SUPPLEMENTARY MATERIAL

Antidiarrhoeic effect of *Eugenia dysenterica* DC (Myrtaceae) leaf essential oil.

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Abstract: Essential oil from *Eugenia dysenterica* leaves was able to inhibit both the diarrhoea and enteropooling induced by castor oil administration, but not the distance travelled by charcoal meal in the intestine. These data suggest that the antidiarrhoeic effect of the essential oil from *E. dysenterica* leaves is related to its ability to inhibit intestinal secretion and/or to increase intestinal absorption.

Keywords: diarrhoea; castor oil; essential oil; *Eugenia dysenterica*.

1. Experimental

1.1. Animals. The experiments were performed using male Swiss mice (35–45 g). The animals were housed in a room at 21°C with 12 h light/dark cycles and were provided free access to tap water and standard chow. All the protocols used were reviewed and approved by the Ethics Committee on Animal Use of the Federal University of Uberlândia (CEUA/UFU; process n° 118/11).

1.2. Plant Material. *E. dysenterica* leaves were collected at the Santa Rita farm located in Lassance city, state of Minas Gerais, Brazil (17°59'14.23''S;44°44'38.00''W), in September, 2011. The species was identified by Dr. Adriana Arantes, and a voucher specimen was deposited at the Herbarium of the Federal University of Uberlândia (Uberlândia, MG, Brazil) with the number HUFU-45956.

1.3. Preparation of the Leaf Essential Oil. Fresh leaves from 3 different trees were pooled and hydrodistilled in a Clevenger-type apparatus for 3 h (yield: 0.34%; v/w). The oil was stored at 4°C and protected from light until use.

1.4. Preparation of the Aqueous Leaf Extract. The leaves were washed and dried in an oven at 40°C for 48 h. The powdered dried leaves (20 g) were extracted with distilled water (final volume: 100 mL) for 48 h at room temperature. The extract was lyophilised and then stored at –20°C and protected from light until use (yield: 0.8%; w/w).

1.5. Castor oil-induced diarrhoea. Nonfasted animals (n = 7–8) were administered orally with saline, loperamide (20 mg/kg; standard antidiarrhoeic drug; vehicle: 0.5% carboxymethylcellulose), or *E. dysenterica* essential oil (3, 30 or 300 mg/kg; vehicle: 10% tween 80), 1 h before castor oil (0.8 mL/mice, p.o.) or saline (0.8 mL/mice, p.o.; control group) administration. Immediately after the administration of the cathartic agent or saline, each animal was placed in a separate cage lined with an absorbent paper sheet. The paper was replaced and the stools were counted each hour for 4 hours. Aimed at evaluating the onset of diarrhoeic faeces, the stools were numerically scored based on their presence and/or consistency as described by Shoba and Thomas (2001) with modification: no stools = 0; normal stool = 1; semi-solid stool = 2; watery stool = 3. The sum of the number of stool pellets scored as 2 and 3 defined diarrhoeic faeces. This experiment was also conducted in animals treated with aqueous extract.

1.6. Gastrintestinal motility evaluation. 24 h fasted mice (n = 6–8) were administered orally with saline, loperamide (20 mg/kg), or *E. dysenterica* essential oil (300 mg/kg), 30 min before the administration of 1.0 mL of charcoal meal (5.0% charcoal in 0.5% carboxymethylcellulose, p.o.). After 30 min, animals were killed by cervical dislocation under anaesthesia (thiopental, 60 mg/kg, i.p.), and the small intestine was excised (from the gastro-aesophageal to the ileocaecal junction). The distance travelled by charcoal meal from the pylorus was measured and expressed as the percentage of total length of the small intestine (Harrison et al. 2004, Uddin et al. 2005).

1.7. Intestinal fluid accumulation. Fluid accumulation was studied by enteropooling assay, as described by Capasso et al. (2002), with some modifications: 24 h fasted mice (n = 7–8) were orally treated with *E. dysenterica* essential oil (300 mg/kg) or vehicle (10% tween 80), or intraperitoneally treated with clonidine (1.0 mg/kg; standard antidiarrhoeic drug), 30 min before the administration of 0.8 mL of castor oil. After 30 min, animals were killed as described above, and the entire small intestine from each animal was isolated and its content carefully withdrawn and weighed. The enteropooling was then calculated as follows: $Pic/Pm \times 1000$, where Pic is the weight of small intestine content (mg) and Pm is the weight of mouse (g).

1.8. GC-MS analysis. The volatile compounds of *E. dysenterica* essential oil (1 μ L) were analysed using a gaseous chromatograph system coupled to a mass spectrometer (Shimadzu GC-MS, GC-17A/QP-5000). The operating conditions were: capillary column J&W, DB-5, 30 m, 0.25 mm i.d., film 0.25 μ m; the temperature program used was: 40°C (5 min), 40 to 60°C (4°C min⁻¹) and 60 to 250°C (8°C min⁻¹); injector at 250°C, interface at 240°C; electronic impact at 70 eV.¹⁰ The fragments were collected from 40 to 650 Daltons. Identification of oil components was based on the Kovats and arithmetic index (Adams 2007), and their mass spectral fragmentation patterns were compared with the Mass Spectra libraries (Wiley 139, 275 and 7 and Nist 127, Pherobase, Adams). The GC-MS quantification was obtained using the total ions chromatogram (TIC) and was expressed as a percentage (%).

1.9. Drugs. Castor oil and tween 80 were purchased from Sigma-Aldrich (USA), loperamide hydrochloride was purchased from Jasen-Cilag Farmacêutica (Brazil), clonidine was purchased from Cristália (Brazil), activated charcoal was purchased from Proquímicos (Brazil), and carboxymethylcellulose was purchased from a local pharmacy.

1.10. Statistical analysis. Data are given as the mean $\pm$ S.E.M for the indicated number of independent experiments and were compared using one-way ANOVA or Kruskal-Wallis with

the Dunnett or Dunn post-hoc test, as appropriated. The statistical analyses were performed using GraphPad® Software, and statistical significance was accepted at $P < 0.05$.

Figure S1. Effects of loperamide (LOP; 20 mg/kg, p.o.) and of the essential oil from *E. dysenterica* leaves (30 – 300 mg/kg, p.o.) on the total number of faeces (A) and the number of diarrhoeic faeces (B) in castor oil-treated mice (0.8 ml/mice). Control group (CTR) received vehicles and saline instead of LOP, essential oil or castor-oil as treatments. *P<0.05 vs. vehicle (Veh)-castor oil. N = 7–8.

Figure S2. Effects of the essential oil from *E. dysenterica* leaves (300 mg/kg, p.o.; EO-300) on charcoal meal intestinal transit (A) and intestinal fluid accumulation (enteropooling) induced by castor oil treatment (0.8 ml/mice) (B). Loperamide (20 mg/kg, p.o.; LOP) and clonidine (1.0 mg/kg, i.p.; CLO) were used as positive controls in A and B, respectively. Enteropooling is expressed as (Pic/Pm)×1000, where Pic is the weight of the small intestine (g) and Pm is the weight of the mouse (g). Control group (CTR) received vehicles (in A and B) and saline instead of castor-oil as treatments (only in B). *P<0.05 vs. CTR in A and *P<0.05 vs. vehicle (Veh)-castor oil in B. N = 6–8.

Table S1. Analysis of *E. dysenterica* leaves essential oil by CG-MS.

Peak	AI	Compound	Area (%)
1	932	Alpha-pinene	1.11
2	974	Beta-pinene	1.86
3	988	Beta-myrcene	2.98
4	1024	Limonene	2.89
5 and 6	1032	Cis-beta-ocimene	19.14
7	1044	Trans-beta-ocimene	4.99
8	1099	Alpha-pinene-oxide	0.71
9	1095	Linalool	5.16
10	1174	1-terpinen-4-ol	0.58
11	1186	Alpha-terpineol	2.91
12	-	(Z,Z)-2,6-dimethyl-3,5,7-octatriene-2-ol	0.72
13	-	(E,E)-2,6-dimethyl-3,5,7-octatriene-2-ol	1.06
14	1249	Trans-geraniol	1.45
15	-	n.i.	0.79
16	-	n.i.	0.91
17	-	n.i.	0.76
18	1417	(E)-caryophyllene	15.36
19	1437	Alpha-guaiene	1.44
20	1452	Alpha-humulene	8.07
21	1489	Beta-selinene	1.29
22	1498	Alpha-selinene	1.15
23	1509	Alpha-bulnesene	2.87
24	-	n.i.	0.81
25	1570	Caryophyllenyl alcohol	1.20
26	1571	(Z)-dihydro-apofarnesol	1.27
27	1582	Caryophyllene oxide	8.23
28	-	n.i.	0.67
29	-	n.i.	1.47
30	-	n.i.	3.08
31	-	n.i.	1.72
32	1639	Caryophylla-4(12),8(13)-dien-5-ol	0.68
33	1638	Tau-cadinol	0.74
34	1652	Alpha-cadinol	1.93
Total % of chromatogram areas identified			90.74

AI = arithmetic index (Adams, 2009); n.i. = not identified.

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