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ORIGINAL ARTICLE

A Validated RP-HPLC–DAD Method for Analyzing Flavonoids in Caatinga Brazilian Green Propolis from *Mimosa tenuiflora* Produced by *Apis mellifera*

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Abstract
There are several types of propolis in Brazil produced by *Apis mellifera* Linnaeus, 1758, Apidae, with the green propolis from the Caatinga biome standing out for its high flavonoid content. In this study, we describe the isolation of flavonoids from Brazilian green propolis of Caatinga *Mimosa tenuiflora* (Willd.) Poir., Fabaceae, and the development of a reliable RP-HPLC quantitative method. This method uses a Shim-pack VP-ODS column (250×4.6 mm i.d., 5 µm) with nonlinear gradient elution and UV detection at 280 nm. Additionally, a sample preparation method for extracting flavonoids using 96% ethanol and caffeic acid as the internal standard was employed. The developed method demonstrated excellent detection response, with limits of detection and quantification ranging from 0.65–2.08 µg/ml and 1.97–6.31 µg/ml, respectively. The maximum relative standard deviation was 4.61%. Thirteen flavonoids were quantified, including santin, ermanin, sakuranetin, quercetin-3-methyl ether, viscosine, eriodictyol-5-*O*-methyl ether, isokaempferide, kaempferide, penduletin, quercetin-3,6,7-trimethyl ether, cirsimaritin, 3,3'-*O*-dimethylquercetin, and luteolin. The developed method met all the parameters set by international guidelines for analytical method development. It is reliable for the quality control of *M. tenuiflora* green propolis and its related products.

Keywords Caatinga · Flavonoids · RP-HPLC-DAD · Analytical method

Introduction

Honey bees (*Apis mellifera* Linnaeus, 1758, Apidae) produce complex mixtures of resin-based compounds, known as propolis, from various plant parts, including buds and exudates (Huang et al. 2014; Santos et al. 2021; Son et al. 2024). Propolis is easily identified by its distinctive appearance, strong scent, and chemical composition, which reflects the local plants of the biome where it is produced. However, its chemical composition can vary with the seasons, even within the same location. Propolis has been used in folk medicine and pharmacological drug development for its numerous beneficial properties, including anticancer, antimicrobial, antioxidant, anti-inflammatory, and vasorelaxant activities (Huang et al. 2014; Son et al. 2024; Jung et al. 2024; Sartori et al. 2024). Green propolis, produced in Brazil's cerrado biome from *Baccharis dracunculifolia* DC., Asteraceae, has gained popularity in the global propolis market. This type of propolis is dry, friable, and can be mechanically ground into a powder (Salatino et al. 2005). The plant's color ranges from deep green to greenish-yellow, and it emits a resinous aroma (Salatino et al. 2005). Most Chinese and European propolis analyses have shown that flavonoids are the predominant resin components. In contrast,

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propolis from Brazil, particularly from the southeast, contains higher concentrations of prenylated phenylpropanoids compared to flavonoids (Salatino et al. 2005; Silva et al. 2012; Rodrigues et al. 2020).

Mimosa tenuiflora (Willd.) Poir., also known as *M. hostilis*, is a perennial shrub native to the Brazilian Caatinga biome in Northeastern Brazil. It is locally called "Jurema preta" and "binho-de-jurema" (Camargo-Ricalde 2000). Previous HPLC-DAD-MS analysis on *M. tenuiflora* green propolis identified flavonoids as the dominant compounds, including flavonols, flavones, chalcones, dihydroflavonols, and dihydroflavones (Ferreira et al. 2017a). Our group's phytochemical study corroborated these results, by isolating several flavonoids from this type of propolis (Son et al. 2022).

In this study, we report the isolation of additional flavonoids from the alcoholic extract of *M. tenuiflora* green propolis and the development of a validated RP-HPLC-DAD method for detecting and quantifying 13 flavonoids in both *M. tenuiflora* green propolis and plant bud extracts. This method was developed and validated under Brazilian National Health Surveillance Agency (Anvisa 2017) guidelines.

Material and Methods

General Experimental Procedures

NMR spectral data (400 MHz for 1H and 100 MHz for ^{13}C NMR) were carried out on a Bruker AVANCE in methanol-d_4. Silica gel (40–63 µm Thermo Fisher, USA) and Sephadex LH-20 (25–100 µm, GE Healthcare, Sweden) were used to perform column chromatography (CC). Thin-layer chromatography (TLC) was performed using pre-coated silica gel 60 F_{254} (Merck) with mobile phase consisting of dichloromethane-methanol (6:1, v/v). Sulfuric acid 5% in ethanol was used for visualizing TLC plates.

HPLC-grade acetonitrile, supplied by SK Chemicals, and formic acid, obtained from Synth, were used for chromatographic method development. Water was purified using a Milli-Q-plus system from Merck Millipore. Caffeic acid and ferulic acid standards were purchased from Sigma-Aldrich. Our research group previously isolated and identified flavonoids from Caatinga Brazilian green propolis, with the purity of the isolated compounds estimated to be greater than 97% by high-performance liquid chromatography (HPLC) and NMR.

Plant and Propolis Samples

Green propolis and buds of *Mimosa tenuiflora* (Willd.) Poir., Fabaceae, were collected in Remanso, Bahia, Brazil, in 2021. The plant material was identified by Prof. Milton Groppo of the University of São Paulo. A voucher specimen (SPFR-15118) was deposited in the herbarium at the Biology Department of the University of São Paulo (USP), Ribeirão Preto, SP, Brazil.

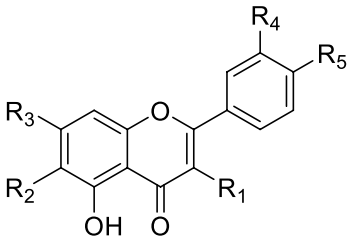
Propolis Extraction and Isolation of Compounds

Green propolis powder (150 g) was extracted with 90% aqueous ethanol (4 × 2 l × 4 h) using a Soxhlet apparatus. After concentration under reduced pressure at 50 °C and lyophilization, 38.50 g of crude extract was obtained. This extract was then subjected to an open silica gel chromatographic column (CC) using hexanes-acetone (1:0 to 0:1, v/v) as the mobile phase, yielding 13 fractions (JS1–JS13). Fraction JS4 (5.51 g) was further chromatographed on an open silica gel CC with chloroform-acetone (15:1 and 9:1, v/v), resulting in four sub-fractions (JS41–JS44). Fraction JS43 (0.8 g) was separated by preparative HPLC-UV [Phenomenex reverse-phase column, 4 µm, 250 × 10 mm, λ_{max} 281 and 335 nm], eluted with acetonitrile-water (6:4, v/v, 0.2% acetic acid), yielding compounds **11** (18.5 mg) and **12** (19.3 mg). Fraction JS6 (2.17 g) was subjected to Sephadex LH-20 CC [100% methanol], yielding three sub-fractions (JS61–JS63). Fraction JS62 (1.5 g) was further fractionated by preparative HPLC-UV at λ_{max} 281 and 332 nm, eluted with acetonitrile-water (7:3, v/v, 0.2% acetic acid), yielding compound **13** (17.5 mg).

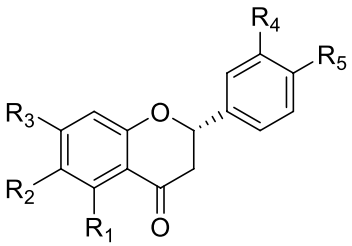
RP-HPLC–DAD Analytical Method Development

Standard Compounds

We report the isolation and identification of compounds **11**, **12**, and **13**. Compounds **1–10**, including santin (**1**), ermanin (**2**), sakuranetin (**3**), quercetin-3-methyl ether (**4**), viscosine (**5**), eriodictyol-5-*O*-methyl ether (**6**), isokaempferide (**7**), kaempferide (**8**), penduletin (**9**), and quercetagetin-3,6,7-trimethyl ether (**10**), were previously reported (Son et al. 2022).



1 R_1=R_2=R_5=OCH_3; R_3=OH; R_4=H
2 R_1=R_5=OCH_3; R_2=R_4=H; R_3=OH
4 R_1=OCH_3; R_2=H; R_3=R_4=R_5=OH
5 R_1=R_2=OCH_3; R_3=R_5=OH; R_4=H
7 R_1=OCH_3; R_2=R_4=H; R_3=R_5=OH
8 R_1=R_3=OH; R_2=R_4=H; R_5=OCH_3
9 R_1=R_2=R_3=OCH_3; R_4=H; R_5= OH
10 R_1=R_2=R_3=OCH_3; R_4=R_5=OH
12 R_1=R_4=OCH_3; R_2=H; R_3=R_5=OH
13 R_1=R_2=H; R_3=R_4=R_5=OH



3 R_1=R_5=OH; R_2=R_4=H; R_3=OCH_3
6 R_1=OCH_3; R_2=H; R_3=R_4=R_5=OH
11 R_1=R_5=OH; R_2=R_3=OCH_3; R_4=H

HPLC Apparatus and Chromatographic Conditions

The analytical method for *M. tenuiflora* buds and Caatinga green propolis was developed using a Shimadzu LC-20AR Prominence HPLC system equipped with a SIL-10AF autosampler, a CTO-20A column oven, a CBM-20A communications bus module, a DGU-20A3R in-line degasser, and an SPD-M20A photodiode array detector. All experiments were conducted in triplicate with temperature control. Chromatographic procedures were performed on a Shimpack VP-ODS analytical column (250 × 4.6 mm i.d., 5 µm; Shimadzu) using acidified water (0.2% acetic acid) as solvent A and acetonitrile as solvent B with the following gradient: 0.01 – 7.00 min, 30 – 36% B; 7.00 – 17.00 min, 36 – 38% B; 17.00 – 25.00 min, 38 – 45% B; 25.00 – 29.00 min, 45 – 55% B; 29.00 – 31.00 min, 55 – 80% B; 31.00 – 32.00 min, 80 – 100% B; 32.00 – 39.00 min, 100% B; and 40.00 – 42.00 min, 30% B. The flow rate was set at 1 ml/min, and UV detection was performed at 280 nm. The column chamber temperature was maintained at 40 °C, and the injection volume was 20.0 µl. Data processing was performed using LabSolutions® software.

Method Validation

The validation was performed according to the guidelines of the Brazilian National Health Surveillance Agency (Anvisa 2017). Parameters such as selectivity, linearity, limits of detection (LOD) and quantification (LOQ), accuracy, precision, recovery, and robustness were evaluated. All validation experiments were conducted in triplicate.

The method's selectivity was established by assessing the separation efficiency between chromatographic peaks, based on chromatographic resolution. Peaks were compared to authentic standards to determine the selectivity of compounds from Caatinga green propolis and *M. tenuiflora*, by evaluating retention time, peak area, resolution, and UV spectra.

Linearity was determined by constructing analytical curves. Solutions for the linearity test were prepared by dissolving 5 mg of analytes **1**, **3**–**9**, and **11**–**12** in 5 ml of acetonitrile, followed by serial dilutions to achieve eight different concentrations: 300, 200, 100, 75, 50, 25, 15, and 10 µg/ml. For compounds **2**, **10**, and **13**, 3 mg of each compound were dissolved in 5 ml of acetonitrile to achieve concentrations of 180, 120, 60, 45, 30, 15, 9, and 6 µg/ml. Caffeic acid was used as the internal standard at a final concentration of 100 µg/ml. Twenty microliters of each solution were injected in triplicate over three consecutive days. The analytical curve was constructed by calculating the ratio between each analyte and the internal standard areas. Linearity was evaluated using the correlation coefficient (R), the coefficient of determination (R^2), and a lack-of-fit test. LOD and LOQ were calculated using the formulae LOD = 3.3σ/S and LOQ = 10σ/S, where σ represents the standard deviation of the response and S represents the slope of the analytical curve.

To evaluate precision, three concentrations were selected: high (100 µg/ml), medium (75 µg/ml), and low (50 µg/ml) for compounds **1**, **3**–**9**, and **11**–**12**, and high (60 µg/ml), medium (45 µg/ml), and low (30 µg/ml) for compounds **2**, **10**, and **13**. Precision was assessed by measuring the repeatability of results at these concentration levels within the same day (intraday precision) and across three consecutive days (interday precision). Accuracy was determined by comparing the theoretical and actual values of the same three concentration solutions.

A standard solution containing compounds **1**, **3**, **5**, **7**, **8**, and **9** at a final concentration of 100 µg/ml each was prepared. Next, 200 mg of propolis matrix was spiked with this solution at three concentration levels: 1 ml (100 µg, high level), 0.75 ml (75 µg, medium level), and 0.25 ml (25 µg, low level).

The spiked matrix solvents were allowed to evaporate at room temperature. An ethanolic solution (70%) containing 75 µg/ml of caffeic acid, used as an internal standard, was employed for sample analysis. Ferulic acid, at 75 µg/ml, was used as a secondary internal standard to quantify caffeic acid. The extraction was performed using a shaker incubator at 35 °C and 140 rpm for 120 min. The samples were analyzed using the developed HPLC method, and the recovery percentage was calculated by comparing the theoretical and actual concentration values. All experiments were performed in triplicate.

Robustness was evaluated using the Plackett-Burman design (Ferreira et al. 2017b), with five factors and eight experiments. The factors tested were flow rate, oven temperature, percentage of organic solvent, detection wavelength, and injection volume. These factors were adjusted to higher and lower levels, as described in Table S1. The effects were calculated using the equation $Ex = \frac{\sum y(+) - \sum y(-)}{n/2}$, and expressed as variation coefficients in percentage. Low, medium, and high concentrations, as used for the precision evaluation, were employed to assess robustness.

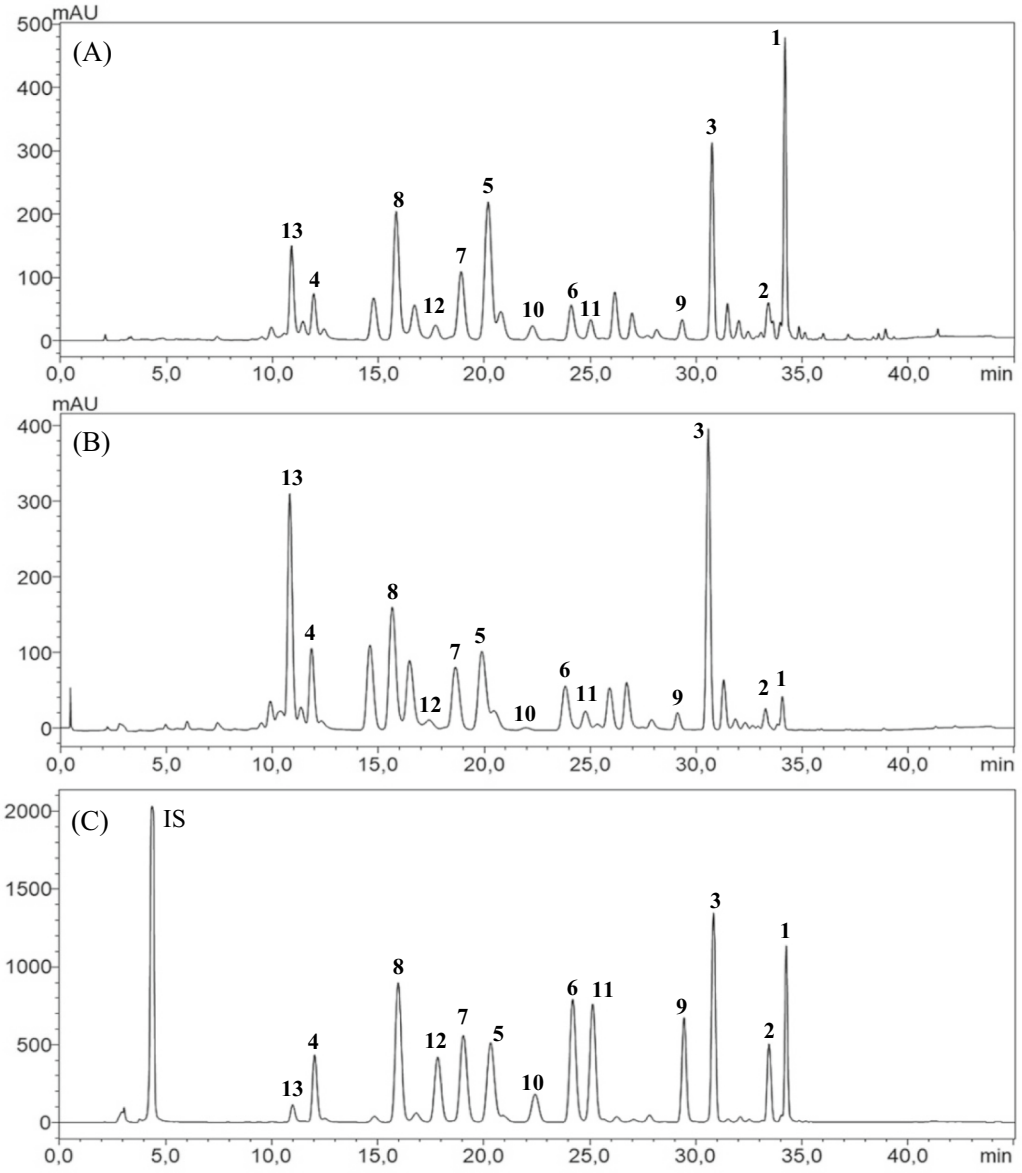


Fig. 1 HPLC-DAD chromatographic profiles: **A**) Propolis extract using the developed method; **B**) Plant extract using the developed method; **C**) Isolated compounds **1–13** and caffeic acid (IS)

Results and Discussion

Phytochemical Isolation

High-purity chromatographic standards are essential for developing accurate chromatographic analytical procedures. To achieve this, it is often necessary to conduct a phytochemical study by isolating and identifying the primary constituents of the sample. The isolation and identification of chemical markers from natural sources are crucial not only for developing analytical methods to ensure the quality control of extracts and products, but also for their use in pharmacological and toxicological studies. These steps are vital for validating the chemical and pharmacological properties of these products in healthcare applications.

In this paper, we report the isolation of the compounds cirsimaritin (**11**), 3,3'-*O*-dimethylquercetin (**12**), and luteolin (**13**), in addition to the 10 compounds previously isolated (Son et al. 2022). The NMR data for compounds **11**–**13** are consistent with literature reports (Figs. S1-S8) (Dutra et al. 2017; Silva et al. 2020; Khan et al. 2021). It should be noted that compounds **11**–**13** were first isolated from caatinga green propolis. Additionally, compound **12** was also detected in the genus *Mimosa* for the first time. The purity of all standard compounds was estimated to be greater than 97% based on both NMR and HPLC analyses.

Compound **11** was previously reported in *Mimosa hamata* Willd. stem, and compound **13** was identified in *M. scabrella* Benth. honeydew (Dutra et al. 2017; Silva et al. 2020; Khan et al. 2021). Notably, compound **12** is being reported for the first time in propolis and *Mimosa* plants. All thirteen compounds **1**–**13** (Fig. 1) belong to the flavonoid class of natural products, which we are reporting for the first time in both Caatinga green propolis and its botanical source, *M. tenuiflora* extracts. These results challenge previous claims that flavonoids were present in high amounts only in Chinese and European propolis (Silva et al. 2012; Rodrigues et al. 2020).

HPLC Method Validation

The compounds were identified in the HPLC chromatographic profiles by comparing retention times (Rt) and UV spectra with those of the thirteen authentic standard compounds. Caffeic acid was used as the internal standard (IS) (Fig. 1).

Based on the retention times of the standard solution, the following compounds were identified in both Brazilian Caatinga green propolis and *M. tenuiflora* plant extracts, including the internal standard: IS (peak 1, Rt 4.37 min), **13** (peak 2, Rt 10.99 min), **4** (peak 3, Rt 12.03 min), **8** (peak 4, Rt 15.97 min), **12** (peak 5, Rt 17.83 min), **7** (peak 6, Rt 19.04 min), **5** (peak 7, Rt 20.32 min), **10** (peak 8, Rt 22.42 min), **6** (peak 9, Rt 24.19 min), **11** (peak 10, Rt 25.13 min), **9** (peak 11, Rt 29.44 min), **3** (peak 12, Rt 30.83 min), **2** (peak 13, Rt 33.44 min), and **1** (peak 14, Rt 34.25 min) (Fig. 1).

Analytical calibration curves for standard compounds **1**–**13** were generated by plotting the ratio of each compound's area to that of the internal standard. The R^2 values (ranging from 0.9992 to 1) demonstrated the linearity of the calibration curves (Table 1). Compounds **1**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **11** and **12** showed linearity over the concentration range of 10–300 µg/ml, while compounds **2**, **10**, and **13** were linear within the range of 6–180 µg/ml. LOD values ranged

Table 1 Linearity, limits of detection and quantification of the method

Compounds	Equation	R^2	R	LOD	LOQ	Minimum observed residual value	Maximum observed residual value	Lack of fit *p* value
1	y = 0.0013x – 0.0014	1	1	0.73	2.22	– 3.90719	2.32396	0.97
2	y = 0.0013x – 0.0013	0.9999	0.9999	0.77	2.35	– 2.36871	3.00570	0.81
3	y = 0.0022x + 0.0036	0.9996	0.9997	1.13	3.44	– 3.96324	3.16934	0.20
4	y = 0.0008x – 0.0003	0.9992	0.9995	2.08	6.31	– 2.9778	3.5319	0.63
5	y = 0.0017x – 0.0008	1	1	0.99	3.01	– 4.65610	1.72288	0.87
6	y = 0.0021x – 0.0057	0.9999	0.9999	1.30	3.96	– 1.61868	1.23401	0.92
7	y = 0.0016x – 0.0016	0.9999	0.9999	1.46	4.43	– 1.97615	1.83148	0.83
8	y = 0.0023x – 0.0034	0.9999	0.9999	1.40	4.25	– 2.06904	1.78612	0.86
9	y = 0.0013x – 0.0015	0.9999	0.9999	1.33	4.05	– 2.48367	1.96646	0.84
10	y = 0.0009x – 0.0009	0.9999	0.9999	0.84	2.56	– 2.41431	1.25577	0.81
11	y = 0.002x + 0.0004	1	1	1.07	3.25	– 2.96662	2.69049	0.86
12	y = 0.0012x – 0.0015	0.9999	0.9999	1.41	4.27	– 2.08408	1.07792	0.53
13	y = 0.0003x + 0.00009	0.9995	0.9997	0.65	1.97	– 2.56112	2.74617	0.36

R^2 Determination coefficient, *R* Correlation coefficient, *LOD* Limit of detection (µg/mL), *LOQ* Limit of quantification (µg/mL)

Table 2 Precision and accuracy of method

Compounds	Level	Precision (RSD)		Accuracy (%)	Error (%)	Recovery (%)
		Intraday	Interday			
	High	0.69	0.49	103.02 ± 0.40	3.02	103.16 ± 0.36
1	Medium	0.86	0.40	101.78 ± 0.92	1.78	103.07 ± 1.24
	Low	0.56	0.75	101.12 ± 0.61	1.12	104.76 ± 1.19
	High	0.42	0.35	104.15 ± 0.23	4.15	
2	Medium	0.58	0.54	102.78 ± 0.60	2.78	
	Low	0.52	0.63	102.62 ± 0.62	2.62	
	High	0.39	0.35	105.19 ± 0.16	5.19	103.24 ± 0.46
3	Medium	0.46	0.51	103.14 ± 0.53	3.14	101.17 ± 1.48
	Low	0.33	0.42	102.47 ± 0.67	2.47	103.22 ± 1.30
	High	3.35	0.28	100.51 ± 0.97	0.51	
4	Medium	3.26	4.61	97.97 ± 3.52	–2.03	
	Low	0.73	2.08	104.87 ± 0.15	4.87	
	High	0.45	0.40	100.36 ± 0.20	0.36	97.20 ± 0.53
5	Medium	0.43	0.61	98.94 ± 0.47	–1.06	95.85 ± 1.63
	Low	0.82	0.52	99.77 ± 1.17	–0.23	98.96 ± 1.29
	High	0.38	0.33	100.56 ± 0.13	0.56	
6	Medium	0.46	0.46	98.97 ± 0.47	–1.03	
	Low	0.40	0.32	99.55 ± 0.72	–0.45	
	High	0.49	0.34	102.27 ± 0.28	2.27	97.08 ± 0.51
7	Medium	0.42	0.58	100.79 ± 0.46	0.79	96.60 ± 1.53
	Low	0.49	0.48	101.42 ± 0.84	1.42	99.41 ± 1.37
	High	0.40	0.36	99.54 ± 0.18	–0.46	93.68 ± 0.55
8	Medium	0.48	0.60	98.10 ± 0.50	–1.90	94.32 ± 1.48
	Low	0.20	0.33	98.81 ± 0.55	–1.19	95.93 ± 1.30
	High	0.45	0.30	96.84 ± 0.24	–3.16	96.91 ± 0.49
9	Medium	0.49	0.54	95.39 ± 0.54	–4.61	95.54 ± 1.39
	Low	0.34	0.36	96.11 ± 0.67	–3.89	99.24 ± 1.38
	High	0.72	0.67	103.47 ± 0.43	3.47	
10	Medium	0.55	0.70	102.25 ± 0.49	2.25	
	Low	2.29	1.46	103.66 ± 2.56	3.66	
	High	0.44	0.32	99.44 ± 0.25	–0.56	
11	Medium	0.48	0.44	98.42 ± 0.53	–1.58	
	Low	0.49	0.30	99.76 ± 0.85	–0.24	
	High	0.31	0.38	98.28 ± 0.08	–1.72	
12	Medium	0.53	0.77	97.24 ± 0.49	–2.76	
	Low	0.63	0.32	98.35 ± 0.96	–1.65	
	High	2.86	2.53	99.19 ± 2.92	–0.81	
13	Medium	0.43	1.09	102.23 ± 0.12	2.23	
	Low	1.91	0.88	102.72 ± 2.29	2.72	
	High					103.06 ± 0.32
I.S	Medium					104.81 ± 1.74
	Low					101.40 ± 3.92

RSD, Relative standard deviation

from 0.65 to 2.08 µg/ml, and LOQ values ranged from 1.97 to 6.31 µg/ml, demonstrating that the developed chromatographic method is reliable and sensitive for the qualitative analysis of the thirteen flavonoids present in Caatinga green propolis.

The precision of the method, expressed as the relative standard deviation (RSD), was less than 4.61%, indicating high precision and low variation among analyses (Table 2) (Santos et al. 2021). Accuracy values ranged from 95.39% to 104.87%. A Soxhlet apparatus was used for exhaustive extraction of the

Table 3 Results of effects (Ex) of the robustness test for HPLC method

High (Concentr.)	1		2		3		4		5	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)
Flow rate	–6.25122	–0.00017	0.044636	2.02E-06	1.993682	3.09E-05	4.417679	0.000191	8.272307	0.00018
Oven temperature	7.050355	0.000195	5.581957	0.000253	6.774437	0.000105	–5.01797	–0.00022	11.64872	0.000254
Organic solvent (%)	8.617159	0.000238	5.842708	0.000264	9.456278	0.000147	–3.41972	–0.00015	10.58513	0.00023
Absorbance	–1.93135	–5.3E-05	–2.14197	–9.7E-05	–3.22319	–5E-05	–16.2452	–0.0007	–4.7509	–0.0001
Injection volume	7.965268	0.00022	4.364622	0.000197	2.783093	4.31E-05	1.191062	5.15E-05	15.12614	0.000329
High (Concentr.)	6		7		8		9		10	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)
Flow rate	–1.33004	–2.4E-05	–3.31986	–7.1E-05	3.56702	5.92E-05	3.083177	8.13E-05	0.913233	5.23E-05
Oven temperature	–0.56428	–1E-05	12.89556	0.000275	–0.03774	–6.3E-07	1.259115	3.32E-05	0.886856	5.08E-05
Organic solvent (%)	13.01058	0.00023	–0.34299	–7.3E-06	10.04881	0.000167	10.41117	0.000275	5.763631	0.00033
Absorbance	–5.06794	–9E-05	–9.95314	–0.00021	–4.7072	–7.8E-05	–2.33608	–6.2E-05	1.784457	0.000102
Injection volume	13.561	0.00024	1.327934	2.84E-05	2.929844	4.87E-05	6.173013	0.000163	4.983639	0.000286
High (Concentr.)	11		12		13					
	Response		Response		Response					
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)				
Flow rate	2.797873	5.6E-05	3.672713	0.000114	22.54561	0.00529				
Oven temperature	–5.86883	–0.00012	–8.67751	–0.00027	–2.81048	–0.00066				
Organic solvent (%)	14.61837	0.000292	13.47224	0.000418	–13.604	–0.00319				
Absorbance	4.376683	8.75E-05	3.622006	0.000112	–7.65237	–0.0018				
Injection volume	6.571126	0.000131	6.56732	0.000204	–7.65237	–0.0018				
Medium (Concentr.)	1		2		3		4		5	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)
Flow rate	–16.7895	–0.00062	–7.423	–0.00045	–11.5154	–0.00024	–11.3666	–0.00065	–9.56236	–0.00028
Oven temperature	12.55169	0.000464	8.888472	0.00054	13.16861	0.000273	–10.7085	–0.00061	8.100103	0.000233
Organic solvent (%)	20.06492	0.000742	13.02407	0.000791	21.76599	0.000451	27.91638	0.001588	22.70072	0.000654
Absorbance	–13.7474	–0.00051	–9.0237	–0.00055	–14.7126	–0.0003	–8.32318	–0.00047	–11.4887	–0.00033
Injection volume	16.87565	0.000624	10.00989	0.000608	14.2168	0.000295	13.31019	0.000757	15.55765	0.000448
Medium (Concentr.)	6		7		8		9		10	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)
Flow rate	–11.24	–0.00027	–13.3749	–0.00039	–8.87856	–0.0002	–19.0888	–0.00066	–5.04385	–0.00039
Oven temperature	6.223198	0.000148	19.01434	0.000554	6.675614	0.00015	18.11813	0.000631	1.585411	0.000123
Organic solvent (%)	23.55326	0.000562	20.63774	0.000601	22.20504	0.000499	11.01176	0.000383	14.34341	0.001116
Absorbance	–14.8214	–0.00035	–15.2762	-0.00044	–12.5879	–0.00028	–10.2839	–0.00036	-5.67281	–0.00044
Injection volume	17.05301	0.000407	16.64517	0.000485	15.37837	0.000345	25.28968	0.000881	9.113805	0.000709
Medium (Concentr.)	**11**		**12**		**13**					
	Response		Response		Response					
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)				
Flow rate	–8.95151	–0.00024	–7.24173	–0.00028	–4.9658	–0.00251				
Oven temperature	2.228139	5.96E-05	0.181249	6.95E-06	9.812812	0.004966				
Organic solvent (%)	24.12923	0.000646	25.81406	0.00099	11.27353	0.005705				
Absorbance	–8.71416	–0.00023	–8.19949	–0.00031	7.678737	0.003886				
Injection volume	15.02624	0.000402	15.20209	0.000583	7.678737	0.003886				
Low (Concentr.)	1		2		3		4		5	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)

Table 3 (continued)

Flow rate	–12.9861	–0.00073	–5.01478	–0.00046	–8.2507	–0.00026	–8.13337	–0.0007	–6.53354	–0.00029
Oven temperature	9.749624	0.000545	5.969793	0.000553	9.476555	0.000297	–5.11397	–0.00044	5.946898	0.00026
Organic solvent (%)	12.73569	0.000712	8.443708	0.000783	14.41786	0.000452	21.88928	0.001875	14.46211	0.000633
Absorbance	–6.68273	–0.00037	–4.20865	–0.00039	–7.14502	–0.00022	–10.9256	–0.00094	–5.80744	–0.00025
Injection volume	10.76237	0.000602	5.587946	0.000518	7.973184	0.00025	8.491591	0.000727	9.409035	0.000412
Low (Concentr.)	6		7		8		9		10	
	Response		Response		Response		Response		Response	
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)
Flow rate	–6.18511	–0.00023	–9.37369	–0.00042	–6.08216	–0.00021	30.40866	0.001492	–3.55072	–0.00042
Oven temperature	5.864135	0.000215	14.08651	0.000625	4.68573	0.000161	50.81104	0.002493	1.420577	0.000168
Organic solvent (%)	13.78246	0.000506	12.97224	0.000575	14.228	0.000488	–23.1497	–0.00114	9.12609	0.001079
Absorbance	–6.4395	–0.00024	–8.18107	-0.00036	–5.90552	–0.0002	30.85286	0.001514	–2.67435	–0.00032
Injection volume	8.835944	0.000324	9.92961	0.000441	8.747892	0.0003	–28.3536	–0.00139	5.234136	0.000619
Low (Concentr.)	11		12		13					
	Response		Response		Response					
Factors	E*x*	E*x* (%)	E*x*	E*x* (%)	E*x*	E*x* (%)				
Flow rate	–6.95358	–0.00028	–10.3092	–0.00067	–1.61884	–0.00126				
Oven temperature	1.653098	6.66E-05	7.573295	0.000495	8.013286	0.006213				
Organic solvent (%)	16.21692	0.000654	9.059472	0.000592	4.144434	0.003213				
Absorbance	–3.83785	–0.00015	–10.6453	–0.0007	5.058086	0.003922				
Injection volume	7.483995	0.000302	3.409843	0.000223	5.058086	0.003922				

propolis to evaluate the recovery method, yielding an extraction efficiency of 80%. The extracted matrix was spiked with the six major compounds (**1**, **3**, **5**, **7**, **8**, and **9**) at three different concentration levels. Recovery percentages for all evaluated compounds ranged from 93.68% to 104.76% across the three levels (Table 2). These compounds were used as representatives for the recovery studies, as they belong to the same class as other compounds present in the extract. Additionally, the recovery of the internal standard ranged from 101 to 103%. Thus, the method can be considered reliable for the extraction of flavonoids from Caatinga green propolis.

Finally, robustness was assessed by making slight variations to five method parameters at three levels (Table 3, Table S1). The RP-HPLC-DAD method showed no significant variations in the concentrations of the analyzed compounds, as changes in flow rate, oven temperature, solvent ratio, absorbance, and injection volume did not significantly impact the chromatographic results (Victor et al. 2023). Therefore, the developed method can be considered reliable for analyzing Caatinga green propolis, *M. tenuiflora* extracts, and their products.

Conclusion

The developed RP-HPLC-DAD method for flavonoid analysis in Brazilian Caatinga green propolis was shown to be accurate and reliable. Thirteen flavonoids—including santin, ermanin, sakuranetin, quercetin-3-methyl ether, viscosine, eriodictyol-5-*O*-methyl ether, isokaempferide, kaempferide, penduletin, quercetagetin-3,6,7-trimethyl ether, cirsimaritin, 3,3'-*O*-dimethylquercetin, and luteolin—were successfully quantified using caffeic acid as the internal standard. This method represents an important tool for researchers and laboratories performing routine analyses of propolis and its products. The studied parameters, such as linearity, precision, accuracy, and robustness—met international guidelines for the development of analytical methods.

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Author Contribution NTS, and VPR: isolation, identification of compounds, analytical method development and writing–original draft; NNL: identification of compounds and writing–original draft; MG: plant identification; JKB: conceptualization, methodology, resources, review editing, project administration and funding acquisition.

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Declarations

Competing Interests There are no competing nor any conflict of interest.

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