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Research Paper

Esenbeckia leiocarpa Engl. inhibits inflammation in a carrageenan-induced murine model of pleurisy

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Abstract

Objectives The aim of this study was to investigate the anti-inflammatory effects of the crude hydroalcoholic extract (CHE) isolated from *Esenbeckia leiocarpa* Engl., and fractions and subfractions derived from it.

Methods Dried *E. leiocarpa* Engl. bark was macerated and extracted with ethanol to obtain the CHE. The *n*-hexane, ethyl acetate, aqueous and alkaloid fractions, as well as two alkaloid subfractions (polar and nonpolar) were obtained from the CHE. A preliminary analysis using thin-layer chromatography was performed. Capillary electrophoresis, physical characteristics and spectral data produced by IR analysis and nuclear magnetic resonance (^{1}H and ^{13}C NMR), and mass spectrometry analysis were used to identify and elucidate the structure of the major compounds. Swiss mice were used in a carrageenan-induced pleurisy model. Pro-inflammatory parameters (leukocyte and exudate concentrations, myeloperoxidase and adenosine-deaminase activity, and nitrate/nitrite, interleukin 1β and tumour necrosis factor α levels) were quantified in exudates at 4 h after carrageenan-induced pleurisy in mice.

Key findings The dihydrocorynantheol alkaloid was isolated as the majority compound in the CHE, ethyl acetate and alkaloid fractions, and in the polar and nonpolar alkaloid subfractions. The CHE, fractions and subfractions inhibited the increases in leukocyte and exudate concentrations, myeloperoxidase and adenosine-deaminase activity, and nitrite/nitrate, interleukin 1β, and tumour necrosis factor α levels ($P < 0.05$) in the fluid secreted from the pleural cavity of the carrageenan-treated mice.

Conclusions *E. leiocarpa* Engl. showed significant *in vivo* anti-inflammatory action by inhibiting the inflammation caused by carrageenan. This effect may be, in part, due to the dihydrocorynantheol alkaloid, which was identified as the majority compound isolated from *E. leiocarpa* bark.

Keywords adenosine-deaminase; anti-inflammatory activity; *Esenbeckia leiocarpa* Engl.; myeloperoxidase; nitric oxide; pro-inflammatory cytokines

Introduction

Unlike synthetic compounds, natural products are very diverse in structure. Of the 1184 new chemical entities reported between 1981 and 2006, 60% were derived from, or were based on, natural products.[1] The continuing search for novel anti-inflammatory substances, especially from plants with historically documented pharmacological properties, holds considerable pharmaceutical promise.[2]

Esenbeckia leiocarpa Engl. is popularly known in Brazil as 'guarantã' and belongs to the *Esenbeckia* genus (family Rutaceae, subfamily Rutoideae), which includes approximately 30 species native to the tropical Americas.[3] Some species in the *Esenbeckia* genus are used in traditional medicine because they have important antimalarial,[4,5] anthelmintic[6] and antimicrobial properties.[7] *Esenbeckia febrifuga* is also used for the treatment of fever by inhabitants of the Brazilian Amazon.[5]

Chemical studies on the *Esenbeckia* genus have indicated the presence of several compounds, such as quinolone alkaloids,[8–11] limonoids,[8,11] coumarins and furocoumarins,[8,11–13] triterpenoids[14] and flavonoids[15]. Furthermore, the following two quinolinone alkaloids

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were isolated from *E. leiocarpa* Engl. leaves: 3-methoxy-1-methyl-2-propyl-4-quinolone and 2(1′-ethylpropyl)-1-methyl-4-quinolone.[10]

Studies *in vivo* and *in vitro* have demonstrated the anti-inflammatory properties of the compounds mentioned above and they have been shown to inhibit oedema and the production of eicosanoids, nitric oxide and/or pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6).[16–22]

The isolation of many compounds from *Esenbeckia*, including alkaloids, limonoids, coumarins, triterpenoids and flavonoids that present important anti-inflammatory properties has been reported.[8–15] However, there are no studies demonstrating the anti-inflammatory effects of *E. leiocarpa* Engl. We therefore decided to investigate whether *E. leiocarpa* Engl. could have anti-inflammatory activity. The present study focused on evaluating the anti-inflammatory effects of the crude hydroalcoholic extract (CHE) and fractions and subfractions derived from *E. leiocarpa* Engl. bark, by analysing their effects on leukocyte migration, exudate concentration, myeloperoxidase and adenosine-deaminase activity, and nitrite/nitrate, interleukin 1β (IL-1β), and tumour necrosis factor α (TNF-α) levels in a carrageenan-induced murine model of pleurisy in mice.

Materials and Methods

Plant material

E. leiocarpa Engl. bark was collected in October 2007 in Arenápolis, Mato Grosso State, Brazil. The material was identified by the botanist Celice Alexandre (State University of Mato Grosso, Tangará da Serra, MT, Brazil). A voucher specimen was deposited in the Herbarium at the Federal University of Mato Grosso and registered with the number 38639.

Extraction and fractionation

The bark of *E. leiocarpa* Engl. was air-dried and protected from light at room temperature (25°C) for 1 week. Subsequently, the dried bark (5400 g) was ground into particles (1.5 mm) using a knife mill (Mill TE-651; Tecnal, Piracicaba, SP, Brazil). The ground material was extracted with 8 litres of 96% ethanol (plant material/ethanol 1 : 8, w/v) at room temperature. After 10 days, the extract obtained was filtered (using Whatman paper no. 1) and the ethanol was removed using a rotary evaporator (Fisatom-802; Fisatom, São Paulo, SP, Brazil) at 55°C under reduced pressure (460 mm Hg; Vacuum Q-355A2; Quimis, Diadema, SP, Brazil). This procedure was repeated three times over a period of 1 month to obtain the maximal yield of the CHE, which was 290 g. A 100-g sample of the CHE was fractionated by liquid-liquid extraction using solvents of increasing polarity resulting in the production of the following fractions: *n*-hexane (9.9 g), ethyl acetate (22 g) and aqueous (68 g).

A preliminary analysis using thin-layer chromatography (TLC) was performed. In this experiment, the CHE was subjected to TLC using silica gel 60 F254 plates (Macherey-Nagel, Düren, Germany) in acetone/hexane/NH_4OH (30 : 65 : 5) as the solvent. The bands on the TLC plates were sprayed with Dragendorff's reagent, which resulted in an orange colour that indicated the presence of alkaloids. After part of the CHE was subjected to acid-base extraction to produce an alkaloid fraction (2.5 g) and a residual aqueous fraction (16.7 g), the alkaloid fraction was further extracted with ethyl ether to yield two alkaloid subfractions: an ether-soluble (nonpolar alkaloid) (280 mg) and an ether-insoluble (polar alkaloid) (720 mg) subfraction. Further, the nonpolar alkaloid subfraction (1 g) was run on a silica gel 60 CC (Vetec-0.063–0.2 mesh) column to isolate the major compound. The *E. leiocarpa* Engl. products that were derived using these extraction procedures are illustrated in Figure 1.

Capillary electrophoresis analysis

The experiments involving separation and optimization were conducted using a capillary electrophoresis system ($HP^{3D}CE$; Agilent Technologies, Palo Alto, CA USA). This system is equipped with a diode array detector set at 215 nm, a temperature control device maintained at 25°C, and data acquisition and treatment software (HP ChemStation, rev B.04.02). Samples of CHE, fractions (*n*-hexane, ethyl acetate, aqueous and alkaloid), and subfractions (polar alkaloid and nonpolar alkaloid) were injected hydrodynamically (50 mbar for 3 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions of +30 kV in the injection side. For all experiments, a fused-silica capillary (Polymicro, Phoenix, AZ, USA) 48.5 cm (40 cm effective length) × 50 μM inner diameter × 375 μM outer diameter was used. Benzylamine was used as the internal standard. The results were expressed as a percentage considering the total mass of the major compound in relation to the mass of the CHE, fractions and subfractions (% m/m).

Elucidation of compound structure

The chemical structure of the isolated compound was determined in the nonpolar alkaloid subfraction because this subfraction yielded a higher amount of the compound than the other fractions and/or subfractions. This procedure was based on the physical characteristics, spectral data produced by infrared (IR) analysis (Perkin Elmer FTIR 16 PC, Beaconsfield, Bucks, England) and nuclear magnetic resonance (^1H and ^{13}C NMR) recorded on a Varian AS-400 spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 400 and 100 MHz, respectively. Optical rotation measurements were performed on a Schmidt-Haensch Polartronic E polarimeter (Berlin, Germany). Finally, the structure of the isolated compound was confirmed by comparison with the reference data previously reported.[23]

Electrospray ionization mass spectrometry $[M + H]^+$ was used to further elucidate the structure of the major compound present in the CHE, fractions and subfractions isolated from *E. leiocarpa* Engl. bark. The mass spectrometer system consisted of a hybrid triple quadrupole/linear ion trap mass spectrometer QTrap 3200 (Applied Biosystems/MDS Sciex, Concord, ON, Canada) coupled to a Harvard Pump 11 Plus (Harvard Apparatus, Holliston, MA, USA) for sample infusion. Mass spectrometry was tuned in the negative and positive modes by infusion of a polypropylene glycol solution. The experiments were performed using the TurboIonSpray source (electrospray ionization) in the positive ion mode. The capillary needle was maintained at 5500 V, and the

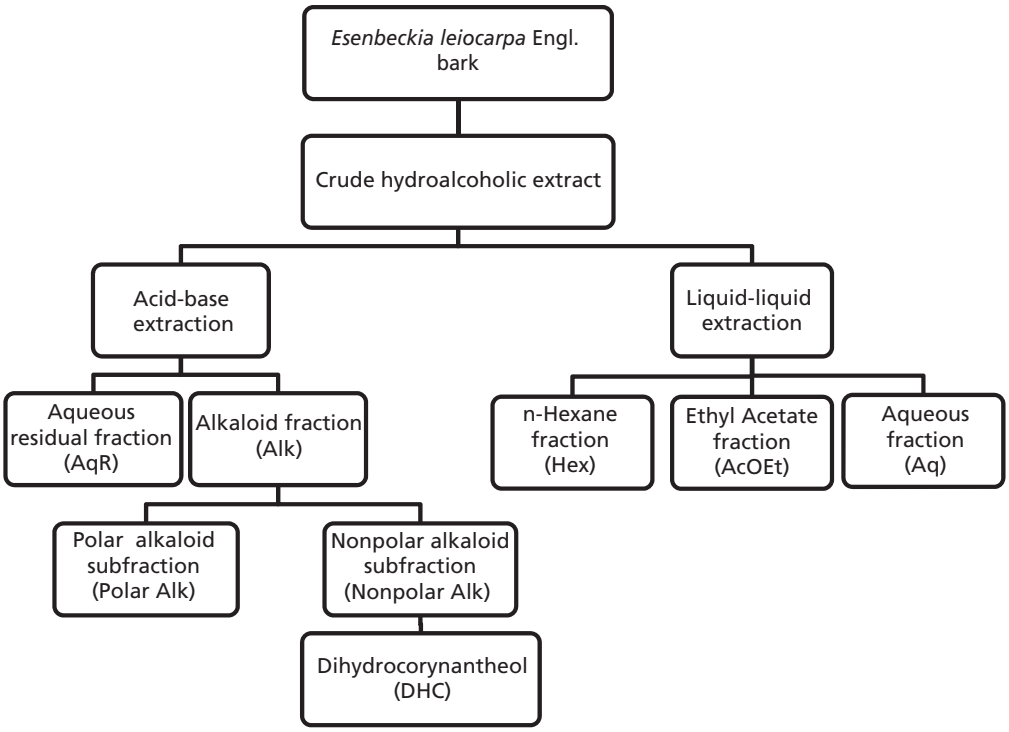

Figure 1 Flow diagram of the *Esenbeckia leiocarpa* Engl. bark extraction process.

declustering potential was set to 56 V. Synthetic air was used as the nebulizer gas (GS1) at a pressure of 15 psi, and nitrogen was used at 10 psi as the Curtain Gas in the interface. A sample solution of the dihydrocorynantheol (DHC) compound was diluted to 5.0 µg/ml in a methanol/water (50/50, v/v) solution and infused at a rate of 10 µl/min.

Animals

Swiss mice (18–25 g) were housed under standard conditions in a room at a constant temperature (22 ± 2°C) with alternating 12-h periods of light and darkness and humidity of 50–60%. Mice were fed with a standard mouse diet and with water *ad libitum*. This study was approved by the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (protocol no. PP00343) and the experiments were performed in accordance with the norms of the Brazilian College of Animal Experimentation (COBEA).

Induction of pleurisy

Pleurisy was induced by a single intrapleural injection of 0.1 ml of sterile saline (0.95% NaCl) containing carrageenan (1%).[24] At 4 h after the induction of pleurisy, animals were killed with an overdose of pentobarbital. The thorax was then opened and the pleural cavity was washed with 1.0 ml of heparin (20 IU/ml)-containing sterile phosphate-buffered saline (pH 7.5) composed of the following components: 130 mM NaCl, 5 mM Na_2HPO_4 and 1 mM KH_2PO_4. Several samples of exudates were collected for further determinations of total and differential leukocyte concentrations, exudate concentration, myeloperoxidase (MPO) and adenosine-deaminase (ADA) activity, and nitrite/nitrate (NO_x), IL-1β and TNF-α levels.

Experimental protocol

Experiments were performed to determine the dose–response curves for the CHE, fractions and subfractions under investigation. Different groups of animals were treated with different doses of CHE (10–100 mg/kg), *n*-hexane (10–50 mg/kg), ethyl acetate (10–50 mg/kg), aqueous (2–10 mg/kg) and alkaloid fractions (1–10 mg/kg), and polar alkaloid (0.5–5 mg/kg) and nonpolar alkaloid (0.1–2 mg/kg) subfractions, administered intraperitoneally 30 min before pleurisy induction with carrageenan. The studied inflammatory parameters were evaluated after 4 h.

Based on the results of this protocol, we selected the following single doses for each of the CHE, fractions and subfractions: 25 mg/kg CHE, 50 mg/kg *n*-hexane fraction, 50 mg/kg ethyl acetate fraction, 5 mg/kg aqueous fraction, 10 mg/kg alkaloid fraction, 1 mg/kg polar alkaloid subfraction and 0.5 mg/kg nonpolar alkaloid subfraction. Each of these was administered at different time points (0.5–2 h) to determine the time course of action on the inflammatory parameters described below.

For some experiments, a group of animals received 0.1 ml of 1% carrageenan administered by the intrapleural route. This group was the positive control group. At the same time, another group of animals received 0.1 ml of sterile saline (0.95% NaCl) by intrapleural injection. This group was used as the negative control group.

Dexamethasone (0.5 mg/kg) and indometacin (5 mg/kg) were used as the reference anti-inflammatory drugs and were administered intraperitoneally 30 min before pleurisy induction.

Quantification of leukocyte migration and exudate concentration

The animals were killed with an overdose of pentobarbital and samples of exudate from the pleural cavity were collected to determine the total and differential leukocyte content and exudate concentration. Total leukocyte counts were determined in a Newbauer chamber and cytospin preparations of exudate were stained with May-Grünwald-Giemsa for the differential count, which was performed using a microscope with an oil immersion objective lens.[24]

The exudate concentration was measured by quantifying the Evans blue dye in the mouse pleural cavity using the enzyme-linked immunosorbent assay (ELISA) plate reader. The animals were challenged 30 min before the induction of inflammation by carrageenan with a solution of Evans blue dye (25 mg/kg) administered intravenously. The Evans blue dye is an indirect marker of exudate extravasation because this dye can bind with albumin, which is an indicator of exudate formation.[24–26] The blue colour quantified in the fluid leaked into the pleural cavity is proportional to the exudate concentration measurement.[24] In this protocol, on the day of the experiment, a batch of stored samples was thawed at room temperature and the amount of dye was estimated by interpolation from a standard curve of Evans blue dye (0.01–50 μg/ml) using colorimetric measurements (620 nm) on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA).

Quantification of MPO and ADA activity, and NO_x levels

In-house assays of MPO and ADA activity, and NO_x levels were done according to methods described in the literature.[27–29]

MPO and ADA activity, and NO_x levels were analysed in samples of exudate from the pleural cavity and were estimated by interpolation from the standard curve derived using colorimetric measurements (absorbance at 520 nm for MPO, 620 nm for ADA, and 450 nm for NO_x) performed on an ELISA plate reader (Organon Tecknica). Results were expressed in units of mU/ml for MPO, U/l for ADA and μM for NO_x.

Quantification of IL-1β and TNF-α levels

Samples of exudate from the pleural cavity were collected and immediately prepared for quantification of cytokine levels. Commercially available kits with monoclonal antibodies for each cytokine were used. The cytokine levels were measured by enzyme immunoassay according to the manufacturer's instructions. The range of values detected by these assays was as follows: 11.72–750 pg/ml for IL-1β and 5–2000 pg/ml for TNF-α. The intra-assay coefficients of variation were $3.6 \pm 0.5\%$ for IL-1β and $7.8 \pm 0.9\%$ for TNF-α, and the interassay coefficients of variation were $9.0 \pm 1.7\%$ for IL-1β and $9.6 \pm 2.1\%$ TNF-α, with sensitivity values of 1.67 pg/ml for IL-1β and 5.0 pg/ml for TNF-α. All cytokine levels were estimated by interpolation from a standard curve defined using colorimetric measurement at 450 nm on an ELISA plate reader (Organon Teknika). The results were expressed in units of pg/ml.

Drugs

Carrageenan (degree IV), human neutrophil myeloperoxidase, indometacin, ortho-dianisidine dihydrochloride (3,3'dimetoxibenzidine), sodium azide, vanadium chloride (III) and sulfanilamide were all obtained from Sigma Chemical Co. (St Louis, MO, USA). Dexamethasone was from Ache Pharmaceutical Laboratories S.A. (São Paulo, SP, Brazil). Sodium hydrogen phosphate, zinc sulfate and hydrogen peroxide were from Vetec (Rio de Janeiro, RJ, Brazil). Sodium hydroxide was from Reagen (Rio de Janeiro, RJ, Brazil). Türk-May-Grünwald dye was from Newprov (Pinhais, PR, Brazil), Giemsa dye was from Laborclin (Pinhais, PR, Brazil) and Evans blue dye was from Acros-Organics (New Jersey, USA). *n*-(1-Naphthyl) ethylenediamine dihydrochloride was from Merck (Darmstardt, Germany). Enzyme-linked immunosorbent assays for the quantitative determination of mouse TNF-α and IL-1β were obtained from BD-Biosciences Pharmingen (San Diego, CA, USA) and IBLImmuno Biological Laboratories Co. Ltd (Fujioka-city, Gunma, Japan), respectively. All other reagents used were of analytical grade and were obtained from different commercial sources.

Statistical analysis

The data are reported as the means ± SEM. Significant differences among groups were determined by two-way analysis of variance followed by Student-Newman-Keuls post-hoc test and significance was set at $P < 0.05$.

Results

Capillary electrophoresis analysis

Figures 2–4 show the electropherograms of the CHE, fractions and subfractions isolated from *E. leiocarpa* Engl. using capillary electrophoresis analysis. The DHC compound was found in the CHE, fractions and subfractions as follows: CHE 8.54% (Figure 2), *n*-hexane fraction 0.067% (Figure 3a), ethyl acetate fraction 14.11% (Figure 3b), aqueous fraction 1.1% (Figure 3c), alkaloid fraction 23.83% (Figure 4a), polar subfraction 20.22% (Figure 4b) and nonpolar subfraction 26.87% (Figure 4c). It is important to note that the *n*-hexane and aqueous fractions presented reduced amounts of DHC (Table 1).

Elucidation of compound structure

Fractionation of the nonpolar alkaloid subfraction from *E. leiocarpa* Engl. bark resulted in the isolation of the indolic DHC alkaloid, not previously reported in this species. The compound was analysed using the IR spectrum and indicated the presence of O-H and N-H groups by absorption at 3460 and 3385 cm^{-1}. No carbonyl band was observed in the IR spectrum and the high resolution electrospray ionization mass spectra showed a molecular ion $[M + H]^+$ peak at m/z 299.4. IR, ^1H NMR and ^{13}C NMR were used to confirm the presence of alkaloids. The comparison of the chemical displacements

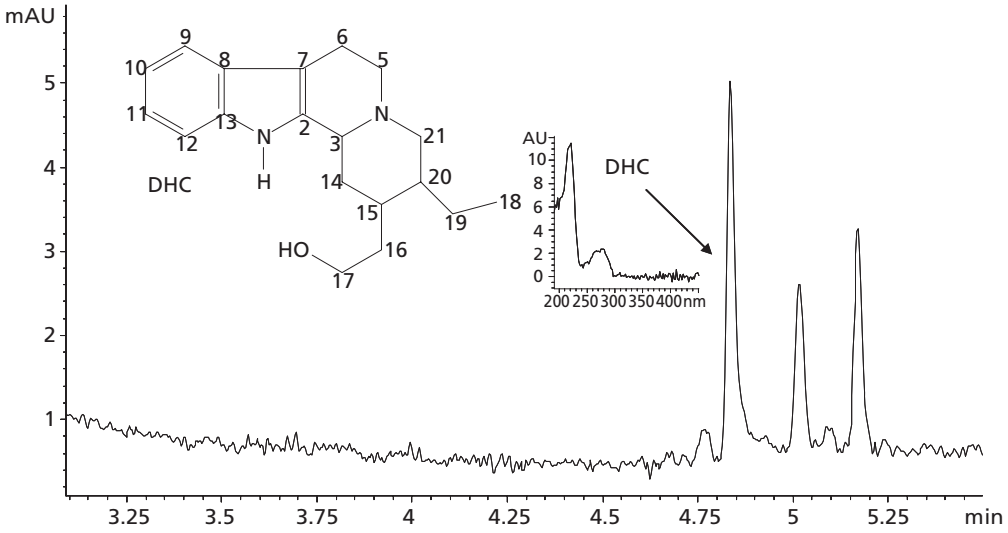

Figure 2 Determination of dihydrocorynantheol in the crude hydroalcoholic extract. Electropherogram of a standard mixture at 50 mg/l for dihydrocorynantheol (DHC) and the internal standard benzylamine. Electrolyte system composed of 40 mmol/l Tris and 50 mmol/l HIBA (pH 4.5). Other conditions: hydrodynamic injection (50 mbar, 3 s); 30 kV applied voltage; 25°C; direct detection at 215 nm.

using ^{1}H NMR and ^{13}C NMR[23] indicated the presence of the DHC compound as an amorphous solid (70 mg), m.p. 173–176°C with $[\alpha]^{25}_{D\ -1}$,52° (c_D 0.05, $CDCl_3$).

The NMR spectrum showed signals corresponding to the aromatic protons (7.45–7.00 ppm, H-9, H-10, H-11, H-12), a proton bonded to the nitrogen of the indolic ring (8.60 ppm), and methyl protons on the side ethyl chain (0.90 ppm *t*, H-18). The 2D-COSY spectrum allowed us to deduce the spin system. First, a CH_2-CH_2 unit was related to the protons at 3.04 (2H), 2.50 and 2.70 ppm. The chemical shift of the last two protons is in agreement with a linkage to a nitrogen atom. This fragment moiety should correspond to the N-C(5)H_2-C(6)H_2.

A convenient point for the second fragment was provided by the 3.05 ppm, which showed a weak coupling with the previous fragment. The connectivity provided the means of assembling the following substructure: CH-CH_2CHCH_2-CH_2. The chemical shifts of the protons of the last methylene in this fragment (3.74 ppm) are in agreement with a linkage to an oxygen atom. The ^{13}C chemical shifts (total decoupling, DEPT and C-H correlation) fully support this structure. The ^{13}C spectrum ($CDCl_3$, 100 MHz) of the DHC compound indicated the presence of 19 carbon atoms. The methyl carbon signal was centred at δ 11.0 ppm, whereas the C-19 methylene protons resonated at δ 23.4 ppm. The downfield methylene signals at δ 53.0, 59.7 and 60.1 ppm were assigned to the C-5, C-21 and C-17 carbons, respectively. The downfield chemical shift was due to the presence of nitrogen and oxygen adjacent to these carbons. The C-6, C-14, and C-16 methylenes appeared at δ 21.5, 35.3 and 34.8, respectively. The chemical shift of C-3 (δ 59.7 ppm), C-5 (δ 53.0 ppm), C-6 (δ 21.5 ppm), C-7 (δ 107.2 ppm), C-2 (δ 135.1), C-8 (δ 127.2), C-9 (δ 117.9), C-10 (δ 119.1), C-11 (δ 121.0), C-12 (δ 111.1) and C-13 (δ 136.1) supported an indole ring. The chemical structure of the DHC compound is depicted in the Figure 2.

Effects of *E. leiocarpa* Engl. on leukocyte migration and exudate concentration

The CHE from *E. leiocarpa* Engl. at doses of 25–100 mg/kg significantly decreased leukocyte migration to various degrees ranging from 41.5 ± 5.9 to 65.4 ± 3.8% ($P < 0.01$). This inhibitory effect was due to decreases in the migration of neutrophils, ranging from 41.6 ± 7.2 to 66.5 ± 3.9% ($P < 0.01$), and mononuclear leukocytes, ranging from 40.9 ± 3.3 to 61.1 ± 5.4% ($P < 0.01$). Furthermore, CHE (10–100 mg/kg) decreased exudation to various degrees ranging from 17.7 ± 3.5 to 33.0 ± 2.5% ($P < 0.05$) (Table 2).

The *n*-hexane fraction also demonstrated important anti-inflammatory action because this fraction, at doses of 25 and 50 mg/kg, significantly suppressed the number of leukocytes (by 53.2 ± 2.2 and 56.2 ± 2.9%, respectively, $P < 0.01$) and neutrophils (by 12.35 ± 4.7 and 19.0 ± 6.8%, respectively, $P < 0.05$). The *n*-hexane fraction (10–50 mg/kg) decreased the number of mononuclear leukocytes to various degrees ranging from 34.1 ± 0.5 to 39.7 ± 5.4% ($P < 0.01$). Further, only the 50 mg/kg dose of this fraction inhibited exudate concentrations (by 27.0 ± 6.6%, $P < 0.01$) (Table 2).

The ethyl acetate fraction demonstrated a similar anti-inflammatory pattern to that of the *n*-hexane fraction, producing a significant inhibition of leukocyte migration (by 56.8 ± 3.3 and 58.3 ± 2.9%, respectively, $P < 0.01$) and neutrophil migration (by 23.6 ± 5.7 and 27.2 ± 4.9%, respectively, $P < 0.01$) at the same doses (25 and 50 mg/kg). The ethyl acetate fraction at doses of 10–50 mg/kg also inhibited the migration of mononuclear leukocytes to varying degrees ranging from 22.3 ± 7.5 to 24.5 ± 8.2% ($P < 0.05$). Moreover, regarding the *n*-hexane fraction, only the highest dose of the ethyl acetate fraction (50 mg/kg) inhibited exudate concentrations (by 26.2 ± 7.7, $P < 0.01$) (Table 2).

The aqueous fraction had greater anti-inflammatory effects than the *n*-hexane and ethyl acetate fractions. Lower doses of

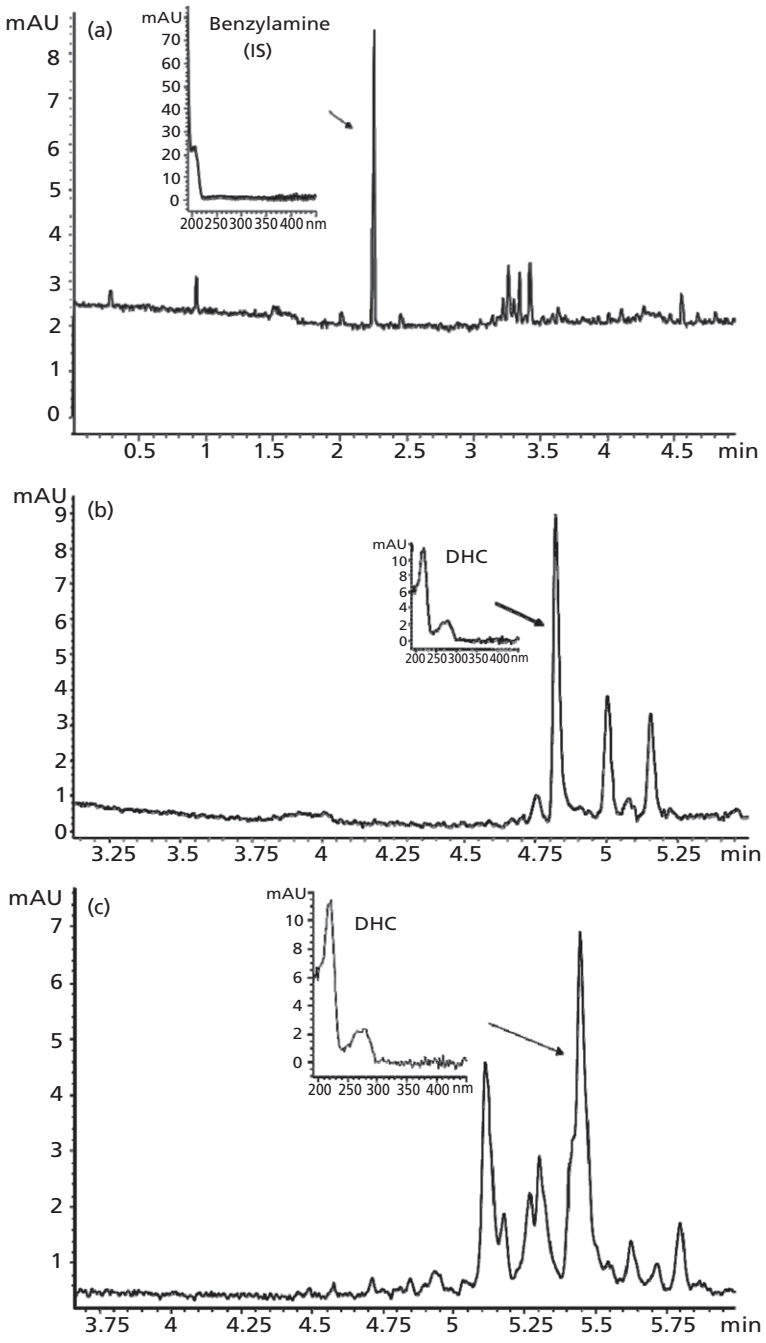

Figure 3 Determination of dihydrocorynantheol in crude hydroalcoholic extract fractions. Dihydrocorynantheol (DHC) alkaloid was determined in the *n*-hexane fraction (a), ethyl acetate fraction (b), and aqueous fraction (c). Electropherogram of a standard mixture at 50 mg/l for DHC and internal standard benzylamine. Electrolyte system composed of 40 mmol/l Tris and 50 mmol/l HIBA (pH 4.5). Other conditions: hydrodynamic injection (50 mbar, 3 s); 30 kV applied voltage; 25°C; direct detection at 215 nm.

the aqueous fraction (5 and 10 mg/kg) caused a significant inhibition of total and differential leukocyte content and exudate concentrations. In this context, the leukocyte numbers were decreased by 24.3 ± 4.0 and 32.4 ± 6.9% for the two doses, respectively ($P < 0.01$). Neutrophil numbers were decreased by 20.1 ± 6.3 and 30.2 ± 9.2% ($P < 0.01$), and exudation was decreased by 23.4 ± 2.9 and 23.6 ± 4.9% ($P < 0.01$). Moreover, the aqueous fraction at doses of 2–10 mg/kg inhibited mononuclear leukocyte migration to various degrees ranging from 41.1 ± 2.2% to 43.5 ± 2.4% ($P < 0.01$) (Table 2).

The alkaloid fraction demonstrated anti-inflammatory effects that were similar to those observed with the aqueous fraction. At doses of 5 and 10 mg/kg, the alkaloid fraction significantly inhibited leukocyte numbers by 67.7 ± 3.8 and 69.7 ± 1.5%, respectively ($P < 0.01$), neutrophil numbers by

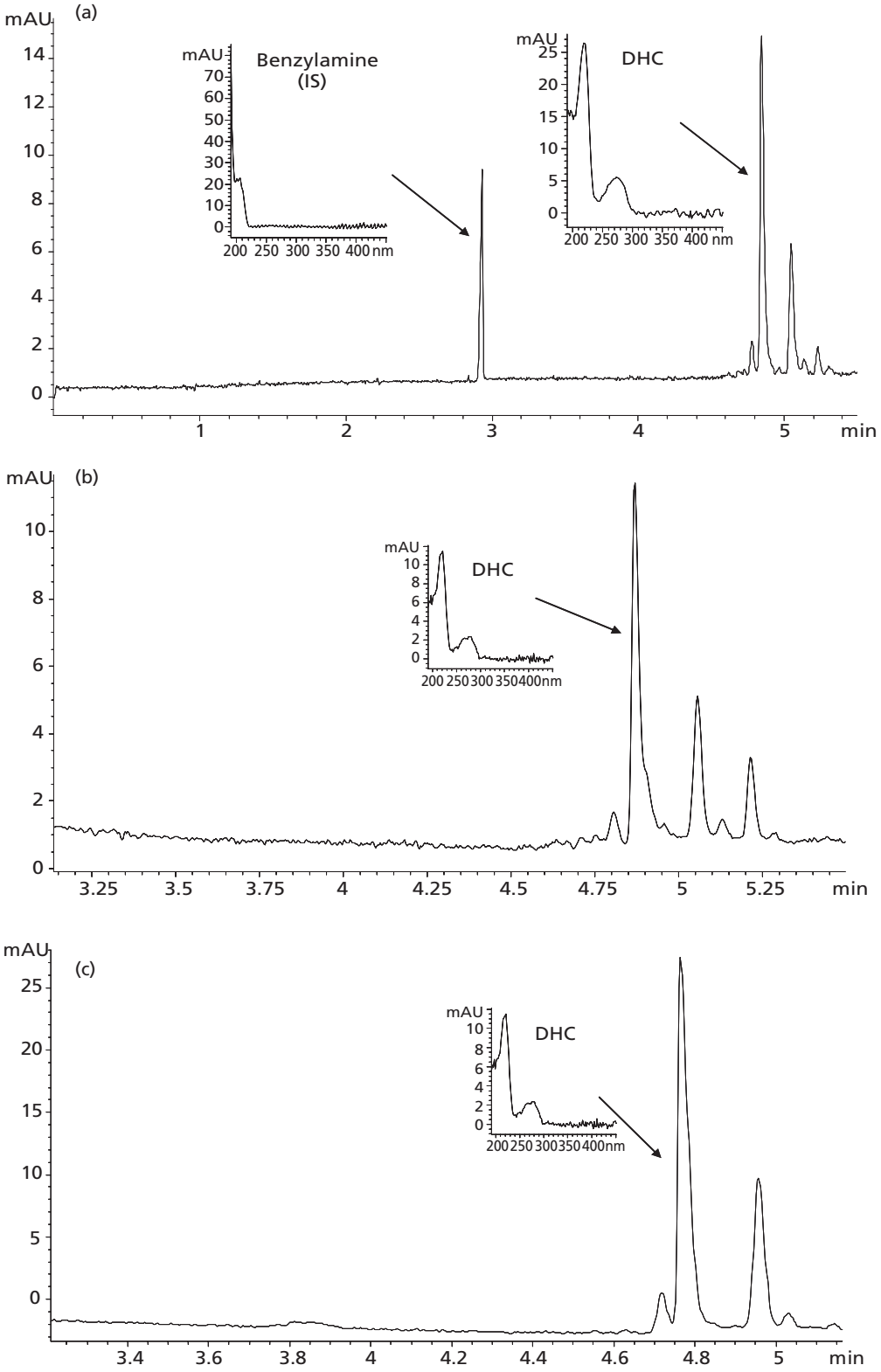

Figure 4 Determination of dihydrocorynantheol in alkaloid fractions and subfractions. Dihydrocorynantheol (DHC) alkaloid was determined in the alkaloid fraction (a), polar alkaloid subfraction (b), and nonpolar alkaloid subfraction (c). Electropherogram of a standard mixture at 50 mg/l for DHC and internal standard benzylamine. Electrolyte system composed of 40 mmol/l Tris and 50 mmol/l HIBA (pH 4.5). Other conditions: hydrodynamic injection (50 mbar, 3 s); 30 kV applied voltage; 25°C; direct detection at 215 nm.

45.9 ± 5.2 and 46.7 ± 4.3%, respectively ($P < 0.01$), and mononuclear leukocyte numbers by 32.3 ± 15.4 and 46.6 ± 7.7%, respectively ($P < 0.05$). At a dose of 10 mg/kg, this fraction also decreased exudation by 15.6 ± 5.1% ($P < 0.05$) (Table 2).

The alkaloid and aqueous fractions demonstrated greater anti-inflammatory activity than the *n*-hexane and ethyl acetate fractions, because lower doses of these fractions (5–10 mg/kg) inhibited the same inflammatory parameters as higher doses of *n*-hexane and ethyl acetate fractions (25–50 mg/kg). The alkaloid and aqueous fractions had similar inhibitory effects on inflammation.

In the next step we isolated polar and nonpolar subfractions from the alkaloid fraction to evaluate these subfractions for potential anti-inflammatory properties. Both polar and the nonpolar subfractions inhibited the inflammation caused by carrageenan. The polar alkaloid subfraction, at doses of 1–5 mg/kg, decreased leukocyte numbers to varying degrees ranging from 40.9 ± 6.1 to 24.5 ± 3.5% ($P < 0.01$), neutrophil numbers to varying degrees ranging from 43.5 ± 7.2 to 23.9 ± 3.1% ($P < 0.01$), and mononuclear leukocyte numbers to varying degrees ranging from 30.1 ± 3.8 to 26.7 ± 10.2% ($P < 0.05$). This fraction, at doses of 0.5–5 mg/kg, was also effective at inhibiting exudate concentrations to varying degrees ranging from 32.0 ± 3.0 to 29.7 ± 2.9% ($P < 0.01$) (Table 3).

In analysing the anti-inflammatory effects of the subfractions under investigation, we observed that the nonpolar alkaloid subfraction had a greater anti-inflammatory effect than the polar alkaloid subfraction, because the nonpolar subfraction produced significant inhibition of leukocyte numbers (inhibition ranging from 28.6 ± 13.5 to 46.6 ± 5.8%, $P < 0.05$), neutrophil numbers (inhibition ranging from 25.1 ± 14.0 to 46.1 ± 6.5%, $P < 0.05$) and mononuclear leukocyte numbers (inhibition ranging from 43.1 ± 12.7 to 48.7 ± 5.1%, $P < 0.01$) at lower doses (0.5–2 mg/kg) than the polar alkaloid subfraction (1–5 mg/kg). The nonpolar alkaloid subfraction (0.1–2 mg/kg) was also effective at inhibiting exudate concentrations to varying degrees ranging from 29.3 ± 4.6 to 35.3 ± 4.6% ($P < 0.01$) (Table 3).

The dose–response curve study showed that the best doses of the CHE, fractions and subfractions for the inhibition of inflammation induced by carrageenan were the following:

Table 1 Dihydrocorynantheol concentration in the crude hydroalcoholic extract, and its derived fractions and subfractions isolated from *Esenbeckia leiocarpa* bark

Extract	Dihydrocorynantheol (% m/m)	RSD
Crude hydroalcoholic extract	8.54	1.14
n-Hexane	0.067	4.7
Ethyl acetate	14.11	3.17
Aqueous	1.1	2.54
Alkaloid	23.83	1.24
Polar alkaloid	20.22	2.62
Nonpolar alkaloid	26.87	1.95

RSD, relative standard deviation.

Table 2 Effects of the crude hydroalcoholic extract isolated from *Esenbeckia leiocarpa* Engl. bark and its derived fractions on leukocyte migration and exudate concentrations in a carrageenan-induced murine model of pleurisy

Group	Leukocytes ($\times 10^6$) (% of inhibition)	Neutrophils ($\times 10^6$) (% of inhibition)	Mononuclear cells ($\times 10^6$) (% of inhibition)	Exudation (μg/ml) (% of inhibition)
Saline	0.6 ± 0.06	0.11 ± 0.01	0.5 ± 0.05	1.9 ± 0.2
Carrageenan (1%)	6.3 ± 0.16	5.09 ± 0.11	1.25 ± 0.06	11.1 ± 0.4
CHE 10 mg/kg	6.0 ± 0.29	5.10 ± 0.19	0.9 ± 0.06	9.2 ± 0.39 (17.7 ± 3.5)*
CHE 25 mg/kg	3.7 ± 0.4 (41.5 ± 5.9)**	3.0 ± 0.4 (41.6 ± 7.2)**	0.7 ± 0.04 (40.9 ± 3.3)**	9.0 ± 0.25 (19.6 ± 2.8)**
CHE 50 mg/kg	3.2 ± 0.3 (49.3 ± 4.6)**	2.6 ± 0.25 (48.6 ± 4.9)**	0.6 ± 0.04 (52.2 ± 3.5)**	8.1 ± 0.3 (27.4 ± 2.6)**
CHE 100 mg/kg	2.2 ± 0.2 (65.4 ± 3.8)**	1.7 ± 0.2 (66.5 ± 3.9)**	0.5 ± 0.07 (61.1 ± 5.4)**	7.5 ± 0.3 (33.0 ± 2.5)**
Hex 10 mg/kg	5.9 ± 0.3	5.1 ± 0.3	0.8 ± 0.01 (34.1 ± 0.5)**	9.6 ± 0.9
Hex 25 mg/kg	5.2 ± 0.2 (53.2 ± 2.2)**	4.45 ± 0.2 (12.35 ± 4.7)*	0.75 ± 0.02 (39.8 ± 1.7)**	9.4 ± 0.9
Hex 50 mg/kg	4.9 ± 0.3 (56.2 ± 2.9)**	4.1 ± 0.3 (19.0 ± 6.8)**	0.8 ± 0.07 (39.7 ± 5.4)**	8.1 ± 0.7 (27.0 ± 6.6)**
AcOEt 10 mg/kg	6.4 ± 0.3	5.4 ± 0.3	1.0 ± 0.1 (22.3 ± 7.5)*	10.6 ± 0.5
AcOEt 25 mg/kg	4.8 ± 0.4 (56.8 ± 3.3)**	3.9 ± 0.3 (23.6 ± 5.7)**	0.9 ± 0.08 (25.9 ± 6.3)*	10.3 ± 0.50
AcOEt 50 mg/kg	4.65 ± 0.3 (58.3 ± 2.9)**	3.7 ± 0.25 (27.2 ± 4.9)**	0.95 ± 0.1 (24.5 ± 8.2)*	8.2 ± 0.85 (26.2 ± 7.7)**
Aq 2 mg/kg	5.9 ± 0.3	5.2 ± 0.4	0.7 ± 0.03 (43.5 ± 2.4)**	10.1 ± 0.7
Aq 5 mg/kg	4.8 ± 0.25 (24.3 ± 4.0)**	4.1 ± 0.3 (20.1 ± 6.3)**	0.7 ± 0.08 (41.1 ± 6.0)**	8.5 ± 0.3 (23.4 ± 2.9)**
Aq 10 mg/kg	4.3 ± 0.4 (32.4 ± 6.9)**	3.55 ± 0.5 (30.2 ± 9.2)**	0.75 ± 0.03 (41.1 ± 2.1)**	8.5 ± 0.5 (23.6 ± 4.9)**
Alk 1 mg/kg	5.7 ± 0.5	4.45 ± 0.48	1.3 ± 0.2	12.4 ± 0.6
Alk 5 mg/kg	3.6 ± 0.4 (67.7 ± 3.8)**	2.75 ± 0.3 (45.9 ± 5.2)**	0.85 ± 0.2 (32.25 ± 15.4)*	10.5 ± 0.4
Alk 10 mg/kg	3.4 ± 0.2 (69.7 ± 1.5)**	2.7 ± 0.2 (46.7 ± 4.3)**	0.7 ± 0.1 (46.6 ± 7.7)**	9.4 ± 0.6 (15.5 ± 5.1)*
Dexamethasone 0.5 mg/kg	1.9 ± 0.2 (70.0 ± 5.6)**	1.3 ± 0.2 (74.4 ± 7.5)**	0.6 ± 0.2 (52.0 ± 6.5)**	6.8 ± 0.5 (39.0 ± 9.5)**
Indometacin 5 mg/kg	2.3 ± 0.3 (64.4 ± 5.0)**	1.8 ± 0.25 (65.0 ± 8.3)**	0.5 ± 0.1 (61.6 ± 8.6)**	7.9 ± 0.6 (28.8 ± 7.8)*

The crude hydroalcoholic extract (CHE) from *E. leiocarpa* Engl. and its derived fractions, *n*-hexane (Hex), ethyl acetate (AcOEt), aqueous (Aq) and alkaloid (Alk), were administered intraperitoneally 0.5 h before carrageenan-induced inflammation. The negative control groups were treated only with sterile saline (0.95% NaCl) by the intrapleural route. The positive control groups were treated only with carrageenan (1%) by the intrapleural route. Dexamethasone and indometacin were used as reference anti-inflammatory drugs and were administered intraperitoneally. The data are reported as the mean ± SEM, n = five animals. *$P < 0.05$ and **$P < 0.01$; significantly different compared with positive control groups (carrageenan 1%).

Table 3 Effects of polar and nonpolar alkaloid subfractions isolated from *Esenbeckia leiocarpa* Engl. bark on leukocyte migration and exudate concentrations in a carrageenan-induced murine model of pleurisy

Group	Leukocytes ($\times 10^6$) (% of inhibition)	Neutrophils ($\times 10^6$) (% of inhibition)	Mononuclear cells ($\times 10^6$) (% of inhibition)	Exudation (μg/ml) (% of inhibition)
Saline	0.6 ± 0.06	0.1 ± 0.01	0.5 ± 0.05	1.9 ± 0.2
Carrageenan (1%)	6.3 ± 0.2	5.1 ± 0.1	1.25 ± 0.06	11.1 ± 0.4
Polar Alk 0.5 mg/kg	6.1 ± 0.35	4.9 ± 0.3	1.2 ± 0.1	7.6 ± 0.3 (32.0 ± 3.0)**
Polar Alk 1 mg/kg	3.75 ± 0.4 (40.85 ± 6.1)**	2.9 ± 0.4 (43.5 ± 7.15)**	0.87 ± 0.05 (30.1 ± 3.8)**	8.2 ± 0.3 (26.7 ± 2.8)**
Polar Alk 2 mg/kg	4.8 ± 0.6 (23.8 ± 9.3)**	3.9 ± 0.4 (22.7 ± 8.7)**	0.90 ± 0.15 (28.1 ± 12.2)*	6.8 ± 0.2 (38.0 ± 1.8)**
Polar Alk 5 mg/kg	4.8 ± 0.2 (24.5 ± 3.5)**	3.9 ± 0.2 (23.9 ± 3.1)**	0.9 ± 0.1 (26.7 ± 10.2)*	7.9 ± 0.3 (29.7 ± 2.85)**
Nonpolar Alk 0.1 mg/kg	6.2 ± 0.2	5.15 ± 0.15	1.1 ± 0.08	7.9 ± 0.5 (29.3 ± 4.5)**
Nonpolar Alk 0.25 mg/kg	6.2 ± 0.65	5.3 ± 0.4	0.9 ± 0.2	7.4 ± 0.8 (33.6 ± 7.2)**
Nonpolar Alk 0.5 mg/kg	4.5 ± 0.9 (28.6 ± 13.5)*	3.8 ± 0.7 (25.1 ± 14.0)*	0.7 ± 0.2 (43.1 ± 12.7)**	6.8 ± 0.4 (39.0 ± 3.5)**
Nonpolar Alk 1 mg/kg	4.0 ± 0.9 (36.9 ± 14.8)**	3.2 ± 0.8 (37.15 ± 16.0)**	0.8 ± 0.1 (35.85 ± 11.5)**	7.4 ± 0.6 (33.75 ± 5.5)**
Nonpolar Alk 2 mg/kg	3.4 ± 0.4 (46.6 ± 5.8)**	2.7 ± 0.3 (46.1 ± 6.5)**	0.6 ± 0.06 (48.7 ± 5.1)**	7.2 ± 0.5 (35.3 ± 4.25)**
Dexamethasone 0.5 mg/kg	1.9 ± 0.2 (70.0 ± 5.6)**	1.3 ± 0.2 (74.4 ± 7.5)**	0.6 ± 0.2 (52.0 ± 6.5)**	6.8 ± 0.5 (39.0 ± 9.5)**
Indometacin 5 mg/kg	2.3 ± 0.3 (64.4 ± 5.0)**	1.8 ± 0.25 (65.0 ± 8.3)**	0.5 ± 0.1 (61.6 ± 8.6)**	7.9 ± 0.6 (28.8 ± 7.8)*

The polar alkaloid (polar Alk) and nonpolar alkaloid (nonpolar Alk) subfractions from *E. leiocarpa* Engl. were administered 0.5 h before carrageenan-induced inflammation. The negative control groups were treated only with sterile saline (0.95% NaCl) by the intrapleural route. The positive control groups were treated only with carrageenan (1%) by the intrapleural route. Dexamethasone and indometacin were used as reference anti-inflammatory drugs and were administered intraperitoneally. The data are reported as the mean ± SEM, n = five animals. $^*P < 0.05$ and $^{**}P < 0.01$; significantly different compared with positive control groups (carrageenan 1%).

25 mg/kg for the CHE, 50 mg/kg for the *n*-hexane fraction, 50 mg/kg for the ethyl acetate fraction, 5 or 10 mg/kg for the aqueous fraction, 10 mg/kg for the alkaloid fraction, 1 or 2 mg/kg for the polar alkaloid subfraction, and 0.5 mg/kg for the nonpolar alkaloid subfraction. In addition, the time-course profile analysis of the crude extract, fractions and subfractions isolated from *E. leiocarpa* Engl. demonstrated that they were effective at inhibiting the inflammatory parameters when administered at 0.5 h, but not at 1 or 2 h, before the carrageenan injection (results not shown). We used the above doses and a pretreatment time of 0.5 h to further analyse the effects of the CHE, *n*-hexane, ethyl acetate, aqueous and alkaloid fractions, and polar and nonpolar alkaloid subfractions on the following inflammatory parameters: MPO and ADA activities, and NOx, IL-1β and TNF-α levels.

As expected, dexamethasone (0.5 mg/kg i.p.) and indometacin (5.0 mg/kg i.p.) significantly inhibited all the inflammatory parameters tested ($P < 0.05$) (Tables 2 and 3).

Effects of *E. leiocarpa* Engl. on MPO and ADA activity, and NO_x levels

MPO and ADA are known to participate in the inflammatory process.[30,31] These enzymes are linked to the activation of neutrophils and mononuclear leukocytes, respectively.[32] Nitric oxide is also involved in leukocyte chemotaxis and exudation formation.[33]

Pretreatment of animals with CHE from *E. leiocarpa* Engl. and its fractions and subfractions, caused a significant decrease in MPO and ADA activities, and NO_x levels: CHE (25 mg/kg) 49.8 ± 6.7, 75.3 ± 0.6 and 67.6 ± 7.5%, respectively; *n*-hexane fraction (50 mg/kg) 35.5 ± 5.1, 57.1 ± 3.8 and 52.2 ± 3.1%, respectively; ethyl acetate fraction (50 mg/kg) 28.9 ± 7.5, 62.9 ± 3.4 and 43.6 ± 15.5%, respectively; aqueous fraction (5 mg/kg) 47.9 ± 9.3, 57.1 ± 5.2 and 67.4 ± 6.4%, respectively; alkaloid fraction (10 mg/kg) 35.9 ± 10.3, 67.3 ± 6.5 and 57.1 ± 8.3%, respectively; polar alkaloid subfraction (1 mg/kg) 60.6 ± 4.0, 45.4 ± 6.4 and 73.4 ± 5.5, respectively; and nonpolar alkaloid subfraction (0.5 mg/kg) 66.7 ± 3.4, 54.3 ± 7.6 and 72.1 ± 6.7%, respectively ($P < 0.05$) (Table 4).

Dexamethasone and indometacin were also effective at inhibiting MPO and ADA activity, and NO_x levels ($P < 0.01$) (Table 4).

Effects of *E. leiocarpa* Engl. on IL-1β and TNF-α levels

To determine whether the modulation of leukocyte migration, exudation and nitrite/nitrate inhibition could be associated with changes in other inflammatory mediators, levels of the cytokines TNF-α and IL-1β were measured.

The CHE of *E. leiocarpa* Engl. and its derived fractions and subfractions caused a significant decrease in IL-1β: % inhibition: CHE (25 mg/kg) 23.4 ± 1.5; *n*-hexane fraction (50 mg/kg) 38.9 ± 4.7; ethyl acetate fraction (50 mg/kg) 58.5 ± 1.3; aqueous fraction (5 mg/kg) 37.2 ± 1.1; alkaloid fraction (10 mg/kg) 47.0 ± 0.7; polar alkaloid subfraction (1 mg/kg) 46.9 ± 1.8; and nonpolar alkaloid subfraction (0.5 mg/kg) 46.3 ± 1.0 ($P < 0.05$). TNF-α levels were also decreased by *E. leiocarpa* Engl. (% of inhibition: CHE (25 mg/kg): 61.9 ± 3.2, *n*-hexane fraction (50 mg/kg): 25.9 ± 2.1, ethyl acetate fraction (50 mg/kg): 89.0 ± 0.4, alkaloid fraction (10 mg/kg): 52.4 ± 4.1 and nonpolar alkaloid subfraction (0.5 mg/kg): 38.4 ± 3.0) ($P < 0.01$) (Table 4). Only higher doses of the aqueous fraction (10 mg/kg) and the polar alkaloid subfraction (2 mg/kg) caused significant decreases in TNF-α levels (by 89.5 ± 1.2 and 75.0 ± 1.9%, respectively, $P < 0.01$) (Table 5).

Dexamethasone and indometacin also significantly decreased the levels of these pro-inflammatory cytokines ($P < 0.01$) (Table 5).

Table 4 Effects of the crude hydroalcoholic extract from *Esenbeckia leiocarpa* Engl. bark, and its derived fractions and subfractions on myeloperoxidase and adenosine-deaminase activities, and nitrite/nitrate levels in a carrageenan-induced murine model of pleurisy

Group	MPO (mU/ml) (% of inhibition)	ADA (U/l) (% of inhibition)	NO_x (μM) (% of inhibition)
Saline	56.9 ± 2.3	2.2 ± 0.2	2.8 ± 0.8
Carrageenan (1%)	414.9 ± 47.5	9.3 ± 0.6	19.0 ± 1.0
CHE 25 mg/kg	192.2 ± 25.6 (49.8 ± 6.7)**	2.3 ± 0.05 (75.3 ± 0.6)**	5.5 ± 1.3 (67.6 ± 7.5)**
Hex 50 mg/kg	267.4 ± 21.15 (35.5 ± 5.1)*	4.0 ± 0.36 (57.1 ± 3.8)**	8.2 ± 0.5 (52.2 ± 3.05)**
AcOEt 50 mg/kg	295.0 ± 30.9 (28.9 ± 7.45)*	3.9 ± 0.45 (62.9 ± 3.4)**	8.0 ± 1.6 (43.6 ± 15.5)**
Aq 5 mg/kg	216.4 ± 38.7 (47.85 ± 9.3)*	4.0 ± 0.48 (57.1 ± 5.2)**	6.2 ± 1.2 (67.41 ± 6.4)**
Alk 10 mg/kg	245.3 ± 39.35 (35.9 ± 10.3)*	3.05 ± 0.61 (67.3 ± 6.5)**	7.3 ± 1.4 (57.1 ± 8.25)**
Polar Alk 1 mg/kg	163.5 ± 16.8 (60.6 ± 4.05)**	5.1 ± 0.6 (45.4 ± 6.4)**	5.05 ± 1.05 (73.4 ± 5.5)**
Nonpolar Alk 0.5 mg/kg	138.4 ± 14.1 (66.7 ± 3.4)**	4.3 ± 0.7 (54.3 ± 7.6)**	5.3 ± 1.3 (72.1 ± 6.7)**
Dexamethasone 0.5 mg/kg	135.8 ± 27.5 (67.3 ± 7.4)**	2.2 ± 0.04 (76.4 ± 5.0)**	5.5 ± 1.3 (71.05 ± 3.5)**
Indometacin 5 mg/kg	120.1 ± 13.7 (71.05 ± 5.0)**	3.5 ± 0.1 (62.7 ± 6.0)**	7.4 ± 0.9 (61.05 ± 2.3)**

ADA, adenosine-deaminase; MPO, myeloperoxidase; NO_x, nitrite/nitrate. The crude hydroalcoholic extract (CHE) from *E. leiocarpa* Engl. and its derived fractions, *n*-hexane (Hex), ethyl acetate (AcOEt), aqueous (Aq) and alkaloid (Alk), and subfractions, polar alkaloid (polar Alk) and nonpolar alkaloid (nonpolar Alk), were administered 0.5 h before carrageenan-induced inflammation. The negative control groups were treated only with sterile saline (0.95% NaCl) by the intrapleural route. The positive control groups were treated only with carrageenan (1%) by the intrapleural route. Dexamethasone and indometacin were used as reference anti-inflammatory drugs and were administered intraperitoneally. The data are reported as the mean ± SEM, n = five animals. *$P < 0.05$ and **$P < 0.01$; significantly different compared with positive control groups (carrageenan 1%).

Table 5 Effects of the crude hydroalcoholic extract from *Esenbeckia leiocarpa* Engl. bark, and its derived fractions and subfractions on pro-inflammatory cytokine levels in a carrageenan-induced murine model of pleurisy

Group	IL-1β (pg/ml) (% of inhibition)	TNF-α (pg/ml) (% of inhibition)
Saline	70.7 ± 9.4	45.25 ± 4.8
Carrageenan (1%)	1156.0 ± 118.9	2858.0 ± 85.8
CHE 25 mg/kg	885.3 ± 17.7 (23.4 ± 1.5)*	1088.0 ± 9.1 (61.9 ± 3.2)**
Hex 50 mg/kg	706.2 ± 54.4 (38.9 ± 4.7)*	2118.0 ± 60.5 (25.9 ± 2.1)**
AcOEt 50 mg/kg	479.5 ± 15.1 (58.5 ± 1.3)**	315.1 ± 12.75 (89.0 ± 0.4)**
Aq 5 mg/kg	726.2 ± 12.2 (37.2 ± 1.1)*	2942.5 ± 70.4
Aq 10 mg/kg	–	299.7 ± 33.5 (89.5 ± 1.2)**
Alk 10 mg/kg	613.0 ± 6.5 (47.0 ± 0.7)*	1360.0 ± 23.1 (52.4 ± 4.1)**
Polar Alk 1 mg/kg	613.7 ± 20.25 (46.9 ± 1.8)*	2114.8 ± 38.7
Polar Alk 2 mg/kg	–	386.8 ± 28.9 (75.0 ± 1.9)**
Nonpolar Alk 0.5 mg/kg	621.2 ± 11.6 (46.3 ± 1.0)*	1759.0 ± 87.1 (38.4 ± 3.0)**
Dexamethasone 0.5 mg/kg	516.0 ± 33.4 (55.4 ± 2.9)**	1375.7 ± 270.3 (53.3 ± 9.2)**
Indometacin 5 mg/kg	763.5 ± 72.2 (34.0 ± 6.2)*	1553.1 ± 140.3 (47.4 ± 4.8)**

IL-1β, interleukin 1β; TNF-α, tumour necrosis factor α. The crude hydroalcoholic extract (CHE) from *E. leiocarpa* Engl. and its derived fractions: *n*-hexane (Hex), ethyl acetate (AcOEt), aqueous (Aq) and alkaloid (Alk), and subfractions, polar alkaloid (polar Alk) and nonpolar alkaloid (nonpolar Alk), were administered 0.5 h before carrageenan-induced inflammation. The negative control groups were treated only with sterile saline (0.95% NaCl) by the intrapleural route. The positive control groups were treated only with carrageenan (1%) by the intrapleural route. Dexamethasone and indometacin were used as reference anti-inflammatory drugs and were administered intraperitoneally. The data are reported as the mean ± SEM, n = five animals. *$P < 0.05$ and **$P < 0.01$; significantly different compared with positive control groups (carrageenan 1%).

Discussion

Excessive synthesis and secretion of pro-inflammatory enzymes such as MPO and ADA, and pro-inflammatory mediators such as nitric oxide, TNF-α and IL-1β are common features of chronic inflammatory diseases.[30,31,34–36]

Despite the vast array of commercially available drugs, of which non-steroidal anti-inflammatory drugs are the most commonly used, efforts to develop improved anti-inflammatory drug are continuing. Among other endeavours, natural or synthetic compounds have been tested *in vivo* and *in vitro* to develop novel, highly selective drugs.

In this study, CHE, *n*-hexane, ethyl acetate, aqueous, and alkaloid fractions, and polar and nonpolar alkaloid subfractions were prepared from *E. leiocarpa* Engl. bark for anti-inflammatory screening in a carrageenan-induced murine model of pleurisy.

The results of our study provide direct evidence that *E. leiocarpa* Engl. has a marked anti-inflammatory effect, inhibiting the release of pro-inflammatory mediators including MPO, ADA, nitric oxide, IL-1β and TNF-α, and reducing leukocyte activation at the site of inflammation.

The anti-inflammatory effect of *E. leiocarpa* Engl. was pronounced, especially with respect to the inhibition of

leukocyte content and exudate concentrations. Also, the aqueous and alkaloid fractions were demonstrated to be more effective in inhibiting these inflammatory parameters that the other fractions studied because lower doses (5 and 10 mg/kg) of these fractions caused decreases in inflammation.

The plants of the *Esenbeckia* genus have high amount of alkaloids.[8–11] For this reason, we isolated two subfractions (polar and nonpolar) from the alkaloid fraction to determine those responsible for the anti-inflammatory action. In this context, the nonpolar subfraction (0.5 mg/kg) was more effective at inhibiting leukocyte content and exudation than the polar subfraction. In contrast to the polar alkaloid subfraction, the effect of the nonpolar subfraction was dose dependent. These results also provided a better anti-inflammatory response. When we compared the nonpolar subfraction with dexamethasone (at the same dose of 0.5 mg/kg), we observed that this subfraction was as efficient as the control anti-inflammatory drug at inhibiting inflammation in this model.

To understand the mechanism of leukocyte modulation by this plant, we studied its effects on MPO and ADA, which are known to be important markers of activated neutrophils and mononuclear leukocytes, respectively.[30] The inhibitory effect on leukocytes found in this study correlated with a significant decrease in the activity of MPO, a key enzyme responsible for oxygen-dependent microbicidal activity and which is released by activated neutrophils during the respiratory burst.[37] In this context, our results revealed that *E. leiocarpa* Engl. inhibited not only leukocyte migration to the site of inflammation but also leukocyte activation.[32]

The CHE of *E. leiocarpa* Engl. and its isolated fractions and subfractions exhibited a similar anti-inflammatory pattern to that observed with the control drugs with respect to ADA and MPO activities. With regard to these parameters, the nonpolar alkaloid subfraction also had a similar anti-inflammatory effect as the reference anti-inflammatory drugs (dexamethasone and indometacin).

E. leiocarpa Engl. showed potent inhibitory action on exudate concentrations. Exudation formation is well correlated to the enhancement of vascular permeability and macrophage activity through nitric oxide production mediated by endothelial and inducible nitric oxide synthase, respectively.[34,38] Nitric oxide is the most important of the endothelium-derived relaxing factors. It is an important pro-inflammatory substance that is released during the acute and chronic inflammatory responses and is related to exudation and cellular chemotaxis.[33] Once again, *E. leiocarpa* Engl. showed a significant anti-inflammatory response. In this context, the nonpolar alkaloid subfraction significantly decreased exudation and NO_x levels. These results were similar to those observed in the animals pretreated with the reference anti-inflammatory drugs.

Inhibition of inflammatory mediators has been linked to the disruption of several pro-inflammatory pathways including the production of pro-inflammatory cytokines. IL-1β and TNF-α are directly related to leukocyte chemotaxis and exudation via activation of adhesion molecules and other mediators related to exudation.[39,40] In our experimental model, *E. leiocarpa* Engl. was found to decrease both IL-1β and TNF-α levels. In this context, the alkaloid and aqueous fractions and the nonpolar alkaloid subfraction had greater inhibitory effects on these pro-inflammatory cytokines than the other fractions and subfractions, and the nonpolar alkaloid subfraction had similar effects to dexamethasone and indometacin.

All the studied fractions and subfractions isolated from *E. leiocarpa* Engl. had important anti-inflammatory effects, and the differences in biological effects among them may be linked to differences in their chemical structures. The nonpolar alkaloid subfraction was demonstrated to be the most effective subfraction at inhibiting the inflammation caused by carrageenan in this experimental model. The DHC compound was identified as the major compound in the CHE, ethyl acetate and alkaloid fractions, and in the polar and nonpolar alkaloid subfractions isolated from *E. leiocarpa* Engl. In this context, we speculated that the DHC compound could be, in part, responsible for the observed anti-inflammatory effects. Other studies have shown anti-inflammatory effects of alkaloid compounds, such as those described by Adams *et al*.[18] who showed that quinolone alkaloids inhibited leukotriene biosynthesis using an *in vitro* assay, and Olajide *et al*.[41] who demonstrated that the cryptolepine alkaloid significantly inhibited carrageenan-induced rat paw oedema and carrageenan-induced pleurisy in rats.

Conclusion

The results of this study suggest that the reported anti-inflammatory activity of *E. leiocarpa* Engl. is mediated via inhibition of the production of pro-inflammatory cytokines (IL-β and TNF-α) related to leukocyte chemotaxis and exudation formation. In this model of inflammation, the effect may be due, in part, to the DHC compound identified as the major compound in *E. leiocarpa* Engl. bark. Owing to the limitations of this model, further study of the anti-inflammatory properties of *E. leiocarpa* Engl. is warranted.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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