Article

Chemical constituents and potential anti-inflammatory activity of the essential oil from the leaves of *Croton argyrophyllus*

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Abstract: Many species from *Croton* genus have been used in traditional medicine and its pharmacological activities demonstrated. *Croton argyrophyllus* Kunth, Euphorbiaceae, is a shrub that grows in the flora of Northeastern Brazilian. The essential oil of *C. argyrophyllus* leaves was tested in rodents (10-100 mg/kg, *p.o.*) in classical models of inflammation (carrageenan-induced paw oedema and peritonitis) and its chemical constituents were determined by GC-MS/FID analysis. Nitric oxide radical-scavenging activity and lipidic peroxidation were determined to evaluate the antioxidant capacity of the essential oil (0.001-100 μg/mL). Forty-two components were identified, among them, bicyclogermacrene (14.60%) and spathulenol (8.27%) were the most abundant ones. *C. argyrophyllus* essential oil reduced significantly the oedema (30 and 100 mg/kg, $p<0.05$) and, besides, reduced the carrageenan increase in mieloperoxidase activity (10, 30, and 100 mg/kg, $p<0.001$). The carrageenan-induced peritonitis was significantly reduced ($p<0.001$) by the essential oil (10, 30, and 100 mg/kg). The essential oil (100 mg/kg) reduces the total peritoneal lavage NOx⁻ concentration ($p<0.01$). Nitric oxide radical generated from sodium nitroprusside was found to be inhibited by the essential oil ($p<0.001$). *C. argyrophyllus* essential oil was able to prevent Fe^{2+}- or Fe^{2+} plus H_2O_2-induced lipid peroxidation ($p<0.001$). This study suggests that the anti-inflammatory effect of the essential oil of *C. argyrophyllus* observed in the present study can be related, at least in part, its antioxidant capacity.

Introduction

Inflammation, an important process for maintenance of biological homeostasis, is a protective response initiated after injury. It is correlates with oxidative process, mainly because they share similar pathways which supply themselves (Kunsch & Medford, 1999). An aberration of these mechanisms may favor the development of various illnesses. Increased free radical level was found in many pathological conditions besides inflammation, such as cancer, ischemic disorders, and dementia (Harput et al., 2012). The use of plant products with known anti-inflammatory and/or antioxidant properties can be of great significance in therapeutic anti-inflammatory treatments.

The genus *Croton*, Euphorbiaceae, is widespread in the Northeast region of Brazil, mainly in the caatinga (semi-arid vegetation). Generally, the genus presents a consistent profile of biological activities and folk use. Various bioactivities, including anti-inflammatory, antinociceptive, gastroprotective, healing, and cardiovascular of the essential oil from this genus were

reported in various animal models (Bighetti et al., 1999; Siqueira et al., 2006; Cavalcanti et al., 2012; Coelho-de-Souza et al., 2013). However, the anti-inflammatory property of the essential oil from *Croton argyrophyllus* remains unknown.

Croton argyrophyllus Kunth [syn: *Croton argyroglossus* Baill. and *Croton micans* var. *argyroglossus* Müll. Arg. (Gomes et al., 2010)] is a shrub that grows in the flora of Northeastern Brazilian. Usually, leaf or flower infusions are traditionally used in the treatment of heart diseases, influenza, and as tranquilizer (Albuquerque et al., 2007; Compagnone et al., 2010). Despite of the large array of data on other *Croton* species, the knowledge about *C. argyrophyllus* is scarce. In the literature, there are few studies involving the phytochemical study of this species. E Silva-Filho et al. (2011) have recently isolated two novel casbane diterpenes of the ethanol extract from *C. argyrophyllus* stem.

Based on the above considerations, the goal of the present study was to determine the chemical constituents and evaluate the anti-inflammatory effect

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of the essential oil (EO) from *Croton argyrophyllus* leaves.

Materials and Methods

Plant collection

The leaves of *Croton argyrophyllus* Kunth, Euphorbiaceae, was collected in the municipality of Canindé de São Francisco-SE, Brazil, in October 2010 (09º39'40"S, 37º55'18"W). The plant was authenticated by Professor Ana Paula Prata, Department of Biology, Federal University of Sergipe. A voucher specimen has been deposited at the Herbarium of the Federal University of Sergipe (number ASE 20,584).

Extraction of the essential oil

The essential oil was obtained by hydrodistillation on a Clevenger-type apparatus for 2 h using 500 g of fresh leaves. The EO obtained was separated from the aqueous phase and kept in freezer until further analysis. The percentage of yield of the EO was 0.76%.

Gas chromatography

Gas chromatograph (GC) analyses were performed using a GC-MS/FID (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an auto-sampler AOC-20i (Shimadzu). Separations were accomplished using an Rtx®-5MS Restek fused silica capillary column (5%-diphenyl-95%-dimethyl polysiloxane) of 30 m x 0.25 mm i.d., 0.25 µm film thickness, at a constant helium (99.999%) flow rate of 1.2 mL/min. Injection volume of 0.5 µL (5 mg/mL) was employed, with a split ratio of 1:10. The oven temperature was programmed from 50 °C (isothermal for 1.5 min), with an increase of 4 °C/min, to 200 °C, then 10 °C/min to 250 °C, ending with a 5 min isothermal at 250 °C. The MS and FID data were simultaneously acquired employing a Detector Splitting System; the split flow ratio was 4:1 (MS:FID). A 0.62 m x 0.15 mm i.d. restrictor tube (capillary column) was used to connect the splitter to the MS detector; a 0.74 m x 0.22 mm i.d. restrictor tube was used to connect the splitter to the FID detector. The MS data (total ion chromatogram) were acquired in the full scan mode (m/z of 40-350) at a scan rate of 0.3 scan/s using the electron ionization with an electron energy of 70 eV. The injector temperature and the ion-source temperature was 250 °C. The FID temperature was set to 250 ºC, and the gas supplies for the FID were hydrogen, air, and helium (30, 300, and 30 mL/min, respectively). Quantification of each constituent was estimated by FID peak-area normalization (%). Compound concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Animals

Young adults Wistar rats (120-180 g) and Swiss mice (20-30 g) of both sexes were obtained from the Central Biotery of Sergipe Federal University (São Cristóvão, Brazil). Animals were maintained at controlled room temperature (21±2 °C) with free access to food and water. The protocols were approved by the Institutional Ethics Committee (CEPA 06/2011) and were carried out in accordance with the current guidelines for the care of laboratory animals.

Measurement of oedema in rat paws

The anti-inflammatory activity of the EO was studied using the paw oedema induced by carrageenan (1%, 0.1 mL), which was administered into the subplantar region of the right hindpaw of the rat (Winter et al., 1962). The EO (10-100 mg/kg, *p.o.*), dexamethasone (2 mg/kg, *s.c.*), or vehicle (0.2% Tween 80, *p.o.*) were administrated 1 h before of the oedematogenic agent (n=6/group). The paw oedema was measured plethysmographically (model 7150, Ugo Basile, Varese, Italy) at 1, 2, 3, and 4 h after the carrageenan. The data obtained were expressed in mL. The percentage inhibition was calculated based on the area under the time-course curves (AUC_{0-4h}).

Myeloperoxidase (MPO) activity was measured in paw tissue samples obtained from animals after the end (4 h) of oedema measurement. These samples were homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl-trimethylammonium bromide. The supernatants were mixed to a solution of o-dianisidine dihydrochloride (0.167 mg/mL, in 50 mM phosphate buffer) containing 0.005% of H_2O_2. The changes of absorbance at 460 nm were measured with a microplate reader. The results were expressed as units of MPO (UMPO)/mg tissue, where one UMPO is defined as the amount of enzyme that degrades 1 µmol of H_2O_2/min (Bradley et al., 1982).

Leukocyte migration into the peritoneal cavity of mice

The leukocyte migration was induced by injection of carrageenan (1%, 250 µL, *i.p.*) into the peritoneal cavity of mice (n=6/group) 1 h after the administration of the EO (10-100 mg/kg, *p.o.*), dexamethasone (2 mg/kg, *s.c.*), or vehicle (0.2% Tween 80, *p.o.*). The mice were euthanized 4 h after the carrageenan, and 3 mL of saline containing EDTA (1 mM) were injected into the peritoneal cavity. The peritoneal lavages were collected and the total number of cells was counted in a Neubauer chamber. Cytospin

preparations were stained with May-Grunwald-Giemsa for the differential leukocyte counts. The results were expressed as the number of leukocytes/mL.

Total nitrate/nitrite (NOx⁻) concentration

The EO (100 mg/kg, *p.o.*), dexamethasone (2 mg/kg, *s.c.*), or vehicle (0.2% Tween 80, *p.o.*) were administrated 1 h before of the carrageenan (*n*=6/group). Six h after the injection of the inflammatory agent, NOx⁻ concentration was determined in the peritoneal lavage submitted to ultrafiltration (10 kDa; Millipore, USA) using the Griess reaction for nitrite, after the nitrate reductase-catalyzed reduction of nitrate to nitrite (Grisham et al., 1996).

Determination of nitric oxide (NO) radical-scavenging activity

In order to determine the NO radical-scavenging activity, sodium nitroprusside (20 mM, in phosphate buffer saline) was mixed with 0.05 mL of the EO (0.001-100 µg/mL) and incubated at 37 ºC for 60 min (Green et al., 1982). After, Griess reagent (2% sulphanilamide, 5% H_3PO_4, and 0.1% napthylethylenediamine dihydrochloride) was added to the samples. Ascorbic acid (0.001-100 µg/mL) was used as a positive control. The absorbance was read at 540 nm in a microplate reader. The percentage of inhibition was calculated.

Determination of lipidic peroxidation

The determination of thiobarbituric acid reactive substances (TBARS) was used to measure the antioxidant capacity (Esterbauer & Cheeseman, 1990) of the EO to prevent lipid peroxidation induced by $FeSO_4$ (0.145 mM) or $FeSO_4$ (0.145 mM) plus H_2O_2 (0.4 M). The EO (0.001-100 µg/mL) was added to phospholipid preparations (0.1%, w/v, 50 mM phosphate buffer, pH 7.4) and incubated for 1 h at 37 ºC (Bligh & Dyer, 1959). Then, 0.3 mL samples were added to 0.6 mL trichloroacetic (20%, v/v) and centrifuged (10,000 x *g*, 10 min, 4 ºC). A supernatant aliquot (0.5 mL) was mixed with thiobarbituric acid (0.67%, 0.5 mL, w/v) and heated at 100 ºC for 30 min. Sample absorbances were measured using a spectrophotometer at 532 nm. Ascorbic acid (0.001-100 µg/mL) was used as a positive control. Results are expressed as the percentage of inhibition.

Statistical analysis

Results of the anti-inflammatory activity are presented as mean±SE (*n* animals per group) and of the antioxidant activity as mean±SD (triplicate). Statistical evaluation of the data was performed using ANOVA followed by Bonferroni's or Newman-Keuls's

tests (GraphPad Prism Co., version 4.0, San Diego, CA, USA). *p* values less than 0.05 were considered significant.

Results

Chemical analysis of the EO

GC-MS/FID analysis of the EO of *C. argyrophyllus* resulted in the identification of 42 compounds, constituting 86.02% of the total oil. Furthermore, bicyclogermacrene (14.60%), spathulenol (8.27%), (*E*)-caryophyllene (6.79%), β-elemene (6.19%), β-phellandrene (5.75%), myrcene (4.81%), and caryophyllene oxide (3.68%) were the main components, comprising 50.09% of the oil.

Carrageenan-induced oedema in rat paws

As observed in Figure 1, the single oral treatment with *C. argyrophyllus* EO at the 100 mg/kg dose was capable of reducing ($p<0.05$) the oedema induced by carrageenan at 3 and 4 h after the injection of the phlogistic agent. Additionally, the EO at 10 and 30 mg/kg reduced ($p<0.05$) the oedema formation induced by carrageenan at 3 h (Figure 1). Likewise, dexamethasone inhibited ($p<0.05$) the oedematogenic response evoked by carrageenan in rats at 1, 2, 3, and 4 h (Figure 1).
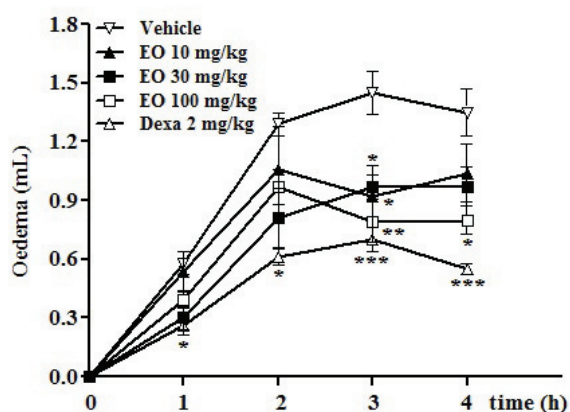

Figure 1. The effect of *Croton argyrophyllus* EO on rat paw oedema. Animals were pre-treated with vehicle, EO (10-100 mg/kg), or dexamethasone (Dexa, 2 mg/kg) before a carrageenan injection. Statistical analysis by ANOVA followed by Bonferroni's test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ *vs*. the vehicle group (n=6/group).

Based on AUC_{0-4h} values, the EO at 30 and 100 mg/kg caused a 32.8 and 35.1% inhibition ($p<0.05$), respectively, of the oedematogenic response compared to vehicle-treated group (3.9±0.3 mL x h). Dexamethasone caused an inhibition of 53.7%

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(*p*<0.001).

  C. argyrophyllus EO (10, 30, and 100 mg/kg) also produced a marked inhibition (*p*<0.001) of carrageenan-induced increase in MPO activity in the paws of rats compared to vehicle-treated group (Table 1). Similarly, dexamethasone caused inhibition (*p*<0.001) of carrageenan-induced MPO activity.

Table 1. The effect of the EO of *Croton argyrophyllus* on myeloperoxidase (MPO) activity.

Treatment	Dose (mg/kg)	UMPO/mg tissue
Vehicle	--	8.57±0.94
EO	10	2.96±0.87*
	30	0.70±0.14*
	100	0.46±0.05*
Dexamethasone	2	0.38±0.06*

UMPO, units of MPO. **p*<0.001 *vs.* vehicle-treated group. All values are mean±SE (*n*=6/group). Statistical analysis by ANOVA followed by Bonferroni's test.

Carrageenan-induced peritonitis in mice

  The carrageenan injection in control animals induced leukocyte migration into the peritoneal cavity after 4 h. The EO at 10, 30, and 100 mg/kg significantly inhibited (47.9, 42.8, and 56.3%, respectively, *p*<0.001) this response (Figure 2). The dexamethasone injection also inhibited (42.8%, *p*<0.001) the carrageenan-induced leukocyte migration (Figure 2).

  The polymorphonuclear (PMN) migration evoked by carrageenan was reduced (*p*<0.001) by 10, 30, and 100 mg/kg EO by 58.6, 62.5, and 70.5%, respectively (Figure 2). Dexamethasone exhibited significant inhibition (58.6%, *p*<0.001) of the PMN migration (Figure 2).
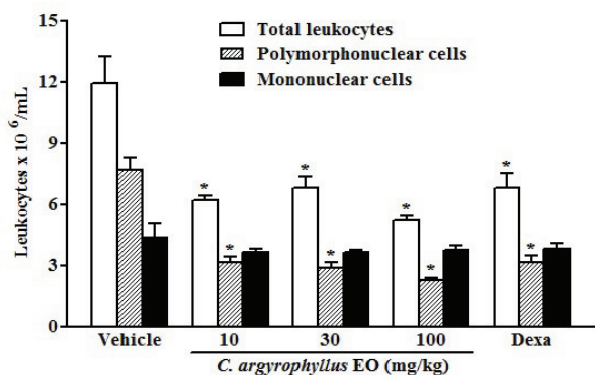

Figure 2. The effect of *Croton argyrophyllus* EO on leukocyte migration. Mice were pre-treated with vehicle, EO (10-100 mg/kg), or dexamethasone (Dexa, 2 mg/kg) before carrageenan-induced peritonitis. Statistical analysis by ANOVA followed by Bonferroni's test. **p*<0.001 *vs.* the respective vehicle group (n=6/group).

Effect of Croton argyrophyllus EO on peritoneal lavage NOx⁻ concentration

  The pre-treatment with the EO at 100 mg/kg significantly decreased (3.99±0.48 μM, *p*<0.01) the total peritoneal lavage NOx⁻ concentration compared to vehicle treated control (7.86±0.94 μM). Treatment with dexamethasone also decreased this concentration (3.09±0.73 μM, *p*<0.01).

Antioxidant activity

  NO radical generated from sodium nitroprusside was found to be inhibited by *C. argyrophyllus* EO at all concentrations (*p*<0.001, Table 2). The EO was also able to prevent Fe^{2+}-induced lipid peroxidation, in a concentration-related manner (*p*<0.001, Table 2).

Table 2. Antioxidant activity of *Croton argyrophyllus* EO.

Sample (μg/mL)	NO radical-scavenging activity	TBARS $(FeSO_4)$	TBARS $(FeSO_4 + H_2O_2)$
	Inhibition (%)		
EO 0.001	57.22±2.65*	51.97±0.66*	75.22±2.61*
EO 0.01	56.35±2.34*	53.51±0.38*	77.65±0.96*
EO 0.1	56.26±3.14*	59.65±0.76*	75.10±1.99*
EO 1.0	51.39±3.26*	60.96±0.38*	78.93±2.33*
EO 10.0	52.09±0.66*	69.96±1.66*	80.72±0.22*
EO 100.0	52.35±0.99*	70.61±0.76*	83.27±0.22*
Ascorbic acid 0.001	78.53±1.20*	49.25±1.26*	70.27±0.92*
Ascorbic acid 0.01	80.82±1.02*	53.92±3.91*	76.11±1.40*
Ascorbic acid 0.1	81.93±0.21*	54.26±5.21*	78.41±0.31*
Ascorbic acid 1.0	82.27±0.12*	60.27±2.52*	78.94±0.31*
Ascorbic acid 10.0	83.38±0.55*	73.62±5.36*	77.70±0.53*
Ascorbic acid 100.0	85.87±0.55*	79.30±1.76*	78.05±0.31*

Values are given as mean±SD (triplicate). **p*<0.001 *vs.* respective system values (ANOVA and Newman-Keuls post hoc test).

Discussion

  This study revealed that *Croton argyrophyllus* Kunth, Euphorbiaceae, EO significantly ameliorated the inflammatory response in rodent models. The anti-inflammatory effect of the EO was investigated by using classical acute inflammatory models, including paw oedema and peritonitis.

  As stated elsewhere, carrageenan-induced inflammation occurs as a non-immune reaction and has been widely used as a pharmacological tool to evaluate the effects of anti-inflammatory and analgesic drugs. The inflammatory response by carrageenan in the rodent hindpaw is a multi-mediated phenomenon: first phase, which lasts up to 2 h, involves the participation of histamine, serotonin (5-HT), and bradykinin (BK);

while the second phase, that is from 3-4 h, is mainly sustained by prostaglandins (PG) and NO release (Di Rosa, 1972; Seibert et al., 1994).

In this study, the intraplantar injection of carrageenan in the rat hindpaw causes a significant and time-dependent oedema formation that peaked at 4 h. Moreover, at the 4 h point the accumulation of MPO levels, used as an indicator of neutrophils, is significantly elevated in the paw tissue, showing that, in addition to plasma extravasation, a significant influx of leukocytes occurred in the hindpaw. However, by treating the animals with the EO, a significant reduction of both paw oedema and increased MPO activity evoked by carrageenan was seen. These data suggest that the EO can act to inhibit increased microvascular permeability (oedema) and leukocyte influx; however, the action mechanisms involved should be further investigated.

To further confirm the role of the EO on leukocyte migration, experiments were carried out in an animal model of carrageenan-induced peritonitis, which is a widely accepted model for induction of a massive influx of leukocytes (mainly neutrophils) to this cavity. As expected, after 4 h of carrageenan injection, a potent influx of leukocytes could be seen in the peritoneal cavity of mice. The EO of *C. argyrophyllus* inhibited both the total migration and the PMN migration induced by carrageenan. It has been reported that the mechanism of action of carrageenan on peritonitis involves synergistic action between PG, leukotriene B_4, and other chemotactic agents, which promote an increase of the vasodilatation, exudation, and recruitment of leukocytes (Foster et al., 1986).

In agreement with the anti-inflammatory effects of the EO, our results show that the production of NO was decreased in animals treated with this oil, as NO metabolites were detected at lower concentration in the peritoneal lavage of these animals.

NO is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, maintenance of vascular wall integrity, control vasodilatation, and control of blood pressure (Hierholzer et al., 1998). NO is a potentially toxic agent with a free radical character, and it is also implicated in diabetes, inflammation, cancer, and other pathological conditions (Moncada et al., 1991). Therefore, inhibitors of NO release may be considered as a therapeutic agent in inflammatory diseases. In this study, NO radical generated from sodium nitroprusside was found to be inhibited by *C. argyrophyllus* EO.

It is known that free radicals may also act on lipids. Lipid peroxidation can be defined as a cascade of biochemical events resulting from the action of free radicals on the unsaturated lipids of cell membranes. This process can lead to destruction of membrane structure, failure of mechanisms of metabolites exchange and, in extreme conditions, cell death (Srinivasan et al., 2007). Ours results show that *C. argyrophyllus* EO was able to prevent Fe^{2+}-induced lipid peroxidation at all concentrations used. The EO presented antioxidant property demonstrated by NO radical-scavenging activity and lipid peroxidation assays.

Usually, the major components reflect the biological activities of essential oils from which they were isolated. The EO of *C. argyrophyllus* is chemically composed of terpenes (sesquiterpenes and monoterpenes) and its biological activities could be attributed to the high concentrations of bicyclogermacrene and spathulenol. However, the anti-inflammatory activity of these compounds from essential oils is unknown, but the anti-inflammatory potential of terpenes has been described (Peana et al., 2002; Fernandes et al., 2007; Chavan et al., 2010; Bastos et al., 2011; Bento et al., 2011; Riella et al., 2012). However, it is possible that the activity of the main components is modulated by other minor components.

In conclusion, our findings show for the first time that the EO from *C. argyrophyllus* presented anti-inflammatory property. Additionally, the observed antioxidant activity of *C. argyrophyllus* EO could be, in part, responsible for their anti-inflammatory effects. *C. argyrophyllus* offers promising active components for the development of potential pharmacological agents to treat inflammatory processes.

Acknowledgements

Authors' contributions

JMOR (PhD student) contributed in collecting plant sample, identification, and confection of herbarium. JMOR, CAS, and DGS contributed in running the laboratory work and analysis of the data. DAS and PBA contributed to chromatographic analysis. CAS and SMT designed the study and supervised the laboratory work. All laboratorial activities were supervised by PBA and SMT. SMT drafted the paper. All the authors have read the final manuscript and approved the submission.

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