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Influence of extraction method and solvent polarity on flavonoid content and antioxidant activity of three Cerrado species

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This study investigated how extraction method and green solvent polarity influence flavonoid content and antioxidant activity in leaf extracts of *Anacardium humile*, *Campomanesia adamantium*, and *Pterodon emarginatus*, native species of the Brazilian Cerrado. Maceration and decoction were performed using ethanol-water mixtures (58 and 77%, v/v), and extracts were evaluated for total flavonoid content and antioxidant activity by aluminum chloride and DPPH assays, respectively. Physicochemical parameters (pH 4.7–6.0; density 0.87–0.94 g/mL; dry residue 1.4–4.4%) showed low variability across species and extraction conditions. Maceration, particularly with 77% ethanol, consistently yielded higher flavonoid contents than decoction, highlighting the dominant role of solvent polarity in flavonoid extraction. *P. emarginatus* exhibited the highest total flavonoid content (4.56 ± 0.17 mg RE/g); whereas *C. adamantium* showed the strongest antioxidant activity, with the lowest DPPH EC₅₀ value (6.89 ± 0.43 mg/mL). Notably, no direct correlation was observed between flavonoid concentration and antioxidant capacity, indicating that antioxidant performance depends primarily on the qualitative chemical profile of extracts rather than flavonoid abundance. Solvent polarity exerted a stronger influence than extraction method on flavonoid extraction, whereas antioxidant activity was driven by compositional complexity. The findings provide a rational basis for optimizing extraction strategies for Cerrado plant species.

Key words: *Anacardium humile*, *Campomanesia adamantium*, *Pterodon emarginatus*, maceration and decoction, flavonoids content, antioxidant activity.

INTRODUCTION

The Cerrado, the second largest biome in Brazil, covers approximately 23.3% of the national territory (Instituto

Brasileiro de Geografia e Estatística [IBGE], 2019) and is recognized as a global biodiversity hotspot due to its

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exceptional species richness and the severe habitat loss caused by agricultural expansion and environmental fragmentation (Claro and Silingardi, 2019; Lima et al., 2021; Neto et al., 2020). The flora of the Cerrado exhibits remarkable adaptive capacity to a wide range of abiotic stresses, including high temperatures, water deficit, intense solar radiation, and recurrent fires. These conditions collectively stimulate the biosynthesis of secondary metabolites, particularly phenolic compounds, which are known for their strong antioxidant properties (Arruda et al., 2022).

Among secondary metabolites, phenolic compounds—especially flavonoids—stand out for their well-established antioxidant capacity. These compounds play a key role in neutralizing reactive oxygen species, primarily by acting as hydrogen donors and radical scavengers (Safe et al., 2021; Tumilaar et al., 2024; Hassanpour and Doroudi, 2023). Scientific interest in medicinal plants rich in phenolic compounds, particularly flavonoids, has grown substantially due to their potential in preventing oxidative damage associated with inflammatory, metabolic, and degenerative diseases (Bailão et al., 2015; Cosme et al., 2020). Among the native species traditionally used in the Cerrado, *Anacardium humile* (cajueiro-do-cerrado), *Campomanesia adamantium* (gabirobeira), and *Pterodon emarginatus* (sucupira) stand out, with several reports describing biological activities from their leaf extracts related to the presence of phenolics and flavonoids (Negri et al., 2014; Lima Júnior et al., 2021; Rocha et al., 2024).

The extraction step is critical in determining both the yield and chemical profile of plant extracts, as it significantly influences the levels of bioactive compounds and the resulting antioxidant capacity (Oliveira et al., 2016a). Factors such as the extraction method and solvent polarity affect the solubilization and stability of phenolic compounds. Consequently, techniques like maceration, decoction, and ultrasound-assisted extraction are frequently compared in the literature (Zhang et al., 2015; Oliveira et al., 2016b; López et al., 2017; Ziani et al., 2019; Haideri et al., 2024).

Conventional methods for extracting phenolic antioxidants from plant parts, including leaves and seeds, often rely on solid-liquid extraction using organic solvents such as methanol, acetone, hexane, and ethanol. These solvents pose significant environmental and toxicological risks to human health (Altemimi et al., 2017; Ameer et al., 2017; Alara et al., 2021). Consequently, there has been a growing emphasis on replacing these hazardous agents with safer and more sustainable alternatives (Pateiro et al., 2021). In this context, aqueous ethanol solutions have emerged as a promising “green” solvent system. Ethanol, classified as GRAS (Generally Recognized as Safe), is suitable for applications in food, pharmaceutical, and cosmetic formulations (Bertan et al., 2025). Moreover, ethanol-water mixtures offer enhanced solvation properties, and the modulation of solvent polarity often results in higher extraction efficiencies for phenolic

compounds compared to pure organic solvents (Pandit and Saha, 2025).

In this context, the present study aimed to evaluate the influence of two extraction processes, maceration and decoction, using green hydroethanolic solvents (58 and 77% ethanol), on the total flavonoid content and DPPH antioxidant activity, in leaf extracts of *C. adamantium*, *A. humile*, and *P. emarginatus*. Comparative studies assessing conventional extraction methods under standardized conditions for Cerrado species remain limited. To date, no study has systematically compared maceration and decoction using ethanol-water mixtures to evaluate their effects on flavonoid content and associated antioxidant activity in these species. This study establishes a framework for selecting extraction conditions—specifically, the method (maceration or decoction) and ethanol concentration (55% and 77%)—based on their impact on the physicochemical properties of the extracts and total phenolic content. These findings are particularly relevant given the growing interest in natural antioxidants for use in phytotherapeutic preparations, as well as in the development of functional foods, beverages, and other plant-based products.

MATERIALS AND METHODS

Plant

Leaves of *A. humile* and *P. emarginatus* were collected in the municipality of Pirenópolis, Goiás, Brazil, at the geographic coordinates 16°07'01.3" S, 48°55'55.3" W and 16°08'04.2" S, 49°00'27.0" W, respectively. Leaves of *C. adamantium* were collected in the municipality of Abadiânia, Goiás, at 16°06'59.3" S and 48°55'39.0" W. All specimens were collected in June 2024 and identified by Professor Dr. Josana de Castro Peixoto. Voucher specimens were deposited in the Herbarium of the State University of Goiás (HUEG), with their respective numbers listed in Table 1.

The collected leaves were dried in a forced-air circulation oven (SOLAB, model SL-102) at 40°C for 12 h. After drying, the plant material was pulverized using a blender (Philips model RI 2024) and stored in sealed plastic bags protected from light until extraction.

Preparation and characterization of extracts

The experiment was conducted as a completely randomized factorial design ($3 \times 2 \times 2$), with plant species (three levels), extraction method (maceration and decoction), and ethanol concentration (58 and 77% v/v) as factors. Leaf extracts were prepared using hydroethanolic solutions as green solvents, and both extraction methods employed 15% (w:v) of dried plant material. Maceration was conducted over 7 days at room temperature. Decoction involved heating the plant material with the solvent under boiling conditions for approximately 10 min. All extracts were prepared in duplicate. After extraction, samples were homogenized, filtered, and adjusted to a final volume of 50 mL. Physicochemical characterization included determination of pH, dry residue, and density, following the procedures described in the 7th edition of the Brazilian Pharmacopoeia (Brazil, 2024).

Qualitative and quantitative determination of flavonoids

The presence of flavonoid glycosides was verified through classical

Table 1. Collected species with their respective botanical data and medicinal uses of the leaves.

Species / Popular names	Family	Voucher number	Medicinal uses
<i>A. humile</i> / Cajueiro-do-cerrado	Anacardiaceae	HUEG 3979	Antifungal; antibacterial; antioxidant; α -amylase inhibition (potential anti-glycation) (Royo et al., 2015; Ferreira et al., 2012; Lima Júnior et al., 2021).
<i>C. adamantium</i> / Gabirobeira	Myrtaceae	HUEG 3689	Anti-inflammatory and antinociceptive; antioxidant; antiproliferative activity; inhibition of adipogenesis/lipogenesis and reduction of pro-inflammatory cytokines (Ferreira et al., 2013; Pascoal et al., 2011, 2014; Coutinho et al., 2008; Campos et al., 2017; Rocha et al., 2024).
<i>P. emarginatus</i> / Sucupira	Fabaceae	HUEG 1544	Antinociceptive (Negri et al., 2014).

Phytochemical Reactions. The following colorimetric tests were performed: reaction with alkaline hydroxides, ferric chloride test, Shinoda test, and aluminum chloride test (Costa, 2002; Matos, 2009; Simões, 2017).

Total flavonoid content was quantified using the aluminum chloride method with rutin as the reference standard, adapted from Figueirêdo et al. (2015). A calibration curve was constructed by correlating absorbance with increasing concentrations of rutin, consisting of five points within the range of 0.003 to 0.024 mg/mL, using Microsoft Excel 2016 (Microsoft Office, USA).

For the analysis, 50 μ L of each extract was transferred into test tubes and mixed with 500 μ L of $AlCl_3$ solution (2.5%, w/v). The volume was then adjusted to 5 mL with methanol. After incubation at room temperature for 30 min, absorbance was measured at 403 nm using a UV-Vis spectrophotometer (Kasvi, model K37-UVVis). "The absorbance values obtained for the extracts were substituted into the variable y of the linear equation ($y = ax + b$), allowing the determination of the corresponding concentration (x) of flavonoids expressed as rutin equivalents (RE) in mg/mL".

Total flavonoid content was subsequently calculated using Equation 1 and expressed as milligrams of rutin equivalents per gram of extract (mg RE g^{-1}) on a wet basis.

$$TFC = C \times 5 / m \quad (1)$$

where C = concentration obtained from the rutin calibration curve (mg/mL); 5= final reaction volume (5 mL); m = mass of extract present in the aliquot used for the assay (g). The mass of extract in the aliquot was estimated from the extract density and the volume of extract solution used in the assay (50 μ L). All analyses were performed in triplicate (n = 3). The analytical blank was prepared using methanol in place of the extract, following the same experimental protocol. Results were subsequently converted to a dry-weight basis using the dry residue of the extracts.

Antioxidant activity assay

Antioxidant activity was assessed for extracts obtained by maceration in 77% ethanol, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, according to the methodology described by Rufino et al. (2007). Initially, each extract was diluted in methanol to obtain a series of five concentrations, prepared by successive dilutions of a stock solution. This procedure generated concentration ranges of 3.306–16.53 μ g mL $^{-1}$ for *A. humile*, 3.225–

16.125 μ g mL $^{-1}$ for *C. adamantium*, and 9.296–46.48 μ g mL $^{-1}$ for *P. emarginatus*, respectively. Under light-protected conditions, 100 μ L of each solution were added to test tubes containing 3.90 mL of DPPH solution (0.0600 mM). The mixtures were incubated for 30 min at room temperature in the absence of light, and absorbance was measured at 515 nm. Methanol served as the blank, and rutin was used as the positive control. All analyses were conducted in triplicate. Antioxidant activity was expressed as EC $_{50}$, defined as the concentration (mg/mL) required to reduce the initial absorbance of DPPH by 50%. Analytical curves were constructed by plotting absorbance values against extract concentrations. Linear regression equations were generated using Microsoft Excel, and EC $_{50}$ values were obtained from the fitted regression line according to the relationship

$$y = -ax + b$$

where y corresponds to half of the initial absorbance of the control and x represents the EC $_{50}$.

Statistical analyses

Statistical analyses were performed using Statistica software (version 7.0, StatSoft Inc., USA). Data for extract pH, density, dry residue, and total flavonoid content were analyzed by three-way analysis of variance (ANOVA) based on a completely randomized factorial design ($3 \times 2 \times 2$), with plant species (three levels), extraction method (maceration or decoction), and ethanol concentration (58 or 77%, v/v) as fixed factors. The model included all main effects and their interactions. Treatment means were compared using Tukey's honestly significant difference (HSD) test at a significance level of 0.05. Different letters indicate statistically significant differences among means.

RESULTS AND DISCUSSION

One of the challenges in phytotherapy is the natural variability of plant raw materials. Differences in genetic factors (such as species, subspecies, or chemotypes) and environmental conditions, including soil characteristics, climate, and cultivation practices, as well post-harvest processing, may lead to considerable variation in the

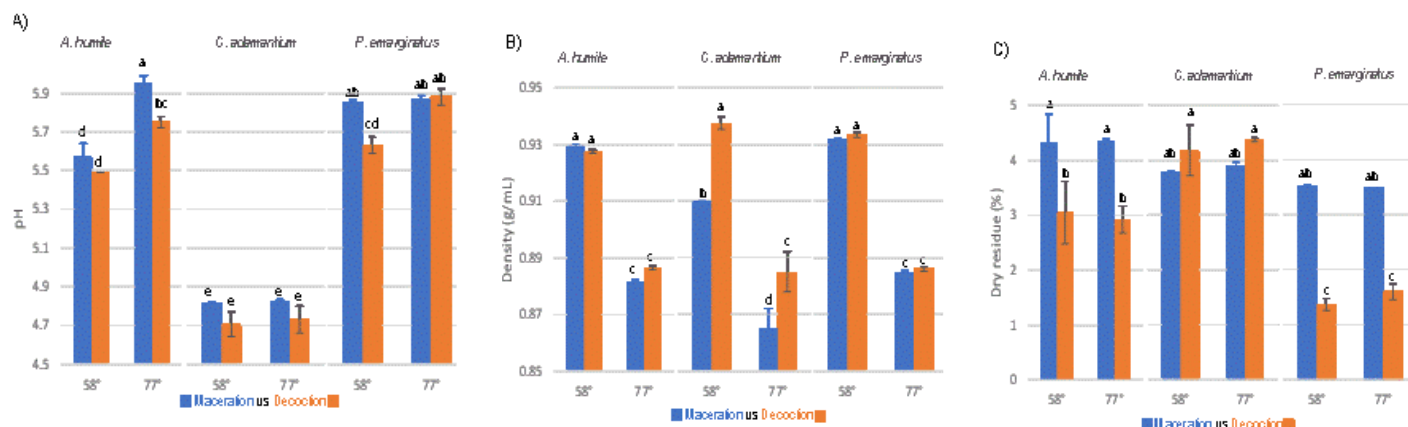


Figure 1. Physicochemical parameters of extracts obtained by different extraction processes (maceration and decoction) using hydroalcoholic solutions at 58 and 77% (v/v). The evaluated parameters include pH (A), density (B), and dry residue (C) of leaf extracts from *A. humile*, *C. adamantium*, and *P. emarginatus*. Data are expressed as mean $\pm$ standard deviation ($n = 2$). Means followed by different letters within each variable indicate statistically significant differences according to Tukey's test ($p < 0.05$).

phytochemical composition of medicinal plants (Srisittirakul et al., 2025). In the present study, plant material was collected during a single period (June 2024), and therefore the results should be interpreted within the context of this specific sampling.

Nevertheless, processing conditions may further contribute to this variability, particularly those related to extraction procedures. Therefore, studies focused on the standardization of extraction conditions remain essential to improve the yield of bioactive compounds and to strengthen the potential of plant-derived phytochemicals. In this context, selecting appropriate extraction techniques and solvent compositions represents an important step toward improving extraction efficiency and maximizing biological activity (Oliveira et al., 2016a; Novaes and Neto, 2026).

Physicochemical characterization of plant extracts provides key parameters for comparing extraction methods and supporting standardization. In this study, pH, density, and dry residue were determined for extracts of *A. humile*, *C. adamantium*, and *P. emarginatus* obtained by maceration or decoction using 58 or 77% ethanol. The effects of plant species, extraction method, and ethanol concentration on these parameters were evaluated using factorial ANOVA models ($3 \times 2 \times 2$) (Tables S1 to S3, Supplementary Material).

The pH values (Figure 1A) ranged from slightly acidic to near neutral, varying between 4.7 and 6.0. As shown in Table S1, pH varied according to plant species and extraction conditions. Factorial ANOVA revealed significant effects of plant species, extraction method, and ethanol concentration on extract pH. Plant species showed a dominant effect ($F = 1600$, $p < 10^{-6}$), with *C. adamantium* extracts presenting markedly lower pH values compared to *A. humile* and *P. emarginatus*. Ethanol concentration also significantly influenced pH ($F = 88.7$, $p = 1 \times 10^{-6}$), with

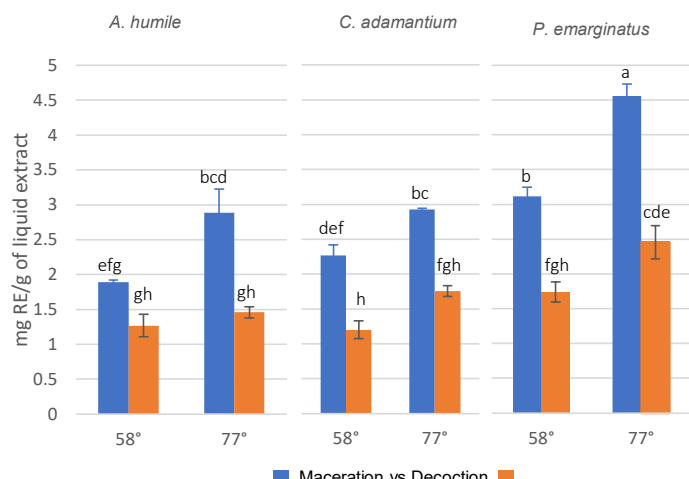
extracts prepared using 77% ethanol generally exhibiting slightly higher pH values.

Extracts of *C. adamantium* exhibited the lowest pH values (4.71 – 4.82), whereas *A. humile* showed the highest (5.5 – 6.0), followed by *P. emarginatus* (5.63 – 5.88). For *A. humile*, extracts prepared with 58% ethanol tended to exhibit slightly lower pH values than those obtained with 77% ethanol; a similar trend was observed for *P. emarginatus* under decoction conditions. Differences between maceration and decoction were small across all species, although the extraction method had a significant effect on pH ($F = 50.3$, $p = 1.3 \times 10^{-5}$). Overall, these results indicate that pH variation is primarily governed by species-specific chemical composition, with secondary contributions from extraction conditions.

Density values (Figure 1B) ranged from approximately 0.87 to 0.94 g/mL. According to a factorial ANOVA model (Table S2), ethanol concentration showed the strongest effect on extract density ($F = 1462$, $p < 10^{-6}$), with extracts prepared using 58% ethanol consistently presenting higher densities than those obtained with 77% ethanol. The extraction method also significantly influenced density ($F = 52$, $p = 1.1 \times 10^{-5}$), with decoction yielding slightly denser extracts than maceration. In addition, significant differences among species were observed ($F = 22$, $p = 9.9 \times 10^{-5}$), with *C. adamantium* exhibiting lower average densities. The decrease in density observed for extracts prepared with 77% ethanol is consistent with the density of the hydroalcoholic solvents themselves, as 58% ethanol has a density of approximately 0.90 g/mL whereas 77% ethanol has a density of about 0.86 g/mL at 25°C. The slightly higher densities measured for the extracts relative to the solvent baseline reflect the presence of dissolved solid constituents extracted from the plant material.

Dry residue values (Figure 1C) ranged from 1.4 to 4.4%. Factorial ANOVA indicated significant effects of plant

A) Wet basis



B) Dry-weight basis

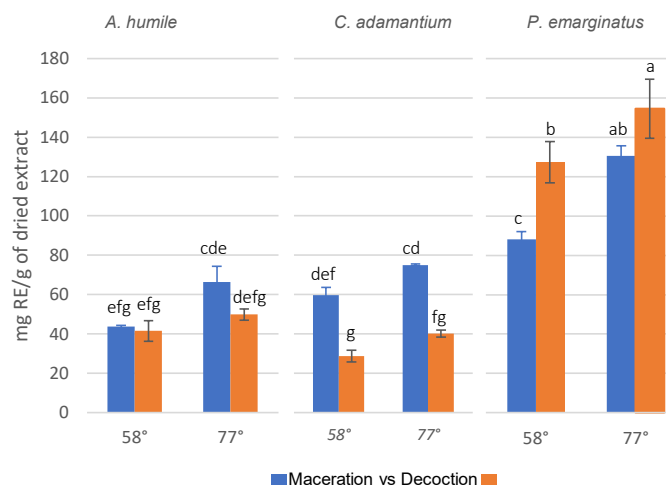


Figure 2. Total flavonoid content of leaf extracts from *A. humile*, *C. adamantium*, and *P. emarginatus*, obtained by maceration and decoction using 58 and 77% ethanol. Results are shown on a wet basis (A) and a dry-weight basis (B). Data are expressed as mean $\pm$ standard deviation of duplicate analyses. Means followed by different letters within each variable indicate statistically significant differences according to Tukey's test ($p < 0.05$).

species and extraction method on dry residue, whereas ethanol concentration did not show a statistically significant effect (Table S3). Extracts of *A. humile* and *P. emarginatus* obtained by maceration generally exhibited higher dry residue values than those obtained by decoction. In contrast, *C. adamantium* showed slightly higher values for decoction, although this difference was less pronounced. Overall, variations in dry residue were primarily associated with species-specific characteristics and extraction method, with no consistent influence of ethanol concentration across the evaluated conditions. Overall, the results indicate that decoction led to lower total extractable solids content in *A. humile* and *P. emarginatus* leaf extracts, as evidenced by the reduced dry residue values. Additionally, *C. adamantium* extracts exhibited a distinct behavior and consistently showed lower pH values than those obtained from the other two species.

Although decoction resulted in lower overall extract yields, this finding raises the hypothesis that, when the levels of bioactive compounds remain relatively high, such extracts may contain these compounds at higher relative concentrations in the dry matter. This hypothesis was subsequently supported by the quantitative results obtained for *P. emarginatus*, but not for *A. humile*, as discussed in the following section.

Qualitative phytochemical assays confirmed the presence of phenolic compounds and flavonoids in all extracts. In alkaline medium, extracts developed a dark brown coloration consistent with the formation of phenolate ions. The ferric chloride assay produced a greenish-brown color, indicative of Fe^{3+} -phenol complex formation, while the aluminum chloride reaction resulted in

enhanced fluorescence due to flavonoid- Al^{3+} complexation under UV light (365 nm). In the Shinoda test, the appearance of a reddish coloration indicated the reduction of flavonoids to anthocyanidins (Simões, 2017).

These qualitative findings are consistent with previous reports describing phenolic compounds in leaf extracts of *A. humile* (Ferreira et al., 2012; Lima Júnior et al., 2021), *C. adamantium* (Coutinho et al., 2008; Castro et al., 2023), and *P. emarginatus* (Negri et al., 2014), and they support the quantitative evaluation of total flavonoid content presented in Figure 2.

Total flavonoid content, expressed in both wet and dry-weight basis, varied according to the extraction method and ethanol concentration, in addition to the expected differences among plant (Tables S4 and S5). Overall, extracts obtained by maceration exhibited higher flavonoid contents than those obtained by decoction (Figure 2), suggesting that extraction procedures without heat exposure favor higher flavonoid content, likely by reducing thermal degradation. Moreover, the longer extraction time of maceration (seven days versus 10 min for decoction) may enhance mass transfer between the plant matrix and the solvent (Chaves et al., 2020). An exception was observed *P. emarginatus* on a dry-weight basis, for which decoction resulted in higher values than maceration.

Total flavonoid content is commonly expressed as milligrams of rutin equivalents (RE) per g of extract, either on a wet basis (liquid extract) or on a dry-weight basis (dried extract) (Djeridane et al., 2006; Esmaeili et al., 2015; Sun et al., 2022). In this study, results were expressed on both wet and dry bases, meaning that flavonoid concentrations were calculated relative to the mass of the

liquid extract (wet basis) and to the mass of the extract obtained after solvent evaporation (dry-weight basis). Because values expressed on a dry-weight basis depend on the total solids content of the extract, variations in dry residue directly affect the calculated concentrations when results are normalized to dried material (Nielsen, 2017; AOAC, 2023).

In *P. emarginatus* extracts, flavonoid content expressed on a dry-weight basis was higher in decoctions than in macerations, a trend not observed for the other species. However, this apparent increase does not indicate a greater extraction efficiency of the decoction process; instead, it reflects the lower dry residue of these extracts, which proportionally elevates flavonoid values when normalized to dry matter.

Similar results were reported for aqueous extracts of *Eugenia dysenterica* leaves, in which maceration proved to be more efficient than decoction for flavonoid extraction (Almeida et al., 2024). Likewise, Luliana et al. (2019) demonstrated that methanolic maceration yielded the highest total phenolic content (338.62 mg GAE/g), followed by Soxhlet extraction with methanol, whereas aqueous infusion resulted in the lowest phenolic levels (144.48 mg GAE/g). Taken together, these findings indicate that non-thermal extraction methods, combined with organic solvents of suitable polarity, are more effective in enhancing the extraction of flavonoids and related phenolic compounds.

The efficiency of phenolic compound extraction is strongly influenced by solvent polarity, and no single solvent is universally considered optimal for recovering polyphenols from plant matrices. In general, solvents with relatively high polarity tend to improve the solubilization of phenolic compounds due to their chemical characteristics (Felhi et al., 2017; Nawaz et al., 2020; Alara et al., 2021).

In this context, mixtures of water and ethanol are frequently reported to be more effective than single solvents, since they provide an adjustable polarity capable of solubilizing a broader range of phenolic constituents (Plaskova and Mlcek, 2023). Additionally, due to its low toxicity and compatibility with human consumption, ethanol is widely used in both laboratory-scale and industrial-scale processes (Martín et al., 2022).

Moreover, ethanol concentrations between 50 and 80% are commonly described in the literature as suitable for extracting phenolic compounds from plant materials (Waszkowiak and Gliszczynska-Swigło, 2016; Bitwell et al., 2023; Teófilo et al., 2026). Based on these reports, ethanol solutions of 58 and 77% were selected in the present study to represent two solvent compositions within this commonly recommended range and to evaluate the influence of solvent polarity on extraction performance.

In this study, the higher flavonoid content obtained with 77% ethanol compared to 58% ethanol can be attributed to a more suitable adjustment of solvent polarity to the chemical profile of the extractable flavonoids, particularly aglycones and less hydroxylated flavonoids, which exhibit

moderately polar characteristics. Increasing ethanol content reduces overall solvent polarity, thereby favoring the solubilization of these constituents, in agreement with previous reports (Simões, 2017; Tzanova et al., 2020). The polarity of the solvent is a key factor for the solubility and extraction efficiency of phenolics, as extensively discussed in previous studies (Felhi et al., 2017; Nawaz et al., 2020).

Solvent polarity also plays a crucial role in extracting antioxidant compounds from plant matrices, as it determines the solvent's ability to dissolve metabolites with diverse chemical characteristics (Nawaz et al., 2020; Mohammed et al., 2022). In general, extracts obtained with more polar solvents tend to exhibit stronger radical-scavenging activity than those obtained with apolar solvents, particularly in DPPH assays (Ghasemzadeh et al., 2011). This effect is attributed to the enrichment of polar fractions in antioxidant compounds capable of neutralizing free radicals, primarily through electron transfer mechanisms (Pool et al., 2021).

In this study, antioxidant activity was evaluated by the DPPH radical scavenging assay using ethanolic extracts obtained with 77% ethanol, which had the highest flavonoid content (Figure 3). Considering that the literature describes a positive correlation between phenolic or flavonoid content and free radical scavenging capacity (Almeida et al., 2024; Haideri et al., 2024), antioxidant activity analysis helps determine whether this trend holds among the studied species. Additionally, focusing the analysis on this extract improved experimental feasibility by prioritizing the condition with the greatest potential bioactivity.

Antioxidant activity showed notable differences between the evaluated extracts (Table 2), reveals that *C. adamantium* had exhibited the highest DPPH radical scavenging capacity ($EC_{50} = 6.89$ mg/mL), followed by *A. humile* ($EC_{50} = 9.11$ mg/mL), while *P. emarginatus* exhibited the lowest DPPH radical neutralization capacity ($EC_{50} = 29.29$ mg/mL), indicating significant variation in antioxidant profiles among species. Herein, no direct correlation was found between total flavonoid content (Figure 2) and antioxidant activity. Although *P. emarginatus* had the highest flavonoid content, it showed the weakest antioxidant effect.

These findings indicate that antioxidant performance depends not only on the total flavonoid content but also on the qualitative composition of the extracts and the possible contribution of other bioactive metabolites. Previous studies support this view, indicating that phenolic profiles and other metabolites can significantly influence antioxidant capacity (Moure et al., 2001; López et al., 2017; Dibacto et al., 2021).

No studies were found in the literature that simultaneously evaluate the influence of maceration and decoction, combined with different ethanol concentrations, on the total flavonoid content of leaf extracts from *A. humile*, *C. adamantium*, and *P. emarginatus*.

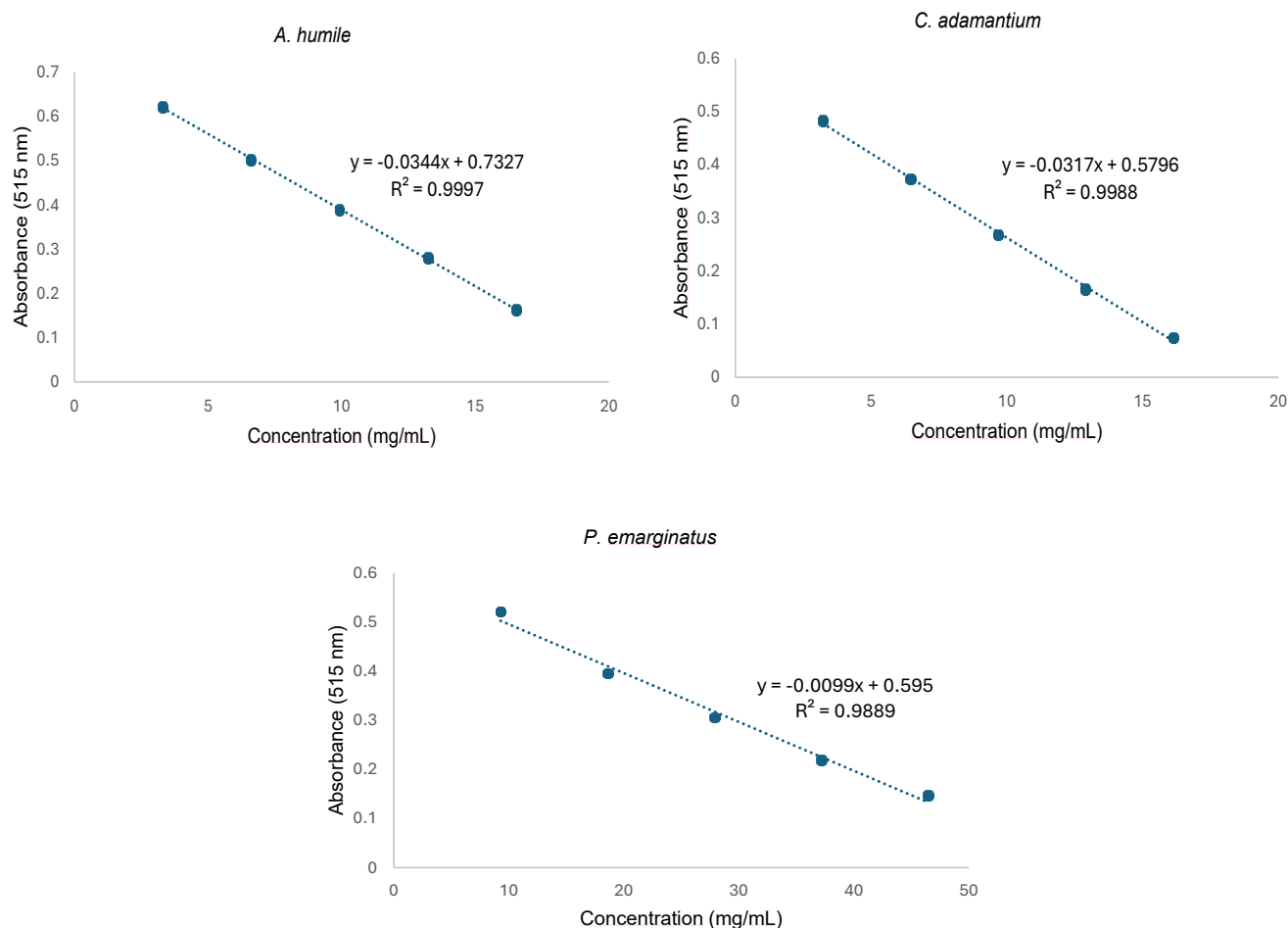


Figure 3. DPPH radical scavenging curves of leaf extracts of *A. humile*, *C. adamantium*, and *P. emarginatus* obtained by maceration with 77% ethanol.

Table 2. Antioxidant activity of the extracts using the DPPH free radical. Extracts were obtained by maceration with ethanol 77%.

Extract	EC ₅₀ (mg/mL)
<i>A. humile</i>	9.11 ± 0.14
<i>C. adamantium</i>	6.89 ± 0.43
<i>P. emarginatus</i>	29.29 ± 2.64

Nevertheless, independent investigations involving these species have reported relevant findings regarding phenolic composition, flavonoid content, and antioxidant activity, although evaluated under specific extraction conditions.

For *A. humile*, the most comprehensive study conducted to date (Lima Júnior et al., 2021) demonstrated that the concentrated ethanolic extract (98% v/v) and its fractions (DCM, EtOAc, ButOH, and aqueous) exhibited expressive levels of both phenolic compounds and flavonoids. The

ethanolic extract showed 165.5 mg GAE/g (gallic acid equivalents) and 28.8 mg QE/g (quercetin equivalents), whereas the DCM, EtOAc, ButOH, and aqueous fractions ranged from 115.2 to 138.6 mg GAE/g for total phenolics and from 10.1 to 58.4 mg QE/g for flavonoids. The DCM and EtOAc fractions displayed notable antioxidant activity, with ORAC values close to 1300 µmol TE/g and strong DPPH radical-scavenging capacity (IC₅₀ ≈ 3 µg/mL), reinforcing the species' high bioactive potential. In another study, hydroethanolic extracts (70% ethanol) obtained by maceration exhibited moderate antioxidant activity, with an EC₅₀ of 95.32 µg/mL (Melo et al., 2017).

For *C. adamantium*, leaves collected from different regions of Mato Grosso do Sul showed substantial variation in bioactive constituents. Extracts obtained by ultrasound-assisted maceration in methanol contained 7.24–21.19 mg GAE/g of total phenolics, as well as wide ranges of chalcones (4.67–232.35 mg/g) and flavanones (15.62–50.71 mg/g). Independent of geographic origin, all extracts demonstrated strong antioxidant activity, with

DPPH radical-scavenging values between 52.0–92.2% at concentrations of 160–1120 µg/mL, and lipid peroxidation inhibition between 14.6–94.2% at 48–336 µg/mL. These results highlight the potent antioxidant capacity associated with the abundance of chalcones and flavanones (Coutinho et al., 2008).

Complementarily, Pascoal et al. (2011) evaluated leaves of *C. adamantium* collected from wild specimens in southern Brazil (Curitiba, Paraná State) and found 15.78–74.83 mg GAE/g of total phenolics, high radical-scavenging activity ($SC_{50} = 7.77\text{--}13.35$ µg/mL, DPPH assay), and antioxidant capacity of 2648–3502 µmol TE/g via ORAC analysis. In the same study, the authors identified flavonoids such as isoquercitrin, quercitrin, quercetin, and myricetin, supporting the conclusion that these compounds play a central role in the antioxidant activity of the species. More recent data of Castro et al. (2023) show that hydroethanolic extracts (25 and 75% ethanol) obtained by static maceration contain significant levels of rutin (63.45–71.23 mg/g). These levels correlated positively with the strong antioxidant activity observed, reflected by low EC_{50} values ($\approx 200\text{--}350$ µg/mL).

For *P. emarginatus*, although most studies focus on fruits and seeds (Dutra et al., 2008; Oliveira et al., 2017), hydroethanolic leaf extracts have revealed a composition rich in C-glycosyl flavonoids, such as vicianin-2, schaftoside, and O-glycosylated derivatives of luteolin, along with triterpenic saponins (Negri et al., 2014).

Several *in vitro* and *in vivo* antioxidant evaluation methods have been applied to different types of samples, including plant extracts, foods, and synthetic antioxidants. Among these, the DPPH radical scavenging assay is one of the most widely used methods, being employed in more than 90% of studies evaluating the antioxidant activity of pure substances, mixtures, or complex matrices (Oliveira, 2015). This method is considered simple, sensitive, rapid, and reproducible, which makes it one of the most convenient and commonly used assays for evaluating the antioxidant capacity of compounds and herbal extracts (Gulcin and Alwasel, 2023).

The assay is based on the spectrophotometric monitoring of the decrease in DPPH absorbance after reaction with antioxidant compounds. The results are commonly expressed as EC_{50} , defined as the concentration required reducing the initial absorbance of DPPH by 50%. Lower EC_{50} values indicate a greater capacity of the sample to scavenge DPPH radicals and, consequently, higher antioxidant activity (Williams et al., 1995; Mishra et al., 2012; Sridhar and Charles, 2019).

However, the determination of EC_{50} presents important limitations, since the percentage of radical scavenging depends on the initial concentration of DPPH used in the assay, which may vary among experimental protocols reported in the literature. This variability may hinder the direct comparison of results obtained in different studies (Fadda et al., 2014). Therefore, *in vitro* antioxidant assays

are generally considered screening methods, useful for the preliminary evaluation of the antioxidant potential of plant extracts Cerrado. In the present study, antioxidant activity was assessed using the DPPH assay as an initial screening approach; however, the absence of complementary antioxidant assays (such as ABTS, FRAP or ORAC), which are commonly employed in similar studies, should be considered a limitation of this work.

The EC_{50} values obtained in this study were within the range reported for Cerrado fruit extracts evaluated by the DPPH assay, such as ethanolic extracts of pequi peel (9.44 µg/mL) and cagaita seeds (14.15 µg/mL) described by Roesler et al. (2007). However, direct comparisons between studies should be interpreted with caution due to methodological differences, including extraction procedures, plant fractions analyzed, assay conditions, differences in sample preparation, and reporting units, as previously discussed.

Conclusion

This study demonstrates that extraction conditions significantly affect total flavonoid content and antioxidant activity in leaf extracts of *A. humile*, *C. adamantium*, and *P. emarginatus*. Among the extraction variables evaluated, solvent ethanol concentration was more influential than the extraction method itself, with maceration using 77% ethanol providing the highest total flavonoid content. Antioxidant activity did not follow the same trend as total flavonoid content, indicating that flavonoid quantity alone is insufficient to predict radical scavenging performance. Notably, *C. adamantium* exhibited superior antioxidant activity under the evaluated conditions, whereas *P. emarginatus* showed lower effectiveness despite its higher flavonoid levels. Overall, the results highlight the need for a rational selection of extraction parameters tailored to each species and support the relevance of comparative studies under standardized conditions for advancing the understanding and sustainable use of Cerrado plant biodiversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

Table S1. Three-way ANOVA ($3 \times 2 \times 2$ factorial design) evaluating the effects of plant species, extraction method, and ethanol concentration on extract pH.

	SS	Degr. of Freedom	MS	F	p
Intercept	705.6842	1	705.6842	434267.2	0.000000
Extraction method	0.0817	1	0.0817	50.3	0.000013
Ethanol concentration	0.1442	1	0.1442	88.7	0.000001
Species	5.2012	2	2.6006	1600.4	0.000000
Extraction method*Ethanol concentration	0.0033	1	0.0033	2.0	0.181680
Extraction method*Species	0.0024	2	0.0012	0.7	0.493833
Ethanol concentration*Species	0.0949	2	0.0474	29.2	0.000025
Extraction method*Ethanol concentration*Species	0.0329	2	0.0165	10.1	0.002646
Error	0.0195	12	0.0016		

Table S2. Three-way ANOVA ($3 \times 2 \times 2$ factorial design) evaluating the effects of plant species, extraction method, and ethanol concentration on the density of extracts.

	SS	Degr. of Freedom	MS	F	p
Intercept	19.65153	1	19.65153	2178059	0.000000
Extraction method	0.00047	1	0.00047	52	0.000011
Ethanol concentration	0.01319	1	0.01319	1462	0.000000
Species	0.00039	2	0.00020	22	0.000099
Extraction method*Ethanol concentration	0.00000	1	0.00000	0	0.936353
Extraction method*Species	0.00067	2	0.00033	37	0.000007
Ethanol concentration*Species	0.00002	2	0.00001	1	0.381495
Extraction method*Ethanol concentration*Species	0.00005	2	0.00003	3	0.093043
Error	0.00011	12	0.00001		

Table S3. Three-way ANOVA ($3 \times 2 \times 2$ factorial design) evaluating the effects of plant species, extraction method, and ethanol concentration on the dry residue of extracts.

	SS	Degr. of Freedom	MS	F	p
Intercept	278.3247	1	278.3247	3712.440	0.000000
Extraction method	5.8115	1	5.8115	77.517	0.000001
Ethanol concentration	0.0234	1	0.0234	0.313	0.586359
Species	10.6217	2	5.3108	70.839	0.000000
Extraction method*Ethanol concentration	0.0084	1	0.0084	0.113	0.743059
Extraction method*Species	6.4299	2	3.2150	42.883	0.000003
Ethanol concentration*Species	0.0447	2	0.0224	0.298	0.747437
Extraction method*Ethanol concentration*Species	0.0415	2	0.0207	0.277	0.763058
Error	0.8997	12	0.0750		

Table S4. Three-way ANOVA ($3 \times 2 \times 2$ factorial design) evaluating the effects of plant species, extraction method, and ethanol concentration on total flavonoid content of extracts (wet basis).

	SS	Degr. of Freedom	MS	F	p
Intercept	126.1702	1	126.1702	4713.288	0.000000
Extraction method	10.0466	1	10.0466	375.308	0.000000
Ethanol concentration	3.4711	1	3.4711	129.668	0.000000
Species	5.5529	2	2.7765	103.719	0.000000
Extraction method*Ethanol concentration	0.4442	1	0.4442	16.594	0.001544
Extraction method*Species	0.6029	2	0.3015	11.262	0.001763
Ethanol concentration*Species	0.3116	2	0.1558	5.820	0.017111
Extraction method*Ethanol concentration*Species	0.1516	2	0.0758	2.832	0.098284
Error	0.3212	12	0.0268		

Table S5. Three-way ANOVA ($3 \times 2 \times 2$ factorial design) evaluating the effects of plant species, extraction method, and ethanol concentration on total flavonoid content of extracts (dry basis).

	SS	Degr. of Freedom	MS	F	p
Intercept	136717.1	1	136717.1	3266.549	0.000000
Extraction method	77.8	1	77.8	1.860	0.197663
Ethanol concentration	2704.5	1	2704.5	64.619	0.000004
Species	29613.7	2	14806.9	353.777	0.000000
Extraction method*Ethanol concentration	189.1	1	189.1	4.518	0.054992
Extraction method*Species	4247.6	2	2123.8	50.743	0.000001
Ethanol concentration*Species	558.8	2	279.4	6.675	0.011251
Extraction method*Ethanol concentration*Species	41.4	2	20.7	0.494	0.621798
Error	502.2	12	41.9		