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Chemical composition, antioxidant activity, anti-inflammatory and neuroprotective effect of *Croton matourensis* Aubl. Leaves extracts obtained by supercritical CO_2

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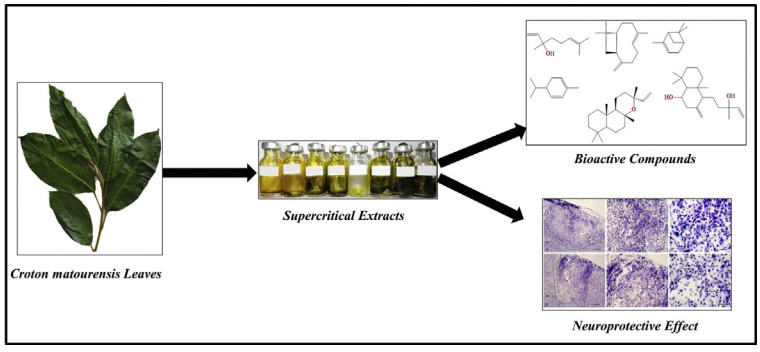
HIGHLIGHTS

- Larixol was found as major compound in SC–CO_2 extracts (24.88–50.57 %).
- The highest extraction yield with SC–CO_2 was at 50 °C/400 bar (5.60 ± 0.06 %).
- The extracts showed high TPC (51.81 ± 2.03–79.53 ± 1.19 mg GAE/g extract).
- All *Croton matourensis* leaves extracts exhibited antioxidant activity.
- SC–CO_2 extract presents anti-inflammatory and neuroprotective effect.

GRAPHICAL ABSTRACT



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ABSTRACT

Croton matourensis leaves extract was studied for its anti-inflammatory and neuroprotective effects after experimental stroke. The study investigated the extracts obtained with supercritical CO_2 (SC–CO_2) under different operational conditions of temperature (40 and 50 °C) and pressure (100–400 bar), assessing their global yield isotherms, chemical composition and antioxidant activity, in comparison to extracts obtained by hydrodistillation (HD) and *n*-hexane (HE). The highest yields were obtained with SC–CO_2 at 50 °C and 400 bar (5.60 ± 0.06 %) and in the HE (5.73 ± 0.26 %). The major compound found in HD and HE was linalool (35.26 and 69.98 %, respectively), whereas in SC–CO_2 extraction was larixol (24.88–50.57 %). The extracts showed high total phenolic contents, total flavonoids and antioxidant activity with maximum percent inhibition of DPPH radical of 83.26 ± 0.58 %. The treatment suggests a potential anti-inflammatory and neuroprotective effect, showing reduction in the injury of the animals treated with the SC–CO_2 extract.

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1. Introduction

Stroke is among the top 10 causes of death worldwide, generating life expectancy reduction in men and women. The damage is caused by the momentary rupture or obstruction of blood flow (ischemia) supplied to the brain, which may cause temporary or irreversible impairment of motor, cognitive and sensory abilities. In the ischemic stroke, the post-injury phase is a neuroinflammation, in which there is an infiltration of inflammatory blood cells followed by the activation of resident cells such as microglia/macrophages releasing pro-inflammatory mediators in the final stages, contributing to secondary damage [1–4]. The search for new neuroprotective agents in anti-inflammatory therapies from medicinal plants has been studied in recent years as an alternative to traditionally used drugs [3,5–7].

Croton matourensis Aubl. (synonym *Croton lanjouwensis* Jabl.), popularly known as "maravuvuia", "orelha de burro", "dima", or "sangra-d'água", has phytogeographic domain in the Brazilian Amazon and in some areas of Central America. In the folk medicine its use is reported in the treatment of infections, fractures and colds [8–11]. Although the genus *Croton* is widely studied, the *C. matourensis* species still has few manuscripts reporting its chemical composition and biological activities [9–13].

Compagnone et al. [9] reported as major compounds fenchol acetate, methyleneugenol, isoelemicine, elemicine, spatulenol and valencene in the essential oil of *C. matourensis* leaves collected in Venezuela. This essential oil presented a potential cytotoxic effect against colon carcinoma (LoVo) and cervical cancer (HeLa). In the essential oil from *C. matourensis* leaves collected in Manaus (Brazil) were reported as major compounds β-caryophyllene, thunbergol, cembrene, *p*-cymene, and β-elemene. This essential oil presented *in vitro* cytotoxicity and *in vivo* antitumoral effect in C.B-17 severe combined immunodeficient mice with HepG2 cell xenografts [10]. Gottlieb et al. [12] determined α-pinene, elemicin, p-cymene and felandrene as the major compounds of the essential oil from the tree bark collected in Pará (Brazil), whereas in the study conducted by Schneider et al. [13] a new seco-labdane diterpene called maravuic acid was isolated from the bark of the *C. matourensis* tree collected in Pará (Brazil) ((12*E*)-3,4-seco-labda-4(18),8(20),12,14-tetraen-3-oic acid) obtained by supercritical carbon dioxide ($SC{-}CO_2$) and then purified by high performance liquid chromatography (HPLC).

The use of supercritical technology has been reported in obtaining bioactive compounds as a promising technique due to its advantages over conventional methods, making it possible to obtain extracts free of toxic agents, which facilitate its applications in biologically active products. The most used supercritical fluid is CO_2 as it is environmentally friendly, inert, non-toxic, non-flammable, has low critical pressure and temperature points (73.7 bar and 31.1 °C) that avoid the degradation of thermosensitive compounds. Moreover, this technology enables an easy separation of the obtained product, reducing post-processing costs [14–16].

Thus, the aim of this study was to evaluate the process variables (temperature and pressure) in the *C. matourensis* leaves extraction with $SC{-}CO_2$, as well as to analyze the effects on the chemical characteristics of the extracts compared to the ones obtained by conventional methods (hydrodistillation and *n*-hexane extraction). Subsequently, it was evaluated the neuroprotective and anti-inflammatory effect of the extract obtained by the best operational condition with $SC{-}CO_2$.

2. Material and methods

2.1. Plant material

The *Croton matourensis* Aubl. leaves were collected in a natural regeneration area of the Degraded Areas Recovery Program (PRAD) of the mining company Paragominas S.A, located in the State of Pará (Brazil), on the Miltônia 3 plateau (02° 02′21.5″S – 020° 22′40 .3″W). Part of the sample was herborized to obtain the exsiccatae that had the HCUFRA6136 voucher as identification.

2.2. Preparation and characterization of the raw material

Leaves were dried in an air recirculation oven (model SL - 102, Solab, Brazil) at 50 °C for 48 h and were comminuted using a knife mill (model MA048, Marconi, Brazil). Then, the moisture content was determined by distillation with xylene (Ecibra, PA-ACS, Brazil) [17]. The granulometric determination was performed on standard Tyler series sieves ranging from 24–48 mesh, coupled to a sieve shaker (Bertel, Brazil). The average particle diameter was determined according to the method from American Society of Agricultural Engineers [18]. The real density of the particles (ρ_r) was determined at the Analytical Center of the Chemistry Institute of the University of Campinas (UNICAMP), by helium pycnometer (model Ultrapyc 1200e, Quantachrome, USA) in triplicate. The bed porosity (ε) was calculated based on the mathematical relation between the real and apparent densities.

2.3. Extraction methods

2.3.1. Hydrodistillation

Hydrodistillation extraction was performed in a Clevenger extractor. In this assay, 100 g of sample and 500 mL of distilled water were deposited in a 1000 mL round-bottomed flask for a period of 10,800 s. The flask was connected to the extraction system with cooling coming from a thermostatic bath (model Q215u2, Quimis, Brazil), and then it was placed in contact with a heating blanket (model Q321A29, Quimis, Brazil). After the extraction period, the sample was centrifuged (model Q-222T18, Quimis, Brazil) for 5 min at 2500 rpm, and then for more 300 s with anhydrous sodium sulfate (PA ACS Anidro, Alphatec, Brazil). The yield was determined on a dry basis using Eq. 1.

$$X_{0_{d.b}}(\%) = \left(\frac{m_e}{m_{sample} \times \left(1 - \frac{U_a}{100}\right)} \right) \times 100 \quad (1)$$

Where: $X_{0_{d.b}}$ is the overall yield of the extract on a dry basis (%), m_e is the extract mass in grams (g), m_{sample} is the sample mass used in grams (g), e U_a is the moisture content of the sample (%).

2.3.2. n-Hexane extraction

The extraction using *n*-hexane (95 %, Vetec, Brazil) was carried out according to the methodology described in the Brazilian Pharmacopoeia [19]. The solute/solvent mixture (1:10, w:w) was kept under reflux at the boiling temperature of the solvent in a Soxhlet apparatus for 10,800 s. After the extraction period, the solvent was evaporated using a rotary vacuum evaporator (Laborota 4000, Heidolph, Germany). The yield of the extract obtained on a dry basis was calculated according to Eq. 1.

2.3.3. Supercritical CO_2 extraction

Extractions using supercritical carbon dioxide ($SC{-}CO_2$) were carried out in the system Spe-ed^{TM}SFE (model 7071, Applied Separations, USA). The volume capacity of extractor vessel used was of 5.74×10^{-5} m^3 (height of 0.3248 m and inside diameter of 0.015 m). Isotherms were determined using 7 g of sample, two temperature values (40 and 50 °C) and four pressure values (100, 200, 300 and 400 bar). Extractions were performed in two periods: static (1800 s) and dynamic (7200 s). In the second period, the outflow of CO_2 was kept constant at 1.40×10^{-4} kg/s. The density of the $SC{-}CO_2$ was calculated using software developed by the National Institute of

Standards and Technology, which uses the state equation developed by Span and Wagner [20]. The calculation of the overall yield on dry basis was carried out according to Eq. 1.

2.4. Characterization of the extracts

2.4.1. Volatile compounds

The chemical composition of the extracts was evaluated according to the methodology reported by Gurgel et al. [21], using gas chromatography/mass spectrometry (QP-2010 plus system, Shimadzu, Japan) under the following conditions: silica capillary column Rtx-5MS (30 m ×0.25 mm, film thickness =0.25 μm), program temperature of 60–240 °C (3 °C/min), injector temperature of 250 °C, helium as drag gas (linear velocity of 32 cm/s, measured at 100 °C), and splitless injection (1 μL of a 2:1000 *n*-hexane solution). Ionization was obtained by the electronic impact technique at 70 eV; the temperature of the ion source and other parts was 200 °C. The compounds were quantified by gas chromatography using a flame ionization detector (FID) (Shimadzu, QP 2010 system) under the same conditions as GC/MS, except that nitrogen was used as the drag gas. The retention index was calculated for all the volatile constituents using a homologous series of *n*-alkanes (C_8–C_{20}). They were identified by comparison of their mass spectra and retention indices to those reported in the literature [22,23].

2.4.2. Total phenolic contents (TPC)

The total phenolic contents was determined according to the methodology described by Singleton et al. [24] and Georgé et al. [25]. For the analysis, it was used 500 μL of the extract diluted in ethanol (95 %, Vetec, Brazil) at 7% (v:v), 2500 μL of Folin-Ciocalteau (Tedia, Brazil) at 10 % (v:v) and 2000 μL of sodium carbonate solution (99.5 %, Vetec, Brazil) at 7.5 % (w:v). After 60 min reaction at room temperature in dark conditions, absorbance was measured at 760 nm using an ultraviolet-visible spectrophotometer (model Evolution 60, Thermo Scientific, USA). Quantification was performed using gallic acid (98.0 %, Vetec, Brazil) as standard for construction of the calibration curve at concentrations of 1, 5, 10, 20, 40, 60, 80 and 100 mg/L. From the line equation, the total phenolic contents were calculated, and the result was expressed in mg of gallic acid equivalent per gram of extract on a dry basis (mg GAE/g extract). The analysis was performed in triplicate.

2.4.3. Total flavonoids (TF)

The analysis of total flavonoids was performed according to the methodology described by Dowd [26] and Meda et al. [27]. For the analysis, 1000 μL of extract diluted in ethanol (95 %, Vetec, Brazil) was added in 1000 μL of ethanolic solution with 2% (w:v) of aluminum chloride (99.0 %, Fluka, Germany). The reading was performed after 10 min reaction at 425 nm on an ultraviolet-visible spectrophotometer (model Evolution 60, Thermo Scientific, USA). Quantification was performed using quercetin (purity ≥ 95.0 %, Sigma-Aldrich, Brazil) to construct the analytical curve at concentrations of 0.5, 1, 5, 10, 15, 20, 25, 30 and 35 mg/L. From the line equation, the calculation was performed, and the result was expressed in milligrams of quercetin equivalent per gram of extract on a dry basis (mg QE/g extract).

2.4.4. Antioxidant activity (AA)

The antioxidant activity was determined by capturing the free radical 2,2-diphenyl-1-picrylhydrazi (DPPH$^{\bullet}$), according to the methodology described by Brand-Williams et al. [28] and modified by Sánchez-Moreno et al. [29]. The samples were diluted in ethanol (99 %, Vetec, Brazil) at concentrations of 8, 10, 12 and 14 mg/mL. Then, 0.1 mL solution of each concentration was added in 3.9 mL of ethanolic solution containing the DPPH$^{\bullet}$ radical (60 μM). The solutions were kept at room temperature and in dark conditions until the stabilization period t_{IC50}, necessary for the sample to reduce 50 % from the initial quantity of DPPH$^{\bullet}$ radical. After this reaction period, the samples were read at 515 nm on an ultraviolet-visible spectrophotometer (model Evolution 60S, Thermo Scientific). The construction of the analytical curve was performed at concentrations of 10–60 μM of the DPPH$^{\bullet}$ radical. Assays were performed in triplicate and calculation of the inhibitory concentration of the capture of free radicals by DPPH$^{\bullet}$ (IC_{50}) was expressed in gram of extract per gram of DPPH$^{\bullet}$ in dry basis (g extract/g DPPH). The result was also expressed by calculating the scavenging activity in terms of percent inhibition using Eq. 2.

$$\text{Inhibition (\%)} = \left(\frac{A_{DPPH} - A_S}{A_{DPPH}}\right) \times 100 \quad (2)$$

Where: A_{DPPH} was the absorbance of control and A_S was the absorbance of test sample.

2.5. Statistical analysis

The essays were performed in triplicate. Thus, the experimental results of mass extraction yield, total phenolic compounds, total flavonoids and antioxidant activity, obtained in the different extraction techniques, had their means and standard deviations calculated. Then, the data were statistically evaluated through analysis of variance with Tukey's post-hoc test with 95 % confidence level, using the STATISTIC 7.0 software (StatSoft, Inc., Oklahoma, USA).

2.6. Experimental model of focal ischemia using Croton matourensis extract obtained by supercritical CO_2

2.6.1. Animals and surgical procedures

Six adult male Wistar rats weighing approximately 0.25 kg were used. Three control animals and three treated animals got focal ischemic injury in the motor cortex. The animals were accommodated in cages containing three animals, each one being properly supplied with water and food. The experimental procedures were performed in accordance with the guidelines established by the Ethics Committee for Research on Animal Experimentation of the Federal University of Pará (CEPAE-UFPA 137−13).

The animals were anesthetized with ketamine hydrochloride (Vetanarcol®, Köning 72 mg/kg) and xylazine hydrochloride (Kensol®, Köning 9 mg/kg) through intraperitoneal injection. After confirming the loss of reflexes, the rats were placed in a stereotactic device. An incision of approximately 1.5 cm was made in the skin of the upper region of the animal's head to expose the skull. Then, the coordinates were removed: 1.2 antero-posterior, 2.5 mid-lateral, 0.5 dorsoventral, and it was injected 80 pmol of endothelin-1 (ET-1). The cranial opening was done with a surgical drill and the incision was closed with non-absorbable cotton suture [30–32].

After surgery, the animals were accommodated in cages supplied with water and food, and then they were kept in a room with controlled temperature at 24 °C during the seven days related to the survival time. After this period, the animals were again anesthetized by intraperitoneal injection and perfused with 0.9 % heparinized saline solution followed by 4 % paraformaldehyde.

2.6.2. Microtomy and immunohistochemical analysis

The brains of the control animals and the brains of the animals treated were removed and cryoprotected in different concentrations of sucrose solution, and then sections with 30 μm thickness were obtained with the aid of a cryostat (CM 1850, Leica). The injury area and the infiltration of inflammatory cells were identified by the violet cresyl technique [2,3].

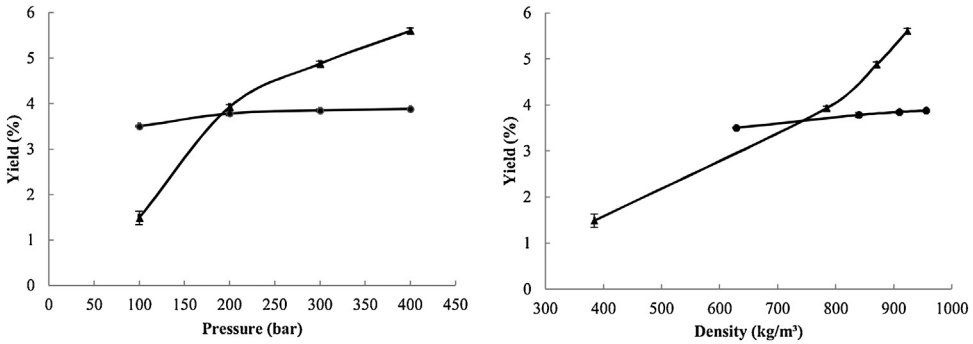

Fig. 1. Overall yield isotherms of *Croton matourensis* leaves, (●) 40 °C and (▲) 50 °C.

2.6.3. Treatment with C. matourensis *extract*

During the seven days related to the survival time, a daily dose of 100 mg/kg of the extract of *C. matourensis* leaves obtained at 50 °C and 400 bar with SC–CO_2 was administered intraperitoneally in the treated group immediately after ET–1 injection. The control group consisted of injured animals that were treated with saline solution with 5 % tween. The extract obtained with supercritical CO_2 was selected to this study because it showed a significant extraction yield, in addition to a composition with a high content of oxygenated diterpenoids (larixol and manool oxide)

3. Results and discussion

3.1. Raw material characterization

The *Croton matourensis* Aubl. leaves presented a moisture content of 10.80 ± 0.13 %, particle size of 3.17×10^{-4} m, particle real density of 1250 ± 10 kg/m^3, apparent density of 2882 kg/m^3 and bed porosity equal to 0.7694.

3.2. Extraction yields by hydrodistillation, n-hexane and supercritical CO_2

Hydrodistillation extraction showed the lowest extraction yield (1.01 ± 0.01 %), while extraction with *n*-hexane (5.73 ± 0.26 %) and extraction with SC–CO_2 at 50 °C and 400 bar (5.60 ± 0.06 %) showed higher yields and were statistically equal. Compagnone et al. [9] carried out the hydrodistillation of *Croton matourensis* leaves and *C. micans* flowers and leaves where it was obtained yields of 1.0 %, 0.13 % and 0.25 %, respectively. Same yield of essential oil from *C. matourensis* leaves was obtained in this study. In the work of Sousa et al. [33] it was done extractions with SC–CO_2, steam distillation and ethanol from *C. zehntneri* leaves, the authors obtained yields of 3.80, 3.06 and 2.68 %, respectively, lower than those obtained in this study by the method of extraction with SC–CO_2 and *n*-hexane. In general, hydrodistillation can present some disadvantages, such as low yield due to the boiling point of the mixture being lower than the boiling point of water and essential oil, occurrence of chemical changes and loss of the most volatile compounds due to the unsaturation thermal degradation or hydrolysis, in addition to a long extraction period [34–36]. Despite the extraction with organic solvent, such as *n*-hexane, often present high yields, it also has some disadvantages such as carrying compounds of greater polarity as chlorophyll, long extraction periods, contamination of the extract by toxic waste of the organic solvent resulting in a less pure product than that obtained by green technologies, such as supercritical extraction [7,15,16].

Fig. 1 shows the overall yield isotherms for *C. matourensis* leaves extracted with SC–CO_2. The yield ranged from 1.49 ± 0.15 %–5.60 ± 0.06 %. The operational condition that showed the lowest yield was 50 °C and 100 bar with a density of 384.33 kg/m^3, while the condition that obtained the highest yield was the one that operated at 50 °C and 400 bar with a density of 923.32 kg/m^3. It can be observed that near to the pressure of 200 bar, the isotherms showed an inflection point, occurring the phenomenon known as retrograde condensation in which the solute vapor pressure prevails with a strong influence of the temperature and pressure on density. Thus, in an isobaric system, below the inflection point, the increase in temperature causes a reduction in the solvation power of the fluid due to the decrease in density. However, with pressure above the inflection point, the increase in temperature can lead to an increase in the efficiency of the extraction even with the reduction of the fluid density, since the vapor pressure of the solute is increased [5,34,37].

3.3. Volatile compounds

Table 1 shows the chemical composition of *Croton matourensis* leaves extracts obtained by the methods of hydrodistillation, *n*-hexane extraction and SC–CO_2 extraction under different operational conditions. Fifty-eight constituents were identified in hydrodistillation, five in *n*-hexane extraction and twenty-eight in SC–CO_2 extraction. The compounds were distributed in the following classes: monoterpenes hydrocarbons, oxygenated monoterpenes, benzenoid, phenylpropanoids, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpenoid hydrocarbons, oxygenated diterpenoid, fatty acid esters, androgens and derivatives, acyclic isoprenoid, and n-alkane.

The extract obtained by hydrodistillation presented in its composition 37.52 % of oxygenated monoterpenes, 27.88 % of monoterpenes hydrocarbons, 27.46 % of sesquiterpene hydrocarbons, 6.23 % of oxygenated sesquiterpenes, 0.45 % of phenylpropanoids, 0.19 % of oxygenated diterpenoid and 0.12 % of benzenoid. The major constituents were linalool (35.26 %), *E*-caryophyllene (9.94 %), α-pinene (8.82 %) and α-phellandrene (6.20 %). Other minority constituents have also been identified as α-humulene (3.56 %), terpinolene (3.09 %), limonene (2.94 %), γ-terpinene (2.80 %), germacrene D (2.60 %) and *p*-cymene (1.98 %). Nine compounds showed concentrations below 0.10 %. The extract showed a chemical composition slightly different from that reported by other authors who also worked with the extract obtained by hydrodistillation of *C. matourensis* leaves. In the study of De Lima et al. [10] were identified as major compounds: β-caryophyllene (12.41 ± 1.02 %), thunbergol (11.74 ± 1.11 %), cembrene (7.12 ± 0.55 %), *p*-cymene (5.05 ± 0.49 %), and β-elemene (4.94 ± 0.35 %); Compagnone et al. [9] obtained as major compounds fenchyl acetate (19.5 %), methyleugenol (14.2 %), isoelemicine (11.3 %), elemicine (7.6 %), spathulenol (6.9 %) and valencene (5.8 %); and Leão et al. [11] identified α-pinene (26.6 %) and α-phellandrene (8.5 %). The differences in chemical composition found in the present work in

Table 1
Comparative chemical composition of *Croton matourensis* leaves extracts obtained by hydrodistillation (HD), *n*-hexane extraction (HE) and different conditions of supercritical carbon dioxide extraction (SC–CO_2).

RI[a]	Compounds			Concentrations (% Area)[2]							
		HD	HE	SC-CO_2							
				40°C				50 °C			
				100 bar	200 bar	300 bar	400 bar	100 bar	200 bar	300 bar	400 bar
	Monoterpenes hydrocarbons										
926	α-Thujene	1.08									
933	α-Pinene	8.82									
1006	α-Phellandrene	6.20									
1017	α-Terpinene	0.97									
1024	*p*-Cymene	1.98									
1029	Limonene	2.94									
1058	γ-Terpinene	2.80									
1089	Terpinolene	3.09									
	Oxygenated monoterpenes										
1099	Linalool	35.26	69.98	0.45		0.25				3.07	8.49
1178	Terpinen-4-ol	0.67									
1183	Naphthalene	0.23									
1192	α-Terpineol	1.03									
	Benzenoid										
1174	1,3-Diisopropylbenzene		6.70								
1185	*m*-Cymen-8-ol	0.12									
1235	Thymol methyl ether										0.51
	Phenylpropanoids										
1288	Safrole										1.11
1358	Eugenol	0.38									
1522	Myristicin			0.98			0.46			0.78	
1603	methoxy-Eugenol			1.97	1.37	0.77	1.14	1.67	1.47	2.08	2.01
	Sesquiterpene hydrocarbons										
1340	δ-Elemene	0.36									
1352	α-Cubebene	0.14									
1379	α-Copaene	1.12									
1388	β-Bourbonene	0.18									
1395	β-Elemene	1.34									
1423	*E*-Caryophyllene	9.94	6.53	1.80	0.36	0.63			1.18	3.55	6.58
1433	β-Copaene	0.27									
1437	γ-Elemene	0.32									
1453	Spirolepechinene	0.13									
1457	α-Humulene	3.56		0.21						0.70	1.32
1479	γ-Muurolene	1.16								0.24	
1485	Germacrene D	2.60		0.40						0.61	1.54
1492	β-Selinene	0.88									
1496	γ-Amorphene	0.11									
1500	α-Selinene	1.51									
1504	α-Muurolene	0.32									
1512	β-Bisabolene	0.77									0.50
1519	γ-Cadinene	0.70									
1526	δ-Cadinene	1.70		0.49		0.11				0.65	1.02
1545	*E*-α-Bisabolene	0.15									
	Oxygenated sesquiterpenes										
1552	Elemol	0.57		0.64	0.41	0.19		0.72	0.38	0.54	0.38
1581	Spathulenol	0.53		0.65	0.39	0.22			0.30	0.71	0.90
1587	Caryophyllene oxide	1.25		1.45	0.88	0.61	0.77		0.94	1.67	2.41
1597	Viridiflorol	0.47									
1609	Ledol	0.14									
1615	Humulene epoxide II	0.23									
1620	110-di-epi-Cubenol	0.21									
1634	Eremoligenol	0.37								0.18	
1637	γ-Eudesmol	0.32									
1646	α-Muurolol	0.25									
1651	*epi*-α-Muurolol	0.12									
1655	β-Eudesmol	0.60		1.20	0.75	0.38	0.57	1.05	0.73	1.10	1.39
1658	α-Eudesmol	1.06		2.11	1.36	0.70	1.19	1.85	1.34	1.88	2.39
1676	Germacra-4(15),510(14)-trien-1-α-ol	0.11									
1818	Cryptomeridiol							0.68			
	Diterpenoid hydrocarbons										
1926	Cembrene			0.47		11.22	13.95			0.63	
	Oxygenated diterpenoid										
2024	Manool oxide	0.19		19.60	17.87	10.25	17.31	26.72	16.79	16.44	17.49
2114	Phytol			2.72	2.76	1.57	2.05	7.47	2.29	2.64	2.43
2277	Larixol		2.93	50.57	43.06	24.88	33.81	49.96	42.91	39.51	48.49
2301	3-α-hydroxy-Manool			1.75	7.09	0.45	1.12	1.47	0.94	1.13	
	Fatty acid ester										
2095	Methyl linoleate						4.50	3.11			
	Androgens and derivatives										

Table 1 (*Continued*)

RI[a]	Compounds	HD	HE	Concentrations (% Area)[2]							
				SC-CO_2							
				40 °C				50 °C			
				100 bar	200 bar	300 bar	400 bar	100 bar	200 bar	300 bar	400 bar
2114	3-α,5-β,17-β-Androstane-317-diol			3.75		0.87	3.74	1.83		1.92	1.04
	Acyclic isoprenoid										
2327	491,317-Tetramethyl-481,216-octadecatetraenal					22.99			14.22	12.38	
	n-Alkane										
2400	Tetracosane		8.84	7.36	18.35	18.81	15.26	2.26	9.08	6.81	
	No identified	0.15	5.02	1.43	5.35	5.10	4.13	1.21	7.43	0.78	
	Other Compounds	0.60									
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[a] Retention index (DB-5 ms column); [2] Concentrações expressas em percentuais relativo área (%Area).

relation to other literatures may be related to extrinsic and intrinsic factors that may include edaphoclimatic factors, geographic location of the material collection, altitude, period of collection, part of the plant studied, preparation of raw material, extraction method used, chemotypes, genetic factors, among others [9,38–43].

The extract obtained with *n*-hexane presented linalool as the major constituent (69.98 %), in addition to other constituents such as tetracosane (8.84 %), 1,3-diisopropylbenzene (6.70 %), *E*-caryophyllene (6.53 %) and larixol (2.93 %). The chemical composition identified in this study was different from other studies that evaluated the hexanic extraction of *Croton* species. Motta et al. [44], in their study of *C. macrobothrys* leaves, determined steroid β-sitosterol (15.4 %) and the triterpenoid β-amyrin (11.0 %) as the major constituents of the extract. In the study of Dey et al. [45] the hexane semi-purified extract of *C. caudatus* leaf showed a positive result for the presence of terpenoids through the Salkowski's assay. Palmeira Jr. et al. [46] studied neutral fractions from hexane extract obtained from the leaves, stems and roots of *C. sellowii*, where they identified as major compounds of the leaves caryophyllene oxide (46.8 %) and *trans*-caryophyllene (40.8 %), in the stems caryophyllene oxide (26.5 %) and cubenol (16.7 %), and in the roots mesitylene (15.2 %), and the sesquiterpenes γ-(7.6 %), α-(6.9 %) and β-eudesmol (5.5 %).

The main constituents identified in the extraction with SC−CO_2 were larixol (24.88–50.57%), manool oxide (10.25–26.72%), 491,317-tetramethyl-481,216-octadecatetraenal (12.38–22.99 %), tetracosane (2.26–18.81 %) and cembrene (0.47–13.95%). Other minority compounds have also been identified, such as linalool under conditions of 40 °C/100 bar (0.45 %), 40 °C/300 bar (0.25 %), 50 °C/300 bar (3.07 %) and 50 °C/400 bar (8.49 %); methyl linoleate identified under conditions of 40 °C/400 bar (4.50 %) and 50 °C/100 bar (3.11 %); phytol was identified in all extraction conditions with concentration ranging from 1.57 to 7.47 %; *E*-caryophyllene (0.36–6.58%); caryophyllene oxide (0.61–2.41%); α-eudesmol (0.70–2.39%); 3-α,5-β,17-β-Androstane-317-diol (0.87–3.75%); and methoxy-eugenol (0.77–2.08 %).

The extracts obtained with SC−CO_2 showed a predominant composition in oxygenated diterpenoids (37.15–85.62%). Schneider et al. [13] also reported in their study the identification of a diterpenoid called maravuic acid ((12*E*)-3,4-seco-labda-4(18),8(20),1214-tetraen-3-oic acid) from extraction with SC−CO_2 and subsequent isolation by HPLC of *C. matourensis* bark. Larixol and manool oxide (second most concentrated compound identified) belong to the class of labdane diterpenes that have industrial, medicinal, pharmacological, and nutraceutical applications [47–51].

It can be seen that the conventional extraction methods through hydrodistillation and extraction with solvent *n*-hexane were more selective for obtaining linalool, which is an oxygenated monoterpene, while SC−CO_2 was more selective for oxygenated diterpenoids such as manool oxide and larixol. This can be associated with the used solvent and the process parameters such as temperature and pressure. Fig. 2 shows the main compounds identified in the different extraction methods.

3.4. Total phenolic contents (TPC)

The TPC results of the extracts from *Croton matourensis* leaves obtained by different methods are presented in Table 2. The extracts showed high TPC content, ranging from 51.81 ± 2.03–79.53 ± 1.19 mg GAE/g extract. The extract obtained with *n*-hexane showed the lowest content (51.81 ± 03.02 mg GAE/g extract), while the extract obtained from the hydrodistillation showed the highest content (79.53 ± 01.19 mg GAE/g extract). Thus, it can be inferred that for the extraction of TPC the solvent polarity index influenced the achievement of a higher content. This result can be explained by the fact that phenolic compounds have, in general, a polar character. However, due to the wide polarity range that these compounds present, other solvents such as *n*-hexane and SC−CO_2 can also be used to solubilize other groups [52,53].

In SC−CO_2 extraction, the extract obtained at 50 °C, 100 bar and density of 384.33 kg/m^3 was the one with the lowest TPC value (58.68 ± 1.99 mg GAE/g extract), while in the conditions of 40 °C, 400 bar and density of 956.07 kg/m^3 and 50 °C, 400 bar and 923.32 kg/m^3 showed higher contents (69.29 ± 0.76 and 67.66 ± 1.65 mg GAE/g extract, respectively) with no statistically significant difference between these two conditions ($p > 0.05$). The results obtained in this work were slightly similar to those obtained by Dutta et al. [54] who studied the hexane extract of *C. bonplandianus* stem and obtained a TPC value of 67.37 ± 0.46 mg GAE/g extract. Dutta et al. [55] who studied the hexane extract of *C. bonplandianus* leaves obtained a value equal to 75.29 ± 3.19 mg GAE/g extract, which are values much higher than the hydroalcoholic extracts (80 % methanol) from leaves of nine Argentinian species of *Croton* (*C. andinus*, *C. argentinus*, *C. catamarcensis*, *C. cordobensis*, *C. curiosus*, *C. lachnostachyus*, *C. lanatus*, *C. saltensis*, and *C. serratifolius*) that ranged from 1.71 to 4.85 mg EAG/g extract [56].

3.5. Total flavonoids (TF)

The TF concentration of *Croton matourensis* leaves extracts obtained by different extraction methods is shown in Table 2. The concentration ranged from 2.50 ± 0.20–6.89 ± 0.45 mg QE/g extract, where the highest values were obtained by the methods of hydrodistillation (6.89 ± 0.45 mg QE/g extract) and extraction with SC−CO_2 (6.53 ± 0.39 mg QE/g extract), which showed no statistically significant difference between them ($p > 0.05$). The results obtained in this study were superior to the extract of *C. bonplandianum* leaves (4.36 ± 0.48 mg/g) [57], to the extract of *C. bonplandianus* stem (3.86 ± 0.12 mg/g) [54], and to the

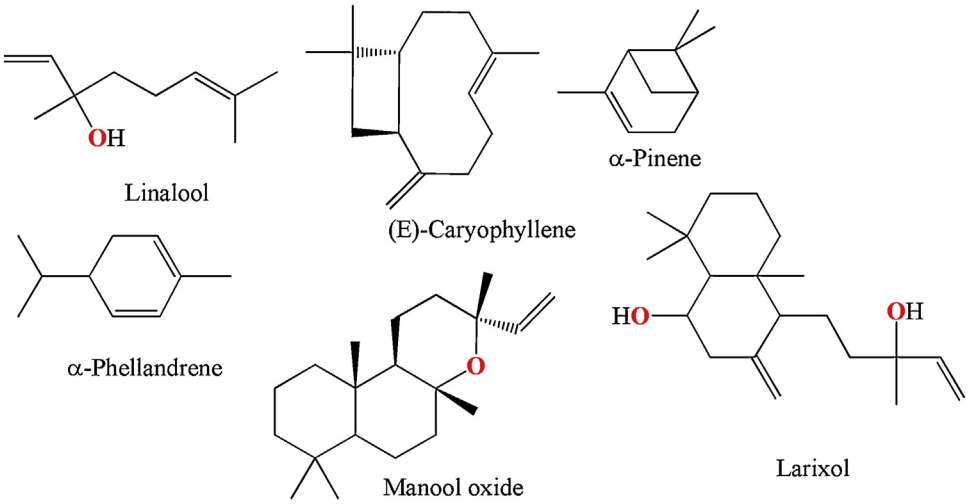

Fig. 2. Chemical structures of the main compounds identified in *Croton matourensis* leaves extracts obtained by hydrodistillation, *n*-hexane extraction and supercritical carbon dioxide extraction.

Table 2
Total phenolic contents (TPC), total flavonoids (TF) and antioxidant activity (AA) of *Croton matourensis* leaves extracts obtained by hydrodistillation (HD), *n*-hexane extraction (HE) and different conditions of supercritical carbon dioxide extraction (SC−CO_2).

Extraction method	Solvent polarity index[a]/ CO_2 density[2] (kg/m^3)	TPC (mg GAE/g extract)	TF (mg QE/g extract)	AA		
				Concentration (mg/mL)	**Inhibition %**	**IC_{50} (g extract/g DPPH)**
HD	9	79.53 ± 1.19^{e}	6.89 ± 0.45^{f}	8	25.76 ± 1.28	1531.34 ± 146.02^{c}
				10	30.41 ± 0.99	
				12	34.76 ± 0.13	
				14	38.90 ± 0.95	
HE	0	51.81 ± 2.03^{a}	5.11 ± 0.57^{c}	8	15.05 ± 0.06	2680.11 ± 109.14^{d}
				10	16.78 ± 1.67	
				12	21.92 ± 0.93	
				14	22.42 ± 0.19	
SC-CO_2						
40 °C/100 bar	628.61	66.51 ± 0.76^{cd}	6.53 ± 0.39^{ef}	8	52.20 ± 0.68	614.73 ± 25.75^{a}
				10	63.04 ± 1.22	
				12	72.20 ± 1.20	
				14	83.26 ± 0.58	
40 °C/200 bar	839.10	66.27 ± 0.80^{cd}	2.50 ± 0.20^{a}	8	42.82 ± 0.09	$763.91 \pm 60.39^{a,b}$
				10	54.24 ± 2.63	
				12	62.66 ± 3.71	
				14	72.97 ± 2.71	
40 °C/300 bar	909.89	65.66 ± 1.75^{cd}	5.70 ± 0.11^{ce}	8	39.19 ± 2.06	$836.06 \pm 12.39^{a,b}$
				10	49.72 ± 2.35	
				12	57.37 ± 2.81	
				14	63.83 ± 0.39	
40 °C/400 bar	956.07	69.29 ± 0.76^{d}	3.18 ± 0.13^{ab}	8	35.04 ± 4.11	$1002.46 \pm 49.23^{a,b}$
				10	42.73 ± 4.60	
				12	49.18 ± 5.57	
				14	53.74 ± 0.19	
50 °C/100 bar	384.33	58.68 ± 1.99^{b}	2.94 ± 0.29^{ab}	8	49.45 ± 6.83	629.83 ± 143.30^{a}
				10	62.20 ± 13.90	
				12	70.66 ± 10.65	
				14	80.13 ± 5.99	
50 °C/200 bar	784.29	65.01 ± 0.76^{c}	5.22 ± 0.22^{c}	8	39.70 ± 0.14	$825.49 \pm 28.57^{a,b}$
				10	50.29 ± 1.11	
				12	58.23 ± 0.25	
				14	68.86 ± 0.35	
50 °C/300 bar	870.43	60.22 ± 1.18^{b}	3.68 ± 0.06^{bd}	8	39.28 ± 1.75	840.80 ± 4.29^{b}
				10	48.11 ± 2.23	
				12	57.03 ± 3.04	
				14	66.66 ± 3.46	
50 °C/400 bar	923.32	67.66 ± 1.65^{cd}	4.09 ± 0.22^{d}	8	31.77 ± 0.25	1065.55 ± 4.32^{b}
				10	38.16 ± 0.91	
				12	47.12 ± 1.68	
				14	52.99 ± 2.71	

Mean ± standard deviation, different letters indicate significant difference between values ($p < 0.05$) by Tukey test.
[a] According to [53]; [2] As described in topic *2.3.3*.

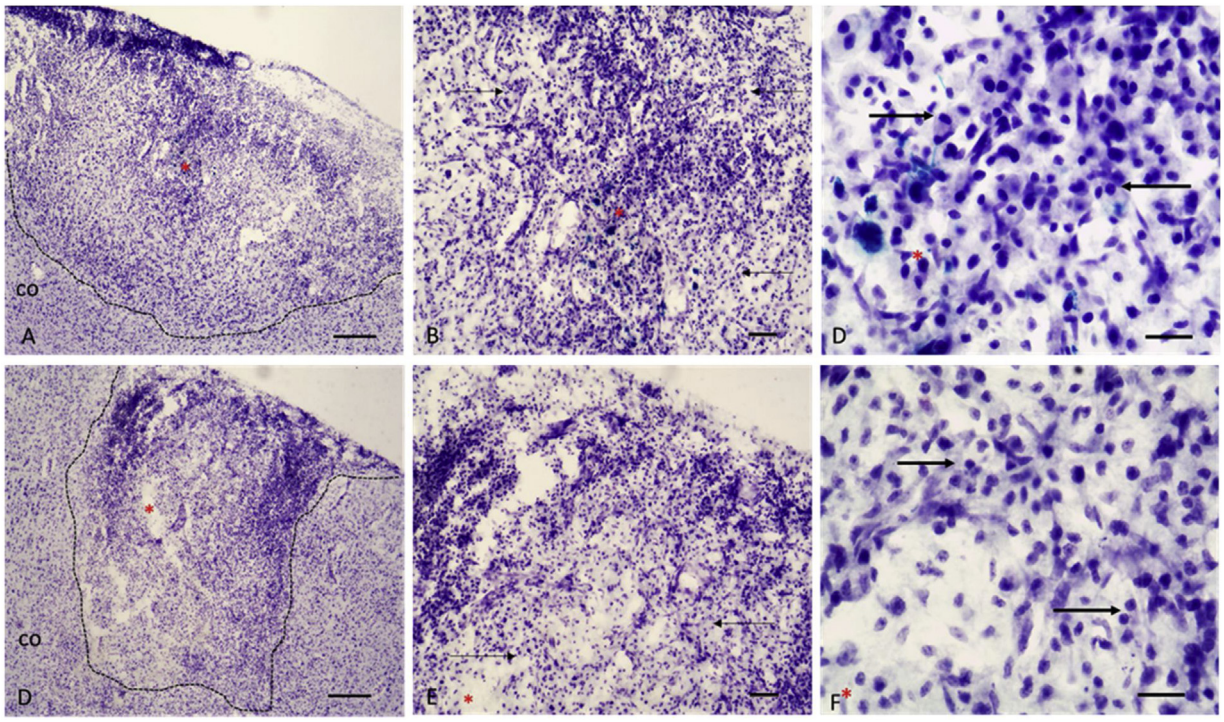

Fig. 3. Histopathological analysis of the ischemic focal lesion area. Coloring: Cresyl violet; **Upper line:** male ischemic control rats after 7 days of survival; **Bottom line:** male ischemic rats treated after 7 days of survival. **Injury area:** dotted line; **Asterisk**: ET-1 injection site; **Arrows:** point to polymorphonuclear cells - neutrophils and / or activated macrophages. **Scale bar:** 40 μm (A, D); 100 μm (B, E); 400 μm (C, F); cortex (CO).

extract obtained with petroleum ether from *C. roxburghii* leaves (4.25 ± 0.35 mg QE/g dry material) [58].

Flavonoids are an important group that is inserted in the class of polyphenols, having more than 600 compounds. TF analysis provides information about the content of its subgroups, such as chalcones, flavones, flavonoids, flavanones, isoflavones, flavan-3-ols (catechins), and anthocyanins, besides providing an estimate of the material's antioxidant activity [7,54].

3.6. Antioxidant activity (AA)

Table 2 shows the free radical scavenging effect of *Croton matourensis* leaves extracts obtained by different techniques. The IC_{50} value represents the amount of sample needed to reduce the initial concentration of the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 50 %. Thus, the lower the sample required, the greater its antioxidant potential [7]. In this sense, the hexane extract showed a higher IC_{50} value (2680.11 ± 109.14 g extract/g DPPH) and lower inhibition percentage (22.42 ± 0.19 %) at a concentration of 14 mg/mL, followed by the extract obtained by hydrodistillation, which presented an IC_{50} equal to 1531.34 ± 146.02 g extract/g DPPH and a inhibition percentage of 38.90 ± 0.95 % at a concentration of 14 mg/mL.

The extracts that showed lower IC_{50} values were those obtained with SC−CO_2 under conditions of 40 °C/100 bar (614.73 ± 25.75 g extract/g DPPH) and 50 °C/100 bar (629.83 ± 143.30 g extract/g DPPH), being statistically equal ($p > 0.05$), and with percent inhibition in the concentration of 14 mg/mL equal to 83.26 ± 0.58 % and 80.13 ± 5.99 %, respectively. Thus, it can be inferred that the extracts which showed greater antioxidant activities were those obtained at lower temperature and pressure, indicating the sensitivity to more abrupt operational conditions. This similar behavior was reported by Almeida et al. [59] and Cunha et al. [60]. The antioxidant activity may be related to the presence of phenolic compounds due to the hydroxyl groups and the double bond between the carbon atoms causing radicals elimination, as well as the synergistic effect of other compounds present in the extracts [7,16,61].

Anti-inflammatory and neuroprotective effect of C. matourensis leaves extract obtained by SC−CO_2 on experimental cerebral ischemia in rats

The cresyl violet staining method revealed the histopathology of ischemic brain tissue after endothelin-1 (ET-1) microinjections internally into the rats' cortex. Fig. 3. A, B and C show the density of cells infiltrated in the lesion area of the control animals that received treatment with 5 % tween solution after the seven-day period, whereas Fig. 3. D, E and F show the animals that received treatment with *Croton matourensis* leaves extract obtained with SC−CO_2 in the same period.

The lesion was characterized by areas with pallor, tissue loss, loss of cells, remarkable infarct area (Fig. 3. A) and infiltrates of inflammatory polymorphonuclear cells at high densities in and around the nucleus of the lesion (Fig. 3. B and C). After the treatment of ischemic animals for a period of seven days with *C. matourensis* leaves extract there was a reduction in the remarkable infarct area (Fig. 3. D). Moreover, there was also a decrease in tissue injury area, the lower cell density, the smaller amount of infiltrates of polymorphonuclear cells (Fig. 3. E and F) and consequent reduction in the area of inflammatory response, compared to control animals that received treatment with tween at 5 % (Fig. 3. A, B and C).

The focal cortical ischemia induced by ET-1 in rats has been established from neurochemical and morphological evidence due to its prolonged and marked vasoconstrictor action that induce precise and reproducible focal ischemic lesions, thus, focal microinjection of ET-1 has been used in the development of a model for focal cortical ischemia to be used for testing anti-stroke drugs [30,31]. Previous studies [3,5–7] report the anti-inflammatory and neuroprotective effects in experimental models of excitotoxicity and stroke from extracts from Amazonian plants obtained with SC−CO_2. The action mechanism of these extracts, as well as those from the *C. matourensis* leaves on the anti-inflammatory and neu-

roprotective response have not yet been elucidated. However, it may be associated with the modulatory effect of extracts on the release of pro-inflammatory substances, neutrophil recruitment and modulation of microglia, macroglia and macrophage activation, leading to reduction in tissue damage due to releasing oxygen radicals, proteases, and cytokines by inflammatory cells [3,7]. Furthermore, biological activity of *C. matourensis* leaves extract obtained by SC−CO_2 can be related to the presence of oxygenated diterpenoids, especially to the major compounds (larixol and manool oxide), which have biological effects reported as anti-malarial, antileishmanial, laxative, antimicrobial, anticonvulsive, cytotoxic, anti-inflammatory, neuroprotective, antiparasitic and analgesic activity [47–51].

In addition, studies involving *Croton* species demonstrate the biological activity effects present in this genus, such as an antitumor effect, effect against colon carcinoma and cervical cancer in essential oils of *C. matourensis* leaves and in flowers and leaves of *C. micans* [9,10]; acetylcholinesterase inhibitory activity by *C. gratissimus* ethyl acetate and butanol fractions [62]; neurodegenerative agents for Alzheimer's disease and other neurological disorders due to nerve growth factor-potentiating activities from the twigs of *C. yanhuii* [63]; antiulcer activity from *C. campestris* root extract [64] and cardioprotective effect of proanthocyanidin-rich fraction obtained from *C. celtidifolius* barks [65].

The authors suggest that in future researches the preservation and quantification of neurons, as well as the identification and quantification of infiltrated cells (microglia/macrophages), are performed in the center and periphery of the lesion after immunostaining.

4. Conclusions

The extraction with supercritical CO_2 (SC−CO_2) is presented as an efficient method for obtaining the extract from *Croton matourensis* leaves. The method showed good selectivity in comparison to conventional methods for obtaining oxygenated diterpenoids (37.15–70.78%), especially larixol (24.88–50.57%), with higher mass yield in the experimental condition of 50 °C/400 bar. The extracts presented high values of total phenolic contents, total flavonoids and antioxidant activity. In the histopathological analysis of ischemic injury in the rats' motor cortex, the extract obtained with SC−CO_2 showed an influence on tissue reconstruction and on the reduction of cell density, suggesting potential neuroprotective effect and anti-inflammatory activity. In this way, *C. matourensis* leaves extract proved to be a source of bioactive compounds with antioxidant, anti-inflammatory and neuroprotective activity, presenting potential to be applied as a supplement in the pharmaceutical and food industries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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