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Propolis from different Brazilian stingless bee species: phenolic composition and antimicrobial activity

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Abstract

Stingless bees (SLB) are insects bread many centuries ago by indigenous people and more than 500 species have already been described. Interest in SLB's propolis has grown as a way to value and preserve native bees, in addition to investigating/prospecting compounds with biological functionality (antimicrobial activity, antioxidant, etc.). The natural active compounds found in propolis come from different plant sources, and consequently, each propolis may show unique biological/pharmacological activity. There is still an important gap about the profile of chemical compounds, biological and pharmacological potential of propolis produced by SLBs. This work aimed to investigate the presence of phenolic and coumaric compounds (HPLC–DAD-FLD) and the antimicrobial activity (microdilution method) of propolis extracts from five different species of SLB reared in different places. The samples from *Melipona quadrifasciata* (82.05 mgGAEg^{-1}) and one from, *Frieseomelitta doederleini* (56.22 mgGAEg^{-1}) showed the highest concentrations of phenolic compounds. It was possible to identify in the propolis samples formononetin, kaempferol, gallic acid and coumarin. Resveratrol was detected in all samples, an unprecedent fact for SLB propolis. *Candida albicans* was susceptible to all tested extracts, while *Escherichia coli* was inhibited only by propolis from *Melipona quadrifasciata*; *Enterococcus faecalis* was inhibited by propolis from *Plebeiadroryana.*, *Melipona quadrifasciata* and *Frieseomelitta doederleini*. It was verified that SLB propolis constitutes a source of different biocompounds, which varies according to the location where the bees are raised, and has mainly antifungal activity, generating possibilities of its use in different biotechnological products.

Keywords Antifungals, Melipona, Natural compounds, Resveratrol, Coumarins

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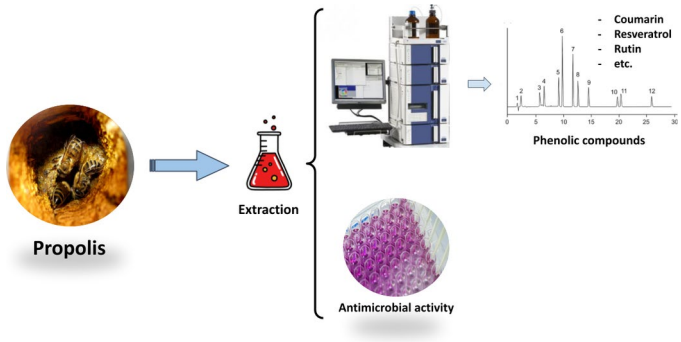
Graphical Abstract



Introduction

Bees play a key role in agriculture as pollinators, and it is estimated that 70% of crops of importance for human consumption are pollinated by these insects around the world; they also preserve biodiversity by ensuring fertilization of several plant species. The role of pollinating animals has already been estimated to represent between 235 to 577 billion dollars (Potts et al. 2016).

The breeding of stingless bees (SLB) is an activity that is easy to handle, has low maintenance costs and economic gains that may be higher than those of *Apis mellifera* bee (Se et al. 2018; Shadan et al. 2018), and more than 500 SLB species have already been described in Latin America, Australia, Africa and Asia (Souza et al. 2021). Knowledge related to propolis from different species of bees from different indigenous peoples around the world has recently begun to be studied and, often, this knowledge is scientifically proven, confirming the biological potential of this matrix (Popova et al. 2021).

Natural and herbal products, such as propolis, have been used by various civilizations (ancient Egyptians, Romans, Greeks, Chinese, and even indigenous populations from South and Central America) throughout history. These communities used bee products (propolis, honey, royal jelly) for the treatment of diseases. Even without scientific knowledge, these people noticed that propolis had antimicrobial and anti-inflammatory properties (Vázquez et al. 2016; Paris et al. 2018). More specifically, stinging bee propolis has long been used in traditional medicine by native populations from Mexico, Brazil, Argentina, India, and Vietnam (Popova et al. 2021). Flores et al. (2018) investigated the use of products from honey-producing insects by populations from northern Argentina, and identified that honey, pollen, wax and propolis from Plebeia sp. nov. had the highest frequency of use.

In recent years, after several studies based on the chemical characterization and in vitro, in silico and animal models assays using propolis, some positive results were published, confirming the knowledge of native populations and ancient civilizations. The use of propolis (dehydrated liquid extracts) in clinical cases of respiratory problems led to negative microbial diagnostic tests after 12 days of treatment (Zorlu, 2021). Fiorini et al. (2021) showed that the use of propolis significantly reduced the intensity of acute kidney injuries. Silveira et al. (2021) used propolis extract as an adjuvant in the treatment of Covid-19 and found a significant reduction in associated clinical symptoms, such as dry cough, shortness of breath, sore throat, chest pain, fever, dizziness, headache, abdominal pain, and diarrhea. Cohen et al. (2004) and Marchisio et al. (2010) obtained a significant reduction (> 50%) in the incidence of cases of upper respiratory tract infection, acute otitis media, pneumonia and tonsillopharyngitis, after treatment with propolis. Ohkuma et al. (2010) also observed the reduction and shortening of symptoms of the common cold in patients undergoing treatments with propolis. Guan et al. 2023 showed that the overall therapeutic effect of propolis extract is better than that of the metformin group, showing that it reduces fasting glycemia in mice by improving the inflammatory reaction, regulating metabolism, and affecting the steady state of the intestinal microbiota.

SLB propolis has gained the attention of researchers over the last 20 years because they are rich sources of phenolic compounds, which have essential activities for the human body (Popova et al. 2021; Rocha et al. 2023). But studies have shown that both the qualitative and quantitative profiles of these components can vary according to the producing species and place of origin (Bueno-Silva et al. 2017; Salatino & Salatino 2021;

Shanahan & Spivak 2021). Stingless bee propolis has a complex and diverse chemical composition, and its constituents include phenolic acids, flavonoids, coumarins, benzophenones, terpenes, steroids, alkaloids, fatty acids, and sugars (Lim et al. 2023; Pereira et al. 2021; Santos-Buelga & González-Paramás 2017).

Phenolic compounds are one of the important chemical substance's classes found in different parts of plants such as leaves, flowers, skin and fruit (Eghbaljoo et al. 2022). It has already been described that some phenolic compounds have immunomodulatory, antioxidant, antiviral and anti-inflammatory activities, and that they can act as an antimicrobial against pathogens of importance to human and animal health (Arung et al. 2023; Santos et al. 2020; Silva-Beltrán et al. 2020, 2022). These compounds are seen as emerging alternatives to the problem of resistance of microorganisms to traditional medicines (Pormohammad et al. 2019). Coumarins represent an important family of naturally occurring benzopyrone compounds, all of which consist of a benzenic ring linked to the pyrone ring. This substance class occur naturally in many plants, natural spices and foods and they can be found in plants seeds, flowers, leaves, roots, and stems (Lončar et al. 2020). However, it is still a class of compounds little discussed in propolis studies, besides having anticoagulant, antimicrobial and antitumor activities (Hroboňová et al. 2013; Rocha et al. 2023). Therefore, different studies have identified several biological activities related to stingless bee propolis such as antimicrobial properties (including bactericidal, fungicidal, and virucidal activities), antioxidant activity, cytotoxicity and anticancer properties, and anti-inflammatory activity. Other biological activities identified include antiemetic, antinociceptive, wound healing, vasomodulator, anti-asthmatic, and anti-mutagenic properties (Lavinas et al. 2019; Zulhendri et al. 2022).

Thus, the work aims at the qualitative and quantitative analysis of phenolic compounds and coumarins, as well as evaluating the antimicrobial activity against *Candida albicans*, *Enterococcus faecalis*, and *Escherichia coli*, of propolis extracts from different Brazilian SLB.

Materials and methods

Propolis samples and extraction

Eleven samples of propolis produced by different SLB species were kindly provided by beekeepers from the state of Bahia, northeastern Brazil (Fig. 1). Samples were taken between spring and summer of 2019 and 2020. The samples were identified as follows: PL1 – produced by *Plebeia droryana*., Palmeiras city, Cerrado biome; PL2 – produced by *Plebeia droryana*., Feira de Santana city, semi-arid biome; TA1 – produced by *Tetragonisca angustula*, Valença city, tropical rainforest biome; TA2 – produced by *Tetragonisca angustula*, Maragogipe city,

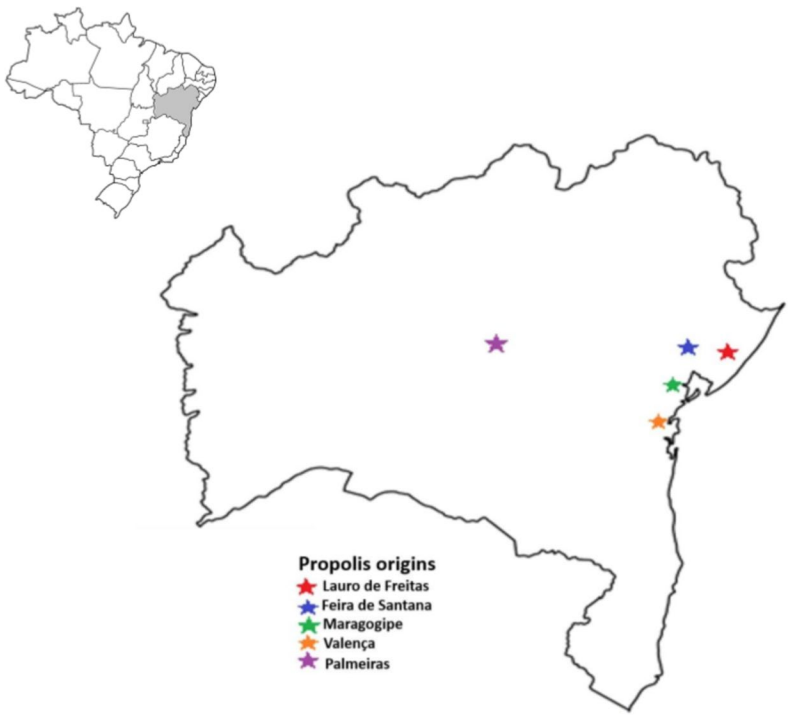


Fig. 1 Samples of propolis from the state of Bahia, northeastern Brazil

tropical rainforest biome; TA3 – produced by *Tetragoniscaangustula*, Feira de Santana city, semi-arid biome; MQ1 – produced by *Melipona quadrifasciata*, Feira de Santana city, semi-arid biome; MQ2 – produced by *Melipona quadrifasciata*, Feira de Santana city, semi-arid biome; FD1 – produced by *Frieseomelitta doederleini*, Feira de Santana city, semi-arid biome; FD2 (viscous propolis) – produced by *Frieseomelitta doederleini*, Feira de Santana city, semi-arid biome; NT1 – produced by *Nannotrigona testaceicornes*, Feira de Santana city, semi-arid biome; NT2 – produced by *N. testaceicornes*, Lauro de Freitas city, tropical rainforest biome.

The extraction was performed as previously described by Escriche and Juan-Borrás, (2018) with adaptations. Briefly, 15 mL of 70% ethanol was added for each 0.5 g of propolis, with subsequent incubation in a water bath at 25 °C for 30 min. The supernatant was removed, filtered, and a new extraction was performed following the same previous steps. The supernatants were then kept in a -20 °C freezer until the time of the analysis.

Determination of the content of total phenolic compounds

The total phenolic content in the propolis samples was determined using a previously described methodology (Singleton et al. 1999), with adaptations. In a dark environment, 0.1 mL of propolis samples were added to a tube with 0.5 mL of Folin-Ciocalteu reagent. After 5 min, 1.5 mL of 20% calcium carbonate and Milli-Q water to a total volume of 10 mL were added. After 30 min, reading was performed in a spectrophotometer at 760 nm (Perkin Elmer, Waltham, MA).

Chromatographic analysis

The analyzes of the phenolic compounds were performed according to a previously described methodology (Silva et al. 2021), with adaptations. The parameters used for analysis can be found in Supplementary Table S1. It was used a high-performance liquid chromatograph with a diode array and fluorescence detector (HPLC–DAD-FLD – Shimadzu, Japan), equipped with a quaternary solvent pumping unit (LC-20AT), an automatic injector (SIL-20AHT), degasser (DGU-205), column oven (CTO-20A), a controller interface (CBM-20A), a detector diode array (SPD-M20A) and a fluorescence detector (RF-20A). The chromatographic separation was performed using a Nucleodur 100–5 C18 ec column (150 mm×4 mm ID; 5 µm particle size – Sigma-Aldrich, Saint Louis, MI) coupled to an Zorbax Eclipse Plus C18 pre-column (4.6 mm×12.5 mm – Agilent, Santa Clara, CA). 5% acetic acid (solvent A) and acetonitrile (solvent B) were used as mobile phase, whose percentages varied throughout the analyses, as follows: 0 to 22.50 min (0–59%); 22.50 at 24 min (59–0%); 24 to 26 min (0%). Chromatographic runs were performed sequentially, using both detectors simultaneously, and had a total duration of 26 min, at a constant flow of 1.00 mL min^{-1}. The oven temperature was 40 °C.

To obtain the analytical curves, a mixture with standards of the 22 analyzed compounds (Gallic acid, Caffeic acid, Epicatequin, *p*-cumaric acid, trans-ferulic acid, Ellagic acid, Rutin, Piceatannol, Myricetin, Resveratrol, Quercetin, trans-cinnamic acid, Naringenin, Kaempferol, Isoliquiritigenin, Formononetin, Biochanin, Kaempferide, Coumaric compounds, 7-hidroxicumarin, Scopoletin, 4-metilumbeliferonone, Coumarin) was made at a concentration of 15 $mg.L^{-1}$ (stock solution) in methanol. Dilutions were performed using the stock solution, and the concentration ranges were considered for the curves in the diode array detector from 0.01 – 1.0 $mg.L^{-1}$ for all analyzed compounds. Five phenolic compounds (epicatechin, trans-ferulic acid, piceatanol, resveratrol and formononetin) and three coumarins (7-hydroxycoumarin, scopoletin, 4-methylumbelliferone) were analyzed in the fluorescence detector and the analytical curves were in the range of 0.01 – 5.0 $mg.L^{-1}$. The limit of detection (LOD) was 0.005 $mg.L^{-1}$ and the limit of quantification (LOQ) was 0.01 $mg.L^{-1}$. As recommended by Thompson et al., (2002) to obtain the LOD of the analytes, the instrumental detection limit of the chromatographic method was considered. Thus, the concentration below the first point of the analytical curves whose chromatographic peak was identified, for each analyte, was considered to be the LOD. Below 0.005 mg.L-1 the chromatographic peaks of the analytes were not identified. Considering the same recommendations, to obtain the LOQ, the lowest concentration values of the analytical curves were considered, taken as a fixed multiple value (typically 2) of the limits of detection obtained for the analytes.

For the analysis of SLB propolis, the extracts were diluted in methanol (1 mg mL^{-1}) and filtered (0.45 µm) before injection into the chromatographic system. All analyzes were performed in triplicate, and the identities of the analytes were confirmed by comparison of the retention times and chromatographic peak profiles of the samples with those of the analytical standards.

Antimicrobial activity

For this experiment, reference strains of a fungus, a Gram-negative and a Gram-positive bacterium, all of medical importance, were chosen. Those were, respectively, *Candida albicans* FIOCRUZ CPF 02508, *Escherichia coli* ATCC 25992 and *Enterococcus faecalis* ATCC 29212.

The antimicrobial activity of SBL propolis ethanolic extracts were evaluated using the broth microdilution assay, as described by the M27-A3 protocol for yeast,

and the M07-A9 protocol for bacteria, both from the Clinical Laboratory Standards Institute (CLSI 2008, 2012). Briefly, the microorganisms were spectrophotometrically adjusted to an optical density of 0.08–0.1 at 600 nm by dilution in RPMI 1640 medium (Sigma-Aldrich) for yeast, and Mueller–Hinton medium (MH – Sigma-Aldrich) for bacteria. The propolis extracts were diluted in concentrations of 8, 16, 32, 64, 128, 256, 512 and 1024 $\mu g.mL^{-1}$. The inoculum was added to a sterile 96-well plate, 100 $\mu.L^{-1}$ per well, followed by the addition of the propolis extracts at different concentrations. As a negative control, RPMI 1640 medium for yeast and MH medium for bacteria added with propolis extracts at the different concentrations, but without the inoculum, were used. As a positive control, culture media with fungal and bacterial inoculum and without any treatment were used. The plates were then incubated for 48 h at 37 °C. Then, the microbial growth was evaluated using a spectrophotometer (Thermo Scientific, USA) at 600 nm. Each combination of inoculum and propolis treatment was performed in triplicate to obtain the minimum inhibitory concentration (MIC100) value, which represented the lowest concentration of propolis extract that inhibited 100% of fungal or bacterial growth. For the determination of the minimum fungicidal concentration (MFC100—minimum concentration of extract capable of killing 100% of the yeasts) and the minimum bactericidal concentration (MBC100—minimum concentration of extract capable of killing 100% of the bacteria), aliquots of each well of the microdilution in broth were seeded in Sabouraud Agar for yeast and MH Agar for bacteria, and incubated at 37 °C for 48 h. Thus, the lowest concentration that showed no visible fungal or bacterial growth was determined to be MFC100 or MBC100. These experiments were repeated three times, and the results are expressed as means of the microbial growth inhibition. The susceptibility of these strains to commercially available antimicrobials was already assessed by Fonseca et al., (2022) and Sokolonski et al. (2021).

Statistical analyses

Phenolic and coumaric concentration results were presented as mean ± standard deviation, and the normality was verified using the Kolmogorov–Smirnov test for all variables. The analysis of variance (ANOVA) was applied to verify significant differences ($P < 0.05$) using the SAS statistical software, and the microbial growth inhibition graphs were prepared using the GraphPad Prism v.8 software.

The Principal Component Analysis (PCA) was performed with the objective to assess the influence of sample origin on the content of phenolic and coumaric compounds, being performed using the PAST software (Paleontological Statistics, Norway) version 3.26; phenolic compounds that did not show levels in any sample were excluded from the analysis. Since the averages related to the aforementioned characterization tests use different units of measurement, the data were normalized in the range of 0 to 1.

Results

Total phenolic compounds

A variation of 13.45 ± 1.64 to 82.05 ± 2.33 $mgGAE.g^{-1}$ in the composition of total phenolic compounds between the propolis produced by the species studied herein was found. There were also propolis producing variations: 30.13 ± 0.0 to 51.90 ± 2.47 $mgGAE.g^{-1}$ for Plebeia droryana.; 29.09 ± 2.63 to 82.05 ± 2.33 $mgGAE.g^{-1}$ for Melipona quadrifasciata; and 13.45 ± 1.64 to 56.22 ± 0.85 mg $GAE.g^{-1}$ for Frieseomelitta doederleini (Table 1). There was a statistical difference between all samples, even propolis belonging to the same SLB species. It is interesting to observe that the samples TA1, TA2 and TA3, and the samples NT1 and NT2, presented absorbances below the Beer-Lambert Law, therefore, there were no detectable results by the methodology employed.

Chromatographic analysis.

The results obtained in the analysis of the propolis composition HPLC–DAD-FLD (Table 2) demonstrated the variety of phenolic compounds. Coumarin was found in 7 of the 11 samples evaluated, with concentrations ranging from 0.07 $mg.L^{-1}$, for the FD1 sample, to 2.34 $mg.L^{-1}$ for the PL2 sample. Scopoletin was another

Table 1 Total phenolic content of the stingless bee propolis extracts included in this study. The results are expressed as means of three independent experiments and their respective standard deviations. The different superscript letters represent significant statistical differences at the ANOVA test at $P < 0.05$. ND – not detected

Species	Sample	Total phenolic compounds ($mgGAEg^{-1}$)
Plebeia droryana	PL1	51.90 ± 2.47c
	PL2	30.13 ± 2.31d
Tetragonisca angustula	TA1	ND
	TA2	ND
	TA3	ND
Melipona quadrifasciata	MQ1	82.05 ± 2.33a
	MQ2	29.09 ± 2.63d
Frieseomelitta doederleini	FD1	13.45 ± 1.64e
	FD2	56.22 ± 0.85b
Nannotrigona testaceicornes	NT1	ND
	NT2	ND

Table 2 Phenolic compounds found at the stingless bee propolis included in this study. The results in mg L^{-1} are shown as the means of three independent experiments and their respective standard deviations. The different superscript letters in the same line represent significant statistical differences at the ANOVA test at $P < 0.05$. ND – not detected

	Stingless bee propolis extracts (mg L^{-1})										
	PL1	**PL2**	**TA1**	**TA2**	**TA3**	**MQ1**	**MQ2**	**FD1**	**FD2**	**NT1**	**NT2**
Phenolic compounds											
Gallic acid	0.04 ± 0.01^{d}	0.02 ± 0.01^{d}	ND	ND	ND	2.06 ± 0.02^{a}	4.38 ± 0.02^{c}	0.09 ± 0.01^{d}	2.57 ± 0.03^{b}	ND	0.13 ± 0.01^{d}
Caffeic acid	ND	ND	ND	ND	ND	0.81 ± 0.01^{a}	0.07 ± 0.01^{d}	ND	0.17 ± 0.01^{b}	0.09 ± 0.01^{c}	0.06 ± 0.01^{d}
Epicatequin	0.07 ± 0.01^{e}	0.08 ± 0.01^{e}	0.15 ± 0.01^{c}	0.13^{d}	0.14 ± 0.01^{cd}	0.15 ± 0.01^{c}	0.17 ± 0.01^{b}	0.2 ± 0.01^{a}	0.18 ± 0.01^{ab}	0.18 ± 0.01^{b}	0.19 ± 0.01^{ab}
p-cumaric acid	ND	ND	ND	ND	ND	2.81 ± 0.14^{a}	0.19 ± 0.01^{b}	0.19 ± 0.01^{b}	1.35 ± 0.1^{b}	0.06 ± 0.01^{c}	ND
trans-ferulic acid	ND	0.31 ± 0.02^{a}	ND	0.07 ± 0.01^{d}	0.07 ± 0.01^{cd}	ND	0.07 ± 0.01^{cd}	0.08 ± 0.01^{c}	0.12 ± 0.01^{b}	0.07 ± 0.01^{cd}	0.07 ± 0.01^{cd}
Ellagic acid	ND	ND	ND	ND	ND	1.2 ± 0.03^{a}	0.23 ± 0.01^{cd}	0.2 ± 0.02^{d}	0.39 ± 0.01^{b}	ND	0.26 ± 0.01^{c}
Rutin	ND	ND	ND	ND	ND	1.52 ± 0.03^{a}	0.18 ± 0.01^{c}	ND	0.30 ± 0.03^{b}	ND	0.17 ± 0.01^{c}
Piceatannol	0.11 ± 0.01^{e}	ND	0.12 ± 0.01^{c}	0.11 ± 0.01^{e}	0.12 ± 0.01^{c}	0.77 ± 0.03^{a}	0.1 ± 0.011^{d}	0.1 ± 0.01^{f}	0.23 ± 0.01^{b}	0.1 ± 0.01^{f}	0.11 ± 0.01^{d}
Myricetin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Resveratrol	0.07 ± 0.01^{c}	0.11 ± 0.01^{b}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}	0.23 ± 0.01^{a}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}	0.06 ± 0.01^{d}
Quercetin	ND	ND	ND	ND	ND	0.16 ± 0.01^{a}	ND	0.11 ± 0.01^{b}	0.12 ± 0.0^{b}	ND	ND
trans-cinnamic acid	ND	0.14 ± 0.01^{d}	ND	0.06 ± 0.01^{g}	0.07 ± 0.01^{f}	1.60 ± 0.02^{a}	0.19 ± 0.01^{c}	0.08 ± 0.01^{e}	0.65 ± 0.03^{b}	0.05 ± 0.01^{h}	0.06 ± 0.01^{g}
Naringenin	ND	0.07 ± 0.01^{de}	0.08 ± 0.01^{c}	ND	0.07 ± 0.01^{e}	0.75 ± 0.05^{b}	0.09 ± 0.01^{cd}	0.11 ± 0.01^{c}	1.00 ± 0.1^{a}	ND	ND
Kaempferol	ND	ND	ND	0.11 ± 0.0^{d}	0.1 ± 0.01^{d}	0.32 ± 0.03^{a}	0.12 ± 0.01^{cd}	0.13 ± 0.01^{c}	0.210 ± 0.01^{b}	0.11 ± 0.01^{d}	ND
Isoliquiritigenin	0.06 ± 0.01^{d}	ND	ND	ND	0.08 ± 0.01^{c}	$0.090.01^{b}$	ND	ND	0.09^{a}	ND	ND
Formononetin	0.12 ± 0.01^{c}	ND	ND	ND	ND	45.40 ± 0.36^{a}	1.28 ± 0.04^{b}	ND	ND	ND	ND
Biochanin	0.0 ± 0.013^{c}	ND	0.02 ± 0.01^{c}	0.02 ± 0.01^{c}	0.02 ± 0.01^{c}	0.48 ± 0.01^{b}	0.02 ± 0.001^{c}	0.02^{c}	0.5 ± 0.04^{a}	0.02 ± 0.02^{c}	0.01 ± 0.01^{c}
Kaempferide	ND	ND	ND	0.2 ± 0.02^{c}	ND	0.33 ± 0.31^{a}	0.2 ± 0.01^{c}	ND	0.26 ± 0.01^{b}	ND	ND
7-hydroxycumarin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Scopoletin	ND	ND	ND	ND	ND	0.1 ± 0.01	ND	ND	ND	ND	ND
4-methylumbeliferonone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Coumarin	0.1 ± 0.01^{de}	2.34 ± 0.03^{a}	0.05 ± 0.01^{e}	ND	ND	0.69 ± 0.01^{b}	0.13 ± 0.07^{d}	0.07 ± 0.01^{de}	0.28 ± 0.04^{c}	ND	ND

coumarin found, but only in the MQ1 sample, at a concentration of 0.10 mg.L^{-1}.

Resveratrol was identified and quantified in all propolis samples; the MQ1 sample presented a content almost four times higher (0.23 mg.L^{-1}) of this compound than the other samples, which presented 0.06 mg.L^{-1}. The sample PL2 had almost twice the concentration of resveratrol when compared to other eight samples (0.11 mg.L^{-1}), while sample PL1 had a content very close to 0.07 mg.L^{-1}. Biochanin could not be quantified only in the PL2 sample, but was present in all other samples, while for formononetin the highest concentration was found in the MQ1 sample (45.40 ± 0.36 mg.L^{-1}). It was also possible to verify that samples TA1, TA2 and TA3 presented similar chemical compositions and concentrations (Table 2).

Principal components analysis

The principal components were determined using the results of the phenolic and coumarins compounds found in the SLB propolis samples, and are shown in Fig. 2. The first two components (F1 and F2) explained 94.28% of the data variation. The F1 had most of its variation (80.47%) due to the qualitative contrast in the phenolic compounds found, while the F2 (13.81%) was better represented by the quantitative variation. A grouping was observed in most of the results of the propolis sample phenolic compounds in the negative left quadrant. The PL2 sample, plotted on the left side, is more related in this analysis with its coumarin profile.

On the other hand, samples PL1, TA1, TA2 and TA3 were positioned in the left portion (negative), correlated with most of the phenolic compounds found herein, especially resveratrol and quercetin. The NT1, FD1, MQ1 and MQ2 samples are located on the right portion (negative), in correlation with rutin. The phenolic compound gallic acid was far from having a relationship with the samples (Fig. 2).

Oliveira et al. (2012), in a study on honeys produced by Melipona bees, after carrying out analysis of main components, they were able to distinguish the species studied through the phenolic composition of the honeys analyzed. This factor indicates possible selectivity of the species in relation to the botanical and geographic origin of the honeys, corroborating the present study, which demonstrated well-defined groups among these phenolics.

Antimicrobial activity

For this experiment, reference strains of a fungus, a Gram-negative and a Gram-positive bacterium, all of medical importance, were chosen. Those were, respectively, Candida albicans FIOCRUZ CPF 02508, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212. These specific strains were chosen since they were previously used in antimicrobial sensitivity assays (Sokolonski et al. 2021; Fonseca et al. 2022; Moradi et al. 2023; Simsek et al. 2023).

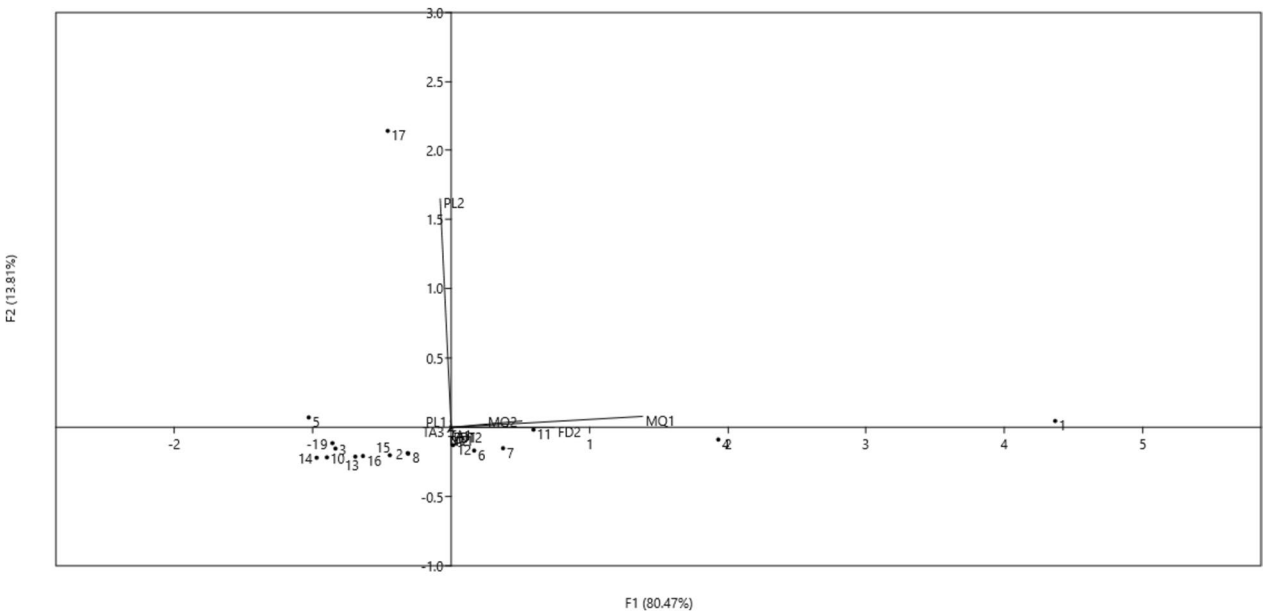


Fig. 2 Principal Component Analysis of the phenolic and coumaric compounds found at the stingless bee propolis extracts. 1- Gallic acid; 2- Caffeic acid; 3- Epicatechin; 4- p-cumaric acid; 5- trans ferulic acid; 6- Ellagic acid; 7- Rutin; 8- Piceatannol; 9- Resveratrol; 10- Quercetin; trans cinnamic acid; 12- Naringerin; 13- Kaempferol; 14- Isoliquiritigenin; 15- Biochanin; 16- Kaempferide; 17- Coumarin

Due to the non-detection of total phenolic compounds in the samples of propolis from N. testaceicornes and Tetragonisca angustula bees, it was decided to test only the other samples (PL1, PL2, MQ1, MQ2, FD1 and FD2). Table 3 presents the MIC and MFC or MBC of the propolis samples on the microorganisms tested herein, and Supplementary Figure S1 indicates the kinetics of inhibition of microbial growth according to the concentrations of propolis. C. albicans was the most susceptible microorganism to SLB propolis extracts, as all samples demonstrated inhibitory and fungicidal capacity, with propolis PL2 and MQ2 being the most efficient, presenting the lowest MIC100 and MFC100, 256 $\mu g.mL^{-1}$.

Regarding the antibacterial activity, none of the SLB propolis samples were able to inactivate 100% of the Gram-positive and Gram-negative bacteria tested herein. Four samples presented MIC100 for E. faecalis (PL2, MQ2, FD1and FD2), while only three were able to inhibit 100% of the E. coli growth (PL2, MQ2 and FD2).

Discussion

The worldwide importance and use of propolis produced by *Apis mellifera* has obfuscated the development and study of propolis produced by SLB. These bees are ease to handle and are present in many different countries in the southern hemisphere. In this work, we were able to verify the composition and the antimicrobial activity of propolis produced by five SLB species, and we found in these propolis many compounds that were not previously identified.

The total phenolics assay consists of the reduction of sodium tungstate and sodium molybdate salts, which are present in the Folin-Ciocalteu reagent. Among the reducing species are phenolic compounds, so it is not a highly accurate method, since other compounds, such as vitamin C, carbohydrates and minerals can interfere with the result, but even so, it is still a test widely performed to determine the phenolic composition of different extracts (Bastola et al. 2017). We could observe an intense variation in the concentration of these compounds in the different samples studied, and these results suggest that the geographic location and the ecosystem interfere with the composition of the product (Salatino & Salatino 2021). In an analysis of *Apis mellifera* bee propolis, Duca et al. (2019) reported a variation from 170.24 to 333.83 mgGAE g^{-1} in the phenolic content between the samples. In another study also with propolis from *Apis bees*, Andrade et al. (2017) found concentrations of total phenolic compounds of 55.74 ± 0.48, 90.55 ± 1.52 and 91.32 ± 0.49 mgGAE g^{-1} for brown, green and red propolis, respectively.

Some studies mention that a greater amount of total phenolics is expected in the polar fraction of extracts from different plants (Kamkar et al. 2014). However, the chromatographic profile of extracts from the use of different solvents are quite different. Thus, to obtain propolis extracts, water does not act as a good solvent to extract phenolic compounds, since it cannot extract important compounds responsible for the therapeutic effects of hydrophilic propolis extracts, also impacting the biological activity of these extracts (Contieri et al. 2022). Thus, in Brazil, propolis extracts are marketed mainly in the form of ethanolic (hydroalcoholic) solutions. Therefore, we chose to use 70% ethanol to obtain propolis extracts in this study, in order to be as close as possible to the way these extracts are marketed.

The values found for *A. mellifera* propolis phenolic concentrations are close to or much higher than those shown in this work (13.45 to 82.05 mgGAE g^{-1}). However, there are studies that report a high content of total phenolics

Table 3 Antimicrobial activity of SLB propolis. It was tested three reference strains of three pathogenic agents: a fungus (C. albicans), a Gram-negative (E. coli) and a Gram-positive (E. faecalis) bacterium in a microdilution assay, along with different propolis concentrations. MIC100—minimum concentration capable to inhibit the growth of 100% of the microbes; MFC100 – minimum concentration capable to inactivate 100% of the fungi; MBC100 – minimum concentration capable to inactivate 100% of the bacteria. The experiment was repeated three times and the results are their averages

Sample	*Candida albicans*		*Escherichia coli*		*Enterococcus faecalis*	
	MIC_{100} ($\mu g.mL^{-1}$)	MFC_{100} ($\mu g.mL^{-1}$)	MIC_{100} ($\mu g.mL^{-1}$)	MBC_{100} ($\mu g.mL^{-1}$)	MIC_{100} ($\mu g.mL^{-1}$)	MBC_{100} ($\mu g.mL^{-1}$)
PL1	512	512	-	-	-	-
PL2	256	256	1024	-	512	-
MQ1	512	512	-	-	-	-
MQ2	256	256	512	-	1024	-
FD1	512	512	-	-	1024	-
FD2	256	512	1024	-	1024	-

for SLB, such as 152.46 ± 55.61 to 327.86 ± 38.15 mg GAE. g^{-1} (Mulyati et al. 2020), and 2192.7 ± 12.3 to 2391.0 ± 16.1 mgGAE.g^{-1} (Abdullah et al. 2020). The data found in the literature show significant variations regarding the total phenolic content for a same SLB species, for example, a variation from 3.87 ± 0.32 to 211 ± 7.5 mgGAE.100 g^{-1} (Campos et al. 2014; Torres et al. 2018) was already described for the genus *Melipona droryana.* The samples of a specific species of this bee, *Melipona quadrifasciata*, presented in this study 82.05 ± 2.33 mgGAE.g^{-1} and 29.09 ± 2.63 mgGAE.g^{-1} in the concentrations of total phenolic compounds, respectively. For the genus *Tetragonisca droryana* it is known that the biological activity of propolis is lower when compared to other species, and this situation is linked to a low diversity of phenolic compounds (Campos et al. 2015). Furthermore, Torres et al. (2018), reported only 1.26 ± 0.17 mgGAE.g^{-1} for the phenolic content of *Tetragonisca angustula* propolis. In our work, the low concentrations of phenolic compounds could not be detected using the Folin-Ciocalteu method for propolis samples produced by bees of this genus.

Coumarins are compounds that have more than one aromatic ring with the presence of an oxygen, and can be formed from a phenolic compound, as is the case of coumarin itself, which can be derived from p-coumaric acid (Dos Anjos et al. 2011). These compounds are present in propolis due to the fact that bees insert resins and other parts of the plants in the production of propolis. Due to the presence of these phytochemicals, propolis may have different activities, such as anti-inflammatory, immunomodulatory, antimicrobial, anticancer and antioxidant (Sanches et al. 2017).

Badiazaman et al. (2019) performed a phytochemical screening on samples of propolis from stingless bees of the *Geniotrigona thoracica* species using thin layer chromatography (TLC). The presence of coumarins was found in all analyzed samples. Additionally, phytochemical screening studies also showed the presence of coumarins in propolis extracts from stingless bees of the *Lepidotrigona terminate* species (Nafi et al. 2016).

Coumarins are a compound class still little discussed in the study of propolis, and they were found to be present in some samples tested in this work, highlighting coumarin and scopoletin. This coumarin has already been identified in honey samples from the *Apis mellifera* bee (Guerrini et al. 2009); however, it has not yet been identified in honey from bees from the Meliponini tribe (Braghini et al. 2022; Guerrini et al. 2009), which presents a more diverse phytochemical profile due to the flora rich environment in tropical regions (Lim et al. 2023). It is worth mentioning that there are no previous reports in the literature of this coumaric derivative for SLB propolis(Popova et al. 2021) mention that chemical studies of Meliponini propolis resulted in the discovery of new natural molecules, some of them with valuable bioactivity, which stimulates the study of its pharmacological properties.

Some of the compounds identified in this work were already reported in the literature, such as biochanin, an isoflavonoid compound found almost exclusively in vegetables of the Leguminosae family and one of the biomarkers of red propolis (Daugsch et al. 2008; Park et al. 2002). Along with biochanin, formononetin is another phenolic compound widely cited as one of the main constituents of red propolis and also as one of the biomarkers of this type of propolis (da Silva Frozza et al. 2014; López et al. 2014). Thus, this situation demonstrates that plants that are foraged by the bee A. mellifera are also targets for SLB, and that perhaps these compounds are not as useful as biomarkers for propolis, as has been widely reported (López et al. 2014). It should be noted that this is the first study to report formononetin for SLB propolis.

With regard to the propolis produced by the *Tetragonisca angustula* SLB species, the results obtained herein reinforce the idea that the propolis of this bee does not have great differences in its composition, even if there is a change in the collection site (Carneiro et al. 2016). These bees are attracted to terpenoids, which restricts the plant types used for propolis production and is a possible cause of this lower variation; terpenoids are one of the main volatile compounds found in propolis (Lavinas et al. 2019). However, the amount of resin produced by a plant does not determine the preference of bees for a particular type of plant (Leonhardt & Blüthgen 2009).

Resveratrol is a compound of great importance for its anticancer, antimicrobial, anti-inflammatory, and mainly antioxidant activities, with great potential for direct use on the skin, since its metabolization and excretion are fast when used orally (Berman et al. 2017). Its presence occurs in a wide range of plants, including *Myrtacea*, which has the eucalyptus as a representative, a plant that has seen great expansion as an agricultural crop, and it is known that SLBs frequently visit this tree (Freitas et al. 2008). Another factor that reinforces eucalyptus as a possible source of resveratrol is that the sample with the highest concentration of this compound was MQ1, produced by *Melipona quadrifasciata* species, and this tree is known to be the main botanical origin of propolis produced by this bee (Martins Ribeiro et al. 2019), in addition to eucalyptus pollen being the most found in bee products (de Souza et al. 2019). There are few reports in the literature regarding this compound in propolis. Of the works referring to resveratrol, the one by (Volpi 2004) can be mentioned, in which it is described the presence of this compound in A. mellifera propolis. Duca et al. (2019) found resveratrol concentrations ranging from

4.90 ± 0.57 to 188.50 ± 42.52 μg.mL^{-1}, and Kasiotis et al. (2017) found concentrations from 0.9 to 1 0.4 μg.g^{-1}. None of these reports refer to propolis from stingless bees, and this work is possibly the first to identify resveratrol in SLB propolis.

We could observe in this work a significant fungicidal and fungistatic effect of propolis produced by SLB. Campos et al. (2014), observed that the *Melipona orbignyi* propolis extract exerted an inhibitory, fungicidal and bactericidal effect against *C. albicans* and *Staphylococcus aureus*, with the bactericidal effect at a concentration of 3.1 mg.mL^{-1} and a fungicide effect at 50 mg.mL^{-1}, but there was no action against *E. coli*, a Gram-negative bacterium. This study partially corroborates with previous results and extends these activities on fungi to propolis produced by other SLB species, being that we observed fungistatic and fungicide effects at lower propolis concentrations.

For *E. coli*, MIC100 results only were observed for PL2 and MQ2 propolis at concentrations of 1024 and 512 μg.mL^{-1}, respectively, and none of the extracts showed detectable MBC100. The difficulty of propolis in achieving antimicrobial activity in Gram-negative bacteria has already been described by other studies, and it is assumed that this type of bacteria has the ability to circumvent the effects of the compounds present in different propolis extracts, which consequently will have little effect or an activity at high concentrations (Abdullah et al. 2019).

De Souza et al. (2018) found an inhibitory activity at *E. faecalis* by the propolis of the bee *Frieseomelitta longipes* at a concentration of 125 μg.mL^{-1}, and for the yeast *C. albicans* the inhibitory concentration ranged from 62.5 to 250 μg.mL^{-1}. Torres et al. (2018) identified a greater sensitivity of Gram-positive bacteria in relation to Gram-negative ones to propolis produced by the bee *Melipona quadrifasciata*, and *E. faecalis* was inhibited by a concentration around 4 mg.mL^{-1}. Dos Santos et al. (2017) also found lower susceptibility of the Gram-negative bacteria *E. coli* and *Pseudomonas areuginosa* than Gram-positive bacteria *Staphylococcus aureus* to *Melipona quadrifasciata* propolis. In our work, we found no bactericide activity against *E. faecalis*, and a bacteriostatic effect was exerted by the sample PL2 at the 512 μg.mL^{-1} concentration, and by the MQ2, PD1 and PD2 samples at the concentration of 1024 ug.mL^{-1}. Silva-Beltrán et al. (2021), cite that propolis is capable of causing bacteriolysis by disorganizing the cytoplasmic membrane and cell wall, which hinders the growth of bacterial cells, and this situation can be a possible explanation for the greater susceptibility of Gram-positive bacteria, since they present a more complex cell wall structure.

The antimicrobial activity of some natural products can be associated with the content of specific phenolic compounds, such as phenolic acids (gallic acid, caffeic acid, *p*-coumaric acid), stilbenes (resveratrol), and flavonoids (quercetin, myricetin, rutin, kaempferol, formononetin), in addition to coumarins (Liu 2004; Takó et al. 2020; Teixeira et al. 2023). However, it is difficult to specifically relate a compound to the antimicrobial activity of a specific propolis sample, since there are compounds that have not yet been identified or have been recently reported, and that may present a promising activity (Oanh et al. 2021). Another fact to be recognized is the synergism of the various compounds, which can constitute and enhance the antimicrobial effects of propolis (Bhargava et al. 2021). However, when analyzing our results, one thing that can be noticed is the presence of gallic acid in some propolis and the antimicrobial activity of these specific samples. Gallic acid is a compound with a recognized antimicrobial activity against Gram-positive and Gram-negative bacteria (Lima et al. 2019), and the present work reports relevant concentrations of this compound for MQ2 (4.38 ± 0.02 mg.L^{-1}) and FD2 (2.57 ± 0.03 mg.L^{-1}) samples, which inhibited the growth of *E. faecalis*. On the other hand, the PL2 sample, which also contains the gallic acid compound, but in a much smaller amount, may have its ability to inhibit *E. faecalis* associated with coumarin, which presented the highest content of this specific compound.

It is noteworthy that studies by Da Cunha et al. (2020) showed that an important 4-phenyl coumarin (cinnamoyloxy-mameisin) isolated from stingless bee geopropolis showed antimicrobial activity against *Staphylococcus aureus*, in addition to preventing microbial adhesion to human cells, biofilm formation and mature biofilm. This substance showed bacteriostatic activity against strains of *S. aureus* susceptible and resistant to methicillin, with MIC of 11.3 μM. In addition, 5.7 μM cinnamoyloxy-mameisin reduced bacterial adherence to human keratinocytes from 1 to 3 h and disrupted biofilm formation, reducing cell viability and architecture.

Some of the phenolic compounds found in the evaluated propolis and also mentioned in the literature (p-coumaric acid, coumarins, naringenin, caffeic acid, pinocembrin, pinobanksin, galantine, artepillin C, ferulic acid, calycosin, kaempferol, catechin, epicatechin, formononetin, isoformononetin, umbellic acid, luteolin, quercetin and others) has been shown to act on the mechanism of bacterial multiplication. These compounds can interfere with protein synthesis, consequently affecting cell division, as well as disorganizing the cell wall and/or cytoplasmic membrane. These effects cause bacteriolysis (change in permeability) (Devequi-Nunes et al. 2018; Gomes Do Nascimento et al. 2019; Herrera-López et al. 2019; Koru et al. 2007; Smyth et al. 2009; Trusheva et al. 2007; Valencia et al. 2012). Antifungal activity of

coumarin against *Candida albicans* (apoptosis-dependent way and depletion of ergosterol content) was reported by Thati et al. (2007) and Jia et al. (2019).

The limitations of this study refer mainly to the extraction methodology of the analyzed compounds, as the same extraction conditions were used for different types of propolis. However, in future studies, the optimization of the extraction method for each type of propolis may result in better extraction yields of the compounds of interest, since it is known that the composition of propolis varies according to the bee species and the ecosystem where the itself is produced. In addition, other more sensitive and more specific analytical techniques, such chromatographic techniques coupled to mass spectrometry, may be used in the future for the identification and quantification of phenolic compounds and coumarins in different types of propolis. This will enable a significant improvement in the evaluation of the presence of new bioactive substances in this matrix, as well as quantify the analytes with lower concentrations than those obtained in this work.

According to the results found in this work, it is possible to conclude that the SLB species and the place of origin influenced the chemical profile of propolis, since there are statistical differences regarding the total phenolics concentrations, and mainly in the quantitative study by chromatographic analysis. The isoflavonoid formononetin was identified in three samples, and the presence of this compound had not yet been reported for SLB propolis. Resveratrol was verified in all propolis samples analyzed herein, and this compound had only been previously described for *Apis mellifera* bee propolis. In addition, it was possible to identify and quantify two coumarins (coumarin and scopoletin) in the analyzed SLB propolis samples. Regarding the antimicrobial activity, the results showed significant fungistatic and fungicidal actions on *C. albicans*. Principal component analysis showed that the compounds resveratrol, quercetin and coumarin are significantly related to the samples and their different origins.

Abbreviations

SLB	Stingless Bees
HPLC–DAD-FLD	High-Performance Liquid Chromatograph with a Diode Array and Fluorescence Detector
PL1	*Plebeiadroryana*, Palmeiras city
PL2	*Plebeiadroryana*, Feira de Santana city
TA1	*Tetragoniscaangustula*, Valença city
TA2	*Tetragoniscaangustula,* Maragogipe city
TA3	*Tetragoniscaangustula*, Feira de Santana city
MQ1	*Meliponaquadrifasciata*, Feira de Santana city
MQ2	*Meliponaquadrifasciata*, Feira de Santana city
FD1	*Frieseomelittadoederleini*, Feira de Santana city
FD2	*Frieseomelittadoederleini*, Feira de Santana city
NT1	*Nannotrigona testaceicornes*, Feira de Santana city
NT2	*Nannotrigona testaceicornes*, Lauro de Freitas city
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
MBC	Minimum Bactericidal Concentration

Supplementary Information

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Additional file 1: Table S1. Phenolic compounds and coumarins with their respective wavelengths analyzed in the diode array detector (HPLC-DAD). **Table S1.2.** Phenolic compounds and coumarins with their respective wavelengths analyzed in the fluorescence detector (HPLC-FLD). **Table S1.3.** Parameters of the analytical curves for the diode array detector. **Table S1.4.** Parameters of the analytical curves for the fluorescence detector. **Figure S1.** kinetics of inhibition of microbial growth.

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Conflicts of interest
The authors declare no conflict of interest.

Sample availability
Samples of the compounds are available from the authors.

Authors' contributions
Conceptualization, VMR, RWP COdS and MAU-G; methodology, RWP, JPdA, MAU-G.; validation, VMR, RWP, JPdA, COdS and MAU-G; investigation, VMR, RWP, LEL, ARS, COdS and MAU-G; data curation, VMR, RWP, LEL, ARS, JPdA, RQN, COdS and MAU-G.; writing—original draft preparation, VMR, RWP, JPdA, COdS and MAU-G.; writing—review and editing, RWP, MAU-G.; visualization, RWP, MAU-G.; supervision, MAU-G.; project administration, MAU-G. All authors have read and agreed to the published version of the manuscript.

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