Supporting Information

The essential oil of *Eucalyptus tereticornis* and its constituents, α- and β-pinene, show accelerative properties on rat gastrointestinal transit

Davi Matthews Jucá¹, Moisés Tolentino Bento da Silva¹, Raimundo Campos Palheta Junior², Francisco José Batista de Lima¹, Willy Okoba¹, Saad Lahlou³, Ricardo Brandt de Oliveira⁴, Armênio Aguiar dos Santos¹, Pedro Jorge Caldas Magalhães¹

Affiliation

¹ Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, CE, Brasil
² Curso de Medicina Veterinária, Universidade Federal do Vale do São Francisco, Petrolina, PE, Brasil
³ Instituto Superior de Ciências Biomédicas, Universidade Estadual do Ceará, Fortaleza, CE, Brasil
⁴ Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Correspondence

*Prof. Pedro Magalhães*
Departamento de Fisiologia e Farmacologia
Faculdade de Medicina
Universidade Federal do Ceará
R. Cel. Nunes de Melo 1127, Rodolfo Teófilo
60430-270, Fortaleza, CE
Brasil
Tel.: +55/85/3366/8334
Fax: +55/85/3366/8333
pjcmagal@ufc.br
Animals

This study was conducted on male Wistar rats (200-250 g) kept under conditions of stable temperature (22 ± 2 °C) and a 12 h light/12 h dark cycle with free access to tap water and food. The present experimental protocols followed the recommendations of the “Guide for the Care and Use of Laboratory Animals”, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). All experimental procedures were reviewed by and had prior approval from the local animal ethics committee (Process number 13/06).

Assessment of the gastric emptying and the gastrointestinal transit in conscious rats

Gastric emptying and gastrointestinal transit was evaluated by means of the dye dilution technique which has been previously employed in our laboratory [15]. Rats were submitted to fasting for 24 h with free access to an oral rehydration solution composed of Na⁺ 75, Cl⁻ 65, K⁺ 20, glucose 75, and citrate 10 mmol/L. Next, they were gavage-fed with vehicle (Tween 80, 1 mL/kg), α-, or β-pinene (100 mg/kg, p.o.), and 30 min later they received 1.5 mL of a liquid test meal labelled with phenol red (0.75 mg/mL in 5% glucose solution). After 10, 20, or 30 min, they were euthanized by cervical dislocation. Each subset consisted of 8 rats. 5-Hydroxytryptamine creatinine sulfate salt monohydrate (purum, ≥98.0%; Sigma-Aldrich) administered by gavage (3 mg/kg, p.o.) was used as positive control. After laparotomy and visceral exeresis, the stomach and the small intestine were quickly ligated at the pylorus, the lower esophageal sphincter and the ileocecal junction. The gut was carefully stretched along a meter stick on a plain table top and divided into four consecutive segments: stomach; proximal (~40%), mid (~30%), and distal (~30%) small intestine. Each
segment and its contents were separately homogenized in 100 mL of 0.1 N NaOH. The homogenate (10 mL) of each segment was centrifuged (2800 rpm) for 10 min. Next, 5 mL of the supernatant was added to 0.5 mL trichloroacetic acid (20% w/v) to precipitate proteins. After a new centrifugation (20 min, 2800 rpm), 3 mL of the supernatant was added to 4 mL 0.5 N NaOH to intensify its colour. The solutions were assayed by spectrophotometry (Spectronic GENESYS 20; Thermo) at 560 nm and expressed as optic densities. In each experiment, a standard dilution curve was obtained by means of plotting the phenol red concentration against the optical density (OD) of the 0.1 N NaOH solution. The linear coefficient ($\alpha$) of the dilution curve allowed to determine the solution concentration (C = OD), as well as the amount of phenol red ($m$) recovered from each segment ($m = C \times$ volume). The value of fractional (%) dye recovery in each gut segment ($x$) was expressed according to the following equation:

$$\frac{\text{Recovery}}{\text{Segment (x)}} = 1 - \left( \frac{\text{amount of phenol red recovered in segment (x)}}{\text{total amount of phenol red recovered from all four segments}} \right) \times 100$$

Assessment of the gastric compliance in anaesthetized rats

A barostat system was used to monitor, by plethysmometer, the gastric volume (GV) changes under increasing distension pressure [25]. Initially, the rats were fasted for 24h with free access to the oral rehydration solution. After urethane anaesthesia (1.2 g/kg, i.p.), the rats were submitted to a midline cervical incision, tracheostomy, and cervical vessels’ cannulation. Next, the rats were treated by gavage with vehicle (1 mL/kg), $\alpha$-, or $\beta$-pinene (100 mg/kg, p.o.). Then, a balloon catheter (~4 mL) made of surgical glove fingertips was inserted orally and positioned at the rat's proximal stomach. Its free end was connected to a U-shaped glass reservoir (ID=2.5 cm, vol=30
mL), creating a communicant vessel system filled with an ionic standard solution (45 mg% of NaCl and 0.3 mL% of Imbebient BBC Ornano®; Comerio), pre-warmed at 37 °C. The changes in the reservoir volume were continuously displayed by a plethysmometer (model 7140; Ugo Basile). Since the total volume of fluid in the system was known, changes in the reservoir volume reflected those of the stomach and could be taken as a gastric compliance index [2S]. The reservoir liquid level was consecutively set every 10 min at 4, 8, and 12 cm above the rat xyphoid appendix. GV values were recorded at every 15 s.

Assessment of the small intestine transit in conscious rats

The intestinal transit index was also evaluated by means of the classical dye dilution technique, as previously described [3S]. Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, a midline laparotomy incision was made using aseptic technique. Each rat had a silastic catheter introduced into the stomach through a small fistula, advanced 1 cm distally to the pylorus, and fixed to the gastric fundus wall with a 6-O silk purse-string suture. Next, the catheter was passed through the abdominal wall musculature and subcutaneous tissue and directed towards the back of the neck where it was exteriorized through an interscapular incision and fixed to the skin with 4-O silk suture. A rubber cap was used to seal the exposed end of the catheter. Rats were individually housed in Bollman's cages and kept fasting for 24 h with free access to the oral rehydration solution. On the occasion of the study, the rats were treated by an intra-duodenal injection of vehicle (1 mL/kg), α-, or β-pinene (100 mg/kg, p.o.), and 20 min later they received via duodenum 1 mL of a liquid test meal labelled with phenol red dye (0.75 mg/mL in 5% glucose solution). Each subset consisted of 8 rats. After 10 min
of feeding, they were euthanized by an i.v. injection of pentobarbital followed by laparotomy and gut exeresis. The stomach and the first 1 cm of the proximal duodenum that contained the cannula comprised segment 1. The remaining intestine was carefully removed and slightly stretched. Obstructive ligatures were performed to obtain five consecutive segments of the small bowel (about 20 cm long). Each gut segment was homogenized and its dye content determined by spectrophotometry as described above. The fractional marker retention was calculated for each gut segment as a ratio between the counts obtained in it to the sum of counts of all gastrointestinal segments, including the gastroduodenal one. The data obtained for each individual segment was then multiplied by the number of the respective segment and summed up to calculate the geometric center of the marker distribution throughout the gastrointestinal tract.

**Assessment of the contractile activity of isolated gastric fundus and duodenum strips**

*In vitro* experiments were carried out on isolated longitudinal strips obtained from gastric fundus and duodenum of rats euthanized by cervical dislocation. After laparotomy, the stomach and a duodenal segment of approximately 8 cm were pulled out, excised and immersed in perfusion medium (Tyrode solution) at room temperature. The stomach was opened along the lesser curvature and its contents rinsed with Tyrode solution. The gastric fundus was cut into strips of approximately 15–20 mm in length and 4–6 mm in width, respecting the direction of the longitudinal smooth muscle, with a maximum of 4 strips from one rat’s stomach. After careful flushing of the duodenal luminal contents with perfusion medium, it was cut in
cylindrical segments of approximately 10 mm, with a maximum of 4 strips from one rat’s duodenum.

Either gastric or duodenal strips were set up under 1 g of tension in 5 mL tissue baths filled with physiological solution. Isometric contractions were recorded by means of an isometric transducer (Grass Model FT03) coupled to a PC-based Dataq acquisition system (PM-1000, CWE Inc.). The tissues were continuously maintained at 37°C and bubbled with air.

After an equilibration period of at least 60 min, the reference contractions were induced by adding to the bath a submaximal concentration (60 mM) of potassium chloride (KCl) solution. When two successive control contractions showed similar amplitudes, the preparations were considered to be in equilibrium. To assess the monoterpenes effects on rat gastrointestinal contractility, concentration–effect protocols were performed by exposing the strips to increasing concentrations (4 – 408 µg/mL) of α- or β-pinene added to the bath and maintained at a given concentration during 5 min. The pinenes were directly added to the buffer solution into the bath chamber in a cumulative manner. To quantify the pinene-induced effect on smooth muscle contraction, the tissues were exposed to α- or β-pinene and a submaximal concentration of a contractile agent was further added to the bath still in the presence of α- or β-pinene.

**Solutions and drugs**

The perfusion medium was a freshly modified Tyrode’s solution (pH 7.4) of the following composition (mM): NaCl 136, KCl 5, MgCl₂ 0.98, NaH₂PO₄ 0.36, CaCl₂ 2, NaHCO₃ 11.9, and glucose 5.5. The α- or β-pinene were dissolved in Tween 80,
attaining similar concentration as the Tyrode’s solution and shaken just before use. Tween 80 concentration did not reach levels higher than 0.2% in bath chamber, a concentration ineffective on this preparation (data not shown). The pure monoterpenes α- and β-pinene (purity of 98% and 99%, respectively) were purchased from Sigma. Acetylcholine (ACh) chloride, carbamylcholine (CCh), 5-hydroxytriptamine (5-HT) creatine sulfate, phenol red, trichloroacetic acid, and Tween 80 were purchased from Sigma and dissolved directly in saline or Tyrode’s solution just before use. All reagents were of analytical purity.

The essential oil of *Eucalyptus tereticornis* (EOET) was obtained by steam distillation from leaves collected at the Horto de Plantas Medicinais of the Federal University of Ceará (Fortaleza, Brazil). The plant’s taxonomic identity was confirmed by Prof. A.G. Fernandes. A voucher specimen (number 24743) is deposited in the Prisco Bezerra Herbarium, Federal University of Ceará. The composition of the EOET sample used in this study was determined by gas chromatography/mass spectrometry (GC/MS) and was already reported [45]. In brief, EOET showed the following compounds (in % of oil yield): eucalyptol 74.6; α-pinene 7.8; β-pinene 7.4; limonene 3.9, other constituents 6.3.

**Supporting References**


4S Coelho-de-Souza LN, Leal-Cardoso JH, de Abreu Matos FJ, Lahlou S, Magalhães PJ. Relaxant effects of the essential oil of Eucalyptus tereticornis and its main constituent 1,8-cineole on guinea-pig tracheal smooth muscle. Planta Med 2005; 71: 1173-1175