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Pharmacognosy
Optimization of phenolic compounds extraction from *Campomanesia lineatifolia* leaves

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Abstract

Campomanesia lineatifolia (gabiroba) is a native edible species found in the Amazon Rainforest. Previous studies have demonstrated the flavonoid nature of ethanolic extract from the *C. lineatifolia* leaves, in addition to gastroprotective activity and TNF inhibition. However, the extraction process used was long and consumed a large amount of solvent. Therefore, the objective of this study was to obtain a bioactive extract rich in phenolics, in an extractive method of simpler, faster, and lower-cost execution. The *C. lineatifolia* leaves were dried and crushed, and the extractions were carried out in different solvents/mixtures (ethanol, methanol, ethyl acetate, and water) under ultrasonic bath (UB), electromagnetic stirring, and continuous reflux extraction (R). The extraction efficiency was evaluated by the flavonoid major compound concentration in the extracts, in a method developed by ultra-high performance liquid chromatography (UHPLC). Injection and pattern matching tests, and UHPLC analyses coupled to ultraviolet spectrometry were conducted to identify catechin and quercitrin. It has been demonstrated that the ethanolic extraction by R and the mixture of ethanol: water (8:2) by UB represented optimized methods in obtaining the flavonoid compounds identified. Thus, the results may contribute to chemical-biological extract standardization for gastric antiulcer activity evaluation.
Key words: *Campomanesia lineatifolia*, extraction, flavonoids, optimization, UHPLC.

Resumo

Campomanesia lineatifolia Ruiz & Pavón (gabiroba) é uma espécie nativa comestível encontrada na Floresta Tropical Amazônica. Estudos prévios têm demonstrado a natureza flavonoídica do extrato etanólico das folhas de C. lineatifolia, além de atividade gastroprotetora e inibição do TNF. No entanto, o processo de extração utilizado foi longo e consumiu uma grande quantidade de solvente. Portanto, o objetivo deste estudo foi obter um extrato bioativo rico em fenólicos, através de um método extrativo simples, rápido e de baixo custo de execução. As folhas de C. lineatifolia foram secas e trituradas e as extrações conduzidas em diferentes solventes / misturas (etanol, metanol, acetato de etila e água) sob banho ultrassônico, agitação eletromagnética e sistema contínuo de refluxo. A eficiência da extração foi avaliada pela concentração do componente flavonoídico majoritário nos extratos, em um método desenvolvido por cromatografia líquida de ultra eficiência. Ensaios de injeção e coinjeção com padrões autênticos, análises em cromatografia líquida de ultra eficiência acoplada à espectrometria ultravioleta foram conduzidos para identificar a presença de catequina e quercitrina. Demonstrou-se que a extração etanólica sob sistema contínuo de refluxo e a mistura de etanol:água (8:2) sob banho ultrassônico representaram métodos otimizados na obtenção dos componentes flavonoídicos identificados. Assim, os resultados obtidos podem contribuir para a padronização químico-biológica do extrato para a avaliação da atividade antiúlcera gástrica.
Palavras-chave: *Campomanesia lineatifolia*, extração, flavonoides, otimização, UHPLC

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Introduction

Campomanesia lineatifolia Ruiz & Pavón (Myrtaceae) originates from the western region of Amazonia and its geographic distribution comprises Amazonas (Brazilian state), eastern Colombia and Peru, and part of Amazonian Bolivia as well as non-flooded regions with hot and humid climate and clayey soils slightly drained, and may also be found in the inter-Andean valleys. It is a tree species that can reach 8 to 10 meters in height (D'eeckenbrugge & Ferla 2000).

It is known by several common names, such as "gabiroba, gaviroba, champa, palillo, guayaba de leche or guayaba de mono". The name "gabiroba", in Guarani, means "tree with acid bark." The fruits are edible and much appreciated, widely used to make sweets, juices, and liqueurs (Villachica *et al.* 1996; D'eeckenbrugge & Ferla 2000).

Barbosa (2009) revealed the flavonoid nature of the chemical constituents in the ethanolic extract of *C. lineatifolia* leaves obtained by percolation. This extract was subsequently subjected to successive partitions with hexane, dichloromethane, ethyl acetate, and n-butanol, obtaining flavonoid-enriched fractions with the ethyl acetate solvent. The extracts and fractions obtained showed a high content of phenols, such as flavonoids and tannins, in addition to high antioxidant activity, suggesting their ethnopharmacological relevance for antidiarrheal, gastric antiulcer, and cicatrizant activity. Also in this study, two substances were isolated and identified by spectroscopic techniques, catechin (tannin monomer) and quercitrin (flavonol). The same ethanolic extract and the ethyl acetate fraction of *C. lineatifolia* protected the gastric mucosa of rats against gastric lesions induced by ethanol and indomethacin (Madalosso *et al.* 2012). In a study of Brazilian medicinal plants in lipopolysaccharide-(LPS-) stimulated THP-1 cells, Henriques *et al.* (2016) demonstrated the *in vitro* tumor necrosis factor (TNF) inhibitory activity of *C. lineatifolia*. It is important to emphasize that, in order to obtain extracts and fractions, the extractive method was long (over 48 hours) and involved the consumption of a large amount of solvents (approximately 2 L) (Madalosso *et al.* 2012; Barbosa 2009). Therefore, the objective of this study was to optimize the extraction of phenolic-rich *C. lineatifolia* leaf extracts, developing simpler, faster, and less costly extraction planning with respect to the amount of solvents and/or solvent mixtures used in the process.

Material and Methods

Plant material

Campomanesia lineatifolia leaves were collected in February 2017, in the state of Minas Gerais, Brazil (19°52'9.87"S, 43°58'12.04"W). The species was identified by Dr. Marcos Sobral from the Botany Department of Instituto de Ciências Biológicas at Universidade Federal de Minas Gerais (UFMG), Belo Horizonte. A voucher specimen (no. BHCB 150.606) was deposited at the UFMG Herbarium. The registration in the National System of Genetic Heritage and Associated Traditional Knowledge Management (SisGen) was carried out and has the code A216C7C.

Extraction

The *Campomanesia lineatifolia* leaves were dried at 40 °C/72 h with forced air circulation. The powdered dry leaves (particle size 0.5 mm) were extracted by different methods in different solvents or mixtures thereof, as described in Table 1. All the tests were performed in triplicate.

5.0 g of dried plant material was extracted using 100 mL of ethanol 96° (EtOH) or extractive solvent mixtures (EtOH:H_2O 8:2, v/v and EtOH: EtOAc 1:1, v/v) by liquid-solid maceration under ultrasonic bath at 27 °C ± 4 °C for 20 min (Ultronique, model Q1.8/40A). The supernatant was centrifuged at 1,400 g (Edutec Centrifuge, model EEQ-9004/B) for 5 min and then filtered. Evaporation of the solvent under vacuum was made in a rotary evaporator (T = 60 °C) (Buchi rotary evaporator, model R-114 and waterbath B-480) or lyophilized (Liotop Liophilizer, model L101) in order to obtain the dry crude extracts.

In the liquid-solid maceration under electromagnetic stirring and in the continuous reflux extraction system, the same extraction conditions, vegetal material proportion, and solvent extractor type were used. In the first method, the material was subjected to extraction by maceration under electromagnetic stirring on a hot plate at 45–55 °C. In the second method, the solution was subjected to reflux method (ball capacitor) under heating (T = 100 ± 5 °C) for 3 cycles of 20 min. The solutions were centrifuged, filtered, and dried in a rotary evaporator and lyophilizer, as described.

Chromatographic analysis by UHPLC

In order to evaluate the efficiency of the extractive method as a function of solvents and extractive solvent mixtures, the flavonoid main

Table 1 – Experimental planning for evaluation of different extractive methods and solvents/solvent mixture, in the extraction of enriched extracts in phenolics from *Campomanesia lineatifolia* leaves.

Test	Solvents / Solvent Mixture	Agitation / Temperature (°C)
1	EtOH	Ultrasonic bath (UB) / 27 °C ± 4 °C
2	EtOH	Electromagnetic stirring (ES) / 50 ± 2 °C
3	EtOH	Continuous reflux extraction (R) / 100 ± 5 °C
4	EtOH:H_2O (8:2)	Ultrasonic bath (UB) / 27 °C ± 4 °C
5	EtOH:H_2O (8:2)	Electromagnetic stirring (ES) / 50 ±2 °C
6	EtOH:H_2O (8:2)	Continuous reflux extraction (R) / 100 ± 5 °C
7	EtOH:AcOEt (1:1)	Ultrasonic bath (UB) / 27 °C ± 4 °C
8	EtOH:AcOEt (1:1)	Electromagnetic stirring (ES) / 50 ±2 °C
9	EtOH:AcOEt (1:1)	Continuous reflux extraction (R) / 100 ± 5 °C

EtOH = ethanol 96°; H_2O = water; AcOEt = ethyl acetate; UB = liquid-solid maceration under ultrasonic bath; ES = liquid-solid maceration under electromagnetic stirring; R = continuous reflux extraction.

compound concentrations in the extracts were analyzed by UHPLC-UV-DAD. The extract solutions obtained from *C. lineatifolia* vegetal drug were prepared in 5 mg/mL concentration, as described next. 5.0 mg of the extracts were weighed into plastic microtubes and 1.0 mL of methanol analytical grade (Dinâmica - Química Contemporânea Ltda) was added. Dissolution was performed under ultrasonic bath for 20 min. Then, the solutions were centrifuged at 10,000 g for 10 min (Cientec Centrifuge, model CT-5000R). The supernatant was filtrated through a Millex (Millipore, Bedford, MA, USA) LCR (pore size, 0.45 lm) polytetrafluoroethylene membrane and transferred into 2.0 ml vials.

Analyses were performed on a Waters UPLC Acquity System® (Milford, MA, USA) equipped with a quaternary pump, autosampler, photodiode array detector, and Empower software for data processing. An Acquity UPLC® BEH C18 column (100 × 2.1 mm · 1.7 µm i.d.), and pre-column VanGuard ™ C18 (2.1 × 5 mm · 1.7 µm i.d.) was used at a temperature of 40 °C, flow rate of mobile phase 0.3 mL/min, and injection volume 2.0 µL. Ultraviolet (UV)-photodiode array detection was performed at λ 270 nm. UV spectra from λ 200 to 600 nm were recorded on-line for peak identification. Mobile phase consisted of two solvents: (A) 0.1% formic acid in ultrapure water (Millipore Direct-Q Water Purifier), and (B) 0.1% formic acid in acetonitrile (LS Chemicals), and the following gradient program was performed: 0 min, 95% A–5% B; 1 min, 92% A–8% B; 22 min, 78% A–22% B; 25 min, 5% A–95% B; 27 min, 5% A–95% B; 30 min, 95% A–5% B.

Identification of major flavonoid compounds by UHPLC-UV-DAD and injection and coinjection assays with authentic standards.

The catechin and quercitrin (≥ 98%, Sigma Aldrich) standards were individually injected into UHPLC-UV-DAD, and prepared in analytical-grade methanol at a concentration of 1.0 mg/mL, as described below. 1.0 mg of the standards were weighed in plastic microtubes and 1.0 mL of analytical-grade methanol was added. Dissolution was performed under ultrasonic bath for 20 min. Then, the solutions were centrifuged at 10,000 *g* for 10 min and the supernatant was transferred to vials. The chromatographic profiles obtained from the standards were compared to that of the EtOH R extract, as a function of their UV spectra obtained online and respective retention times.

Coinjection assays were performed by UHPLC-UV-DAD. A solution of 5.0 mg/mL EtOH extract (prepared as described in the section "Chromatographic analysis") fortified with 100 µL of standard quercitrin solution (1.0 mg/mL) was analyzed. Chromatographic conditions described previously were employed. The injection volume was 2.0 µL, and the extract solubilization was made in analytical-grade methanol.

Statistical analysis

Statistical analyses were performed using the Graph Prism version 6.0 program. All results were expressed as mean ± standard error values. For the comparison of more than two groups and the relationships of independent factors, One-way ANOVA or Two-way ANOVA tests were used, respectively, followed by Tukey's post-test for multiple comparisons of small samples. The choice of these tests was performed according to the data relative to descriptive statistics and $p < 0.05$ was considered significant.

Results and Discussion

Flavonoids are found in plants, especially in their glycosylated form, being the most common sugars *O*-glycosylated, and *C*-glycosylated, D-glucose, and L-rhamnosis (Hermann 1988; Erlund 2004). The effect of glycosylation makes the flavonoids have a greater solubility in water and, therefore, aqueous and hydroalcoholic solutions can be considered more suitable for the extraction of these compounds. Less polar flavonoid aglycones, such as isoflavones, flavanones, flavones, and flavonols, can be extracted using low to medium polar solvents such as chloroform, dichloromethane, ethyl ether, or ethyl acetate (Andersen & Markham 2006).

For a preliminary analysis to obtain enriched flavonoid extracts, different solvents (ethanol, methanol, ethyl acetate, and water) and solvent mixtures (ethanol: water in the proportions 8:2, 7:3, 1:1, 3:7, 2:8, v/v), as well as liquid-liquid (dichloromethane followed by ethyl acetate) and solid-liquid (ethyl acetate) partitions of *C. lineatifolia*, were evaluated by HPLC. The results showed that EtOH and EtOH:H_2O extracts (8:2 and 7:3, v/v) obtained by ultrasonic bath maceration, and the ethyl acetate fraction defatted with dichloromethane followed by ethyl acetate obtained by liquid-liquid partition presented efficiency in extracting phenolic compounds, previously identified by Barbosa (2009), catechin and quercitrin (supplementary material, available at <https://doi.org/10.6084/m9.figshare.12252368.v1). It is important to note that although the preliminary tests demonstrated the efficiency described by the US extraction method, no solvent or solvent mixture was able to optimize the obtainment of all selected flavonoid compounds to evaluate the extraction efficiency. Even though the ethyl acetate fraction showed efficiency in the extraction of these substances, the method used in the partition was long and laborious, and with higher consumption of solvents, approximately 2 L (Barbosa 2009).

The EtOH solvent and EtOH:H_2O (8:2) solvent mixture were maintained for the new extractive methods proposed in the experimental design (Tab. 1), in view of the efficiency demonstrated in obtaining rich extracts in phenolics (previously evaluated by HPLC and UHPLC), as well as good solubility of the phenolic compounds in these solvents (Daneshfar *et al.* 2008; Barbosa 2009; Vuong *et al.* 2011; Madalosso *et al.* 2012; Cuevas-Valenzuela *et al.* 2014). As there was no statistical difference between the EtOH: H_2O (8:2) and (7:3) mixtures, the latter was not included in the planning, since it is more difficult to remove the water that is in the highest proportion in this extract. In addition, a new solvent mixture was included in the planning, ethanol:ethyl acetate (1:1) [EtOH:EtOAc (1:1)], considering that the ethyl acetate solvent has demonstrated efficiency in phenolic compound extraction, as well as the fact that the ethyl acetate fraction has shown a good result (Barbosa 2009; Daneshfar *et al.* 2008; Vuong *et al.* 2011; Ferreira *et al.* 2013).

The extractive methods included in this study were selected to develop sustainable strategies to use less labor intensive, less solvent, as well as the use of safer solvents (which include ethanol) than that previously used by our research group (Barbosa 2009). The inclusion of ethyl acetate solvent is due to the fact that ethyl acetate fraction presented gastroprotective activity in models *in vivo*, also tested by our research group (Barbosa 2009; Madalosso *et al.* 2012; Machado *et al.* 2017). These *in vivo* assays will be conducted with the optimized extract.

According to the literature, several extractive methods have been proposed to obtain enriched phenolic extracts. Machado *et al.* (2017) developed and optimized an extractive method to obtain good yields of phenolic compounds in guaraná extracts, employed liquid-solid maceration using a solvent mixture of ethanol: water (8:2, v/v) with diluted acid (H_3PO_4 0.1% in water, v/v) under electromagnetic stirring, temperature between 75–78 °C, three successive extractions in 10 min each. Other studies have focused on the phenolic compound extraction using solvents that are accepted by the pharmaceutical and food industries, *e.g.* water and ethanol, in combination with assisted extraction techniques such as pressurized liquid

extraction, microwave extraction, and extraction by ultrasound (Duba *et al.* 2015; Xu *et al.* 2015; Bodoira *et al.* 2017a, b; Caleja *et al.* 2017), besides the high temperatures used to improve the phenolic constituent solubility of the extract, increasing the extractive process efficiency (Daneshfar *et al.* 2008; Srinivas *et al.* 2010; Cuevas-Valenzuela *et al.* 2014; Bodoira *et al.* 2019).

To analyze the results of different extractive methods and solvents or solvent mixtures used,

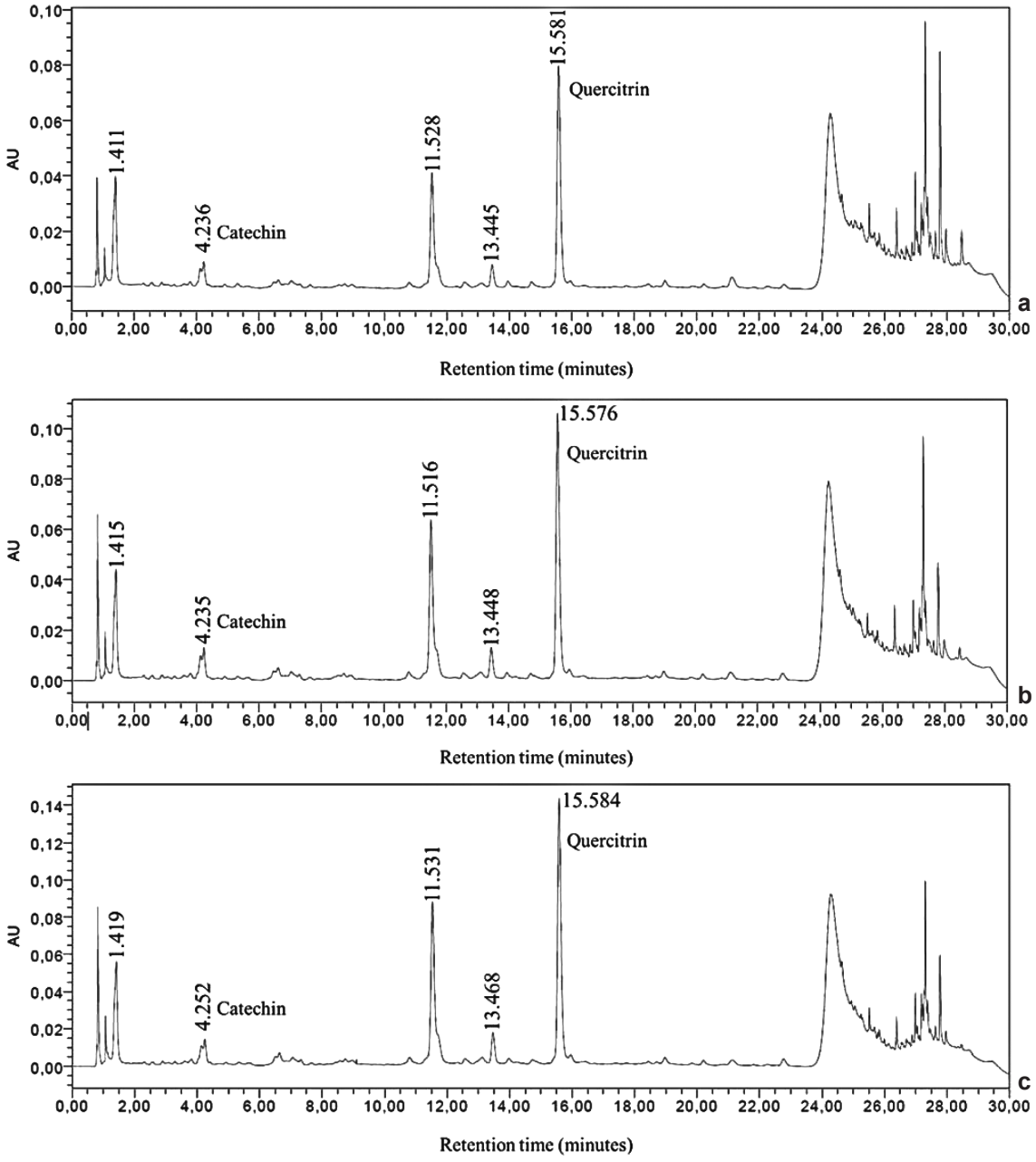

Figure 1 – a-c. Chromatographic profile obtained by UHPLC-UV- DAD, in λ = 270 nm, for EtOH *C. lineatifolia* extract, in different extractive methods – a. extract EtOH UB; b. extract EtOH ES; c. extract EtOH R. Legend: UHPLC-UV- DAD = ultra-high performance liquid chromatography coupled to ultraviolet-photodiode array detector; EtOH = ethanol; UB = maceration under ultrasonic bath; ES = maceration under electromagnetic stirring; R = extraction in continuous reflux system.

chromatographic profiles were obtained. In these chromatograms, five substances with a flavonoid profile, called peaks 1 to 5 (Fig. 1), were selected.

It was demonstrated, from the chromatogram (Fig. 1), that the five selected peaks presented close retention times for all extracts of 1.4 (peak 1), 4.2 (peak 2), 11.5 (peak 3) , 13.4 (peak 4), and 15.5 (peak 5) minutes. The UV spectra obtained on-line for these peaks indicated absorption at two similar wavelengths, around 250–280 nm and 340–350 nm, compatible with the Bands II and I characteristics of flavonoids respectively (Mabry *et al.* 1970). The 2 (peak 2) and 5 (peak 5) compounds showed the same wavelength absorption of Bands II and I, compared to the spectra obtained by Barbosa (2009), catechin and quercitrin, respectively. Injection assays of the catechin standard, and injection and coinjection of the quercitrin standard confirmed the presence of these compounds in *C. lineatifolia* leaf extracts.

The respective peak areas were compared for the different solvent/solvent mixture and extractive methods tested. When analyzing the relationship between solvent variables and solvent mixtures, no statistical difference was observed in the extractive profile between peaks 1, 2, and 4 (Fig. 2). Peaks 3 and 5 have already shown statistical difference in different extraction solvents and methods.

For the extraction of compound 3 (peak 3), the reflux method using ethanol (EtOH R) was statistically different from the UB EtOH systems; EtOH ES; EtOH:EtOAc (1:1) UB, R, and ES, but without statistical difference when EtOH:H_2O solvents (8:2) were used for all methods (Fig. 3a).

For the extraction of compound 5 (peak 5), the maceration UB method using solvent mixture EtOH:H_2O (8:2) was statistically different in relation to the EtOH:H_2O systems (8:2) ES and R; EtOH UB and ES; and EtOH:EtOAc (1:1) UB, ES, and R, but without statistical difference when the solvent ethanol was used, in the reflux system (EtOH R), according to Figure 3b.

Thus, extractions by EtOH R and EtOH: H_2O (8:2) UB represented optimized methods to obtain the selected major flavonoid compounds, peaks of 1 to 5 in *C. lineatifolia* leaves. The extractive methods then optimized by the solid-liquid extraction technique represent an extraction commonly used in vegetal materials, using liquid solvents, due to their wide applicability, efficiency, and ease of use (Sharma & Gupta 2015; Safdar *et al.* 2017). In general, the technique involves the drying and milling of plant material and the choice of a solvent and a suitable procedure for the compounds of interest extraction (Sharma & Gupta 2015), including here the optimized techniques observed, ultrasonic bath maceration and continuous reflux system. Albu *et al.* (2004) used ultrasound to extract phenolic compounds from rosemary and demonstrated that the operating time was decreased using this extractive method. The thermal effects of ultrasound occur when ultrasonic waves are converted to heat and absorbed by the plant tissue, while the mechanical effects cause acoustic cavitation, causing a bubble to grow resulting in cellular rupture, with consequent improvement of the solvent penetration in plant material and intracellular content release by cell wall rupture. In

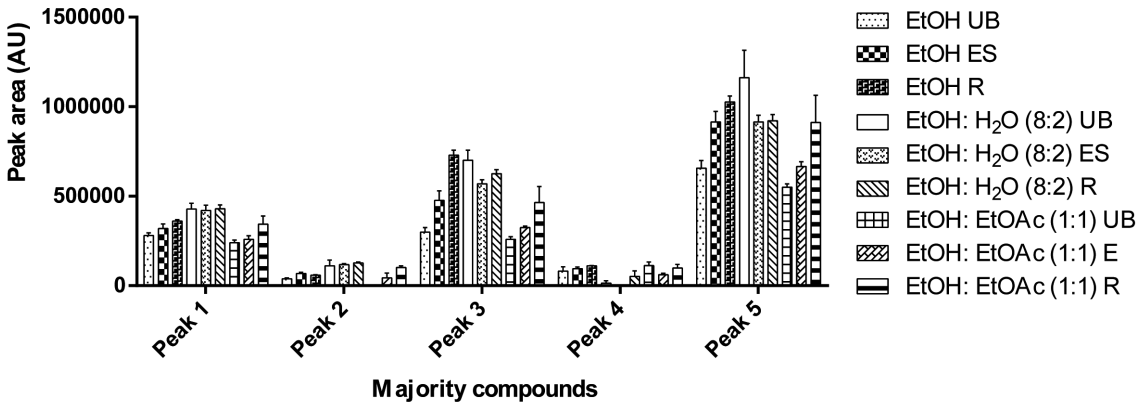

Figure 2 – Peak areas of the major compounds (peaks 1 to 5) of *C. lineatifolia* extracts obtained by UHPLC-UV-DAD in different extractive methods and solvents. Each bar represents the mean ± SEM, Two-way ANOVA, with Tukey's post-test comparison (n = 3), $p < 0.05$. Legend: EtOH = ethanol; H_2O = water; EtOAc = ethyl acetate; UB = maceration under ultrasonic bath; ES = maceration under electromagnetic stirring; R = extraction in continuous reflux system.

addition, the bioactive compound extraction under ultrasonic irradiation offers high reproducibility in short periods of time, simplified handling, reduced solvent consumption, use of lower temperatures, and lower energy consumption (Khan *et al.* 2010).

In the reflux extraction technique, a higher temperature (100 ± 5 °C) was used in a continuous maceration extraction system to allow solvent reflux. The temperature of the extractive process is another parameter that must be taken into account, since the continuous reflux system was presented as an efficient method in the extraction with pure solvent. Several studies have shown that increasing temperatures improve the solubility of the phenolic compounds in extractive solvents and optimizes the extraction of these compounds from plant raw materials (Daneshfar *et al.* 2008; Srinivas *et al.* 2010; Cuevas-Valenzuela *et al.* 2014; Bodoira *et al.* 2019), which may justify the results obtained in this study.

Organic polar solvents, such as ethanol, and hydroalcoholic mixtures are cheaper options and have lower toxicity, as well as a large capacity for dissolution and extraction of various plant constituents (Xu *et al.* 2017). In addition, it is important to highlight that safer solvents (here included water and ethanol) are being prioritized in routine laboratory analyses to establish the chromatographic fingerprints of numerous plant samples. This approach is particularly useful for the medicinal plant analysis due to the program created by the Brazil's Ministry of Health to enable the use of herbal medicines by the Brazilian Unified National Health System (SUS) (Brasil 2009; Funari *et al*. 2014) and to comply with the National Sanitary Vigilance Agency (ANVISA) regulations for the safe use of herbal medicines.

Conclusions

Our results demonstrated the influence of isolated polar solvents (EtOH) and hydroalcoholic mixture (EtOH:H_2O 8:2, v/v) on the flavonoid extraction, relating the solubility profile of these compounds in polar solvents. The extractive methods by maceration under ultrasonic bath and by continuous reflux system proved to be effective methods to obtain *C. lineatifolia* flavonoid-enriched extracts. Injection and coinjection assays with authentic standards allowed confirming the presence of catechin (flavan-3-ol) and quercitrin (flavonol), which may contribute to the chemical-biological validation of *C. lineatifolia* leaf ethanolic extract, in relation to its gastric antiulcer activity.

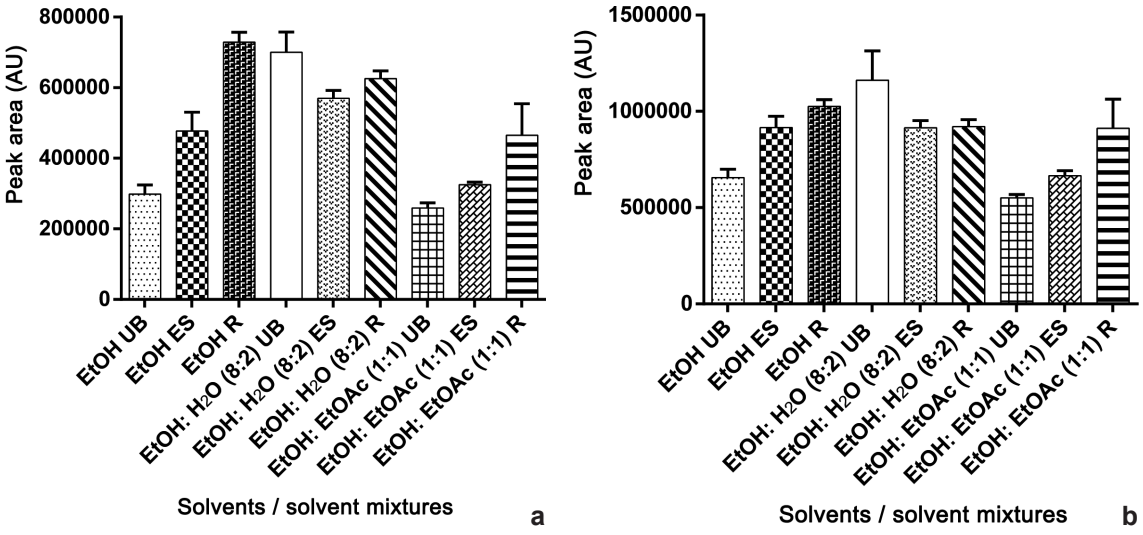

Figure 3 – Evaluation of extractive methods of *C. lineatifolia* extracts, according to chromatogram area obtained by UHPLC-UV-DAD, for compound 3 (a) e 5 (b). Legend: EtOH = ethanol; H2O = water; EtOAc = ethyl acetate; UB = maceration under ultrasonic bath; ES = maceration under electromagnetic stirring; R = extraction in continuous reflux system. Test: Two-way ANOVA, followed by Tukey's post-test (n = 3). Each bar represents the mean ± SEM, and a, b, c represent means that differ from each other ($p < 0.05$).

In current times, where there is a growing concern about developing sustainable strategies, the use of less labor intensive, less solvent extraction methods, as well as the use of safer solvents, has become a practical and sustainable choice for driving future studies to evaluate the biological activity of *C. lineatifolia* extracts.

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