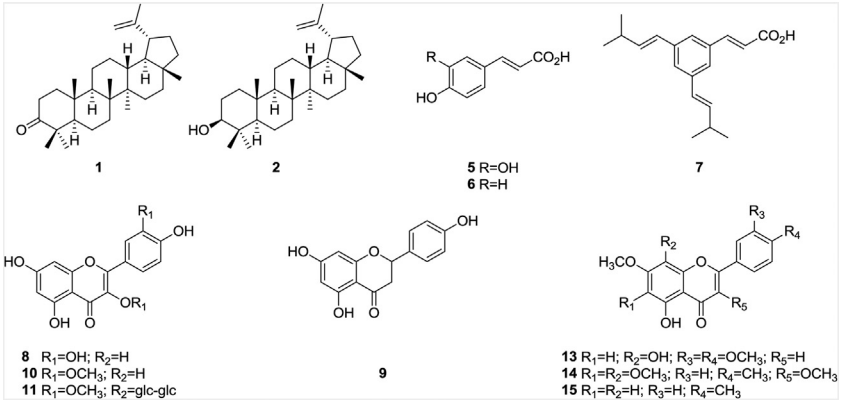
The antioxidant properties of fractions isolated from the ethyl acetate extract are shown in Table 3. Antioxidant activity on DPPH method ranged from 14.95 ± 1.11 (F3) to 112.12 ± 2.78 mg EQ g^{-1} (F7). On the other hand, the activity of the fractions ranged from 31.13 ± 0.00 (F6) to 36.28 ± 0.29 mg EQ g^{-1} (F5) with the β-carotene method, while F1–F4 were inactive (Table 3).

The main constituents of the most actives fractions F5–F7 derived from the ethyl acetate extract were isolated and their antioxidant properties were evaluated. The results are shown in Table 3. With the DPPH method, the antioxidant activity ranged from 2.51 ± 2.20 for diprenylcinnamic acid (**9**) to 572.86 ± 2.89 mg EQ g^{-1} for isorhamnetin (**10**). On the other hand, with the β-carotene method, the antioxidant activities ranged from 13.94 ± 0.64 for 4,2′,4′-trihydroxy-2-methoxychalcone (**12**) to 49.35 ± 0.00 mg EQ g^{-1} for quercetin (**8**).

Anti-HIV activity: HIV-1 reverse transcriptase colorimetric assay

The preliminary screening for HIV-1 reverse transcriptase inhibition was performed using 200 μg/ml of constituents and extracts (Woradulayapinij et al., 2005). At this concentration, hexane, chloroform and ethyl acetate extracts were inactive and crude methanol extract showed weak activity (2.56 ± 0.06%) regarding the inhibition of the HIV-1 reverse transcriptase. Fractions F2–F6 did not inhibit the HIV reverse transcriptase, and F1 and F7 showed weak activity (3.64 ± 0.73% and 22.54 ± 1.71%, respectively).

On the other hand, the isolated compounds were more effective, varying from 17.88 ± 4.04 [*p*-coumaric acid (**6**)] to 56.99 ± 3.91%



Scheme 1. Structures of constituents of a propolis sample from Salitre (state of Paraná, northeast Brazil). Digits correspond to compounds listed in Tables 1 and 2.

Table 3
Antioxidant activity of fractions isolated by column chromatography of the ethyl acetate extract and constituents isolated from these fractions in the ethyl acetate extract of a sample of propolis from Salitre (state of Ceará, northeast Brazil). The results are expressed as mg of equivalent quercetin per gram (EQ g^{-1}).

Fraction	β-Carotene method	DPPH method
F1	Inactive	17.83 ± 1.20
F2	Inactive	20.46 ± 0.79
F3	Inactive	14.95 ± 1.11
F4	Inactive	25.18 ± 0.00
F5	36.28 ± 0.29	28.33 ± 3.34
F6	31.13 ± 0.00	98.75 ± 1.67
F7	32.47 ± 1.02	112.12 ± 2.78
Isolated compounds		
p-Coumaric acid (**6**)	31.84 ± 3.12	374.96 ± 2.33
Diprenyl cinnamic acid (**7**)	Inactive	2.51 ± 2.20
Naringenin (**9**)	28.74 ± 2.56	35.44 ± 3.70
Isorhamnetin (**10**)	42.38 ± 2.16	572.86 ± 2.89
4,2′,4′-Trihydroxy-2-methoxychalcone (**12**)	13.94 ± 0.64	32.69 ± 3.47
Myricetin-3,3′,4′-trimethyl ether (**14**)	41.87 ± 0.48	559.97 ± 1.64

Table 4
HIV-1 reverse transcriptase inhibition of isolated constituents from the ethyl acetate of a sample of propolis from Salitre (state of Ceará, northeast Brazil).

Compound	% Inhibition
p-Coumaric acid (**6**)	17.88 ± 4.04
Diprenyl-cinnamic acid (**7**)	41.59 ± 2.59
Quercetin (**8**)	43.41 ± 4.56
Naringenin (**9**)	44.22 ± 1.71
Isorhamnetin (**10**)	56.99 ± 3.91
4,2′,4′-Trihydroxy-2-methoxychalcone (**12**)	34.01 ± 4.12
Myricetin-3,3′,4′-trimethyl ether (**14**)	35.35 ± 3.99

[isorhamnetin (**10**)], as can be seen in Table 4. The inhibitory effect on HIV reverse transcriptase can be classified as strong (>90%), moderate (>50–90%) and weakly active (<50%). According to this classification, the most active compound was isorhamnetin (**10**, Table 2) with a moderate activity (56.99 ± 3.91%, Table 4). According to a study carried out by Cole et al., flavonoids that contain either a α- or β-hydroxy-carbonyl motif within their structure can exhibit high anti-HIV activity.

Discussion

There are many studies about green propolis from southeast region of Brazil and red propolis from the northeast region. However, there is little information about propolis from Ceará State (Albuquerque et al., 2007; Gutierrez-Gonçalves and Marcucci, 2009). It is known that the chemical composition of propolis varies according to phytogeographic regions (Kasiotis et al., 2017). On the other hand, the chemical composition of propolis is often similar in a determined geographic region (Pasupuleti et al., 2017; Kocot et al., 2018).

The propolis sample analyzed in this study was harvested in the municipality of Salitre, in the Atlantic forest region of the Araripe plateau, Ceará State. Albuquerque et al. (2007) analyzed the chemical composition of a propolis from Ceará, harvested in Alto Santo, located within a Caatinga region. The Atlantic forest region of the Araripe plateau possesses distinct soil, weather patterns and flora, in comparison with the Caatinga region. In propolis from Alto Santo, canaric acid, the triterpenes lupeol, lupenone, germanicone and the flavonoids quercetin, kaempferol and acacetin were identified as main constituents. The botanical resin source of propolis from Alto Santo, although unknown, probably contains canaric acid as one of the main constituents (Albuquerque et al., 2007). As can be seen in Tables 1 and 2, lupenone (**1**), lupeol (**2**) and quercetin (**8**) were detected in Salitre propolis. However, these compounds are widely distributed in plants. Among the other constituents detected, chalcones (for example **12**) have been detected also in Brazilian red propolis (Bueno-Silva et al., 2017). The main polar constituents detected in propolis from Salitre were methoxylated flavonols, such asmyricetin-3,3′,7-trimethyl ether. A new type of green propolis harvested from Rio Grande do Norte State possesses methoxylated flavonols and chalcones as main constituents (Ferreira et al., 2017). However, myricetin-3,3′,7-trimethyl ether (**14**) and 5-hydroxy-3,3′,4′,7,8-pentamethoxyflavone (**15**) suggest that plants so far unreported contribute as resin sources for Salitre propolis. The presence of a chalcone (compound **12**, Table 2, and Scheme 1) as a relevant constituent in Salitre propolis suggests that a plant from the Fabaceae family might be one of its resin sources (Ferreira et al., 2017), although not belonging to the subfamily Faboideae. Plants of the latter group of Fabaceae often contain isoflavonoids, as is the case of *D. castaphyllum*, the resin source of Brazilian red propolis (Rufatto et al., 2017; Salatino and Salatino, 2018). Isoflavonoids were not detected in the present study.

The biological activity of propolis has been assigned to the antioxidant activity of phenolic compounds. Free radicals and other oxidative agents can produce many cell toxins that induce oxidative damage in biomolecules (Silva-Carvalho et al., 2015; Zheng et al., 2017). Flavonoids and other phenolic compounds protect cells against the damage caused by oxidation, acting as potent inhibitors of oxidative stress, which is involved in the pathogenesis of neurodegenerative disorders (Zheng et al., 2017). In addition, the intracellular free radical scavenging capacities of phenolic compounds can protect cell membranes against lipid peroxidation (Daleprane and Abdalla, 2013; Galeotti et al., 2018). Phenolic compounds exert antioxidant activity through the donation of hydrogen

atoms from an aromatic hydroxyl group, leading to sequestration of free radicals (Zaccaria et al., 2017; Amorati et al., 2017).

The antioxidant activity of the propolis sample from Salitre was evaluated by two assays, DPPH and β-carotene/linoleic acid. The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay measures the hydrogen atom donation capacity of phenolic compound to scavenge the DPPH radical (Table 3). The β-carotene bleaching method evaluate the ability of phenolic compound to prevent the oxidation of β-carotene, protecting it from the free radicals generated during the peroxidation of linoleic acid (Zheng et al., 2017). The ethyl acetate extract exhibited the best antioxidant activity in both assays. Ethyl acetate extracts of propolis from several Algerian regions exhibited high antioxidant activity by scavenging free radicals and preventing lipid peroxidation (Boufadi et al., 2014).

The configuration, substitution, and total number of hydroxyl groups in flavonoids can influence the mechanisms of radical scavenging. The B ring hydroxyl configuration determines the scavenging of reactive oxygen species (ROS) through the donation of hydrogen, producing relatively stable flavonoid radicals (Amorati et al., 2017). The suppression of ROS formation is performed either by inhibition of enzymes or by chelating trace elements involved in free radical generation, scavenging ROS and upregulation or protection of antioxidant defenses, involved in mechanisms of antioxidant action (Amorati et al., 2017). Quercetin exhibits high antioxidant activity, because possess an ortho catechol group on the B ring, which enables the generation of intra- and intermolecular hydrogen bonds (Treml and Šmejkal, 2016). As can be seen in Table 3, quercetin (**8**), isorhamnetin (**10**) and myricetin-3,3′,7-trimethyl ether (**14**) exhibited high antioxidant activity, followed by *p*-coumaric acid (**6**). Quercetin, a constituent of many types of propolis (Zheng et al., 2017), revealed important cytotoxicity processes against cultured human cells (Treml and Šmejkal, 2016; Amorati et al., 2017) and can be used as a novel therapeutic agent for neurodegenerative diseases induced by oxidative stress (Bao et al., 2017).

The propolis sample from Salitre exhibited moderate activity relative to the inhibition of HIV-1 reverse transcriptase (Table 4). This enzyme converts the viral RNA to viral DNA, which is integrated into the host genome by HIV integrase, responsible for cleaving the translated viral proteins required for formation of new HIV particles, which are released to infect others host cells (Pasetto et al., 2014; Saravanan et al., 2015; Li. et al., 2016). Flavonoids act as bactericidal and bacteriostatic agents by damaging cytoplasmic membranes, inhibiting energy metabolism and synthesis of nucleic acids. Thus, it can be used as antiretrovirals for HIV therapy, due to higher antiviral activity and low toxicity (Ahmad et al., 2015; Saravanan et al., 2015). The inhibition of the HIV-1 reverse transcriptase decreased in the following order: isorhamnetin (**10**), naringenin (**9**), quercetin (**8**), diprenylcinnamic acid (**7**), myricetin-3,3′,7-trimethyl ether (**14**, Fig. 3) and 2′,4,4′-trihydroxy-2-methoxychalcone (**12**, Fig. 3). In general, the presence of prenyl groups may increase anti-HIV activity. A flavonoid containing a 5,7-dihydroxy-6,8-diprenyl system on the A ring exhibited high anti-HIV activity (Meragelman et al., 2001; Kurapati et al., 2016). A highly active flavonoid against HIV-1 protease is 6,8-diprenylgenistein, an isoflavone with two prenyl groups on the A ring and one hydroxyl group at the 4′ position of the B-ring (Lee et al., 2009).

Quercetin exhibited moderate activity against HIV-1, as reported by Kurapati et al. (2016). Myricetin, with an additional hydroxyl group on the 5′ position, is a stronger inhibitor of HIV-1 reverse transcriptase, indicating that the presence of either the unsaturation between positions 2 and 3 of the flavonoid pyrone ring and three hydroxyl groups are important requisites for inhibition of reverse transcriptase activity (Ono et al., 1990). Myricetin showed promising results against different strains of HIV-1, while also showed insignificant cytotoxic effects (Pasetto et al., 2014). Myricetin 3-*O*-rhamnoside and myricetin 3-*O*-(6-rhamnosylgalactoside) inhibited the reverse transcriptase activity. The glycosylated moiety enhanced the anti-HIV-1 activity of myricetin (Ortega et al., 2017). However, in propolis sample from Salitre, the hydroxyl groups of myricetin are in methoxylated form and myricetin-3,3′,7-trimethyl ether exhibited weak activity as inhibitor of reverse transcriptase HIV-1 ($35.35 \pm 3.99\%$). Coherently, the inhibitory activity against HIV-1 reverse transcriptase was shown to decrease proportionally with the degree of methoxylation of flavones (Ortega et al., 2017).

Conclusions

The studied propolis sample from Salitre, a locality in the Atlantic Forest region of the state of Ceará, possesses chemical profile distinct from all propolis types of Brazilian propolis reported so far. The finding of a chalcone among the relevant constituents suggests a leguminous plant as resin source. The propolis sample possesses high antioxidant activity, as revealed by analysis of its ethyl acetate extract. Fractions F5–F7 and isolated compounds, such as *p*-coumaric acid (**6**) quercetin (**8**), isorhamnetin (**10**) and myricetin3,3′,7-trimethyl ether (**14**) were shown to be effective antioxidants. In addition, isorhamnetin (**10**) exhibited moderate inhibition of HIV-1 reverse transcriptase, while other isolated constituents were active in lower degree. The results obtained strengthen the view that propolis research is an effective means to detect bioactive substances of plant origin and that much further work is needed aiming the determination of the botanical origin of propolis produced in Brazil.

Author's contributions

This study is part of doctoral thesis of CCFS; AS and MLFS were the mentors and revise the manuscript; GN and LBM were work collaborators. All the authors made important contributions in the accomplishment of the work, read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank CNPq and FAPESP for provision of funds used in the development of this research; as well as Apiário Bosco for provision of the Salitre propolis sample.

References

Ahmad, A., Kaleem, M., Ahmed, Z., Shafiq, H., 2015. Therapeutic potential of flavonoids and their mechanism of action against microbial and viral infections – a review. Food Res. Int. 77, 221–235.

Al-Ani, I., Zimmermann, S., Reichling, J., Wink, M., 2018. Antimicrobial activities of European propolis collected from various geographic origins alone and in combination with antibiotics. Medicines 5, http://dx.doi.org/10.3390/medicines5010002.

Albuquerque, I.L., Alves, L.A., Lemos, T.L.G., Braz-Filho, R., 2007. Ácido canárico (3,4-seco derivado do lupano) em própolis do Ceará. Quim. Nova 30, 828–831.

Ali Khan, M.S., Ahmed, N., Misbah, Arifuddin, M., Zakaria, Z.A., Al-Sanea, M.M., Khundmiri, S.U.K., Ahmed, I., Ahmed, S., Mok, P.L., 2018. Anti-nociceptive mechanisms of flavonoids-rich methanolic extract from *Terminalia coriacea* (Roxb.) Wight & Arn. leaves. Food Chem. Toxicol. 115, 523–531.

Amorati, R., Baschieri, A., Cowden, A., Valgimigli, L., 2017. The antioxidant activity of quercetin in water solution. Biomimetics, http://dx.doi.org/10.3390/biomimetics2030009.

Andrade, J.K.S., Denadai, M., de Oliveira, C.S., Nunes, M.L., Narain, N., 2017. Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region. Food Res. Int. 101, 129–138.

Bao, D., Wang, J., Pang, X., Liu, H., 2017. Protective effect of quercetin against oxidative stress-induced cytotoxicity in rat pheochromocytoma (PC-12) cells. Molecules 22, http://dx.doi.org/10.3390/molecules22071122.

Berretta, A.A., Arruda, C., Miguel, F.G., Baptista, N., Nascimento, A.P., Marquele-Oliveira, F., Hori, J.I., Hernane da Silva Barud, H., Damaso, B., Ramos, C., Ferreira, R., Bastos, J.K., 2017. Functional properties of Brazilian propolis: from chemical composition until the market. In: Waisundara, V., Shiomi, N. (Eds.), Agricultural and Biological Sciences "Superfood and Functional Food – An Overview of Their Processing and Utilization"., http://dx.doi.org/10.5772/65932.

Bertelli, D., Papotti, G., Bortolotti, L., Marcazzan, G.L., Plessi, M., 2012. ^{1}H-NMR simultaneous identification of health-relevant compounds in propolis extracts. Phytochem. Anal. 23, 260–266.

Bittencourt, M.L.F., Ribeiro, P.R., Franco, R.L.P., Hilhorst, H.W.M., de Castro, R.D., Fernandez, L.G., 2015. Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: use of correlation and multivariate analyses to identify potential bioactive compounds. Food Res. Int. 76, 449–457.

Boufadi, Y.M., Soubhye, J., Riazi, A., Rousseau, A., Vanhaeverbeek, M., Nève, J., Boudjeltia, K.Z., Van Antwerpen, P., 2014. Characterization and antioxidant properties of six Algerian propolis extracts: ethyl acetate extracts inhibit myeloperoxidase activity. Int. J. Mol. Sci. 15, 2327–2345.

Bueno-Silva, B., Marsola, A., Ikegaki, M., Alencar, S.M., Rosalen, P.L., 2017. The effect of seasons on Brazilian red propolis and its botanical source: chemical composition and antibacterial activity. Nat. Prod. Res. 31, 1318–1324.

Cao, X., Weia, Y., Ito, Y., 2009. Preparative isolation of isorhamnetin from stigma maydis using high-speed countercurrent chromatography. J. Liq. Chromatogr. Relat. Technol. 32, 273–280.

Castro Ishida, V.F., Negri, G., Salatino, A., Bandeira, M.F.C.L., 2011. A new type of Brazilian propolis: prenylated benzophenones in propolis from Amazon and effects against cariogenic bacteria. Food Chem. 125, 966–972.

Cuesta-Rubio, O., Frontana-Uribe, B.A., Ramírez-Apan, T., Cárdenas, J., 2002. Polyisoprenylated benzophenones in cuban propolis; biological activity of nemorosone. Z. Naturforsch. C 57, 372–378.

Daleprane, J.B., Abdalla, D.S., 2013. Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. Evid. Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/175135.

de Oliveira Dembogurski, D.S., Silva Trentin, D., Boaretto, A.G., Rigo, G.V., da Silva, R.C., Tasca, T., Macedo, A.J., Carollo, C.A., Silva, D.B., 2018. Brown propolis-metabolomic innovative approach to determine compounds capable of killing *Staphylococcus aureus* biofilm and *Trichomonas vaginalis*. Food Res. Int. 111, 661–673.

Engels, C., Gräter, D., Esquivel, P., Jiménez, V.M., Gänzle, M.G., Schieber, A., 2012. Characterization of phenolic compounds in jocote (*Spondias purpurea* L.) peels by ultrahigh-performance liquid chromatography/electrospray ionization mass spectrometry. Food Res. Int. 46, 557–562.

Fernandes, F.H., Guterres, Z.R., Violante, I.M.P., Lopes, T.F.S., Garcez, W.S., Garcez, F.R., 2015. Evaluation of mutagenic and antimicrobial properties of brown propolis essential oil from the Brazilian Cerrado biome. Toxicol. Rep. 2, 1482–1488.

Fernandes-Silva, C.C., Salatino, A., Negri, G., Breyer, E., Salatino, M.L.F., 2013. Chemical profiling of six samples of Brazilian propolis. Quim. Nova 36, 237–240.

Ferreira, J.M., Fernandes-Silva, C.C., Salatino, A., Negri, G., Message, D., 2017. New propolis type from north-east Brazil: chemical composition, antioxidant activity and botanical origin. J. Sci. Food Agric. 97, 3552–3558.

Galeotti, F., Maccari, F., Fachini, A., Volpi, N., 2018. Chemical composition and antioxidant activity of propolis prepared in different forms and in different solvents useful for finished products. Foods 7, http://dx.doi.org/10.3390/foods7030041.

George, M., Sebastian, V.S., Reddy, P.N., Srinivas, R., Giblin, D., Gross, M.L., 2009. Gas-phase Nazarov cyclization of protonated 2-methoxy and 2-hydroxychalcone: an example of intramolecular proton-transport catalysis. J. Am. Soc. Mass Spectrom. 20, 805–818.

Gutierrez-Gonçalves, M.E.J., Marcucci, M.C., 2009. Antimicrobial and antioxidant activities of propolis from Ceará State. Fitos 4, 81–86.

Hashemi, J.M., 2016. Biological effect of bee propolis: a review. Eur. J. Appl. Sci. 8, 311–318.

Huang, S., Zhang, C.-P., Wang, K., Li, G.Q., Hu, F.-L., 2014. Review, recent advances in the chemical composition of propolis. Molecules 19, 19610–19632.

Hussein, S.A.M., Hashem, A.N.M., Seliem, M.A., Lindequist, U., Nawwar, M.A.M., 2003. Polyoxygenated flavonoids from *Eugenia edulis*. Phytochemistry 64, 883–889.

Kajiyama, K., Demizu, S., Hiraga, Y., Kinoshita, K., Koyama, K., Takahashi, K., Tamura, Y., Okada, K., Kinoshita, T., 1992. Two prenylatedretrochalcones from *Glycyrrhiza inflata*. Phytochemistry 31, 3229–3232.

Karar, M.G.E., Kuhnert, N., 2015. UPLC–ESI-Q-TOF-MS/MS characterization of phenolics from *Crataegus monogyna* and *Crataegus laevigata* (Hawthorn) leaves, fruits and their herbal derived drops (Crataegutt Tropfen). J. Chem. Biol. Ther. 1, 102.

Kasiotis, K.M., Anastasiadou, P., Papadopoulos, A., Machera, K., 2017. Revisiting Greek propolis: chromatographic analysis and antioxidant activity study. PLOS ONE 12, e0170077.

Khamsan, S., Liawruangrath, S., Teerawutkulrag, A., Pyne, S.G., Garson, M.J., Liawruangrath, B., 2012. The isolation of bioactive flavonoids from *Jacaranda obtusifolia* H. B. K. ssp. *rhombifolia* (G. F. W. Meijer) Gentry. Acta Pharm. 62, 181–190.

Kocot, J., Kiełczykowska, M., Luchowska-Kocot, D., Kurzepa, J., Musik, I., 2018. Antioxidant potential of propolis, bee pollen, and royal jelly: possible medical application. Oxid. Med. Cell. Longev., http://dx.doi.org/10.1155/2018/7074209.

Koleva, I.I., van Beek, T., Linssen, J.P.H., de Groot, A., Evstatieva, L.N., 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13, 8–17.

Koo, I., Kim, S., Zhang, X., 2013. Comparative analysis of mass spectral matching-based compound identification in gas chromatography–mass spectrometry. J. Chromatogr. A 1298, 132–138.

Kranjc, E., Albreht, A., Vovk, I., Makuc, D., Plavec, J., 2016. Non-targeted chromatographic analyses of cuticular wax flavonoids from *Physalis alkekengi* L. J. Chromatogr. A 1437, 95–106.

Kumar, S., Singh, A., Kumar, B., 2017. Identification and characterization of phenolics and terpenoids from ethanolic extracts of *Phyllanthus* species by HPLC–ESI-QTOF-MS/MS. J. Pharm. Anal. 7, 214–222.

Kumazawa, S., Murase, M., Momose, N., Fukumoto, S., 2014. Analysis of antioxidant prenylflavonoids in different parts of *Macaranga tanarius*, the plant origin of Okinawan propolis. Asian Pac. J. Trop. Med. 7, 16–20.

Kurapati, K.R., Atluri, V.S., Samikkannu, T., Garcia, G., Nair, M.P., 2016. Natural products as anti-HIV agents and role in HIV-associated neurocognitive disorders (HAND): a brief overview. Front. Microbiol., http://dx.doi.org/10.3389/fmicb.2015.01444.

Lee, J., Oh, W.K., Ahn, J.S., Kim, Y.H., Mbafor, J.T., Wandji, J., Fomum, Z.T., 2009. Prenyl isoflavonoids from *Erythrina senegalensis* as novel HIV-1 protease inhibitors. Planta Med. 75, 268–270.

Lemos da Silva, L.A., Faqueti, L.G., Reginatto, F.H., Conceicão dos Santos, A.D., Barison, A., Biavatti, M.W., 2015. Phytochemical analysis of *Vernonanthura tweedieana* and a validated UPLC-PDA method for the quantification of eriodictyol. Rev. Bras. Farmacogn. 25, 375–381.

Li, S., Liu, S., Pi, Z., Song, F., Jin, Y., Liu, Z., 2017. Chemical profiling of Fufang-Xialian-Capsule by UHPLC–Q-TOF-MS and its antioxidant activity evaluated by *in vitro* method. J. Pharm. Biomed. Anal. 138, 289–301.

Li, A., Li, J., Johnson, K.A., 2016. HIV-1 reverse transcriptase polymerase and RNase H (Ribonuclease H) active sites work simultaneously and independently. J. Biol. Chem. 291, 26566–26585.

Machado, C.S., Mokochinski, J.B., de Lira, T.O., Evangelista de Oliveira, F.C., Cardoso, M.V., Ferreira, R.G., Sawaya, A.C.H.F., Ferreira, A.G., Pessoa, C., Cuesta-Rubio, O., Monteiro, M.C., de Campos, M.S., Torres, Y.R., 2016. Comparative study of chemical composition and biological activity of yellow, green, brown, and red Brazilian propolis. Evid. Based Complement. Altern. Med., http://dx.doi.org/10.1155/2016/6057650.

Martinotti, S., Ranzato, E., 2015. Propolis: a new frontier for wound healing? Burns Trauma, http://dx.doi.org/10.1186/s41038-015-0010-z.

Mendonça, I.C.G., de Moraes Porto, I.C.C., do Nascimento, T.G., de Souza, N.S., dos Santos Oliveira, J.M., dos Santos Arruda, R.E., Mousinho, K.C., dos Santos, A.F., Basílio-Júnior, I.D., Parolia, A., Barreto, F.S., 2015. Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. BMC Complement. Altern. Med., http://dx.doi.org/10.1186/s12906-015-0888-9.

Meragelman, K.M., McKee, T.C., Boyd, M.R., 2001. Anti-HIV prenylated flavonoids from *Monotes africanus*. J. Nat. Prod. 64, 546–548.

Miguel, M.G., Antunes, M.D., 2011. Is propolis safe as an alternative medicine? J. Pharm. Bioallied Sci. 3, 479–495.

Moreno, M.I., Isla, M.I., Sampietro, R., Vattuone, M., 2000. Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. J. Ethnopharmacol. 71, 109–114.

Nina, N., Quispe, C., Jiménez-Aspee, F., Theoduloz, C., Giménez, A., Schmeda-Hirschmann, G., 2016. Chemical profiling and antioxidant activity of Bolivian propolis. J. Sci. Food Agric. 96, 2142–2153.

Ono, K., Nakane, H., Fukushima, M., Chermann, J.C., Barré-Sinoussi, F., 1990. Differential inhibitory effects of various flavonoids on the activities of reverse transcriptase and cellular DNA and RNA polymerases. Eur. J. Biochem. 190, 469–476.

Ortega, J.T., Suárez, A.I., Serrano, M.L., Baptista, J., Pujol, F.H., Rangel, H.R., 2017. The role of the glycosyl moiety of myricetin derivatives in anti-HIV-1 activity *in vitro*. AIDS Res. Ther., http://dx.doi.org/10.1186/s12981-017-0183-6.

Palacios, C.E.J., Negri, G., Salatino, A., 2015. Esters and other constituents of the foliar cuticular wax of a soybean variety. Biochem. Syst. Ecol. 63, 198–200.

Pasetto, S., Pardi, V., Murata, R.M., 2014. Anti-HIV-1 activity of flavonoid myricetin on HIV-1 infection in a dual-chamber in vitro model. PLOS ONE 9, e115323.

Pasupuleti, V.R., Sammugam, L., Ramesh, N., Gan, S.H., 2017. Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits. Oxid. Med. Cell. Longev., http://dx.doi.org/10.1155/2017/1259510.

Paula, V.F., Barbosa, L.C.A., Errington, W., Howarth, O.W., Cruz, M.P., 2002. Chemical constituents from *Bombacopsis glabra* (Pasq.) A. Robyns: complete ^{1}H and ^{13}C NMR assignments and X ray structure of 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone. J. Braz. Chem. Soc. 13, 276–280.

Pereira Beserra, F., Xue, M., Maia, G.L.A., Leite Rozza, A., Helena Pellizzon, C., Jackson, C.J., 2018. Lupeol, a pentacyclic triterpene, promotes migration, wound closure, and contractile effect *in vitro*: possible involvement of PI3K/Akt and p38/ERK/MAPK pathways. Molecules 23, http://dx.doi.org/10.3390/molecules23112819, pii:E2819.

Petelinc, T., Polak, T., Demšar, L., Jamnik, P., 2013. Fractionation of phenolic compounds extracted from propolis and their activity in the yeast *Saccharomyces cerevisiae*. PLOS ONE 8, e56104.

Popova, M., Dimitrova, R., Talib Al-Lawati, H., Tsvetkova, I., Najdenski, H., Bankova, V., 2013. Omani propolis: chemical profiling, antibacterial activity and new propolis plant sources. Chem. Centr. J., http://dx.doi.org/10.1186/1752-153X-7-158.

Popova, M., Trusheva, B., Antonova, D., Cutajar, S., Mifsud, D., Farrugia, C., Tsvetkova, I., Najdenski, H., Bankova, V., 2011. The specific chemical profile of Mediterranean propolis from Malta. Food Chem. 126, 1431–1435.

Popova, M.P., Graikou, K., Chinou, I., Bankova, V.S., 2010. GC–MS profiling of diterpene compounds in Mediterranean propolis from Greece. J. Agric. Food Chem. 58, 3167–3176.

Reutrakul, V., Ningnuek, N., Pohmakotr, M., Yoosook, C., Napaswad, C., Kasisit, J., Santisuk, T., Tuchinda, P., 2007. Anti-HIV-1 flavonoid glycosides from *Ochna integerrima*. Planta Med. 73, 683–688.

Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A., 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J. Sci. Food Agric. 91, 2363–2370.

Righi, A.A., Negri, G., Salatino, A., 2013. Comparative chemistry of propolis from eight Brazilian localities. Evid. Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/267878.

Ristivojević, P., Trifković, J., Andrić, F., Milojković-Opsenica, D., 2015. Poplar-type propolis: chemical composition, botanical origin and biological activity. Nat. Prod. Commun. 10, 1869–1876.

Rufatto, L.C., Santos, D.A., Marinho, F., Pêgas Henriques, J.A., Roesch Ely, M., Moura, S., 2017. Red propolis: chemical composition and pharmacological activity. Asian Pac. J. Trop. Biomed. 7, 591–598.

Salatino, A., Salatino, M.L.F., 2018. Brazilian red propolis: legitimate name of the plant resin source. MOJ Food Process. Technol. 6, 21–22.

Salatino, A., Teixeira, E.W., Negri, G., Message, D., 2005. Origin and chemical variation of Brazilian propolis. Evid. Based Complement. Altern. Med. 2, 33–38.

Santos, D.C., David, J.M., David, J.P., 2017. Composição química, atividade citotóxica e antioxidante de um tipo de própolis da Bahia. Quim. Nova 40, 171–175.

Saravanan, D., Thirumalai, D., Asharani, I.V., 2015. Anti-HIV flavonoids from natural products: a systematic review. Int. J. Res. Pharm. Sci. 6, 248–255.

Silva, M.D.S.S., Citó, A.M.D.G.L., Chaves, M.H., Lopes, J.A.D., 2005. Triterpenóides tipo cicloartano de própolis de Teresina – PI. Quim. Nova 28, 801–804.

Silva-Carvalho, R., Baltazar, F., Almeida-Aguiar, C., 2015. Propolis: a complex natural product with a plethora of biological activities that can be explored for drug development. Evid. Based Complement. Altern. Med., http://dx.doi.org/10.1155/2015/206439.

Singh, K., Marchand, B., Kirby, K.A., Michailidis, E., Sarafianos, S.G., 2010. Structural aspects of drug resistance and inhibition of HIV-1 reverse transcriptase. Viruses 2, 606–638.

Sun, J., Song, Y., Sun, H., Liu, W., Zhang, Y., Zheng, J., Zhang, Q., Zhao, Y., Xiao, W., Tu, P., Li, J., 2017. Characterization and quantitative analysis of phenolic derivatives in Fufang-Xialian-Capsule by HPLC–DAD-IT-TOF–MS. J. Pharm. Biomed. Anal. 145, 462–472.

Tada, A., Ishizuki, K., Yamazaki, T., Sugimoto, N., Akiyama, H., 2014. Method for the determination of natural ester-type gum bases used as food additives via direct analysis of their constituent wax esters using high-temperature GC/MS. Food Sci. Nutr. 2, 417–425.

Taddeo, V.A., Epifano, F., Fiorito, S., Genovese, S., 2016. Comparison of different extraction methods and HPLC quantification of prenylated and unprenylated phenylpropanoids in raw Italian propolis. J. Pharm. Biomed. Anal. 129, 219–223.

Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid. Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/697390.

Treml, J., Šmejkal, K., 2016. Flavonoids as potent scavengers of hydroxyl radicals. Comprehensive reviews. Food Sci. Food Saf. 15, 720–738.

Trusheva, B., Popova, M., Koendhori, E.B., Tsvetkova, I., Naydenski, C., Bankova, V., 2011. Indonesian propolis: chemical composition, biological activity and botanical origin. Nat. Prod. Res. 25, 606–613.

Woradulayapinij, W., Soonthornchareonnon, N., Wiwat, C., 2005. *In vitro* HIV type 1 reverse transcriptase inhibitory activities of Thai medicinal plants and *Canna indica* L. rhizomes. J. Ethnopharmacol. 101, 84–89.

Xu, F., Huang, X., Wu, H., Wang, X., 2018. Beneficial health effects of lupenone triterpene: a review. Biomed. Pharmacother. 103, 198–203.

Zaccaria, V., Curti, V., Di Lorenzo, A., Baldi, A., Maccario, C., Sommatis, S., Mocchi, R., Daglia, M., 2017. Effect of green and brown propolis extracts on the expression levels of microRNAs, mRNAs and proteins, related to oxidative stress and inflammation. Nutrients 9, http://dx.doi.org/10.3390/nu9101090.

Zhang, H.J., Rumschlag-Booms, D., Guan, Y.F., Liu, K.L., Wang, D.Y., Li, W.F., Nguyen, V.H., Cuong, H.M., Soejarto, D.D., Fong, H.H.S., Rong, L.J., 2017. Anti-HIV diphyllin glycosides from *Justicia gendarussa*. Phytochemistry 136, 94–100.

Zhang, J.-Y., Li, N., Che, Y.-Y., Zhang, Y., Liang, S.-X., Zhao, M.-B., Jiang, Y., Tu, P.-F., 2011. Characterization of seventy polymethoxylated flavonoids (PMFs) in the leaves of *Murraya paniculata* by on-line high-performance liquid chromatography coupled to photodiode array detection and electrospray tandem mass spectrometry. J. Pharm. Biomed. Anal. 56, 950–961.

Zheng, Y.-Z., Deng, G., Liang, Q., Chen, D.-F., Guo, R., Lai, R.-C., 2017. Antioxidant activity of quercetin and its glucosides from propolis: a theoretical study. Sci. Rep. 7, 7543.

Zou, G.-A., Su, Z.-H., Zhang, H.-W., Wang, Y., Yang, J.-S., Zou, Z.-M., 2010. Flavonoids from the stems of *Croton caudatus* Geisel. var. *tomentosus* Hook. Molecules 15, 1097–1102.