A high performance liquid chromatography with ultraviolet method for *Eschweilera nana* leaves and their anti-inflammatory and antioxidant activities

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ABSTRACT

Background: *Eschweilera nana* Miers is a tree widely distributed in Cerrado, Brazil. **Objective:** In this study, we aimed to describe its phytochemical properties and antioxidant and topical anti-inflammatory effects for the first time, as well validate an high performance liquid chromatography with ultraviolet/visible (HPLC-UV-Vis) method for the separation and quantification of the main components (hyperoside and rutin) in the hydroalcoholic extract of *E. nana* leaves. **Materials and Methods:** Structural identification of compounds in *E. nana* extract was performed by analysis of spectral data by ^{1}H nuclear magnetic resonance, ^{13}C nuclear magnetic resonance and/or ESI/EM. The HPLC-UV-Vis method was validated according International Conference on Harmonization (ICH) parameters. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) method were used for determination of *in vitro* antioxidant activities and the croton oil-induced inflammation for evaluation of *in vivo* anti-inflammatory effects. **Results:** Hyperoside, rutin, α-amirin, β-amirin, β-sitosterol, and stigmasterol were identified in the hydroalcoholic extract of *E. nana* leaves. HPLC-UV-Vis was validated according to ICH parameters. Furthermore, *in vitro* and *in vivo* assays demonstrated that the hydroalcoholic extract and methanol fraction showed significant antioxidant and topical anti-inflammatory effects, as they were able to reduce ear edema induced by croton-oil application. **Conclusions:** This research showed the first phytochemical study of *E. nana* extract and their biological activities may be associated with the presence of flavonoids in the extracts.

Key words: Antioxidant activity, *Eschweilera nana* Miers, flavonoids, phytochemical composition, topic anti-inflammatory

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INTRODUCTION

Eschweilera nana Miers is a member of the family Lecythidacea, and it is widely distributed in Cerrado, the region of Atlantic Forest and Amazonian, Brazil. It is popularly known as "ovo frito" referring to the aspect of the flower, however, there are regions that are also known as "tucari," "tucari-do-campo," "sapucaia" and "sapucainha." In traditional medicine, this species have been administered for colic and dysentery.[1,2]

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Phytochemical studies have identified triterpenes,[3,4] saponins,[4] active and inactive ellagic acid derivatives, flavonoids,[5] and sterols[3] as chemical constituents present in members of the genus *Eschweilera*. However, until the moment, there are no phytochemical studies and no evaluation of some pharmacological properties of *E. nana*.

So by virtue of shortage studies about this species, in this study, we performed a phytochemical study to identify the main active components of and evaluate the topical anti-inflammatory activity and antioxidant capacity of extracts of *E. nana* leaves and validate an High performance liquid chromatography with ultraviolet/visible (HPLC-UV-Vis) methodology for the separation and quantification of the main compounds of *E. nana* extract in order to ensure chemical integrity and hence its biological effects.

MATERIALS AND METHODS

Chemicals and reagents

Silica gel 60 (70–230 mesh), silica gel 60 (230–400 mesh), and silica gel plates F254 (0.25-mm thick) were obtained from Merck®. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals®. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox (purity ≥98%), l-ascorbic acid (purity ≥99%), rutin (purity ≥95%), hyperoside (purity ≥97%), croton oil and dexamethasone were obtained from Sigma-Aldrich®. Potassium persulfate P.A was obtained from Vetec®. All HPLC grade solvents were purchased from J. T. Baker®. Purified water, produced by a Milli-Q purification system, was used throughout the study.

Plant material

Eschweilera nana Miers leaves were collected from Nova Mutum, Mato Grosso, Brazil, in August 2007. The species was identified by Dra. Cássia Mônica Sakuragui from the Botanical Garden of Rio de Janeiro, and the voucher specimen was deposited in the Herbarium of the State University of Maringá, under the registration number HUEM - 13323.

Extraction and fractionation

Dried and milled leaves of *E. nana* (691.2 g) were extracted with ethanol: water (9:1 v/v), in the proportion of 10% (w/v), by maceration for dynamic stirring until exhaustion. The filtrate was concentrated in a vacuum evaporator at 40°C and then lyophilized to yield 132.63 g of *E. nana* dry extract (EE). EE (40.75 g) was used for chromatography in a column with silica gel 60 (70–230 mesh, ASTM) eluted with hexane, dichloromethane, dichloromethane: ethylacetate (1:1), ethyl acetate, and methanol, and 5 fractions were obtained: Hexane fraction (HF) (0.7 g), dichloromethane fraction (DF) (4.83 g), dichloromethane:ethyl acetate fraction (DEF) (2.51 g), ethyl acetate fraction (EF) (3.29 g), and methanol fraction (MF) (29.4 g), respectively. The fractions were analyzed by thin-layer chromatography, visualized with UV light, and developed using Godin reactive.

The DF (1.19 g) was purified on a silica gel 60 (70–230 mesh ASTM) eluted with hexane, hexane: dichloromethane (8:2; 1:1; 3:7), dichloromethane, dichloromethane:ethyl acetate (1:1), ethyl acetate: methanol (1:1), and methanol, to yield two subfractions, A (12.5 mg) and B (3 mg). The MF (0.99 g) was purified on a sephadex LH-20 gel eluted with methanol: acetone (1:1), and 65 subfractions were obtained. Fraction 34 was purified using adsorption chromatography under pressure on silica gel (230–400 mesh ASTM) eluted with ethyl acetate: methanol (7,5:2,5; 6:4; 1:1; 4:6), methanol: acetone (1:1), and methanol, to yield two subfractions, C (8.9 mg) and D (6.4 mg).

The chemical structure of the compounds in the subfractions were identified by ^{1}H nuclear magnetic resonance (^{1}H NMR; 300 MHz), ^{13}C nuclear magnetic resonance (^{13}C NMR; 75 MHz), both with Varian® Gemini 2000 BB spectrometers in $CDCl_3$, internal standard TMS (^{1}H NMR) and solvent signal (^{13}C NMR), and/or the mass spectrometry of ESI/EM, negative ion mode, Micromass® Quattro LC. The results were analyzed and compared with data from the literature. The compounds present in subfraction A were identified as a mixture of α-amirin and β-amirin, and in subfraction B, as a mixture of β-sitosterol and stigmasterol. In subfraction C and D, the compounds were identified as quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-rutinoside (rutin), respectively.

High performance liquid chromatography with ultraviolet/visible analysis

The chromatographic conditions were in accordance with the methodology described by Wang *et al*.[6] The analyses were performed using HPLC (Shimadzu® LC20AT) with a UV-Vis detector (Shimadzu® SPD-20A), C-18 column (250 × 4.60 mm i.d., 5 μm) (Phenomenex® Luna), and 100 RP-18/5 μm guard column (4.0 × 3.0 mm i.d., 5 μm) (Phenomenex®). The isocratic mobile phase consisted of a tetrahydrofuran/acetonitrile/0.05% phosphoric acid solution (20/3/77, v/v/v), with a flow rate of 1 mL/min. The analysis time was 25 min for the rutin and hyperoside standards and 35 min for EE. UV detection was performed at a wavelength of 360 nm, and the column temperature was maintained at 25°C. Data integration was done using LC-solution software (Shimadzu®). All samples were filtered through a membrane of 0.45-μm FH (Millipore®) before injection, which was performed using a 20-μL loop.

Method validation

The analytical method was evaluated using the parameters recommended by ICH.[7] Statistical analysis was performed using Microsoft Excel® (Microsoft Corporation) with a significance level of 5%, and the results were analyzed according to AOAC.[8] A mixture of rutin (50%) and hyperoside (50%) was used as the standard solution. All analyses were performed in triplicate.

Specificity

The methanolic solution of EE (1 mg/mL) was subjected to the UV absorption spectrum, in the range of 200-400 nm, by using HPLC (Waters® 600E) with a photodiode array (PDA) detector to verify if other compounds coeluted with rutin and hyperoside.

Linearity

The methanolic solution of the mixture of rutin and hyperoside standards was diluted to six solutions with concentrations of 1.25–50 mg/mL. Analysis of

variance (ANOVA) of the linear regression and testing of the validity and lack of fit of the analytical equation was determined by the Fisher method, with a significance level of 5%.

Limits of detection and quantification

The methanolic solution of the mixture of rutin and hyperoside standards was diluted to a series of appropriated concentrations with methanol, and an aliquot was used for HPLC analysis. Limits of detection (LD) and quantification (LQ) were determined at signal/noise (S/N) of 3 and 10, respectively.

Precision

A methanolic stock solution of EE was prepared and diluted to three concentrations (0.5, 1, and 1.5 mg/mL). The repeatability was evaluated in terms of concentration of rutin and hyperoside obtained at each level in a short period. After 7 days, the procedure was repeated for evaluation of the intermediate precision. Relative standard deviation (RSD) was calculated for repeatability and intermediate precision.

Accuracy

The recovery was evaluated by the standard addition method, adding the mixture of flavonoids in three different levels of known concentrations to the extractive solution of *E. nana,* before lyophilization. All concentrations were prepared independently in three replicates. The recovery data were determined according to recommendations of ICH.[7]

Robustness

Robustness was assessed by changing the HPLC-UV-Vis initially used (Shimadzu®) with Waters® 600 E and a PDA detector. The chromatographic profiles were visually compared, taking the retention time of the flavonoids.

In vitro antioxidant activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

Determination of EE was performed by capturing the $ABTS^{\bullet+}$ radical, according to Re *et al.*[9] with minor modifications. The ABTS radical cation ($ABTS^{\bullet}$+) was produced and diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm. The ethanolic solutions of EE, ascorbic acid, and Trolox were prepared at different concentrations. The sample solution (30 μL) was added to 3 mL of diluted $ABTS^{\bullet+}$ solution and allowed to react at room temperature in the dark. After 6 min, the absorbance values were measured at 734 nm by using the UV-Vis spectrophotometer (Shimadzu® UV1650) against a blank. Ascorbic acid and Trolox served as the positive control and standard, respectively. The activity results were expressed as Trolox equivalent antioxidant capacity (TEAC) units, which are calculated by dividing the gradient of the plot of percentage inhibition of absorbance versus the final concentration plot for the antioxidant in question by the gradient of plot for Trolox.

1,1-diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant activity of EE, MF, and subfractions C and D were performed using the stable DPPH radical, according to the procedure previously reported[10] with minor modifications. Methanolic solutions of the concentrations and ascorbic acid (positive control) were prepared and diluted in different concentrations. The methanolic solution of DPPH at 0.3 mM (1 mL) was added to the sample (2 mL) and kept in the dark for 30 min. The absorbance values were measured at 517 nm by using the UV-Vis spectrophotometer against a blank. The scavenging activity of the samples was expressed in IC_{50}, the concentration necessary to scavenge 50% of DPPH radicals.

Topical anti-inflammatory effect of *Eschweilera nana*

Animals

The experimental procedures were approved by the Ethics Committee of the State University of Maringá (protocol number 032/2007). The topical inflammation was established using male Swiss mice (weight: 25–30 g). The animals were housed at 22°C ± 2°C under a 12-h light/12-h dark cycle with free access to food and water.

Croton oil-induced ear edema in mice

Edema was induced by 20 μL of croton oil (200 μg) diluted in a solution of acetone: water (7:3) applied to the inner surface of both ears, according to the Van Arman method;[11] 20 μL of acetone: water (7:3) solutions of EE (5.0 mg/ear), MF (0.625; 1.25; 2.5 mg/ear), or dexamethasone (0.08 mg/ear) was applied to the inner surface of the left ear. On the right ear, only 20 μL of the vehicle (acetone: water 7:3) was applied (as a control). After 6 h, the animals were sacrificed, and each ear was perforated with a metal punch to provide a 6-mm-diameter disc. Edema (E) was assessed by E = (wc–wt); wc: the weight of the disc from the right control ear, wt: the weight of the disc from the left treated ear), thus determining the percentage of edema inhibition.

Statistics analysis

Data are presented as means ± standard error of the mean (SEM). Results were subjected to ANOVA and Tukey's *post-hoc* test, using Microsoft Excel® (Microsoft Corporation). $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of *Eschweilera nana* extract

Substances such as hyperoside, rutin, α-amirin, β-amirin, β-sitosterol, and stigmasterol were identified from the hydroalcoholic extract of *E. nana* leaves. Structural identification of these compounds was performed by analysis of spectral data by ^{1}H NMR, ^{13}C NMR, and/or ESI/EM, and by comparison with those previously reported in the literature.[3,12-16]

All these substances were already identified in other plant species,[14,16-21] however, this is the first report in the *E. nana* species.

In the *Eschweilera* genus, the triterpenes α-amirin and β-amirin, the sterols β-sitosterol and stigmasterol were isolated and identified from the leaves and bark of *E. longipes*, respectively[3] and the β-amirin also identified in the extract of leaves of *E. rabeliana*.[22] However, the identification of the flavonoids hyperoside and rutin were not reported in this genus at the present moment.

The chromatographic profile of EE [Figure 1a] by using HPLC-UV-Vis, showed the major presence of rutin and hyperoside, with retention times of 12.870 min and 18.851 min, respectively; this result was confirmed by comparison with respective reference patterns in the chromatogram and retention time. Other peaks could not be identified.

The quantitative analysis was performed, indicating that EE has 1.51% ± 0.01% (w/w) of rutin and 0.39% ± 0.01% (w/w) of hyperoside.

Taking into account that the flavonoids were associated with several pharmacological properties such as antioxidant,[23] anti-inflammatory,[24] antiviral, antimicrobial, antifungal,[25] vasodilator,[26] anticarcinogenic and cardioprotective,[27] the subsequent experiments used EE and/or the MF, since it showed a high yield mass (72.15% w/w) when compared with other fractions and because rutin and hyperoside were identified in this fraction.

Validation of high performance liquid chromatography with ultraviolet/visible method for rutin and hyperoside in *Eschweilera nana extract*

For quantitative analysis of rutin and hyperoside, validation of analytical methodology was performed according to the requirements of ICH.[7] Specificity was evaluated using a methanolic solution of EE. The markers rutin and hyperoside were subjected to an absorption spectrum by a PDA detector (Waters® 2998), and results show that the absorption spectra remained the same [Figure 1b and c], which indicated that no other compounds were quantified with these flavonoids.

Linearity was investigated by analyzing rutin and hyperoside simultaneously in six concentrations (1.25–50 mg/mL). The calibration curves showed an excellent correlation coefficient (r). To verify the validity of the linear regression and linear adjustment, the *F*-test was performed at 95%

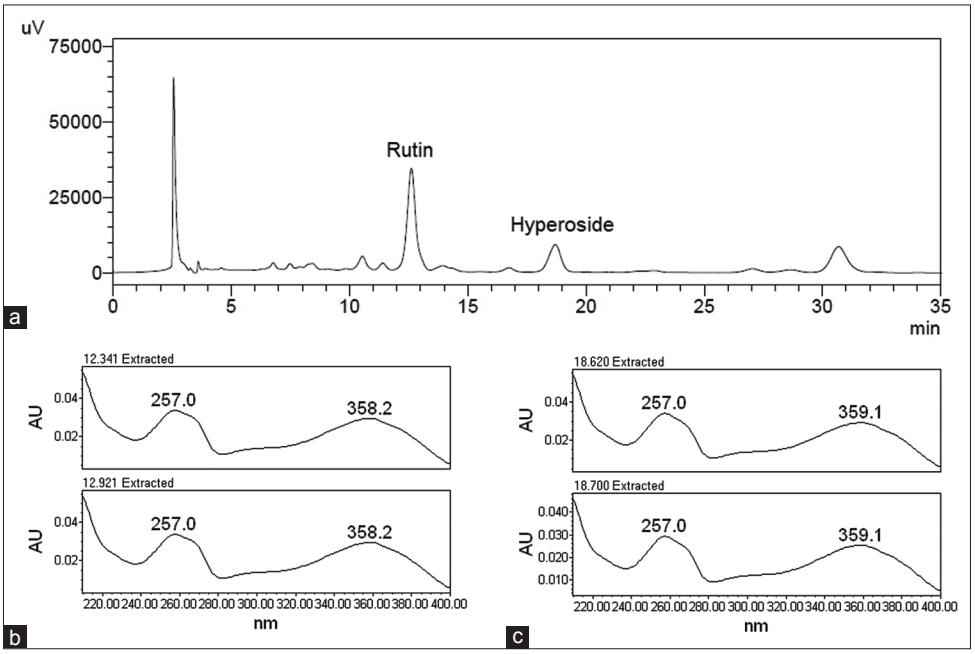

Figure 1: Chromatographic profile of hydroalcoholic extract of *Eschweilera nana* leaves (rutin: retention time of 12.870 min and hyperoside: retention time of 18.851 min) by High performance liquid chromatography with ultraviolet/visible method (a), and the absorption spectra by photodiode array detector of rutin (b) and hyperoside (c)

confidence level, and the results indicated that the slope was significant and the linear regression model did not show a lack of fit [Table 1].

The LD and LQ were determined by the S/N ratio, which was performed by comparing the measured signals of the sample at low concentrations with the blank sample; this established minimum detectable and quantifiable concentrations. The results in Table 1 indicate that the proposed method exhibits a good sensitivity for the quantification of these flavonoids in EE.

Precision was assessed in terms of repeatability and intermediate precision data at low, medium, and high concentrations and [Table 2] indicated that the method was precise regardless of the concentrations tested, since the RSD values obtained were lower than those established by AOAC,[8] which included data of estimated precision in function of the analyte concentration with an RSD up to 7.3% and 11% for 10 ppm (10 μg/mL) and 1 ppm (1 μg/mL) analyte concentrations, respectively.

Accuracy was evaluated using the standard addition method. According to AOAC[8] recovery values of 90–107% and 80–110% for analyte concentrations of 100 ppm (100 μg/mL) and 10 ppm (10 μg/mL), respectively, are acceptable. The results obtained [Table 3] show that the analytical method applied is satisfactory.

Table 1: Linearity, LD and LQ results of rutin and hyperoside determined by HPLC-UV-Vis method

	Rutin	Hyperoside
Linearity range (μg/mL)	1.25-50.0	1.25-50.0
Correlation coefficient (*r*)	0.9997	0.9997
Intercept ± SE	−7822.59±7241.60	−16594.13±8620.18
Slope ± SE	62425.68±310.78	87455.04±369.94
F calculated regression (F critical=4.20)	40349.00	55887.08
F calculated residue (F critical=2.78)	0.1206	0.3380
LD (ng/mL)	12.5	12.5
LQ (ng/mL)	25.0	25.0

LD: limits of detection; LQ: limits of quantification; HPLC-UV-Vis: high performance liquid chromatography with ultraviolet/visible; SE: standard error

Despite changing the analysis equipment, the chromatographic profiles of rutin and hyperoside methanolic solutions, obtained by HPLC, were similar, with little variation in retention time; this indicated that the analytical method was robust.

In vitro antioxidant activity

Among the chemical methods available to detect antioxidant capacity, ABTS and DPPH are the most popular because of their simplicity and speed.[28] The ABTS method measures the total antioxidant capacity of hydrophilic and lipophilic substances,[29] and thus was chosen initially to evaluate the activity of EE. Trolox, a water-soluble analogue of Vitamin E,[29] was used as the standard to report the results in TEAC and the higher this value, the greater the activity of the sample. The TEAC means obtained for EE and ascorbic acid (the positive control), were 0.77 ± 0.01 and 1.43 ± 0.05, respectively. When comparing these values, the antioxidant activity of EE does not seem high; however, compared with the TEAC values in several other studies,[30-32] this plant can be seen to possess significant activity.

After verification of the high antioxidant capacity of EE, the antiradical capacity of this extract, MF and subfractions C and D were determined using the DPPH method. The results obtained were compared with each other and the reference standard, ascorbic acid. The results were expressed in IC_{50} (the lower the value, greater the antioxidant capacity of the compound) as shown in Table 4. All samples presented antioxidant activity with significant differences at $P < 0.05$. EE, despite being a mixture of compounds, showed good antioxidant activity, with values close to the reference standard.

The results published previously[33] also demonstrate that EE has significant activity when compared with the IC_{50} values of the crude extracts of other species. The MF and subfractions C and D also presented good antioxidant potential, with IC_{50} values up to 17.06 μg/mL. Subfraction C showed better antioxidant activity than subfraction D, in which contained hyperoside and rutin, respectively.

Table 2: Repeatability and intermediate precision results of the HPLC-UV-Vis method

	Concentration of *Eschweilera nana* extract (mg/mL)	Repeatability		Intermediate precision	
		Concentration (μg/mL)*	RSD (%)	Concentration (μg/mL)*	RSD (%)
Rutin	0.5	6.65±0.03	0.41	6.73±0.09	1.31
	1.0	13.23±0.09	0.68	13.14±0.14	1.04
	1.5	21.98±0.32	1.47	22.34±0.48	2.14
Hyperoside	0.5	2.44±0.02	0.64	2.47±0.03	1.12
	1.0	4.81±0.10	2.13	4.86±0.09	1.76
	1.5	7.42±0.09	1.24	7.42±0.12	1.63

*Mean±SEM (*n*=3). HPLC-UV-Vis: high performance liquid chromatography with ultraviolet/visible; SEM: standard error of mean; RSD: relative standard deviation

Subfraction C also presented a higher capacity than the positive control, ascorbic acid.

According Seyoum *et al*.,[34] flavonoids and other polyphenols are great scavengers of free radicals because they easily donate hydrogen atoms due to the presence of OH grouping. Thus, the antioxidant activity present in EE, MF, and subfraction C and D was hypothesized to be due to the presence of flavonoids, including hyperoside and rutin, since they possess hydroxyl groups.

Topical anti-inflammatory effect of *Eschweilera nana*

Evaluation of the topical anti-inflammatory activity of EE and MF was performed using croton oil-induced inflammation, which increases phospholipase A_2 activity,[35] which results in the release of arachidonic acid and biosynthesis of prostaglandins and leukotrienes.[36,37]

The data [Figure 2] showed that EE at a dose of 5 mg/ear significantly inhibited swelling, probably due to the decrease of vascular permeability. Despite being a mixture of compounds, EE inhibited 45% of the edema in 6 h after application of the inflammatory agent. MF at doses of 0.625, 1.25, and 2.5 mg/ear also showed significant inhibition of inflammation [Figure 2], with mean percentages of 41, 52, and 67%, respectively. The positive control, dexamethasone, at a dose of 0.1 mg/ear presented with 93% edema inhibition.

Methanol fraction at a dose of 2.5 mg/ear showed a higher topical anti-inflammatory activity than the EE (an increase of around 49%), indicating that higher the concentration of polar compounds, higher the anti-inflammatory activity. Flavonoids have been correlated with the anti-inflammatory activity of many plant extracts and have been hypothesized to inhibit inflammatory mediators such as cyclooxygenase and/or lipoxygenase, which are involved in arachidonic acid release.[27,38] Furthermore, hyperoside and rutin, the major compounds in EE, demonstrated significant anti-inflammatory activity, which can be related by the inhibition of phospholipase A2 activity, which has an important role in the arachidonic acid cascade.[39-41]

Coutinho *et al*.[42] describes some of the structural factors that positively influencing anti-inflammatory activity of flavonoids, among them are the unsaturation in C-ring (positions 2–3), number and position of the OH groups, carbonyl group at C-4 (B-ring), and the absence glycosylation of the molecule. Most of these items are found in the structure of hyperoside and rutin, which explain the anti-inflammatory activity of them.

The results published previously[43] and obtained in this test indicate that EE and MF had satisfactory inhibitory activity against edema, probably due to the presence of flavonoids, including, the hyperoside and rutin that were identified, previously. The results do not explain the exact pharmacological mechanism involved; however, it may be related to the inhibition of different mediators of the inflammatory response, such as involved in arachidonic acid cascade.

Table 3: Accuracy results of the HPLC-UV-Vis method

	Theoretical amount (µg/mL)	Mean of determined amount ±SEM (µg/mL) (*n*=3)	Recovery ±RSD (%)
Rutin	10.0	9.99±0.05	99.95±0.45
	25.0	25.41±0.59	101.64±2.31
	50.0	50.01±0.48	100.00±0.95
Hyperoside	5.7	5.72±0.06	100.37±1.09
	20.7	21.02±0.46	101.53±2.18
	45.7	45.71±0.36	100.03±0.79

HPLC-UV-Vis: High performance liquid chromatography with ultraviolet/visible; SEM: standard error of the mean; RSD: relative standard deviation

Table 4: Comparison of IC_{50} value obtained of hydroalcoholic EE leaves, MF, subfraction C, subfraction D and ascorbic acid by DPPH method

	IC_{50} (µg/mL)
EE	10.87±0.03[a]
MF	12.63±0.13[c]
Subfraction C	7.38±0.09[d]
Subfraction D	17.06±1.22[e]
Ascorbic acid	9.62±0.01[f]

Values expressed in mean±SEM (*n*=3). Different subscript - One-way ANOVA *post-hoc* Tukey's test, specimens differ at $P<0.05$. EE: extract of *Eschweilera nana*; MF: methanol fraction; DPPH: 1,1-diphenyl-2-picrylhydrazyl; IC_{50}: inhibitory concentration 50%

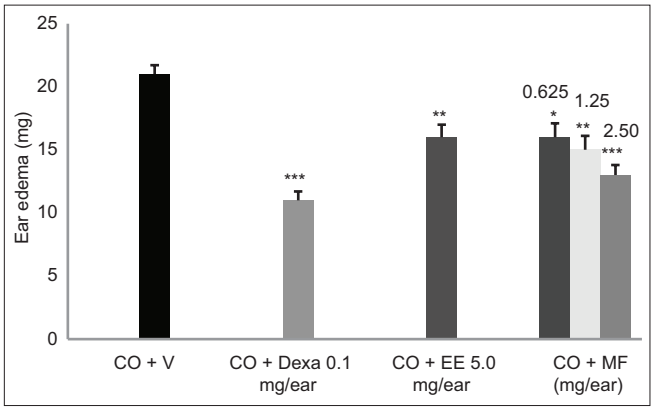

Figure 2: Effect of extract of *Eschweilera nana (EE)*, methanol fraction (MF) and dexamethasone (dexa) on edema of the ear induced by croton oil (CO). (*$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with the control group [CO + V] [analysis of variance, Tukey's *post hoc* test])

CONCLUSION

We identified hyperoside, rutin, α-amirin, β-amirin, β-sitosterol, and stigmasterol in EE, through the first

phytochemical study of this plant species. HPLC-UV-Vis for simultaneous detection and quantification of major compounds, hyperoside and rutin, in EE was validated. Furthermore, *in vitro* and *in vivo* assays demonstrated that EE and MF showed significant antioxidant and topical anti-inflammatory effects, possibly associated with flavonoids.

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