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Chemical variability of phenolic compounds in *Phyllanthus niruri*

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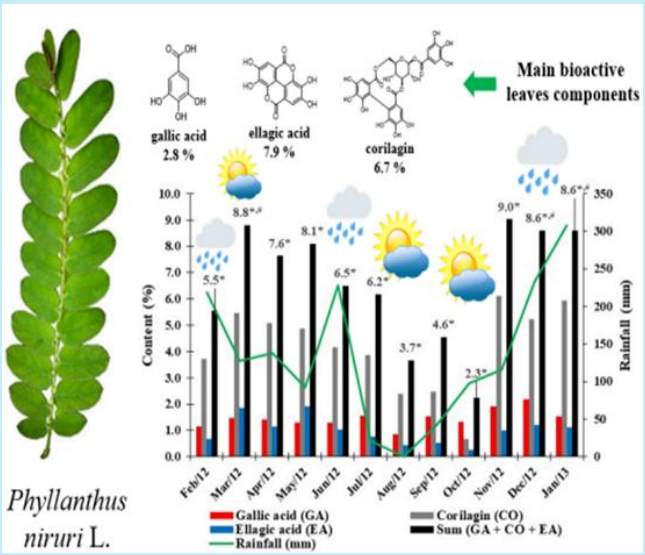
ABSTRACT: *Phyllanthus niruri* L. has been used in folk medicine to treat hepatitis, diabetes, urinary tract disorders, and renal calculi. Here, the chemical differences among its organs and the seasonal chemical variability in the aerial parts were evaluated. The chromatographic profile and gallic acid, corilagin, and ellagic acid contents were determined by ultrahigh performance liquid chromatography with photodiode array ultraviolet (UHPLC-PDA/UV) method, which showed adequate resolution and a short analysis time. The contents of gallic acid, corilagin and ellagic acid in the leaf extract were 2.8, 6.7 and 7.9%, respectively, whilst their contents in the stem and root extracts were lower (< 0.2%). Thus, using the whole plant or aerial parts for herbal medicines can produce different biological responses. The chemical profile of the aerial parts showed only quantitative variation over 12 months. The seasonal content of gallic acid showed no correlation with monthly rainfall, but the contents of corilagin and ellagic acid were positively correlated with rainfall.



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1. Introduction

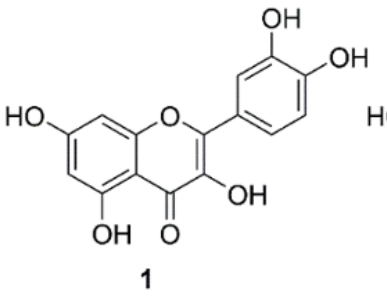
Phyllanthus niruri Linn. (Euphorbiaceae) is an herbaceous plant found in subtropical and tropical areas in Latin America and Asia and known as *quebra-pedra* in Brazil or *chanca-piedra* in Latin America (Klein-Junior *et al*., 2017; Moreira *et al*., 2013). In folk medicine, it is mainly used to treat hepatitis, diabetes, and urinary tract disorders (bladder and kidney), especially for removing kidney stones (Bagalkotkar *et al*., 2006; Santos *et al*., 1995; Wang *et al*., 1995). The traditional use of its aerial parts in urolithiasis treatment and the results of preclinical and clinical trials using its aqueous extract have confirmed its action in preventing the formation of calcium oxalate renal stones. In addition, its extracts also presented antispasmodic, antihyperalgesic, anti-Alzheimer's disease, antioxidant, hepatoprotective, hypoglycemic, anti-inflammatory, antiviral, and antimicrobial activities (Bagalkotkar *et al*., 2006; Barros *et al*., 2006; Moreira *et al*., 2013; Pathania *et al*., 2022; Rajamanickam and Manju, 2022).

Thus, literature data support its therapeutic use as herbal medicine. The Brazilian Health Surveillance Agency (ANVISA, 2021) included aqueous and ethanolic preparations of its dried aerial parts or the whole plant in the *Brazilian Herbal Medicines Form* (2nd edition), indicated for the treatment of urinary disorders.

P. niruri secondary metabolites (Fig. 1) include flavonoids as quercetin (**1**) and quercitrin (**2**), hydrolysable tannins as corilagin (**3**) and geraniin (**4**), phenolic acids as gallic acid (**5**) and ellagic acid (**6**), securinine-type alkaloids as nirurine (**7**) and securinine (**8**), lignans as phyllanthin (**9**) and phyltetralin (**10**), monoterpenes as *p*-cymene (**11**) and limonene (**12**), and triterpenes as lupeol (**13**) and phyllantheol (**14**) (Kumar *et al*., 2017).

Seasonality is one of the essential factors in the variability of secondary plant metabolism since the amount and, sometimes, even the nature of the secondary metabolites are variable during the year, including their bioactivity. The chemical differences among plant organs are also well-established for many species (Gobbo-Neto and Lopes, 2007; Kiazolu *et al*., 2016).

Seasonality, the circadian cycle, organ type, location, environmental factors, temperature, rainfall, soil composition, relative humidity, and processing are essential factors that influence the secondary plant metabolites content, and consequently, their bioactivity (Carvalho *et al*., 2021; Gobbo-Neto and Lopes, 2007; Kiazolu *et al*., 2016). This work aimed to contribute to the standardization of *P. niruri* herbal medicines, through the evaluation of the seasonal dynamics of its phenolic compounds and the comparison of the chemical composition of its different organs, including the quantification of the pharmacopeial chemical markers corilagin (main tannin) and gallic acid (ANVISA, 2021). The chemical analyzes were performed by a developed ultrahigh performance liquid chromatography with photodiode array ultraviolet (UHPLC-PDA/UV) method.



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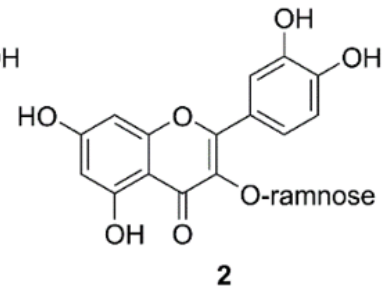

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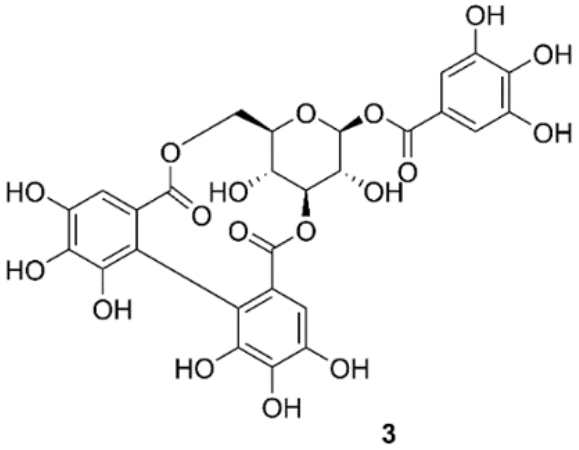

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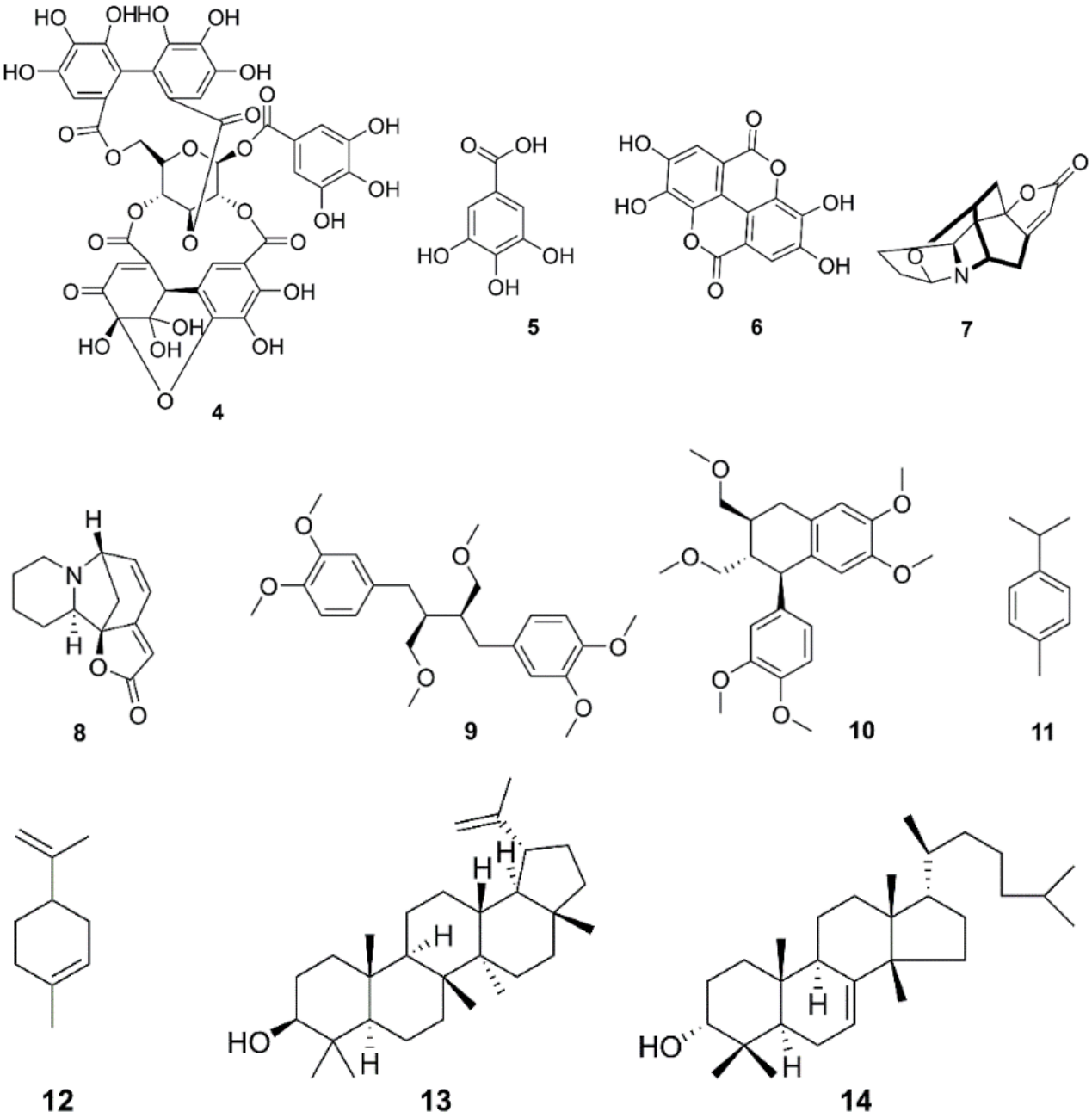

Figure 1. Chemical structure of main components identified in *P. niruri* aerial parts.

2. Experimental

2.1 Reagents and solutions

Ultrapure water (Elga System, Brazil) was used in this work. Acetonitrile and methanol HPLC-grade were purchased from Merck (Germany). Chromatographic standards: gallic acid, corilagin (Chromadex, USA), ellagic acid (Sigma Aldrich, Brazil), and rutin (United States Pharmacopoeia, USA).

2.2 Plant material and extracts

The aerial parts (leaf plus stems) from six specimens of *P. niruri* were harvested monthly between February 2012 and January 2013 at Anidro Extrações do Brasil Ltda, Centroflora Group (Botucatu city, São Paulo state, Brazil, 22°87'33"6 S, 48°48'10"7 W). Leaves, stems, and roots were collected in August 2012 from the same specimens for comparison between the organs. This study was registered in the National System for the Management of Genetic Heritage and Associated

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Traditional Knowledge of Brazil (SisGen) under the code AEFB157. Climatic data were acquired from the Meteorological Institute (INMET, 2013). The plant material was dried in an oven with air circulation at 60 °C for 5 days (Fanem 320 SE, Brazil), and the dried material was powdered in a knife mill (Tecnal TE-650, Brazil).

The dried and powdered *P. niruri* aerial parts (10.0 g) were extracted with deionized water (100.0 mL) at 65 °C for 1 h, in a stainless-steel extractor shaking every 10 min. The extractive solutions were concentrated at 60 °C under reduced pressure (Tecnal, Brazil).

2.3 Ultrahigh performance liquid chromatography with UHPLC-PDA/UV analysis

The leaf, stems, and roots extracts pretreatment (10.0 mg mL^{-1}, methanol: water 50:50) was performed by solid phase extraction using a C18 cartridge (500 mg; 6 mL; Perkin Elmer) and eluting with 4.0 mL of methanol: water 50:50 (high polar interfering compounds), and 5.0 mL of methanol: water 95:05 (sample concentrated in the phenolic compounds). After drying, 1.0 mL of the samples generated (2.0 mg mL^{-1}, methanol: water 60:40) were filtered through 0.22 µm membrane filters (Merck Millipore, Germany) for injection into the chromatographic system. The aerial part (leaf plus stems) extracts for seasonal evaluation (10.0 mg mL^{-1}, methanol: water 60:40) were only filtered through 0.22 µm membrane filters (Merck Millipore, Germany).

Chromatographic analyses were performed on a UPLC H-Class Acquity system (Waters, USA) equipped with a quaternary pump QSM, an FTN autosampler (20 µL-loop), column oven, and PDA detector, controlled with Empower 3 software. The analytical method was developed based on the transfer of HPLC chromatographic parameters (Colombo *et al*., 2009) to UHPLC conditions according to the Acquity UPLC Columns Calculator software. The chromatographic resolution was improved through the experimental mobile and stationary phase modifications. Chromatographic conditions: HSS T3 column (100 × 2.1 mm i.d., 1.8 µm, Waters, USA) at 30 ± 1 °C; segmented linear gradient with acetic acid 0.1% (v/v) (solvent A) and acetonitrile (solvent B), 0–4.80 min 13–37% B, 4.81–5.20 min 37–100% B, 5.21–6.00 min 100% B, 6.01–6.40 min 100–13% B; 6.41–8.00 min 13% B; flow rate of 0.150 mL min^{-1}; samples were kept at 25 ± 1 °C, the pre injection volume was 346 µL and sample injection volume was 1.7 µL; monitoring detection at 267 nm.

Gallic acid, corilagin, and ellagic acid were quantified (triplicate) in the extract by the standard analytical curve using gallic acid (4.0–8.0 µg mL^{-1}), corilagin (15.0–150.0 µg mL^{-1}), and ellagic acid (7.0–63.0µg mL^{-1}) in a UHPLC-PDA/UV. Theoretical limits of detection (LOD) and quantification (LOQ) were determined through the analytical curve equations (ANVISA, 2017). Gallic acid, ellagic acid, and corilagin contents were expressed as percentages (%, w/w) on a dry weight basis.

2.4 Data analysis

The UHPLC-PDA/UV results were presented as average ± standard error. Statistical analyses were performed through statistical software (GraphPad Prism, Version 3, USA) using analysis of variance followed by Tukey's post-test being *p*-values less than 0.05 significant, and Pearson correlation analysis being *p*-values less than 0.05 significant.

3. Results and discussion

Despite the relevance of harvesting medicinal plants (Gobbo-Neto and Lopes, 2007) and the extensive therapeutic use of *P. niruri*, there is only one report on seasonal variations of secondary metabolites in *P. niruri* described in the scientific literature (Couto *et al*., 2013a). Here, the qualitative and quantitative chemical profiles of aqueous extracts from *P. niruri* aerial parts (six specimens) harvested during 12 months were determined. As well as the differences among plant organs.

The extract yield was higher in the aerial parts (15.8%), followed by the stems (6.2%) and roots (4.7%). Higher extract yields (w/w) were observed in February (14.3%), April (13.9%), and June (12.9%), whilst the lowest ones were observed in October, December (6.5%), January (7.5%), and September (8.0%). The extract yield monthly (Fig. 2) average was 10 ± 3%, and, according to the literature data, the aqueous extract yield of *P. niruri* ranged from 1.8 to 26.2% (Asare *et al*., 2011; Giribabu *et al*., 2014; Markom *et al*., 2007). The monthly average temperature and rainfall did not correlate with the extract yield over the 12 months and thus did not influence the extract yield.

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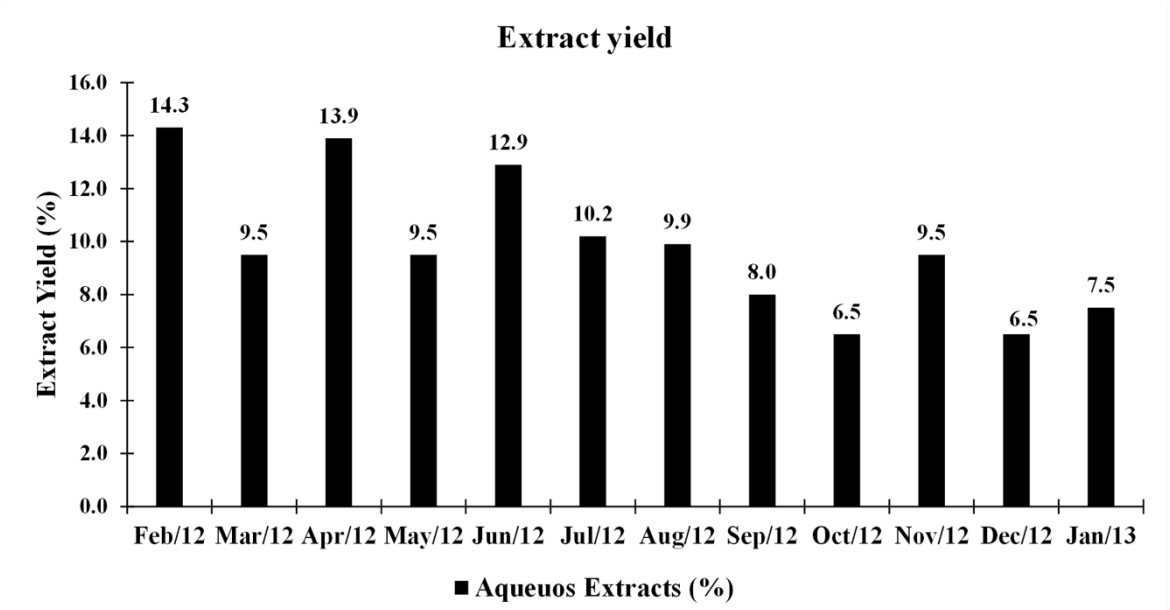

Figure 2. Seasonal variability of the yields (w/w, %) of the aqueous extracts of the aerial parts of *P. niruri* (six specimens mixed).

The phenolic compounds gallic acid, ellagic acid, and corilagin have demonstrated biological activities and may be related to the pharmacological activities of *P. niruri* extracts. According to the 6th edition of the *Brazilian Pharmacopeia* (ANVISA, 2019), gallic acid (minimum content 0.15%) and tannins (minimum content 6.5%) are the analytical markers for quality control of *P. niruri* aerial parts. The developed UHPLC-PDA/UV method showed suitable resolution (Rs > 1.5) for these compounds and others in a short analysis time, 6 min (plus 2 min of conditioning time). Linear regression analysis presented a good fit for gallic acid and corilagin according to r values greater than 0.99 (Table 1). Except for the *r* values of ellagic acid, the other values are in accordance with the minimum required for method validation by the National Health Surveillance Agency (ANVISA, 2017).

Table 1. Data from analytical curves of gallic acid, ellagic acid, and corilagin.

Patterns	Regression equation	*r*	LOD (µg/L)	LOQ (µg/L)
Gallic acid	$y = 3.28 \times 10^4 x + 2.82 \times 10^4$	0.9990	0.459	1.530
Corilagin	$y = 2.03 \times 10^4 x - 1.37 \times 10^4$	0.9996	0.946	3.153
Ellagic acid	$y = 3.35 \times 10^4 x - 1.89 \times 10^5$	0.9813	1.460	4.867

LOD: Limit of detection, LOQ: Limit of quantification, and *r*: Coefficient of correlation.

The UHPLC-PDA/UV analyzes determined the contents of gallic acid, corilagin, and ellagic acid in the leaf's aqueous extract as 2.8 ± 0.1, 6.7 ± 0.3, and 7.9 ± 0.4%, w/w in dry basis (Fig. 3a). The aqueous extract of the stems (Fig. 3b) showed content for gallic acid < 0.10 ± 0.05% and for corilagin 0.1 ± 0.0%; however, ellagic acid was not detected. In the root aqueous extract (Fig. 3c), only corilagin (0.2 ± 0.1%) was detected. The root's chemical profile was previously described as different from other organs (Couto *et al.*, 2013b).

The chemical variability between the organs may produce different pharmacological responses, which is a concern for herbal medicines (Gobbo-Neto and Lopes, 2007; Kiazolu *et al.*, 2016). Considering the contents of the analytical markers corilagin (tannin) and gallic acid, the leaves or aerial parts must be preferred instead of the whole plant to comply with the pharmacopeial specifications (ANVISA, 2021).

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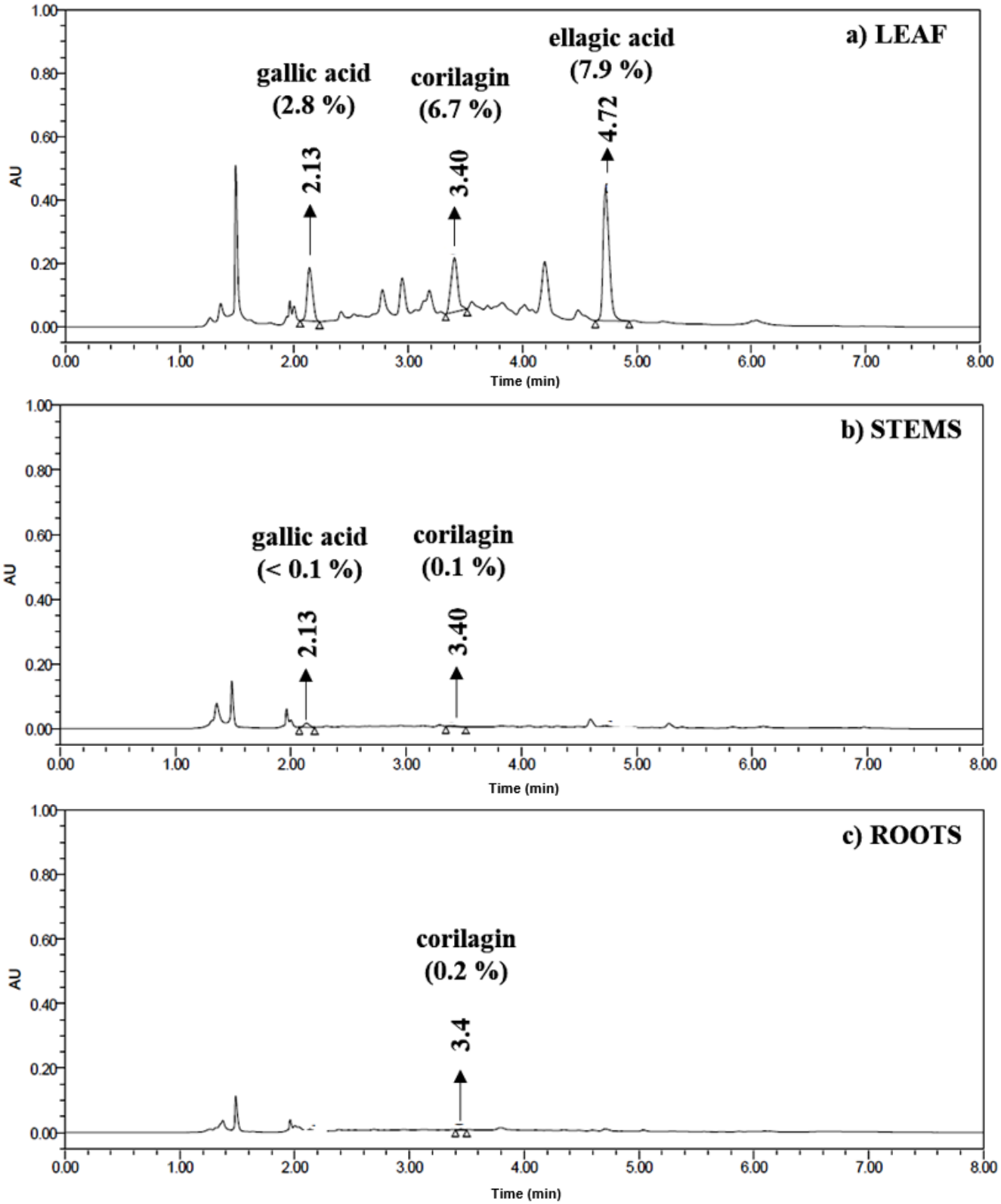

Figure 3. Chromatograms (UHPLC-PDA/UV) of *P. niruri* aqueous extracts: **(a)** leaf, **(b)** stems and **(c)** roots. Gallic acid, corilagin, and ellagic acid contents in the extracts were expressed as w/w (%) in dry basis. Chromatographic conditions: HSS T3 column (100 × 2.1 mm i.d., 1.8 µm) at 30 °C; segmented linear gradient with acetic acid aqueous solution 0.1% (A) and acetonitrile (B), 0–4.80 min 13–37% B, 4.81–5.20 min 37–100% B, 5.21–6.00 min 100% B, 6.01–6.40 min 100–13% B; 6.41–8.00 min 13% B; flow rate 0.150 mL min^{-1}; monitoring detection at 267 nm.

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The UHPLC-PDA/UV chromatograms of the leaves presented identical qualitative profiles (t_R) in the seasonal chemical variability evaluation, except for minor peaks in some months (Fig. 4). For the time course analyzed here, the identified compounds in the extracts were gallic acid, corilagin, rutin, and ellagic acid (t_R: 2.1, 3.3, 4.0, and 4.7 min, respectively). The monthly extract contents of gallic acid, ellagic acid, and corilagin and the associated climatic factors data (monthly average rainfall) are shown in Fig. 5. The lower contents of these three compounds were observed from August to October 2012. Higher ellagic acid and corilagin contents were observed in December, January, and March to June, while the higher gallic acid content was noted in November and December. On the other hand, the gallic acid content was higher in *P. niruri* leaves in the dry season (winter), which was associated with pruning fluence (Couto *et al*., 2013b).

The gallic acid monthly content presented no correlation with the monthly average rainfall through the Pearson correlation (r = –0.3366; p > 0.05). However, the contents of corilagin (r = 0.6077; p < 0.05), ellagic acid (r = 0.7550; p < 0.01), and the sum of these three compounds (SUM; r = 0.7067; p < 0.05) presented a positive correlation with the monthly average rainfall. We verified that corilagin and ellagic acid contents as well as sum showed significative (p > 0.05) decrease from August to October (2.3–4.6%), which may be due to lower rainfall index (< 100 mm), supporting the Pearson correlation and suggesting seasonal variability (Gobbo-Neto and Lopes, 2007; Kiazolu *et al*., 2016). These results were similar to literature data, which reported a positive correlation between tannins/phenolic compounds and the rainy season for the leaves of *Eugenia uniflora* L. (Santos *et al*., 2011). Moreover, the lowest contents of total tannins (condensed tannins) and total phenols in the *Stryphnodendron adstringens* (Mart.) Coville bark collected in Goiânia city, Goiás state (Brazil), were observed for months with the lowest rainfall (< 100 mm), which corroborates this study (Santos *et al*., 2006). The Pearson correlation showed that the contents of gallic acid (r = –0.2871; p > 0.05), corilagin (r = 0.0510; p > 0.05), ellagic acid (r = 0.2212; p > 0.05), and sum (r = 0.1663; p > 0.05) did not demonstrate correlation with the monthly average temperature.

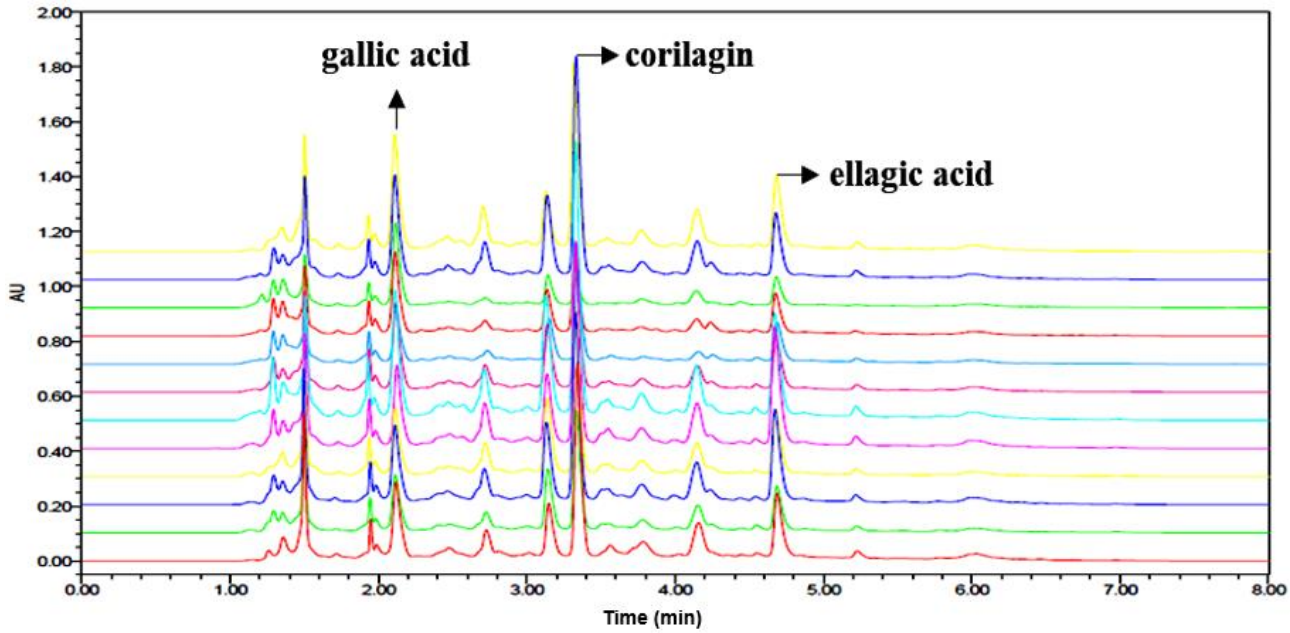

Figure 4. Chromatograms (UHPLC-PDA/UV) of *P. niruri* aqueous extracts of the aerial parts (six specimens) overlaid from February 2012 to January 2013 (t_R: gallic acid = 2.1 min, corilagin = 3.3 min, and ellagic acid = 4.7 min). Gallic acid, corilagin, and ellagic acid contents in the extracts were expressed as w/w (%) in dry basis. Chromatographic conditions: HSS T3 column (100 × 2.1 mm i.d., 1.8 µm) at 30 °C; segmented linear gradient with acetic acid aqueous solution 0.1% (A) and acetonitrile (B), 0–4.80 min 13–37% B, 4.81–5.20 min 37–100% B, 5.21–6.00 min 100% B, 6.01–6.40 min 100–13% B; 6.41–8.00 min 13% B; flow rate 0.150 mL min^{-1}; monitoring detection at 267 nm.

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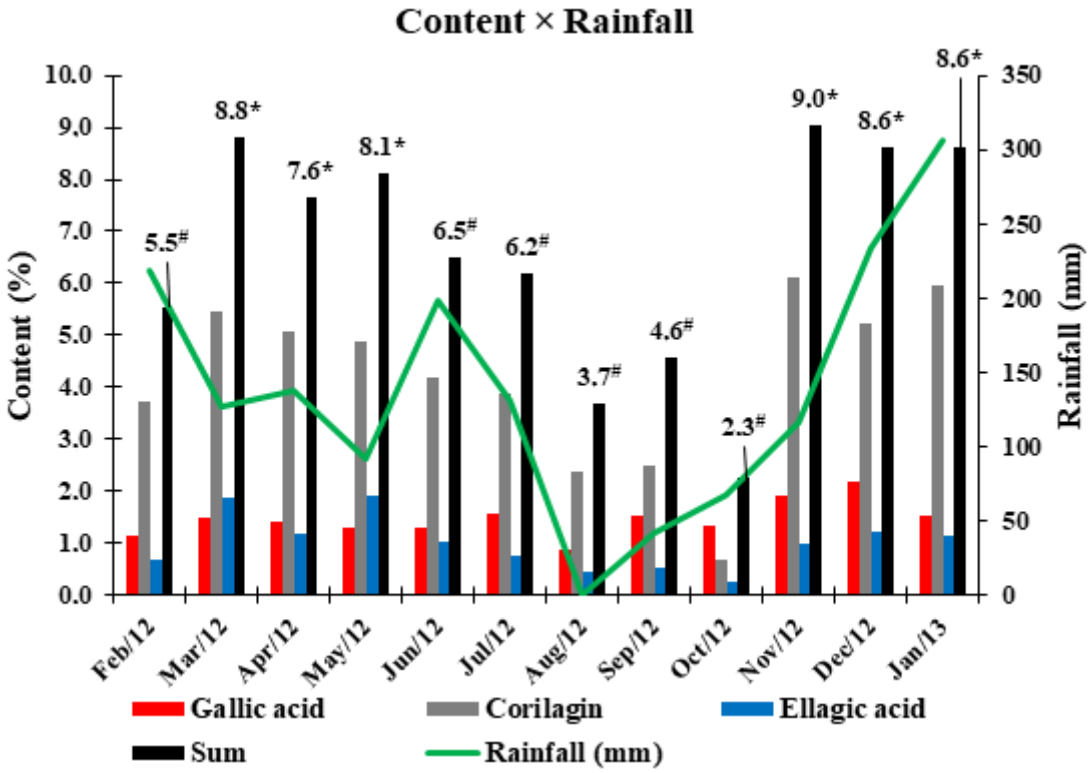

Figure 5. Seasonal variability of gallic acid (**5**), corilagin (**3**), ellagic acid (**6**) contents (w/w,%), and the sum of their contents of the aqueous extracts of the aerial parts of *P. niruri* (six specimens mixed) and climatic factors data - monthly average rainfall (mm). Samples were harvested from February 2012 to January 2013.
*Present statistical significance when compared with other months: $p < 0.05$, and #did not present statistical significance between themselves: $p > 0.05$.

4. Conclusions

The developed UHPLC-PDA/UV method showed adequate resolution and short analysis time, useful for research and quality control, following green chemistry principles. The qualitative and quantitative chemical profiles of the organs (leaves, stems, and roots) of *P. niruri* were different, showing that the use of the leaves, aerial parts (leaves plus stems), or whole plant (aerial parts plus roots) can produce different biological responses. According to the contents of the analytical markers in *P. niruri* leaves, stems, and roots, we suggest that the use of leaves or aerial parts is better than the whole plant, because only the leaves contents comply with the Brazilian pharmacopeial specifications, and the stems and roots have low contents of these compounds, or they were not detected.

The seasonal chemical profile of aerial parts showed only quantitative variation. The higher contents of ellagic acid and corilagin in November to January and March to June may be influenced by the rainy season, whilst gallic acid showed higher contents in November and December without correlation with the rainfall rate.

Authors' contribution

Conceptualization: Santos, A. G.
Data curation: Albrecht, I.; Silva, N. C.; Carvalho, F. A.; Oda, F. B.; Santos, A. G.
Formal Analysis: Carvalho, F. A.; Oda, F. B.; Santos, A. G.
Funding acquisition: Not applicable.
Investigation: Albrecht, I.; Silva, N. C.
Methodology: Albrecht, I.; Silva, N. C.
Project administration: Santos, A. G.
Resources: Albrecht, I.; Silva, N. C.; Santos, A. G.
Software: Albrecht, I.
Supervision: Santos, A. G.
Validation: Albrecht, I.; Oda, F. B.
Visualization: Santos, A. G.
Writing – original draft: Carvalho, F. A.; Oda, F. B.; Santos, A. G.
Writing – review & editing: Carvalho, F. A.; Oda, F. B.; Santos, A. G.

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Data availability statement

Data will be available upon request.

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