Supporting Information

Antinociceptive and antispasmodic effects of the essential oil of *Ocimum micranthum*: potential anti-inflammatory properties

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Plant material, extraction, and chemical analysis

Plants were seeded for 60 days in stonecutters under agronomic conditions in the Amazonian municipality of Santa Isabel do Pará, State of Pará, Brazil. Plant identification was confirmed by comparison with a voucher specimen (N° 165833; taxonomic identification conducted by Elisabeth van den Berg) which is deposited at the herbarium of the Museu Paraense Emílio Goeldi, Belém, Pará, Brazil. EOOM was obtained from leaves and stalks of *O. micranthum* and was kindly provided by the Laboratory of Chemistry of the Center for Exact and Natural Sciences of the Federal University of Pará. It was prepared by steam distillation (0.2% yield, v/w) and analyzed chemically by gas chromatography and mass spectrometry (GC/MS; model 5971; Hewlett-Packard) using a mass spectral library search and Kovat’s indices [1S, 2S].

Animals

Swiss mice of both sexes (20-35 g) and male Wistar rats (200-250 g) were obtained from colonies maintained at the Evandro Chagas Institute (Belém, Brazil) and at the Department of Physiology and Pharmacology, Federal University of Ceará (Fortaleza, Brazil), respectively. They were kept under conditions of constant temperature (22 ± 2 °C) with a 12 h light/12 h dark cycle and free access to food and water. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals [3S]. All procedures described herein were reviewed by and had prior approval from the local animal ethics committee (protocol number CEPA #14/10; approval date: 03-Mar-2010).

Acute toxicity evaluation

For acute toxicity evaluation, fasted overnight mice were randomly divided in 3 groups of 10 animals treated with either EOOM (1 or 5 g.kg\(^{-1}\)) or saline, administered orally by gavage. Animals were evaluated for mortality and behavioral parameters up to 72 h after gavage.

Acetic acid-induced writhing in mice
Counting of writhing movements was taken using acid acetic-treated mice as originally described by Koster and co-workers [4S]. The method was chosen to evaluate the peripheral analgesic properties of the EOOM. Groups of 10 mice were fasted overnight with free access to water. EOOM (15 to 100 mg.kg$^{-1}$, p.o.), indomethacin (5 mg.kg$^{-1}$, used as positive control), or vehicle (NaCl 0.9%, 10 mL.kg$^{-1}$) were administered 30 min prior to the injection of acetic acid (0.6%). The mice were then placed in boxes and the number of squirms was counted for 20 min.

**Formalin test in mice**

The formalin test was performed injecting 20 µL of a 1% (v/v) solution of formalin into the subplantar region of the right paw [5S]. The total time spent by the animal licking its own paw during the first 5 min (1$^{st}$ phase) and from the 20$^{th}$ to the 30$^{th}$ (2$^{nd}$ phase) min after injection was recorded. The test was carried out at room temperature maintained at 22-26°C, and great care was taken to avoid environmental disturbances. Morphine (4 mg.kg$^{-1}$, i.p.) was used as positive control.

**Hot plate test in mice**

Hot plate test was performed accordingly to the method described by Woolfe and McDonald [6S]. Hot plate test was performed in mice that were pre-selected on the hot plate at 55.0 ± 0.5°C. Then, selected mice were treated with EOOM (50 or 100 mg.kg$^{-1}$, p.o.), morphine (10 mg.kg$^{-1}$), or vehicle (NaCl 0.9%, 10 mL.kg$^{-1}$). The reaction time (in seconds) for each mouse was determined on the hot plate before and after drug administration at intervals of 30 min. Naïve animals showing a reaction time (latency for licking the hind feet or jumping) greater than 20 s were discarded, and a total period of 45 s was followed while measuring reaction time.

**Experiments with rat isolated trachea**
Male rats were euthanized by an intraperitoneal (i.p.) injection of chloral hydrate (400 mg.kg$^{-1}$). After careful dissection, the trachea was cut transversely in 3-4 rings which were attached to two steel wire triangular pieces (0.3 mm diameter) mounted in a 5 mL glass organ bath containing physiological solution at 37 °C, gassed with 5% CO$_2$ in O$_2$, pH 7.4, under an initial resting load of 1 g. Contractions were recorded through force transducer (Grass model FT03) connected to a data acquisition system (Windaq, PM-1000). After an equilibration period of at least 60 min, control contractions were induced by adding 60 mM KCl directly to the bath. This procedure was repeated until two consistent reproducible contractions were elicited in each tissue, when the preparations were considered to be equilibrated.

Concentration-effect curves were obtained by exposing the preparations to increasing concentrations of either EOOM or its isomers constituents (E)- or (Z)-methyl cinnamate, all applied at a concentration range of 1-1000 µg.mL$^{-1}$. Effects of EOOM, (E)- or (Z)-methyl cinnamate were assessed in preparations maintained under resting tonus or in tracheal rings precontracted with either KCl (60 mM) or carbachol (CCh; 0.3 µM). On the sustained phase of a K$^+$- or CCh-induced contraction, increasing concentrations of EOOM, (E)- or (Z)-methyl cinnamate were added to construct a concentration-effect curve.

Additional experiments were also performed with tracheal rings of ovalbumin (OVA)-sensitized rats challenged by inhalation of saline or OVA (see below). Concentration-effect curves were obtained by exposing tracheal rings to increasing concentrations of KCl (10-120 mM) or CCh (0.001-30 µM). Maximum response ($E_{\text{max}}$) was obtained when the increase in KCl or CCh concentration did not induce significant additional response. Concentration-effect curves were repeated in the presence of either 30 or 100 µg.mL$^{-1}$ EOOM.

**Ovalbumin-sensitized animals**

Rats were actively sensitized to OVA on days 1, 3, and 5 by an intraperitoneal (i.p.) injection of chicken egg albumin (grade II, Sigma Chemical) diluted in saline (0.5 mL, 10 mg.kg$^{-1}$). They
were challenged to the antigen 21 days after the last immunization. Conscious rats were challenged into a plastic box (21 x 20 x 30 cm) by inhaling, via an ultrasonic nebulizer (RespiraMax; NS Indústria de Aparelhos Médicos), OVA (firstly with 1 mg.mL\(^{-1}\) and secondly with 5 mg.mL\(^{-1}\) for 15 min each) or vehicle only (saline for 30 min). Each rat was euthanized 12 h later under chloral hydrate (400 mg.kg\(^{-1}\), i.p.) anesthesia to assess tracheal responsiveness to contractile agents in vitro. In order to verify that the animals were properly immunized, a separated group of tracheal rings were exposed in bath chamber to OVA (10 µg.mL\(^{-1}\)) following a Schultz-Dale reaction. If immediate contractions were evident after OVA addition, the tissue was considered OVA-sensitized. In a separate set of animals, OVA-sensitized rats were placed into the plastic box for inhalation of a single dose of EOOM aerosolized by ~5 min from a solution at 300 µg.mL\(^{-1}\) via an ultrasonic nebulizer (RespiraMax; NS Indústria de Aparelhos Médicos). Control animals inhaled saline instead of EOOM. Immediately (5 min) after treatment with EOOM or saline, animals were challenged by inhalation with OVA as previously described.

**Solutions and drugs**

The physiological salt solution used in the present study had the following composition (in mM): NaCl 118.0; KCl 4.7; CaCl\(_2\) 2.5; MgSO\(_4\) 1.2; NaHCO\(_3\) 25.0; KH\(_2\)PO\(_4\) 1.2; glucose 10.0. Solutions with high KCl content involved the direct addition of appropriate amounts of a 3 M KCl solution (in distilled water) directly to the organ bath. EOOM was dissolved directly in the physiological solution containing Tween 80, and sonicated β-caryophyllene, (E)- and (Z)-methyl cinnamate were purchased pure (≥98%) from Sigma Chemical Co. and were also dissolved directly in the physiological solution containing Tween 80 and sonicated just before use. The maximum concentration of the vehicle in organ bath was 0.06% (v/v). Morphine sulphate (DIMORF®; Cristália Co.), isoproterenol (Sigma Chemical), and indomethacin (Sigma Chemical) were used as positive control. Salts (all of analytical grade) were purchased from Sigma Chemical or Merck.
Statistical analysis

All data are means ± SEM of a number of experiments. Contractile responses were showed as absolute values of contraction (in g) or as % of the basal tonus, as % of the last response to KCl (60 mM) obtained after equilibration period, or as % of the CCh (0.3 µM)-induced contraction, as appropriately indicated. Significance of the results was determined using Mann-Whitney test, unpaired t test, or ANOVA and, when significant, it was followed by a multiple comparison test as appropriately indicated. Statistical significance was accepted when p < 0.05.

References

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