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Bioactive Compounds and antioxidant potential of *Rhamnidium elaeocarpum* (Rhamnaceae)

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Abstract: This study investigated the antioxidant potential and chemical composition of ethanolic extracts from *Rhamnidium elaeocarpum*. The objective was to characterize the main bioactive constituents and evaluate their biological activity. Ethanolic extracts were prepared and subjected to antioxidant assays, including DPPH radical scavenging, ABTS assay, and inhibition of lipid peroxidation. Phytochemical analysis was performed using chromatographic techniques, leading to the identification of flavonoids and sterols as principal constituents. The extracts exhibited significant antioxidant activity, demonstrating strong free radical scavenging capacity and substantial inhibition of lipid peroxidation. These findings highlight the potential of *R. elaeocarpum* extracts as promising sources of bioactive compounds for therapeutic applications.
Keywords: *Rhamnidium elaeocarpum*, antioxidant activity, flavonoids, sterols, DPPH, ABTS, lipid peroxidation

Introduction

The Rhamnaceae family, predominantly distributed in tropical and subtropical regions, comprises 58 genera and approximately 900 species. Within this family, 47 species belonging to 12 genera have been recorded in a specific region (Richardson et al., 2004). Several Rhamnaceae species are traditionally employed in folk medicine due to their anti-inflammatory, antibacterial, antipyretic, anticonvulsant, and antiviral properties (Alarcón & Cespedes, 2015). *Rhamnidium elaeocarpum* Reissek is an endemic South American species, broadly distributed from Peru to eastern Brazil and Argentina. It is commonly known as “little goat” or “little coffee” (Lima & Giulietti, 2014; Pott & Pott, 1994; Lorenzi, 1998). In traditional medicine, it is used to relieve gingival irritation in teething children and to treat stomach discomfort (Oliveira et al., 2010).

A previous study on the stem bark of *R. elaeocarpum* reported the isolation of eleocarpanthraquinone and chrysophanol, two anthraquinones that act as potent and selective inhibitors of cathepsins B and L, supporting the antitumor potential of these metabolites (Kauffmann et al., 2020). The present study aims to describe the isolation and structural characterization of chemical constituents from the stem bark and leaves of *R.*

elaeocarpum, as well as to evaluate the inhibitory effect of its hydroethanolic extracts on lipid peroxidation and oxidative stress.

Material and Methods

Plant material

Leaves and stem bark of *Rhamnidium elaeocarpum* were collected in March 2018 in Poconé, Mato Grosso, Brazil (16°28′07.1″S; 57°02′00.32″W). The species was identified by botanist Temilze Gomes Duarte (Universidade Federal de Mato Grosso), and a voucher specimen (no. 43615) was deposited in the herbarium of the institution. Genetic access was registered in the SISGEN system under code AAC7246.

Extraction and isolation

The botanical material was dried at room temperature, yielding 1.0 kg each of leaves and stem bark. Both materials were subjected separately to cold maceration in ethanol with occasional stirring for five-day cycles. After each cycle, the extracts were filtered and concentrated under reduced pressure using a rotary evaporator, resulting in crude ethanolic extracts of the leaves (150.0 g) and stem bark (340.0 g). The crude leaves extract was suspended in a MeOH:H_2O mixture (7:3) and partitioned sequentially with hexane, dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc). After solvent removal under reduced pressure, the respective fractions were obtained: hexane (32.8 g), CH_2Cl_2 (4.0 g), and EtOAc (13.8 g). The CH_2Cl_2 fraction was subjected to column chromatography on silica gel (70–230 mesh), eluted with increasing polarity using hexane, CH_2Cl_2, EtOAc, and MeOH. Ninety-six fractions (125 mL each) were collected and recombined based on TLC analysis. Fractions 52–95 (505.3 g) were rechromatographed on silica gel (230–400 mesh) using CH_2Cl_2, EtOAc, and MeOH in a polarity gradient. From the 34 collected subfractions (25 mL each), fractions 20–27 (28.5 mg) were further purified by Sephadex LH-20 chromatography with methanol as the mobile phase, yielding kaempferol (**1**, 20.6 mg). The EtOAc fraction of the leaves extract was also chromatographed on Sephadex LH-20 (isocratic MeOH), producing 25 fractions (125 mL each). Fraction 18 yielded a yellowish amorphous solid, which was identified as quercetin (**2**, 124.0 mg) by NMR analysis. For the stem bark extract, a solid–liquid partition was carried out using silica gel (70–230 mesh) as the stationary phase, eluted with CH_2Cl_2 and EtOAc, affording fractions of 5.3 g and 18.4 g, respectively. The CH_2Cl_2 fraction was chromatographed on silica gel (70–230 mesh), using a gradient of increasing polarity with hexane, CH_2Cl_2, EtOAc, and MeOH. Based on TLC analysis, the 48 collected fractions (125 mL each) were grouped into nine main fractions. Fraction 4 yielded a white amorphous powder that tested positive in the Liebermann–Burchard test, corresponding to a mixture of stigmasterol (**3**) and β-sitosterol (**4**) (46.0 mg). The EtOAc fraction was chromatographed on Amberlite XAD-2 resin using water, MeOH, and EtOAc as eluents. Forty-one fractions (50 mL each) were collected, dried, and analyzed by TLC, and grouped into five main fractions. Group 4 (302.0 mg), after methanol washing, afforded a white amorphous powder identified as glycosylated β-sitosterol (**5**, 226.0 mg).

Kaempferol (1). Yellow amorphous solid. ^{1}H NMR (500 MHz, CD_3OD, δ ppm, J in Hz): 6.16 (1H, d, *J* = 2.0, H-6), 6.37 (1H, d, *J* = 1.5, H-8), 8.08 (2H, d, *J* = 8.85, H-2′/H-6′), 6.90 (2H, d, *J* = 8.85, H-3′/H-5′). ^{13}C NMR (125 MHz, CD_3OD, δ ppm): 148.0 (C-2), 137.2 (C-3), 177.4 (C-4), 160.7 (C-5), 94.8 (C-6), 162.6 (C-7), 99.7 (C-8), 158.5 (C-9), 104.4 (C-10), 123.9 (C-1′), 130.7 (C-2′/C-6′), 116.4 (C-3′/C-5′), 166.8 (C-4′).

Quercetin (2). Yellow amorphous solid. ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm, *J* in Hz): 12.48 (1H, s, 5-OH), 6.17 (1H, d, *J* = 2.0, H-6), 6.39 (1H, d, *J* = 2.0, H-8), 7.66 (1H, d, *J* = 2.0, H-2′), 6.87 (1H, d, *J* = 8.5, H-5′), 7.53 (1H, dd, *J* = 8.5, 2.0, H-6′).
^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 146.7 (C-2), 135.7 (C-3), 175.8 (C-4), 160.7 (C-5), 98.2 (C-6), 164.0 (C-7), 93.3 (C-8), 156.0 (C-9), 102.9 (C-1′), 115.0 (C-2′), 147.7 (C-3′), 145.0 (C-4′), 115.6 (C-5′), 119.1 (C-6′).

Stigmasterol (3). White amorphous powder. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm, *J* in Hz): 5.37 (2H, dd, *J* = 2.7, H-6), 5.17 (1H, dd, *J* = 15.0, 8.8, H-23), 5.03 (1H, dd, *J* = 14.8, 9.3, H-22), 3.54 (1H, m, H-3), 0.69 (3H, s, H-18). ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.0 (C-17), 51.7 (C-14), 50.1 (C-9), 45.8 (C-24), 42.3 (C-4), 42.2 (C-13), 39.7 (C-12), 37.2 (C-1), 36.1 (C-20), 36.5 (C-10), 31.9 (C-7), 31.8 (C-8), 31.6 (C-2), 29.1 (C-25), 28.2 (C-16), 24.3 (C-15), 23.0 (C-28), 21.0 (C-11), 19.4 (C-19), 18.7 (C-21), 19.8 (C-27), 19.0 (C-26), 11.9 (C-18), 11.8 (C-29).

β-Sitosterol (4). White amorphous powder. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm, *J* in Hz): 5.37 (2H, dd, *J* = 2.7, H-6), 3.54 (1H, m, H-3), 0.69 (3H, s, H-18). ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.0 (C-17), 51.7 (C-14), 50.1 (C-9), 45.8 (C-24), 42.3 (C-4), 42.2 (C-13), 39.7 (C-12), 37.2 (C-1), 36.1 (C-20), 36.5 (C-10), 33.9 (C-22), 31.9 (C-7), 31.8 (C-8), 31.6 (C-2), 29.1 (C-25), 28.2 (C-16), 26.0 (C-23), 24.3 (C-15), 23.0 (C-28), 21.0 (C-11), 19.8 (C-27), 19.4 (C-19), 19.0 (C-26), 18.7 (C-21), 11.9 (C-18), 11.8 (C-29).

Glycosylated β-sitosterol (5). White amorphous poder. ^{1}H NMR (500 MHz, pyridine-d_5, δ ppm, *J* in Hz): 5.07 (1H, d, *J* = 7.5, H-1′), 4.59 (1H, m, H-2′), 4.44 (1H, m, H-3′), 4.32 (1H, m, H-4′), 4.09 (1H, m, H-5′), 4.00 (2H, m, H-6′). ^{13}C NMR (125 MHz, pyridine-d_5, δ ppm): 37.9 (C-1), 30.7 (C-2), 78.5 (C-

3), 39.8 (C-4), 141.3 (C-5), 122.3 (C-6), 32.6 (C-7), 32.5 (C-8), 50.8 (C-9), 37.4 (C-10), 21.7 (C-11), 40.4 (C-12), 42.9 (C-13), 57.3 (C-14), 24.9 (C-15), 29.0 (C-16), 56.7 (C-17), 12.4 (C-18), 19.6 (C-19), 36.8 (C-20), 19.4 (C-21), 34.6 (C-22), 26.8 (C-23), 46.5 (C-24), 30.4 (C-25), 19.8 (C-26), 20.4 (C-27), 23.8 (C-28), 12.6 (C-29), 103.0 (C-1′), 75.8 (C-2′), 79.1 (C-3′), 72.1 (C-4′), 78.9 (C-5′), 63.3 (C-6′).

Structural elucidation of the compounds

^{1}H NMR (500 MHz), DEPT-Q (125 MHz), HSQC, and HMBC spectra were recorded at room temperature on a Bruker Ascend™ 500 NMR spectrometer, using $CDCl_3$, CD_3OD, DMSO-d_6, and pyridine-d_5 (Sigma-Aldrich) as solvents. Chemical shifts were referenced to residual solvent signals, with tetramethylsilane (TMS) as the internal standard.

Lipid peroxidation in vitro TBARS

The antioxidant activity of the crude ethanolic extracts from the leaves and stem bark of *Rhamnidium elaeocarpum* was evaluated at concentrations of 1, 50, 100, and 200 µg/mL using the TBARS (thiobarbituric acid reactive substances) assay, as described by Ohkawa, Ohishi, and Yagi (1979). This method is based on the detection of lipid peroxidation end products, primarily malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) in an acidic medium to form a colored complex measured spectrophotometrically at 532 nm. Homogenates of brain, liver, and kidney tissues were prepared in Tris buffer (pH 7.4), centrifuged at 2,500 rpm for 10 minutes, and the supernatants were used in lipid peroxidation assays. Lipid peroxidation was induced by pre-incubating the samples with EDTA (500 µM) and ferrous sulfate (1.4 mM) for 1 hour at 37 °C. Subsequently, TBA (0.8%), acetic acid buffer (pH 3.4), and sodium dodecyl sulfate (8.1%) were added, and the mixtures were incubated for an additional 2 hours. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test (n = 3). Results were expressed as ηmol of malondialdehyde (MDA) formed per milligram of tissue.

DPPH radical scavenger activity assay

The DPPH assay is widely employed to evaluate the antioxidant capacity of compounds to scavenge free radicals, which are major contributors to biological damage caused by oxidative stress (Parthasarathy et al., 2009). This method is based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical characterized by a deep purple color, to its non-radical yellow form upon receiving an electron or hydrogen atom. The assay was carried out following the protocol described by Sharma and Bhat (2009), using different concentrations (1, 10, 50, and 100 µM) of the crude ethanolic extracts from the leaves and stem bark of *R. elaeocarpum*. In 96-well microplates, varying concentrations of the extracts were pipetted and mixed with a methanolic DPPH solution (50 µM) under low-light conditions. The mixtures were incubated for 30 minutes at room temperature, and the absorbance was subsequently measured at 517 nm using a spectrophotometer.

Results and discussion

Chemical investigation of the crude ethanolic extracts from the leaves and stem bark of *R. elaeocarpum* led to the isolation and identification of the flavonoids kaempferol (**1**) and quercetin (**2**), a mixture of steroids stigmasterol (**3**) and β-sitosterol (**4**) and the saponin glycosylated β-sitosterol (**5**) (Figure 1). These compounds, identified for the first time in the genus *Rhamnidium*, were characterized by one- and two-dimensional (1D and 2D) NMR spectroscopy and mass spectrometry, with structural elucidation based on comparisons with literature data (Aderogba et al., 2012; Kojima et al., 1990; Silva et al., 2020).

In the bioassays, oxidative damage was induced using an Iron/EDTA system and assessed by quantifying thiobarbituric acid reactive substances (TBARS) at 532 nm. This procedure evaluates cellular damage caused by oxidative stress by measuring malondialdehyde (MDA), a product of lipid peroxidation. As shown in Figures 2 and 3, the damage induced (groups 2C and 3C) was distinctly different from the control group in all experiments, allowing the proper evaluation of the protective effect of the extracts against lipid peroxidation. The crude leaves extract of *R. elaeocarpum* exhibited a significantly greater protective effect compared to the stem bark extract. The stem bark extract showed notable activity only at 100 µg/mL in liver and kidney tissues (Figure 2), achieving values close to those of the control group. In contrast, the leaves extract was effective at lower concentrations (10, 50, and 100 µg/mL), demonstrating superior inhibition of lipid peroxidation in both liver and kidney tissues (Figure 3). No significant protective effect was observed in brain tissue assays. In the DPPH radical scavenging assay, both extracts exhibited significant antioxidant activity at concentrations of 10, 50, and 100 µM (Figure 4). The crude leaves extract (Figure 4a) also displayed significant activity at 1 µM (19.75% inhibition), with a statistically significant reduction in DPPH activity compared to the control group. Increasing the extract concentrations to 10, 50, and 100 µM resulted in inhibition rates of 46.6%, 83.2%, and 80.75%, respectively. According to Silva et al. (2023), ascorbic acid, used as a positive control, significantly reduced DPPH activity at concentrations of 0.25 and 1 µM, reinforcing the relevance of the observed effects. These results suggest that even at low concentrations, the crude leaves extract possesses substantial antioxidant potential. The antioxidant activity of natural products is mainly attributed to the presence of polyphenolic compounds, such as flavonoids, cinnamic acid derivatives, and phosphatidic acids (Huang et al., 2005). During the fractionation of the crude leaves extract, the flavonoids kaempferol (**1**) and quercetin

(**2**) were isolated, suggesting that these compounds may contribute to the antioxidant and protective effects observed for the leaves extract.

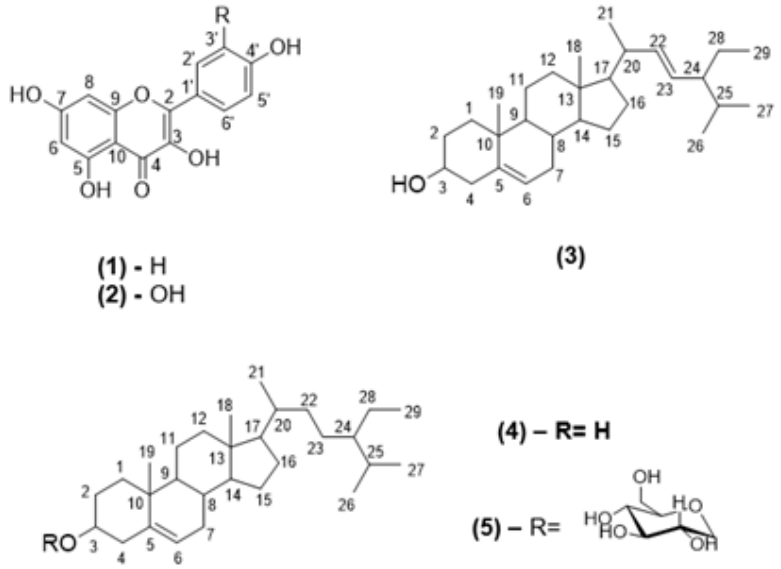

Figure 1. Structural formulas of Kaempferol (**1**), Quercetin (**2**), Stigmasterol (**3**), β-sitosterol (**4**), glycosylated β-sitosterol (**5**).

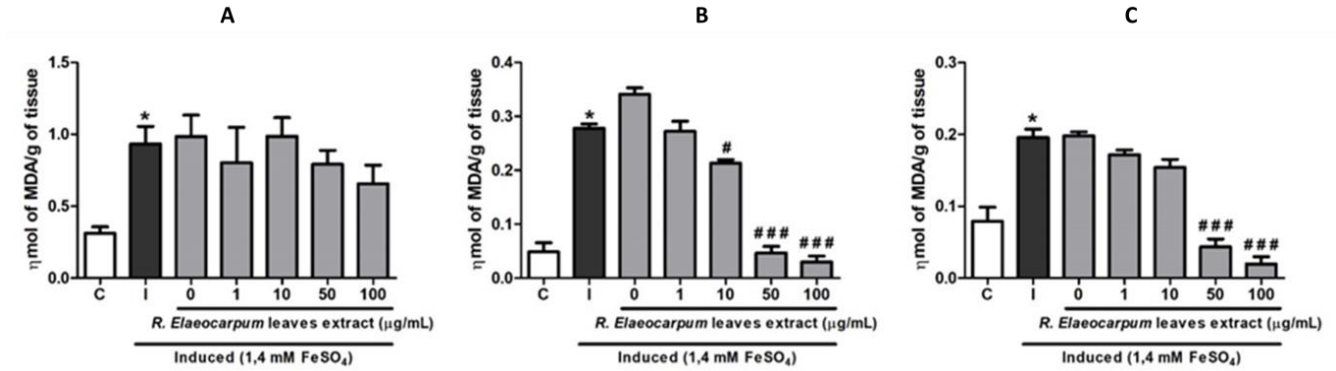

Figure 2. Effect of crude extract of leaves obtained from *R. elaeocarpum* on iron/EDTA-induced lipid peroxidation in brain (A), liver (B), and kidney (C) of mice in vitro.Data are expressed as mean ± SEM (n=3). Asterisks represent the statistical difference in relation to the control group: (*) P<0.001; sharps represent the statistical difference in relation to the induced group: (#) P<0.05 and (# # #) P<0.001.

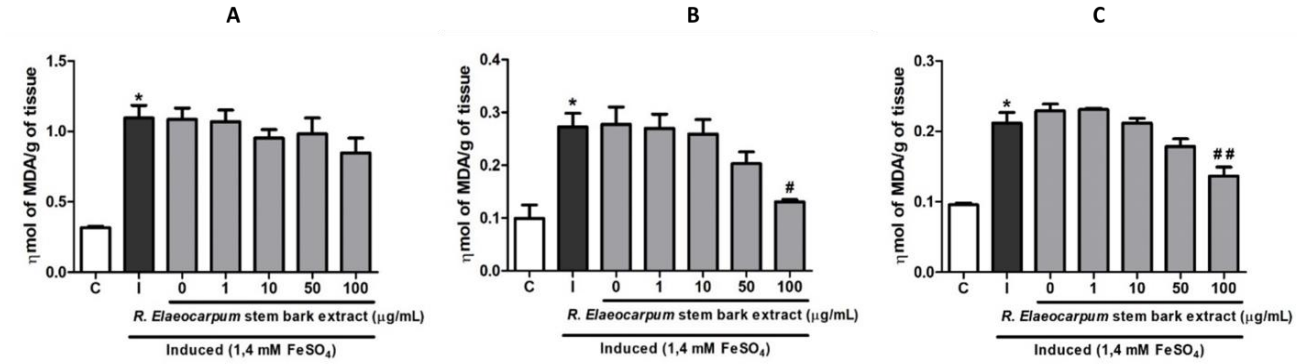

Figure 3. Effect of crude extract of stem bark obtained from *R. elaeocarpum* on iron/EDTA-induced lipid peroxidation in brain (A), liver (B), and kidney (C) of mice in vitro. Data are expressed as mean ± SEM (n=3). Asterisks represent the statistical difference in relation to the control group: (*) P<0.001; sharps represent the statistical difference in relation to the induced group: (#) P<0.05 and (# # #) P<0.001.

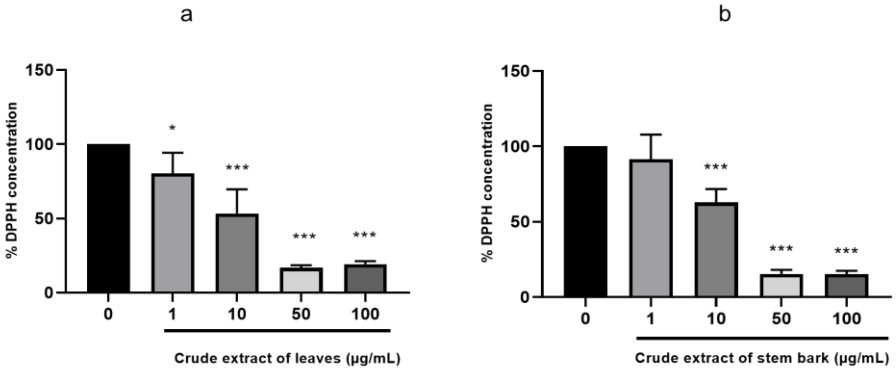

Figure 4. Effect of crude extract of leaves (a) and stem bark (b) obtained from *R. elaeocarpum* in assay DPPH in vitro. Data are expressed as mean ± SEM (n=6). Asterisks represent the statistical difference in relation to the control group: (*) P<0. 0,05 e (***) P<0,0001.

Conclusion

This study reports, for the first time, the identification of kaempferol, quercetin, stigmasterol, β-sitosterol, and glycosylated β-sitosterol in *R. elaeocarpum*. The leaves extract exhibited significant antioxidant activity, effectively inhibiting lipid peroxidation at concentrations of 10, 50, and 100 µg/mL, demonstrating superior results compared to the stem bark extracts. These findings support the traditional medicinal use of this species and highlight its potential as a source for the development of antioxidant therapies.

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