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Attenuation of adjuvant-induced arthritis in rats by phonophoresis with an aqueous gel of the Amazonian plant *Elaeoluma nuda* (Sapotaceae)

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ABSTRACT

Background: Various species of the genus *Pouteria* (*Elaeoluma*) are used by the native population of Brazil because of, among other factors, their anti-inflammatory properties. The anti-inflammatory properties of the extract of the Amazonian plant *Elaeoluma nuda* were recently identified in prospective pharmacological studies.
Objectives: The objective of this study was to assess the anti-inflammatory effect of phonophoresis with aqueous gel extract of *E. nuda* in rat adjuvant-induced arthritis.
Methodology: Arthritis was induced in Lewis rats with an adjuvant. Phonophoresis with *E. nuda* gel was then administered daily and the results compared with those obtained with phonophoresis of diclofenac diethylammonium gel and ultrasound therapy without phonophoresis. Arthritis in the different groups was evaluated by plethysmometry. Proinflammatory cytokines TNF-α and IL-1α were quantified by cytometric bead array (CBA).
Results: The effect of phonophoresis of aqueous gel with *E. nuda* extract on arthritis in rats' paws (a 33% reduction compared with the controls) was the same as that produced by phonophoresis with diclofenac diethylammonium. Ultrasound therapy without phonophoresis produced no significant effect on the 21st day of therapy. There was a significant reduction in TNF-α and IL-1α levels in the group treated with phonophoresis with *E. nuda* gel ($p = 0.0042$; $p = 0.0003$, respectively).
Conclusion: Our results demonstrate the anti-inflammatory effect of phonophoresis with *E. nuda* gel on cytokines TNF-α, IL-1α and adjuvant-induced arthritis.

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1. Introduction

Many South American plant species, particularly those from the Amazon, have been studied and have proven to have good prospects for application in human health. In Brazil, various species from genus *Pouteria* are used because of their disinfectant, laxative, vermifugal, aphrodisiac and anti-dysenteric properties [1]. Genus *Pouteria* belongs to the family Sapotaceae and includes approximately 80 species. *Elaeoluma nuda* (Baehni) Aubrév, whose known basionym is *Pouteria nuda*, is a Sapotaceae that was identified in the Adolpho Ducke Forestry Reservation, in Manaus, AM.

Species from genus *Pouteria* have also been investigated for antiretroviral potential due to the HIV cell entry inhibition on classical viral assays and also due the triterpenoids in the extracts that inhibits the virus replication [2]. The ethanolic and methanolic extracts are active against *Trypanossoma cruzi* (*in vitro*) and *Plasmodium bergei* reducing the induced infection in rats [1]. *In vitro* assays have shown that many *Pouteria* species has antibacterial activity for *Staphylococcus aureus*, *Pseudmonas aeruginosa* and *Mycobacterium smigamatis* [3]. Antifungal against *Candida albicans* was also detected [3]. Anti-inflammatory effects on carrageenan on rat air pouch have been described [4].

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Phonophoresis is the application of topical drugs to the external layer of the skin with the aid of pulsed ultrasound [5,6]. The use of low-frequency ultrasound in phonophoresis increases the concentration of the drug in the tissue being treated by increasing the permeability of the skin to molecules with low or high molecular weights [6]. This property appears to be especially significant when low-frequency ultrasound is used [7]. Low-frequency phonophoresis has also been successfully used with plant extracts in animal models of inflammation [8–10].

Adjuvant-induced arthritis in Lewis rats is an inflammation model used for investigating the immunopathogenesis of autoimmunity and assessing new therapies [11–13]. In light of the ethnopharmacological importance of the genus *Pouteria* in Brazil and the pharmacological potential of *E. nuda* in *in vitro* studies, this study sought to investigate the anti-inflammatory effect of phonophoresis with *E. nuda* gel on arthritis induced by Freund's adjuvant.

2. Methodology

2.1. Aqueous extract of E. nuda

The aqueous extract used in this study was obtained from the Amazonian plant *E. nuda*, which was collected in the Adolpho Ducke Forestry Reservation (Amazonas State, Brazil). The exsiccata is stored at the National Amazonian Institute for Research (INPA) under reference no. 179316. The extract was obtained by a method described elsewhere [3,14]. Briefly, the aqueous extract was obtained using the dried ground material by infusion followed by filtration and total evaporation of the filtrate.

2.2. Preparation of the gel

The gel was prepared at INPA and had the following composition: 87.65% of demineralized water, 0.15% Nipagin, 4.00% propylene glycol, triethanolamine, 5% deionized water and 2% aqueous extract of lyophilized *E. nuda*, which were mixed until completely dispersed. Next, 1.2% carbopol was pulverized, added slowly and left to hydrate for 30 min. The substances were then mixed and heated to no more than 60 °C. Triethanolamine and 5% demineralized water were added and homogenized to a final pH of 7.0.

2.3. Animals

Thirty 8-week-old Lewis rats weighing between 250 and 350 g, donated and maintained by the biotherium at the Federal University of Amazonas (UFAM), were used in the experiment. The animals had *ad libitum* access to water and food and were kept in a light-controlled (12 h light/dark cycle) and temperature-controlled (22 °C) environment. Thirty animals were divided into five groups and two animals were kept in each cage. All the experiments were carried out with the approval of the Institutional Committee for Ethics in Animal Experiments under reference no. 001/2010 (CEEA) UFAM.

The Anti-inflammatory Effect of *E. nuda* phonophoresis on Arthritis Induced with Freund's complete adjuvant.

For this experiment the animals were divided into five groups: Group 1 – negative control (no arthritis and no treatment); Group 2 – positive control (arthritis without treatment); Group 3 – ultrasound with an aqueous placebo gel (UST); Group 4 – phonophoresis with diclofenac diethylammonium (DDP); and Group 5 – phonophoresis with 2% *E. nuda* gel (ENP).

Arthritis was induced on the first day of the experiment by ID injection of 0.1 mL of Freund's complete adjuvant (Difco®, USA) at the base of the tail [12] after brief inhalatory anesthesia with isoflurane. From the 15th day of the experiment, when signs of arthritis became evident, treatment with phonophoresis was started. This was administered daily to both rear paws by a qualified physiotherapist. On the 21st day of the experiment the animals were sacrificed by inhalation of isoflurane, and blood samples was collected by heart puncture [15].

The 2% *E. nuda* gel was administered transdermally to the plantar region of the rear paws with therapeutic ultrasound equipment (Sonacel Expert 1 MHz with a reduced head, Bioset®, Brazil) using slow circular movements and the following parameters: application time – 1 min; frequency – 1.0 MHz; intensity – 0.5 W/cm^2; ERA - 0.8 cm^2; pulse mode; one daily session in the afternoon; total number of sessions per animal – 10. The diclofenac diethylammonium gel (Cataflan Emulgel®, Novartis) was also applied in ten sessions using the same protocol. While the animal was being treated it was gently restrained by a trained researcher so that it was subjected to as little stress as possible.

2.4. Assessment of edema in the paw

Paw swelling was assessed by a trained observer who had not been involved in the planning of the experiment (blind assessment). Paw volumes (mL) were measured on days 0, 7, 14 and 21 of the experiment with a digital plethysmometer (Insight®, Brazil).

2.5. Quantification of cytokines TNF-α and IL-1α

Serum concentrations of cytokines TNF-α and IL-1α were measured by cytometric bead array using CBA Flex Sets® for TNF-α and IL-1α (BD Biosciences) in accordance with the manufacturer's instructions. A FACSCalibur® flow cytometer (BD Biosciences) was used to read the samples. The concentrations and mean fluorescence intensities (MFIs) of each cytokine were calculated using FCAP Array™ version 1.0.1 software.

2.6. High cytokine producer signatures

High-cytokine producer signatures were identified by taking the median MFI value obtained in the flow cytometry for each cytokine for the whole population, as described previously [15,16]. These values were used as cut-off points, and each animal in each group was then classified as a high or low cytokine producer.

2.7. Statistical analysis

Results were expressed as means and standard deviations, and a 95% confidence interval was used. One-way ANOVA was used to compare means, and Holm–Sidak's test with correction for multiple comparisons was used for post hoc analysis. For the last test, adjusted *p*-values were reported for each comparison. The linear trend test was applied to investigate whether there was a significant trend line among the mean cytokine concentrations [16]. A significance level of $p = 0.05$ was used. In the analysis to identify high cytokine producers, the results were considered statistically

Table 1
Volume of paws means on the 21st day of the experiment as measured by plethysmometry.

Group	Average ± SD (mL)	95% CI
No treatment	2.14 ± 0.390	1.51–2.75
UST	1.80 ± 0.350	1.25–2.31
DDP	1.35 ± 0.058	1.25–1.44
ENP	1.35 ± 0.060	1.26–1.45
No arthritis	1.03 ± 0.095	0.87–1.18

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