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Melipona mondury produces a geopropolis with antioxidant, antibacterial and antiproliferative activities

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ABSTRACT

Geopropolis is a special type of propolis produced by stingless bees. Several pharmacological properties have been described for different types of geopropolis, but there have been no previous studies of the geopropolis from *Melipona mondury*. In this study, we investigated the antioxidant, antibacterial, and antiproliferative activities of *M. mondury* geopropolis, and determined its chemical profile. The antioxidant activity was determined using *in vitro* $ABTS^{\bullet+}$, $^{\bullet}DPPH$, and β-carotene/linoleic acid co-oxidation methods. The antibacterial activity was determined using a microdilution method with *Pseudomonas aeruginosa, Staphylococcus aureus*, and methicillin-resistant *S. aureus*. The antiproliferative effect was determined in tumor cell lines using the Alamar Blue assay. The chemical profile was obtained using UHPLC-MS and UHPLC-MS/MS. The butanolic fraction had the highest concentration of phenolic compounds and more potent antioxidant properties in all assays. This fraction also had bacteriostatic and bactericidal effects against all bacterial strains at low concentrations, especially *S. aureus*. The hexane fraction had the highest antiproliferative potential, with IC_{50} values ranging from 24.2 to 46.6 μg/mL in HL-60 (human promyelocytic leukemia cell) and K562 (human chronic myelocytic leukemia cell), respectively. Preliminary chemical analysis indicates the presence of terpenes and gallic acid in the geopropolis. Our results indicate the therapeutic potential of geopropolis from *M. mondury* against inflammatory, oxidative, infectious, and neoplastic diseases.

Key words: antibacterial, antioxidant, antiproliferative, geopropolis, *Melipona mondury*.

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INTRODUCTION

Geopropolis is a mixture of resin, wax, and soil, and it is an uncommon type of propolis produced by native stingless bees from the Meliponini tribe, which are widely distributed in tropical and subtropical areas worldwide (Sforcin and Bankova 2011, Souza et al. 2014). The geopropolis from *Melipona mondury* (Hymenoptera: Apidae: Meliponini) was investigated in the present study because this bee species is distributed extensively in Brazil, especially in Bahia state in the northeast of the country (Melo 2003). *M. mondury* is found exclusively in warm and humid climates in about 24% of the municipalities of Bahia state (Souza et al. 2012). In addition to their importance as efficient pollinators of native flora, stingless bees yields products such as honey, pollen, and geopropolis, which are sources of food, medicine, and income for the rural population (Melo 2003).

Studies of different types of geopropolis have demonstrated their significant antinociceptive (Souza et al. 2014), anti-inflammatory (Franchin et al. 2013, Campos et al. 2015), antioxidant (Souza et al. 2014, Campos et al. 2014, 2015), immunomodulatory (Liberio et al. 2011), antibacterial (Liberio et al. 2011, Cunha et al. 2013, Campos et al. 2014, 2015), and antiproliferative (Cunha et al. 2013, Campos et al. 2014, 2015) activities. Their chemical compositions are extremely complex and their diversity depends on the flora visited and the season (Marcucci 1995, Bankova et al. 2000, Sforcin and Bankova 2011). Indeed, numerous potential bioactive metabolites have been reported previously in geopropolis, such as phenylpropanoids, flavonoids (Souza et al. 2013), gallotannins, ellagitannins (Dutra et al. 2014), benzophenones (Cunha et al. 2013, 2016), coumarins (Cunha et al. 2016) terpenes, and gallic acid (Bankova et al. 2000, Souza et al. 2013, Dutra et al. 2014). These findings have encouraged the exploration of *M. mondury* geopropolis as a potential source of novel bioactive compounds.

The biological activities and chemical compositions of geopropolis have been reported for many different stingless bee species (Liberio et al. 2011, Franchin et al. 2013, Cunha et al. 2013, Campos et al. 2014, 2015). However, there have been no previous pharmacological and chemical analyses of geopropolis from *M. mondury*. Therefore, we performed the first study of the *in vitro* antioxidant, antibacterial, and antiproliferative activities of geopropolis from *M. mondury*, as well as determining the chemical profile of its ethanolic extract and fractions. This study obtained reliable information, which may add value to this natural product.

MATERIALS AND METHODS

GEOPROPOLIS SAMPLE AND FRACTIONATION

Fresh samples of *M. mondury* geopropolis were collected in Nova Ibiá city, Bahia state, Brazil (13°48'36'' S, 39°37'32'' W) during August 2015. The geopropolis (100 g) was extracted three times with absolute ethanol for 72 h and filtered to obtain the ethanolic extract of geopropolis (EEGP = 10 g). EEGP was fractionated via liquid-liquid partitioning with hexane, ethyl acetate, and butanol solvents in order of increasing polarity to obtain hexane (HFGP = 0.5 g), ethyl acetate (EAFGP = 1.6 g) and butanol fractions of geopropolis (BFGP 0.5 g).

TOTAL PHENOLIC CONTENTS

The total phenolic contents of the extract and fractions was determined according to the Folin-Ciocalteu procedure (Slinkard and Singleton 1977, Piccinelli et al. 2004). Briefly, the samples at 1 mg/mL (125 µL) was mixed with 125 µL of Folin-Ciocalteau's reagent and 1 mL of distilled water. After 3 min, 125 µL of saturated sodium carbonate solution was added and the mixture was incubated

for 30 min at 37°C before measuring the absorbance at 765 nm. A calibration curve was obtained with gallic acid (0.5, 5, 10, 15 and 25 µg) and the results were expressed as µg gallic acid equivalent (GAE) per mg of sample.

DETERMINATION OF ANTIOXIDANT ACTIVITIES

The antioxidant activities of geopropolis samples were evaluated using three well-known methods: $^{\bullet}$DPPH (2,2–diphenyl-1-picrylhydrazyl) free radical scavenging (Piccinelli et al. 2004), $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radical) decolorization (Re et al. 1999), and β-carotene-linoleic acid cooxidation (Miller 1971, with minor modifications) assays.

In the DPPH assay, the samples (0-100 µg/mL) were incubated to 2 mL of an ethanolic solution of $^{\bullet}$DPPH (70 µM). After 20 min at 25°C, the absorbance was measured at 517 nm. In the $ABTS^{\bullet+}$ assay, we first prepared ABTS radical cation by reacting ABTS salt (7 mM) concentration with potassium persulfate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The stock $ABTS^{\bullet+}$ solution was diluted with ethanol (absorbance of 0.7 at 734 nm) and incubated to samples (0-100 µg/mL) in a final volume of 2 mL. The absorbance was measured at 734 nm after 7 min of incubation. In both experiments, the values were expressed as the concentration of sample necessary to reduce 50% of the free radicals (IC_{50}).

In the β-carotene-linoleic acid cooxidation assay, a stock solution of β-carotene/linoleic acid was initially prepared by dissolving 2 mg of β-carotene in 100 µL of chloroform. Ten microliters of β-carotene solution were mixed to 40 mg of linoleic acid and 530 µL of Tween 40. The chloroform was rotaevaporated and aerated distilled water was added to the mixture until an initial absorbance of 0.65 at 470 nm. Two milliliters of β-carotene/linoleic acid emulsion were mixed with samples at 74 µg/mL. The reactions were incubated at 50°C for 2h before taking another absorbance reading. The data were expressed as the percentage inhibition of β-carotene bleaching by geopropolis extract or fraction (% IO).

In all assays gallic acid and trolox were used as positive controls.

DETERMINATION OF ANTIBACTERIAL ACTIVITY

Bacterial strains

The bacterial strains used in this study were *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and methicillin-resistant *Staphylococcus aureus* (CA-MRSA). The CA-MRSA strain was cultured at the Microbiology Laboratory in the Multidisciplinary Institute of Health, Federal of University of Bahia. This strain was isolated from raw human milk (LC2). Phenotypic and genotypic tests, identification of the *nuc* gene (Brakstad et al. 1992), detection of the *mecA* gene (Perez-Hoth et al. 2001), and chromosomal cassette typing *mec* (*ssmec*) (Boye et al. 2007) were conducted to identify the strains. The isolated LC2 was positive for the *nuc* gene and *mecA* as well as *ssmec*, and thus typed as IV, which characterized it as a CA-MRSA strain.

Susceptibility testing

The antibacterial activities of EEAGP and other fractions were examined by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the Institute of Clinical and Laboratory Standards (CLSI 2006, Cunha et al. 2013). To determine the MIC, 5×10^5 CFU/mL diluted in brain heart infusion medium (Difco) were incubated with EEGP and fractions (1–1000 µg/mL) in 96-well microplates for 24 h at 37°C. The vehicle control was ethanol (final ethanol concentration = 5%, v/v). MIC was defined as the lowest concentration

of EEGP or fraction that allowed no visible growth after incubation with 0.01% resazurin dye (Sigma-Aldrich) for 60 min at room temperature. MBC was determined by sub-culturing 10 µL of each incubated well that had a concentration higher than the MIC on Müller-Hinton agar. The MBC was then treated as the lowest concentration of each sample with no visible colony growth on agar plates. Tetracycline and ceftriaxone were used as positive controls for Gram-positive and -negative bacteria, respectively.

ANTIPROLIFERATIVE ASSAY

Cells

Antiproliferative effects were determined in tumor cell lines: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia), and HL-60 (human promyelocytic leukemia). All of the cell lines were kindly provided by Hospital A.C. Camargo (São Paulo, SP, Brazil) and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco) medium supplemented with 10% fetal bovine serum (Cultilab), 2 mM L-glutamine (Vetec Química Fina), and 50 µg/mL gentamycin (Novafarma). All cell lines were cultured in cell culture flasks at 37°C in 5% CO_2 and subcultured every 3–4 days to maintain exponential growth (Rodrigues et al. 2015)

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy, non-smoker donors using a Ficoll-Paque density gradient (GE Healthcare). PBMCs were washed and resuspended at a concentration of 0.3×10^6 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and 50 µg/mL gentamycin at 37°C with 5% CO_2. Next, the cells were incubated with a T-lymphocyte-specific mitogen, concanavalin A (10 µg/mL, Sigma Chemical Co.), for 24 h before the experiments to obtain a culture of human lymphoblasts (Berthold 1981, Brown and Lawce 1997). In all experiments, cell viability was determined using the Trypan blue assay (>90%).

The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (#031019/2013). All blood donors signed a written informed consent form to participate in the study.

In vitro antiproliferative activity assay

Cell viability was quantified using the Alamar Blue assay, as described previously by Ahmed et al. (1994) with minor modifications (Rodrigues et al. 2015). Non-tumor and tumor cells were placed in 96-well plates (7×10^4 cells/mL for adherent cells or 3×10^5 cells/mL for suspended cells in 100 µL of medium) and incubated with EEGP or its fractions (0.39–50 µg/mL) for 72 h at 37°C under a CO_2 (5%) atmosphere. Cell viability was quantified based on the ability of living cells to reduce Alamar Blue dye (52 µg/mL; Sigma Aldrich) to a red resorufin product where the absorbance was measured at 570 and 600 nm (DTX-880, Beckman Coulter) after 2h at 37°C under CO_2 (5%) atmosphere. Doxorubicin (purity ≥ 95%, doxorubicin hydrochloride, Laboratory IMA) was used as the positive control (0.08–5 µg/mL).

CHEMICAL ANALYSIS

Chromatographic analyses of samples were performed using a UPLC Acquity chromatograph coupled with a TQD Acquity mass spectrometer (Micromass-Waters), with an electrospray ionization (ESI) source in the negative mode. The column was a C18 BEH Waters Acquity (2.1 mm x 50 mm x 1.7 µm particle size). The mobile phases were 0.1% ammonium hydroxide (phase A) and methanol (phase B). The flow rate was 0.2 mL/min with a linear gradient starting at 75% B and increasing to up 100% methanol in 9 min, before holding until 10 min, and then returning to the initial

conditions, followed by column re-equilibration. The ESI conditions were: capillary = 3.00 kV, cone = 30 V, source temperature = 150°C, desolvation temperature = 350°C, and collision energy = 30 V, with data acquisition between *m/z* 100 and 1000.

The (ESI(-)-MS) fingerprints were obtained by direct injection in duplicate using 0.01 mL of EEGP and the fractions (1 mg/mL) by flow insertion with a solvent mixture comprising methanol:water (90:10 v/v) at flow rate of 0.1 mL/min. The components of geopropolis samples were putatively identified by comparing their *m/z* values and fragmentation patterns with previous reports.

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard deviation or IC_{50} values based on three independent experiments. Significant differences ($p < 0.05$) were detected by one-way ANOVA with Tukey's post-test using GraphPad 4.00.

RESULTS AND DISCUSSION

The chemical composition of propolis is very complex and closely related to its biological activity (Bankova et al. 2000, Sforcin and Bankova 2011, Cunha et al. 2016). Phenolic compounds, terpenes, and aromatic acids are considered the main bioactive constituents of propolis (Bankova et al. 2000, Souza et al. 2013, Dutra et al. 2014). The total phenolic compounds and antioxidant activities of geopropolis samples produced by *M. mondury* are shown in Table I. BFGP contained the highest amount of phenolic (303.1 ± 0.1 µg GAE / mg), which differed significantly from that in the other fractions ($p < 0.05$). HFGP contained a low concentration of phenolics (GAE 40.1 ± 0.4 µg/mg), as expected. In our study, the amount of reducing compounds was much higher than that found by Souza et al. (2013) in *M. subnitida* geopropolis from Paraiba state, Brazil (ranging from 25.6 ± 0.5 to 115.8 ± 0.8 µg GAE/mg). This suggests that the bee species and/or its habitat influence the chemical composition of geopropolis (Campos et al. 2015).

Three different methods were employed to determine the antioxidant properties of the geopropolis: scavenging of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, and inhibition of β-carotene bleaching. All samples exhibited some radical scavenging activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Table I). BFGP was more effective fraction at reducing $DPPH^{\cdot}$ and $ABTS^{\bullet+}$ (IC_{50} ~ 2.2 and 0.9 µg/mL, respectively). BFGP (74 µg/mL) inhibited β-carotene bleaching at around 78.6%, which was similar to that with the same concentration of trolox standard. Unexpectedly, the hexane fraction had weak activity in the β-carotene/linoleic acid bleaching assay. Nevertheless, the antioxidant profile of *M. mondury* geopropolis was higher compared with that of *M. subnitida* geopropolis, which is known as *Jandaíra* in northeastern Brazil (Souza et al. 2013). Previously, it has been suggested that reducing compounds such as phenolics are responsible for the antioxidant activity of geopropolis (Souza et al. 2013, 2014, Campos et al. 2014, Dutra et al. 2014), which may explain why the BFGP had the highest antioxidant activity in all the assays.

Infectious diseases are important causes of morbidity and mortality among humans, especially in developing and poor countries. *S. aureus* and *P. aeruginosa* are particularly important because they are often associated with nosocomial infections, and they have increased resistance to many clinically available antibiotics, thereby stimulating the search for alternative treatments (Cinegaglia et al. 2013, Sampaio et al. 2013). Geopropolis has also been proposed as an alternative for prevention and/or treatment of infectious diseases because it has broad antimicrobial activities. In fact, several types of geopropolis have antibacterial properties (Liberio et al. 2011, Cunha et al. 2013, Campos et al. 2014, 2015).

According to this study, *M. mondury* geopropolis had antibacterial activities against

TABLE I
TOTAL PHENOLICS AND ANTIOXIDANT ACTIVITIES OF THE *Melipona mondury* GEOPROPOLIS EXTRACT AND FRACTIONS.

Sample	Total Phenolics µg GAE/mg	$^{\bullet}$DPPH IC_{50} (µg/mL)	$ABTS^{\bullet+}$ IC_{50} (µg/mL)	β-carotene-linoleic acid cooxidation (% IO)
EEGP	144.4 ± 0.01^{a}	6.91 ± 017^{a}	5.96 ± 0.08^{a}	27.99 ± 0.2^{a}
HFGP	40.12 ± 0.4^{b}	20.22 ± 0.2^{b}	20.51 ± 0.15^{b}	4.7 ± 0.001^{b}
EAFGP	140.9 ± 0.28^{a}	6.58 ± 0.04^{c}	5.5 ± 0.028^{c}	26.45 ± 0.4^{a}
BFGP	303.1 ± 0.14^{c}	$2.23 \pm 0{,}05^{d}$	0.87 ± 0.003^{d}	78.57 ± 0.3^{c}
Trolox	-	1.54 ± 0.01^{e}	1.20 ± 0.035^{e}	83.69 ± 0.7^{d}
Gallic acid	-	1.45 ± 0.01^{e}	$1.08 \pm 0.04^{d,e}$	54.7 ± 0.4^{e}

EEGP = ethanolic extract of geopropolis, HFGP = hexane fraction of geopropolis, EAFGP = ethyl acetate fraction of geopropolis, BFGP = butanol fraction of geopropolis. For the β-*carotene-linoleic acid* cooxidation method, samples were incubated at 74 µg/mL. Values represent the mean ± standard deviation based on triplicate measurements. The same superscript letters in the same column indicate that the values are not different, whereas different superscript letters denote that the values are significantly different (Tukey's test, $p < 0.05$).

Gram-positive and -negative bacteria (Table II). EEGP exhibited bactericidal activity against *P. aeruginosa,* a Gram-negative bacillus (MIC = MBC = 250 µg/mL). BFGP inhibited the growth of *S. aureus*, CA-MRSA, and *P. aeruginosa* at concentrations of 5–10, 250, and 500 µg/mL, respectively. The MBC values showed that BFGP had bactericidal activities at concentrations of 25 and 1000 µg/mL against *S. aureus* and *P. aeruginosa*, respectively. The MIC and MBC values for BFGP against all bacterial strains were comparable to those of standard antibiotics, such as ceftriaxone (MIC = 3 µg/mL and MBC = 10 µg/mL) and tetracycline (MIC = MBC = 3 µg/mL), considering that BFGP is a complex sample. The MIC values of EAFGP against *S. aureus*, CA-MRSA, and *P. aeruginosa* were 25, 500, and 250 µg/mL, respectively. The EAFGP had a bactericidal effect only on *S. aureus* at 1000 µg/mL. EEGP and its fractions had no bactericidal effects on CA-MRSA at the test concentrations (MIC > 1000 µg/mL). These results demonstrate that all of the samples were bactericidal agents (MIC/MBC ≤ 4), except for EAFGP (MBC/MIC = 40), according to French (2006).

Previously, Campos et al. (2014) reported MIC and MBC values > 1000 µg/mL against *S. aureus* and *E. coli* for an ethanolic extract of *M. orbignyi* geopropolis collected in Mato Grosso do Sul state, Brazil. Velikova et al. (2000) also demonstrated that *M. quadrifasciata* geopropolis had antibacterial activities against *S. aureus* and *E. coli*. Campos et al. (2015) found that the ethanolic extract of *Tetragonisca fiebrigi* geopropolis (known as *Jataí*) had antibacterial effects on two strains of *S. aureus* (MIC = 0.55–0.65 mg/mL and MBC > 1000 µg/mL) and *P. aeruginosa* (MIC and MBC > 1000 µg/mL). In these studies, it was suggested that prenylated benzophenones (Cunha et al. 2013), diterpene kaurenoic acid (Velikova et al. 2000), cinnamic acid (Campos et al. 2015), and *p*-coumaric acid (Campos et al. 2015) in the different types of geopropolis were responsible for their antibacterial properties.

The ethanolic extract of geopropolis from *M. scutellaris* (known as *Uruçu verdadeira*) and its hexane fraction had strong antibacterial activities against *S. aureus* and *S. aureus* MRSA strains (MIC = 6.25–12.5 µg/mL and MBC = 25–50 µg/mL), but no activity against *P. aeruginosa*. By contrast, our

BFGP had similar MIC and MBC values against *S. aureus* to the hexane fraction of *M. scutellaris* geopropolis (Cunha et al. 2013). It should be noted that we determined all the MIC values only after incubating with resazurin for 60 min because shorter incubation periods overestimated the results (data not shown). Unfortunately, the incubation time employed by Cunha et al. (2013) was not stated.

Propolis has also been reported to have cytotoxic effects against several tumor cell lines (Cunha et al. 2013, 2016, Cinegaglia et al. 2013, Campos et al. 2015). Thus, we evaluated the *in vitro* antiproliferative potential of *M. mondury* geopropolis in four cell tumor lines: B16-F10, HepG2, HL-60, and K562 (Table III). EEGP had antiproliferative activities against tumor cells with IC_{50} values ranging from 33.48 to 48.67 µg/mL using HL-60 and HepG2. HFGP had IC_{50} values ranging from 24.24 to 46.62 µg/mL using HL-60 and K562. EAFGP had IC_{50} values ranging from 29.79 to 45.90 µg/mL using B16-F10 and HepG2. BFGP was not tested because it exhibited no antiproliferative activity at 50 µg/mL in preliminary screening using HepG2 and HL-60 cells. According

TABLE II
MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) VALUES FOR THE GEOPROPOLIS ETHANOLIC EXTRACT AND ITS FRACTIONS.

Bacterial strains	EEGP[a]		HFGP[a]		EAFGP[a]		BFGP[a]	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus ATCC 29213	250	1000	500	[b]	25	1000	5–10	25
Staphylococcus aureus (CA-MRSA)	[b]	[b]	[b]	[b]	500	[b]	250	[b]
Pseudomonas aeruginosa ATCC 27853	250	250	1000	[b]	250	[b]	500	1000

EEGP = ethanolic extract of geopropolis, HFGP = hexane fraction of geopropolis, EAFGP = ethyl acetate fraction of geopropolis, BFGP = butanol fraction of geopropolis. [a]concentration at µg/mL. [b]values >1000 µg/mL.

TABLE III
***In vitro* ANTIPROLIFERATIVE ACTIVITIES OF *Melipona mondury* GEOPROPOLIS ETHANOLIC EXTRACT AND ITS FRACTIONS.**

Samples	IC_{50} values (µg/mL)				
	B16-F10	HepG2	HL-60	K562	Human lymphoblast
EEGP	35.70 32.60–39.11	48.67 37.93–62.46	33.48 30.48–36.78	46.93 35.96–61.25	>50
HFGP	28.00 25.54–30.71	39.41 36.46–42,60	24.24 21.54–27.27	46.62 35.95–60.45	>50
EAFGP	29.79 26.74–33.19	45.90 40.89–51.53	35.57 31.71–39.89	42.19 35.14–50.65	47.67 41.40–54.89
DOX	0.20 0.17–0.24	0.19 0.15–0.25	0.18 0.16–0.21	0.29 0.23–0.37	2.19 1.08–4.43

IC_{50} values in µg/mL and their 95% confidence interval obtained by nonlinear regression based on three independent experiments performed in duplicate, which were measured based on the Alamar Blue assay after incubation for 72 h. Doxorubicin (DOX) was used as the positive control. EEGP = ethanolic extract of geopropolis, HFGP = hexane fraction of geopropolis, EAFGP = ethyl acetate fraction of geopropolis, BFGP was not tested because it exhibited no antiproliferative activity in preliminary screening. Tumor cells: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia), and K562 (human chronic myelocytic leukemia). Non-tumor cell: human peripheral blood mononuclear cells triggered with concanavalin A = human lymphoblast.

to Suffness and Pezzuto (1990), extracts with IC_{50} values <30 µg/mL are promising cytotoxic agents for use against neoplastic cells. In addition, EEGP and HFGP had no cytotoxic effects on non-tumor cells (human lymphocyte T) whereas EAFGP had an IC_{50} value of 47.67 µg/mL in lymphoblast cells. Therefore, HFGP is a promising source for the isolation and identification of antiproliferative compounds.

To determine the chemical profile of *M. mondury* geopropolis, the EEGP and its fractions were analyzed by direct infusion negative ion mode electrospray ionization mass spectrometry (ESI(-)-MS). The most abundant anions $[M-H]^-$ in all samples were at *m/z* 169, 475, 569, and 601. We also observed other less intense ions (*m/z* 301, 373, 401, and 569) in the raw extract and fractions (Figure 1). We then performed chromatographic analysis using UHPLC-MS and obtained the UHPLC-MS/MS spectra of selected peaks based on the main ions in the fingerprints (Table IV).

The ion at *m/z* 169 and its fragments (Figures 2a and b) were characteristic of gallic acid based on comparisons with previous studies (Abdel-Hameed et al. 2013, Saldanha et al. 2013). In addition, this compound was previously identified in *Myrcia bella*, which is a source of resin for *M. mondury* (Z.S. Lopes et al., unpublished data).

The ion at *m/z* 301 and its fragments (Figures 2c and d) were compatible with *E/Z* communic acid isomers, which were identified previously in some types of Brazilian propolis and *Araucaria heterophylla* resins (Marcucci et al. 2008). *E/Z* communic acid is a diterpene (Barrero et al. 2012) with several known pharmacological properties, including antimicrobial, antitumoral, anti-inflammatory, and antioxidant activities (Velikova et al. 2000, Popova et al. 2009, Barrero et al. 2012, Campos et al. 2015).

Previous studies of propolis produced by native bees from different Brazilian regions found ions at *m/z* 373 (Figures 2e and f) and 401 as diagnostic of terpenes with acid groups present in *Schinus terebinthifolius* resin (e.g., Sawaya et al. 2006, 2007, 2009). *M. mondury* geopropolis contained the same ions, which suggests that this bee also collects resin from *S. terebinthifolius* (e.g., Sawaya et al. 2007, 2009). This plant occurs naturally on the coastline of Brazil, in the remaining areas of the Atlantic Forest (Carvalher-Machado et al. 2008), which is an area inhabited by *M. mondury* (Melo 2003, Souza et al. 2012).

Finally, the ions at *m/z* 601, 475, and 569 (Figure 3) could not be identified, although they were present in all the samples produced by *M. mondury* tested in this study.

UHPLC-MS has been used widely for analyzing propolis (Bankova et al. 2000, Sawaya et al. 2011, Novak et al. 2014, Wali et al. 2015, Ristivojević et al. 2015, Sagi et al. 2016), but it has limitations in terms of the identification of uncommon substances because there is no database for making comparisons among geopropolis samples. Therefore, the unambiguous characterization of the compounds corresponding to the ions at *m/z* 601, 475, and 569 may be possible after their isolation and further analysis using other spectrometric methods (e.g., NMR), in addition to MS experiments.

In this study, for the first time, we demonstrated that the geopropolis produced by *M. mondury* has antioxidant, antibacterial, and antiproliferative properties, thereby indicating the therapeutic potential of this natural product for the prevention and/or treatment of inflammatory, oxidative, and infectious diseases as an unexplored source of new antiproliferative compounds. The chemical composition of geopropolis appears to be partially polar because its activities were concentrated in the butanol fraction. Therefore, *M. mondury* geopropolis is a promising source for identifying new molecules with therapeutic properties.

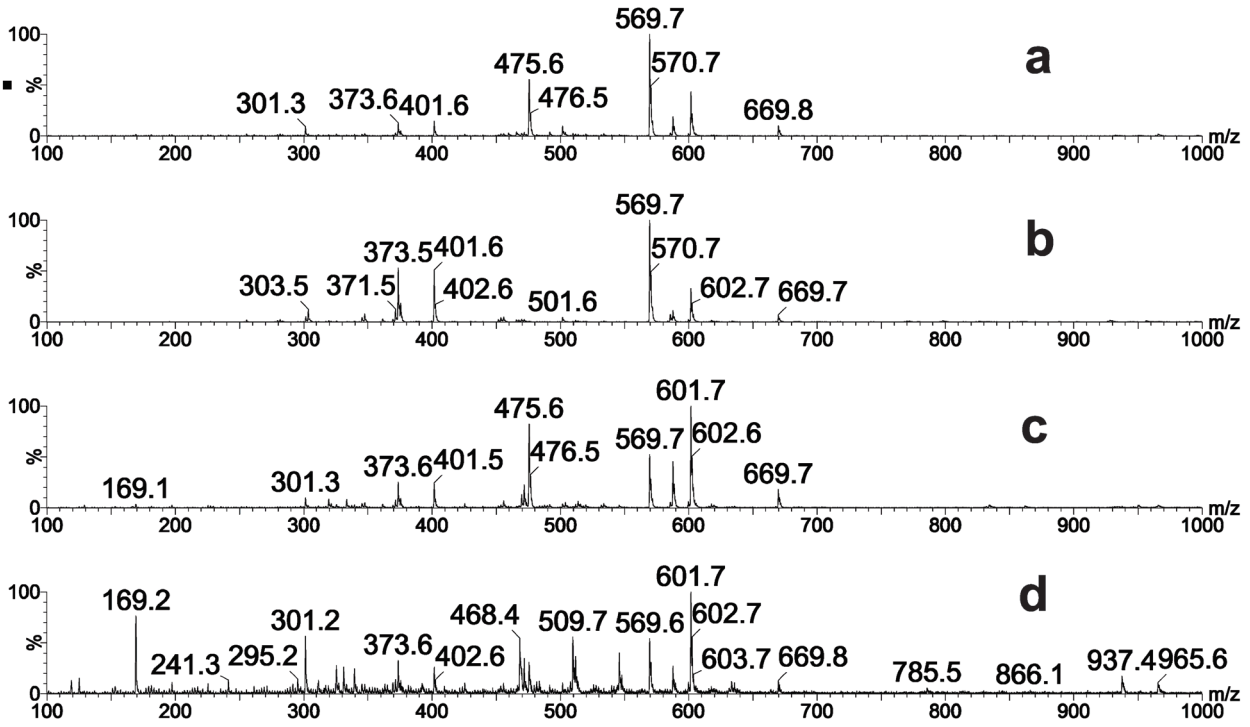

Figure 1 - ESI-MS fingerprints of *M. mondury* geopropolis extract and fractions determined in the negative ion mode. **a**) EEGP: ethanolic extract; **b**) HFGP: hexane fraction; **c**) EAFGP: ethyl acetate fraction; **d**) BFGP: butanol fraction.

TABLE IV
RETENTION TIMES AND MS/MS FRAGMENTS OF MAIN IONS IN *M. mondury* GEOPROPOLIS DETERMINED BY UHPLC-ESI(-)-MS/MS IN THE NEGATIVE ION MODE.

Retention time (min)	$[M-H]^-$	Major fragments (*m/z*)
0.7	169	125, 106
3.9	301	220, 205, 109
2.7	373	329
1.5	475	407, 390, 347, 335
3.4	475	407, 399
4.8	475	399
1.8	569	501
1.7	601	550, 491, 109

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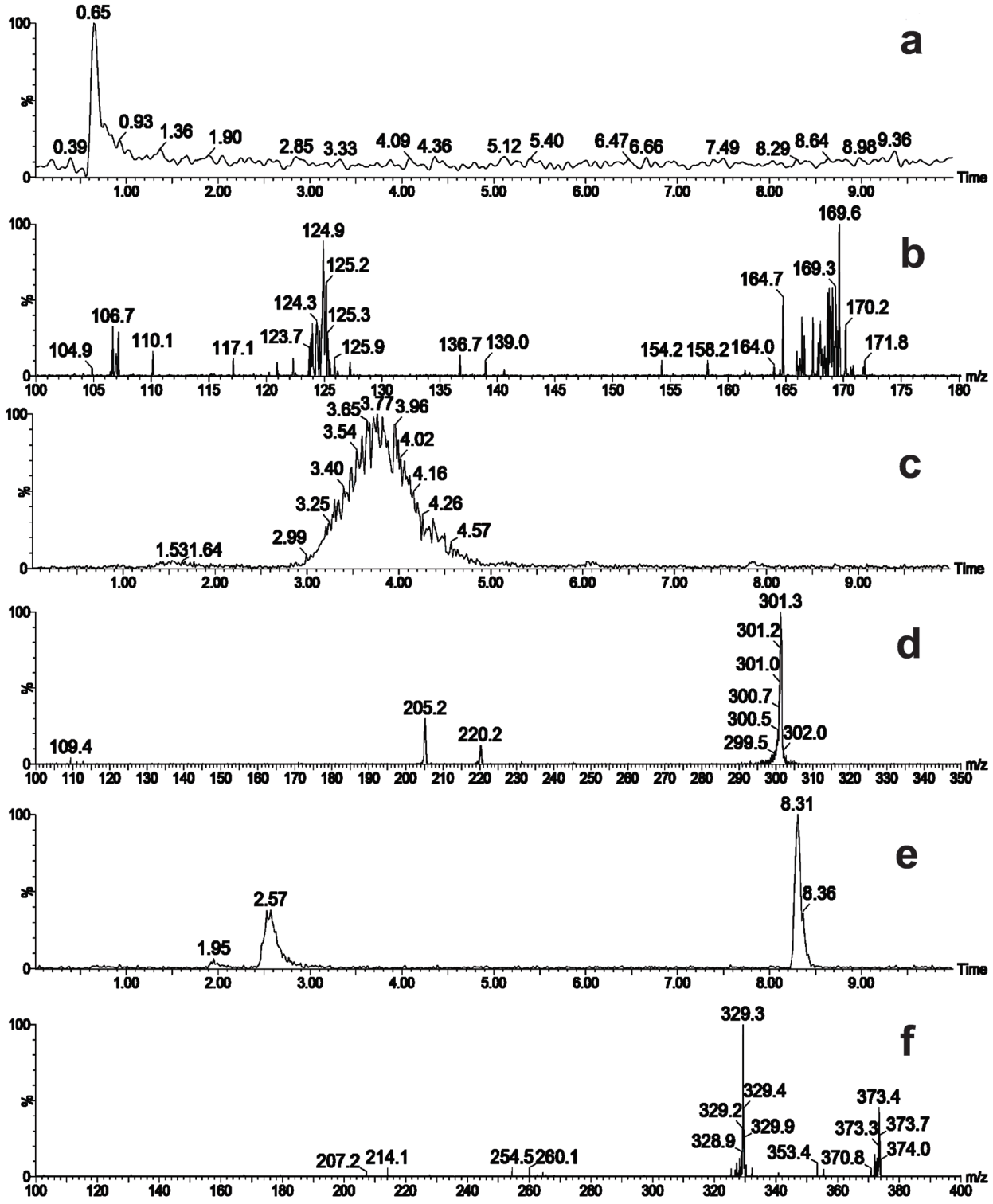

Figure 2 - Extracted ion UHPLC-MS chromatograms for (**a**) *m/z* 169, (**c**) *m/z* 301, and **(e)** *m/z* 373. MS/MS fragments for ions (**b**) *m/z* 169, **(d)** *m/z* 301, and (**f**) *m/z* 373.

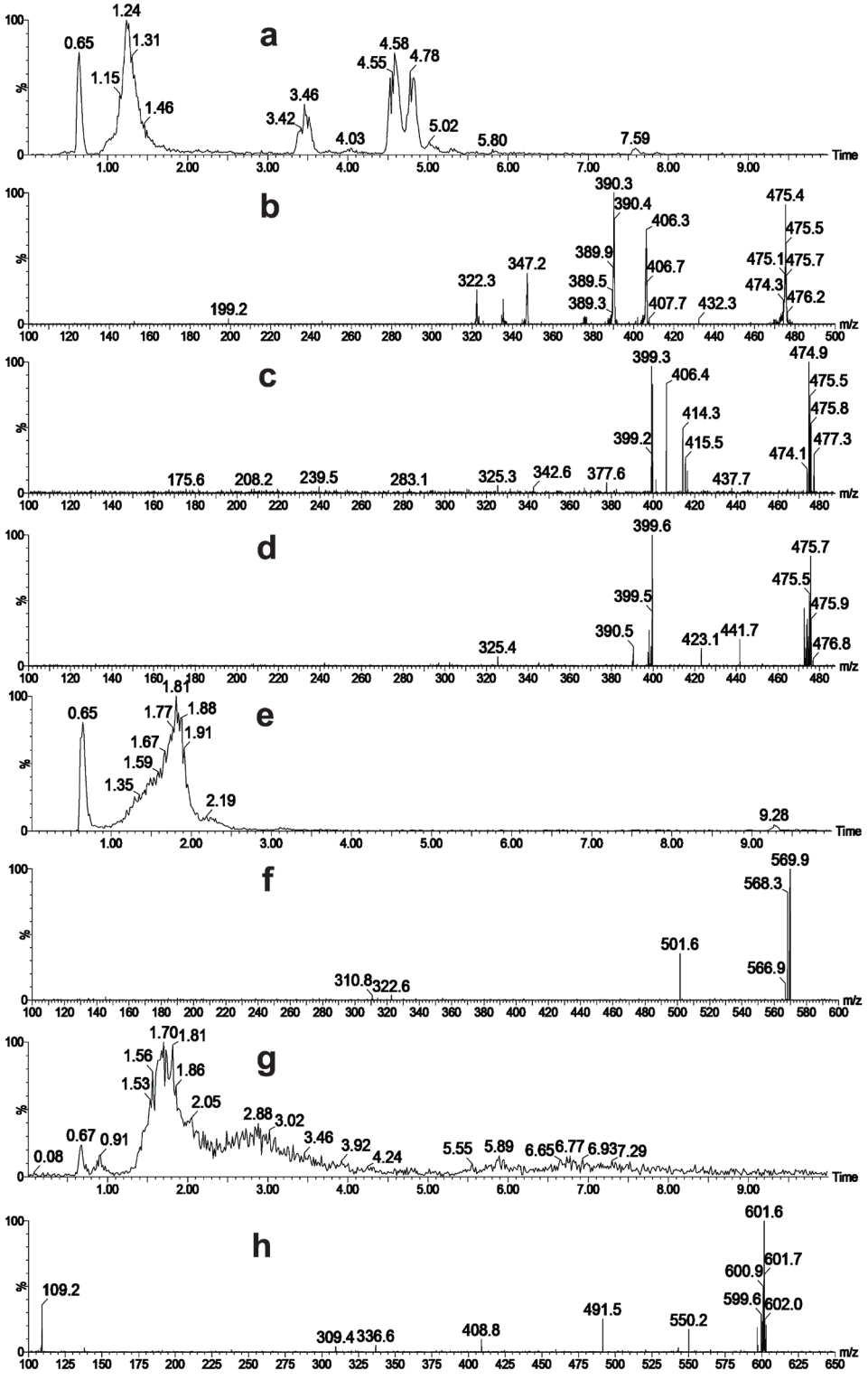

Figure 3 - Extracted ion UHPLC-MS chromatograms for (**a**) *m/z* 475, (**e**) *m/z* 569, and **(g)** *m/z* 601. MS/MS fragments for ions (**b**) *m/z* 475, retention time 1.5 min; (**c**) *m/z* 475, retention time 3.5 min; (**d**) *m/z* 475, retention time 4.8 min; (**f**) *m/z* 569; and (**h**) *m/z* 601.

REFERENCES

ABDEL-HAMEED ES, BASAID SA AND SALMAN MS. 2013. Characterization of the phytochemical constituints of Taif Rose and its antioxidant and anticancer activities. Biomed Res Int 2013: 345465.

AHMED SA, GOGAL RM AND WALSH JE. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes an alternative to [3H] thymidine incorporation assay. J Immunol Meth 170: 211-224.

BANKOVA V, CASTRO SL AND MARCUCCI MC. 2000. Propolis: recent advances in chemistry and plant origin. Apidologie 31: 3-15.

BARRERO AF, HERRADOR MM, ARTEAGA P, ARTEAGA JF AND ARTEAGA AF. 2012. Communic acids: occurrence, properties and uses as chirons for the synthesis of bioactive compounds. Molecules 17: 1448-1467.

BERTHOLD F. 1981. Isolation of human monocytes by ficoll density gradient centrifugation. Blut 3: 367-371.

BRAKSTAD O, KJETILL A AND MAELND JA. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc Gene. J Clin Microbiol 30: 1654-1660.

BROWN MG AND LAWCE HJ. 1997. Peripheral blood cytogenetic methods. In: Barch MJ, Knutsen T and Spurbeck JL (Eds), The AGT Cytogenetics Laboratory Manual. Lippincott-Raven Publishers, Philadelphia, 3nd ed., p. 77-171.

BOYE K, BARTELS MD, ANDERSEN IS, MOLLER JA AND WESTH H. 2007. A new multiplex PCR for easy screening of methicillin–resistant *Sthaphylococcus aureus* SCC mec types I-V. Clin Microbiol Infect 13: 725-727.

CAMPOS JF, SANTOS UP, MACORINI LF, MELO AM, BALESTIERI JB, PAREDES-GAMERO EJ, CARDOSO CA, SOUZA KP AND SANTOS EL. 2014. Antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae). Food Chem Toxicol 65: 374-380.

CAMPOS JF, SANTOS UP, ROCHA PS, DAMIÃO MJ, BALESTIERI JBP, CARDOSO CAL, PAREDES-GAMERO EJ, ESTEVINHO LM, SOUZA KP AND SANTOS EL. 2015. Antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities of propolis from the stingless bee *Tetragonisca fiebrigi* (Jataí). Evid Based Complement Alternat Med 2015: 296186.

CARVALHER-MACHADO SC, ROSAS EC, BRITO FA, HERINGE AP, OLIVEIRA RR, KAPLAN MA AND HENRIQUES MG. 2008. The anti-allergic activity of the acetate fraction of *Schinus terebinthifolius* leaves in IgE induced mice paw edema and pleurisy. Int Immunopharmacol 8: 1552-1560.

CINEGAGLIA NC, BERSANO PRO, ARAÚJO MJAM, BÚFALO MC AND SFORCIN JM. 2013. Anticancer effects of geopropolis produced by stingless bees on canine osteosarcoma cells *in vitro*. Evid Based Complement Alternat Med 2013: 737386.

CLSI - CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. CLSI, Wayne, PA, USA, CLSI document M07-A7, 7.

CUNHA MG, FRANCHIN M, GALVÃO LCC, RUIZ ALTG, CARVALHO JE, IKEGAKI M, ALENCAR SM, KOO H AND ROSALEN PL. 2013. Antimicrobial and antiproliferative activities of stingless bee *Melipona scutellaris* geopropolis. BMC Complement Alternat Med 13: 23.

CUNHA MG, ROSALEN PL, FRANCHIN M, DE ALENCAR SM, IKEGAKI M, RANSOM T AND BEUTLER JA. 2016. Antiproliferative constituints of geopropolis from the bee *Melipona scutellaris*. Planta Med 82: 190-194.

DUTRA RP, ABREU BV, CUNHA MS, BATISTA MCA, TORRES LMB, NASCIMENTO FRF, RIBEIRO MNS AND GUERRA RNM. 2014. Phenolic acids, hydrolyzable tannins, and antioxidant activity of geopropolis from the stingless bee *Melipona fasciculata* Smith. J Agric Food Chem 62: 2549-2557.

FRANCHIN M, CUNHA MG, DENNY C, NAPIMOGA MH, CUNHA TM, KOO H, ALENCAR SM, IKEGAKI M AND ROSALEN PL. 2013. Bioactive fraction of geopropolis from *Melipona scutellaris* decreases neutrophils migration in the inflammatorlimy process: involvement of nitric oxide pathway. Evid Based Complement Alternat Med 2013: 907041.

FRENCH GL. 2006. Bactericidal agents in the tratament of MRSA infections- the potential role of daptomycin. J Amtimicrob Chemother 58: 1107-1117.

LIBERIO SA ET AL. 2011. Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith. BMC Complement Alternat Med 11: 108.

MARCUCCI MC. 1995. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26: 83-99.

MARCUCCI MC, SAWAYA ACHF, CUSTÓDIO AR, PAULINO N AND EBERLIN MN. 2008. Chapter 3: HPLC and ESI-MS typification: new approaches for natural therapy with Brazilian própolis. In Scientific Evidence of the Use of propolis in Ethnomedicine. Transworld Research Network, Kerala, India, p. 33-54.

MELO CAR. 2003. Notas sobre meliponíneos neotropicais (Hymenoptera, Apoidae). Com a descrição de três novas

espécies. Apoidea Neotropical. Homenagem aos 90 anos de Jesus Santiago Moure 2003: 85-92.

MILLER HE. 1971. A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc 48: 91.

NOVAK EM, SILVA MSC, MARCUCCI MC, SAWAYA ACHF, LÓPEZ BGC, FORTES MAHZ, GIORGI RR, MARUMO KT, RODRIGUES RF AND MARIA DA. 2014. Antitumoral actitivity of Brazilian red propolis enriched with xanthochymol and formononetin: an *in vitro* and *in vivo* study. J Funct Foods 11: 91-102.

PEREZ-HOTH E, CLAVERIE-MARTÍN F, VILLAR J AND MÉNDEZ-ÁLVAREZ S. 2001. Multiplex PCR for simultaneous identification of *Sthaphylococcus aureus* and detection of methicillin. J Clin Microbiol 39: 4037-4041.

PICCINELLI AL, DE SIMONE F, PASSI S AND RASTRELLI L. 2004. Phenolic constituents and antioxidant activity of *Wendita calysina* leaves (burrito), a folk Paraguayan tea. J Agric Food Chem 52: 5863-5868.

POPOVA MP, CHINOU IB, MAREKOV IN AND BANKOVA VS. 2009. Terpenes with antimicrobial activity from Cretan propolis. Phytochem 70: 1262-1271.

RE R, PELEGRINI N, PROTEGGENTE A, PANNALA A, YANG M AND RICE-EVANS C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26: 1231-1237.

RISTIVOJEVIĆ P, TRIFKOVIĆ J, GAŠIĆ U, ANDRIĆ F, NEDIĆ N, TEŠIĆ Ž AND MILOJKOVIĆ-OPSENICA D. 2015. Ultrahigh-performance Liquid Chromatography and Mass Spectrometry (UHPLC–LTQ/Orbitrap/MS/MS) study of phenolic profile of serbian poplar type propolis. Phytochem Anal 26: 127-136.

RODRIGUES AC, BOMFIM LM, NEVES SP, MENEZES LRA, DIAS RB, SOARES MB, PRATA APN, ROCHA CAG, COSTA EV AND BEZERRA DP. 2015. Antitumor properties of the essential oil from the leaves of *Duguetia gardneriana*. Planta Med 81: 798-803.

SAGI S, AVULA B, MASOODI MH , FAROOQ WALI A AND IA KHAN. 2016. Identification and quantification of chemical constituents from Indian bee propolis extracts using HPTLC and UHPLC-DAD-MS. Planta Med 83: 5.

SALDANHA LL, VILEGAS W AND DOKKEDAL AL. 2013. Characterization of Flavonoides and phenolic acids in *Myrcia bella* Cambess. Using a FIA-ESI-IT-MS and HPLC-PAD-ESI-IT-MS combined with NMR. Molecules 18: 8402-8416.

SAMPAIO CPL, DIAS IM, FARIA FM AND OLIVEIRA MVM. 2013. Principais bactérias causadoras de infecção hospitalar. Rev Dig Buenos Aires 10: 182.

SAWAYA ACHF, CALADO JCP, SANTOS LC, MARCUCCI MC, AKATSU IP, SOARES AEE, ABDELNUR PV, CUNHA IBS AND EBERLIN MN. 2009. Composition and antioxidant activity of propolis from three species of *Scaptotrigona* stingless bees. JAAS 1: 37-42.

SAWAYA ACHF, CUNHA IBS AND MARCUCCI MC. 2011. Analytical methods applied to diverse types of Brazilian propolis. Chem Cent J 5: 27.

SAWAYA ACHF, CUNHA IBS, MARCUCCI MC, AIDAR DS, SILVA ECA, CARVALHO CAL AND EBERLIN MN. 2007. Electrospray ionization mass spectrometry fingerprinting of propolis of native Brazilian stingless bees. Apidologie 38: 93-103.

SAWAYA ACHF, CUNHA IBS, MARCUCCI MC, RODRIGUES RFO AND EBERLIN MN. 2006. Brazilian propolis of *Tetragonisca angustu*la and *Apis mellifera*. Apidologie 37: 398-407.

SFORCIN JM AND BANKOVA V. 2011. Propolis: is there a potential for the development of new drugs? J Ethnopharmacol 133: 253-260.

SLINKARD K AND SINGLETON VL. 1977. Total phenol analyses: automation and comparison with manual methods. Am J Enol Vitic 28: 49-55.

SOUZA HAC, VIANA MVC, ALVES RMO, PEREIRA DV AND WALDSCHMIDT AM. 2012. Distribution of *Melipona mondury* Smith1863 (Hymenoptera: Apidae, Meliponini) from state of Bahia. Semana Entomológica da Bahia. Magistra, Cruz das Almas 24: 99-104.

SOUZA SA, CAMARA CA, SILVA SEM AND SILVA TMS. 2013. Composition and antioxidant activity of geopropolis collected by *Melipona subnitida* (Jandaíra) bees. Evid Based Complement Alternat Med 2013: 801383.

SOUZA SA, DIAS TLMF, SILVA TMG, FALCÃO RA, ALEXANDRE-MOREIRA MS, SILVA EMS, CAMARA CA AND SILVA TMS. 2014. Chemical composition, antinociceptive and free radical-scavenging activities of geopropolis from *Melipona subnitida* Ducke (Hymenoptera: Apidae: Meliponini). Sociobiology 61: 560-565.

SUFFNESS M AND PEZZUTO JM. 1990. Assays related to cancer drug discovery. In: Hostettmann, Keditor. Methods in Plant Biochemistry: Assays for Bioactivity. Academic Press: London, p. 71-133.

VELIKOVA M, BANKOVAA V, TSVETKOVAB I, KUJUMGIEVB A AND MARCUCCIC MC. 2000. Antibacterial ent-kaurene from Brazilian propolis of native stingless bees. Phytotherapie 71: 693-696.

WALI AF, AVULA B, ALI Z, KHAN IA, MUSHTAQ A, REHMAN MU, AKBAR S AND MASOODI MH. 2015. Antioxidant, hepatoprotective potential and chemical profiling of propolis ethanolic extract from Kashmir Himalaya region using UHPLC-DAD-QToF-MS. Biomed Res Int 2015: 393462.