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Antileishmanial Potentialities of *Croton linearis* Leaf Essential Oil

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The essential oil was obtained from *Croton linearis* Jacq. leaves. Its chemical composition was analyzed by Gas Chromatography-Mass Spectrometry. A total of 82 compounds were identified, being the principal components: guaiol (7.93%); eudesma-4(15),7-dien-1β-ol (4.94%) and guaia-3,10(14)-dien-11-ol (4.52%). Antimicrobial activity was determined against six *Candida* spp., three bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomona aeruginosa*) and two parasites (*Leishmania amazonensis* and *Trypanosoma cruzi*). The selectivity index (SI) was computed through the determination of it cytotoxicity on peritoneal macrophages from BALB/c mice and murine fibroblasts L929 cells. Colorimetric microdilution methods as well as direct counting in optical microscopes were performed. The essential oil showed remarkable activity against *L. amazonensis* ($IC_{50Promastigote}$: 20.0 ± 4.9 µg/mL; $IC_{50Amastigote}$: 13.8 ± 4.3 µg/mL), moderate activity against *T. cruzi* ($IC_{50Trypomastigote}$: 197.26 ± 8.7 µg/mL) and weak activity against *P. aureginosa*. Cytotoxicity values were CC_{50}= 89.1 ± 3.4 µg/mL for macrophages BALB/c and CC_{50}= 306 ± 110 µg/mL for L929 cells, rendering a SI of 6 for amastigote form of *L. amazonensis*. *Croton linearis* Jacq. leaf essential oil, therefore, could be a potential candidate for future investigations regarding Leishmaniasis treatment.

Keywords: *Croton linearis*, Essential oil, Antileishmanial potential.

In recent years, a rapid increase of pathogenic micro-organisms resistant to antimicrobial drugs worldwide has been appreciated, representing a global public health problem [1]. The clinical effectiveness of many existing antibiotics is being threatened by rapid emergence of multidrug-resistant pathogens. Infectious diseases, a leading cause of untimely death worldwide, have become a global concern [2]. Over 700 000 deaths worldwide, including 214 000 neonatal sepsis deaths, are attributable to resistant pathogens bacteria each year [3]. Many infectious diseases have been identified to be treated with products from medicinal plants throughout the history of mankind. Nowadays natural products provide us with a large amount of opportunities for the development of new drugs and/or products with therapeutic potential to treat infectious diseases [4]. In the last few years, the discovery and development of new antimicrobial compounds with diverse chemical structures and novel mechanisms of action against pathogens has been a priority [5]. Essential oils (EO) and their components have been largely employed in the pharmaceutical, cosmetic and food industry as natural antimicrobial agents long since. The *in vitro* antimicrobial activity of essential oils has been well archived against a wide range of microorganisms [6], in particular, bacteria [7], fungi [8] and parasites [9].

Croton is one of the largest genera of Euphorbiaceae family, comprising around 1,300 species of trees, shrubs and herbs, distributed in tropical and subtropical regions of Asia, Africa and America [10]. Several species of these genera have proven pharmacological activity such as: acaricidal [11], antimicrobial [12], antihypertensive [13], vasorelaxant [14], anti-inflammatory, hypoglycemic, antitumoral, hypolipidemic, antioxidant, among others [15]. Many of these activities are associated to the diversity of metabolites that are present in *Croton* genus, mainly diterpenes, alkaloids, flavonoids, tannins and essential oils [16].

Essential oils from *Croton* species contain terpenoids (mono and sesquiterpenes) and rather low amounts of aliphatic compounds of non-terpene origin, and phenylpropanoids [17]. Several reports of EO and/or their components from *Croton* species against a wide of microorganism (bacteria, fungi and parasites) [18] support the pharmacological potentialities for the development of new natural products to treat infectious diseases. In Cuba, a total 47 species of *Croton* have been reported, which are used by Cuban people for different ailments due to theirs antispasmodic, analgesic, anti-catarrhal, astringent, antimalarial, antipyretic, diaphoretic and detersive properties [19]. *Croton linearis* Jacq. is an aromatic shrub endemic from Bahamas and Florida widely distributed in coastal regions of Caribbean countries such as Jamaica [20] and Cuba [19]. Ethnobotanical information refers the use as tea to relieve menstrual pains, of childbirths and associated with rheumatism. In addition, it is used for treating the symptoms of colds [20], as antipyretic [19], insecticidal, sedative and for circulation [21]. According of these facts and the interest to discover new antimicrobial therapeutic agents, the present study aims to evaluate the EO from *C. linearis* leaves against bacteria, fungi and parasites, as well as to determine its cytotoxicity.

The EO from *C. linearis* leaves was obtained by hydrodistillation-cohobation method using Clevenger apparatus, with a yield of 1.6%

Table 1. Chemical composition of essential oil from *Croton linearis* Jacq. leaves.

No.	Constituents	RI[a]	RI[b]	%	No.	Compuesto	RI[a]	RI[b]	%
1	Sabinene	962	965	0.21	43	Germacren-D-4-ol	1575	1574	1.56
2	Eucalyptol	1033	1031	2.45	44	Spathulenol	1579	1578	3.74
3	γ-Terpinene	1051	1056	0.13	45	Himachalene oxide	1588	1577	0.48
4	Linalool	1093	1097	0.04	46	**Guaiol**	1599	1600	**7.93**
5	*trans*-Sabinol	1114	1140	0.10	47	Viridiflorol	1604	1605	0.60
6	Terpinen-4-ol	1176	1180	0.52	48	1,10-di-epi-Cubenol	1612	1614	2.54
7	α-Terpineol	1196	1190	1.11	49	1-epi-Cubenol	1614	1617	0.82
8	Thymol methyl ether	1222	1235	0.13	50	γ-Eudesmol	1627	1632	0.09
9	*trans*-Verbenyl acetate	1269	1282	0.05	51	Selin-11-en-4-α-ol	1643	1650	0.28
10	Bornyl acetate	1277	1283	0.05	52	Intermedeol	1659	1660	0.74
11	Thymol	1285	1287	0.05	53	**Guaia-3,10(14)-dien-11-ol**	1667	1672	**4.52**
12	δ-Elemene	1321	1327	0.04	54	Cadin-4-en-10-ol	1672	1673	3.96
13	β-Terpinyl acetate	1338	1359	0.09	55	α-Germacra-4(15),5,10(14)-trien-1-ol	1676	1678	2.67
14	Cycloisosativene	1359	1362	0.17	56	*trans*-14-Hydroxy-9-epi-β-caryophyllene	1679	1682	1.98
15	Isoledene	1365	1372	0.02	57	epi-α-Bisabolol	1683	1684	0.59
16	Longicyclene	1372	1374	0.47	58	**Eudesma-4(15),7-dien-1β-ol**	1694	1690	**4.94**
17	**β-Elemene**	1384	1392	**4.13**	59	Unidentified	1699		0.15
18	Unidentified	1403	-	0.91	60	7-Hydroxyeudesm-4-en-6-one	1704	1703	2.80
19	Unidentified	1405	-	0.11	61	Eudesma-4,11-dien-2-ol l	1710	1714	0.66
20	β-Caryophyllene	1412	1416	1.22	62	Cedrenone	1718	1722	0.20
21	γ-Elemene	1423	1425	1.33	63	Isolongifolol	1726	1723	0.92
22	α-Humulene	1450	1451	1.49	64	γ-Costol	1730	1732	0.28
23	Alloaromadendrene	1453	1453	0.50	65	*trans*-Humul-(9*E*)-en-2,6-dione	1737	1739	1.10
24	2-epi-(*E*)-β-Caryophyllene	1461	1469	0.42	66	Drimenol	1743	1745	2.97
25	β-Acoradiene	1470	1472	0.25	67	β-Bisabolenol	1760	1753	0.65
26	α-Amorphene	1476	1480	3.68	68	*trans*-α-Atlantone	1768	1773	0.57
27	δ-Selinene	1485	1485	3.11	69	14-Hydroxy-α-muurolene	1779	1776	0.83
28	ε-Amorphene	1488	1497	0.58	70	(*E*)-Isovalencenol	1783	1783	0.34
29	α-Muurolene	1494	1497	1.43	71	epi-Cryptomeridiol	1790	1790	0.18
30	δ-Amorphene	1499	1510	1.88	72	14-Hydroxy-δ-cadinene	1802	1803	0.30
31	γ-Cadinene	1510	1513	1.97	73	8-oxo-Neoisolongifolene	1811	1808	0.82
32	Cadina-1,4-diene	1515	1515	3.48	74	Nootkatone	1819	1820	0.15
33	δ-Cadinene	1517	1518	0.43	75	Cryptomeridiol	1823	1822	0.14
34	Unidentified	1520	-	1.77	76	4,6-Eudesmadiene-3,8-dione	1829	1833	0.05
35	7-epi-α-Selinene	1524	1522	2.93	77	Zerumbone epoxide	1836	1830	0.06
36	**Selina-4(15),7(11)-diene**	1531	1534	**4.19**	78	Polygodial	1844	1853	1.25
37	α-Cadinene	1533	1536	0.09	79	Zizanoic acid	1849	1860	0.18
38	α-Calacorene	1537	1542	0.85	80	Pimaradiene	1937	1941	0.55
39	α-Elemol	1545	1551	1.56	81	Neocembrene	1950	1961	0.09
40	β-Calacorene	1556	1555	1.04	82	*p*-Camphorene	1973	1977	0.12
41	(*E*)-Nerolidol	1559	1562	0.18	83	Kaurene	2047	2048	0.30
42	(*E*)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	1568	1565	0.76	84	Abietadiene	2088	2084	0.98
Monoterpene hydrocarbons		2 (0.34 %)							
Oxygenated monoterpenes		10 (4.55 %)							
Sesquiterpene hydrocarbons		24 (35.52 %)							
Oxygenated sesquiterpenes		40 (54.54 %)							
Diterpene Hydrocarbons		5 (2.04 %)							

[a]Experimental Retention indices; [b]Reported Retention indices

The EO showed no activity against any of the strains *Candida* spp. tested, only was active against *P. aureginosa* at the highest concentration (1000 µg/mL). In contrast, EOs of other *Croton* spp. has showed antifungal and antibacterial activity. Fontenelle et al. report that essential oils from leaves of *C. zehntneri* (estragole 72.9% and anethole 14.3%), *C. nepetaefolius* (methyl-eugenol 15.7%) and *C. argyrophylloides* (spathulenol 20.3% and bicyclogermacrene 11.7%) were active against *Microsporum canis*, but only *C. zehntneri* showed some activity against *C. tropicalis* [16]. In addition, EO from *C. zehntneri* was effective against *S. aureus* and *E. coli* [24]. In other study, Alviano et al. observed that the EO from leaves of *C. cajucara* (linalool 41.2%), inhibits the growth of *C. albicans*, *Lactobacilus casei*, *Porphyromonas gengivalis*, *S. aureus* and *Streptococcus mutans*, all involved in diseases of the oral cavity [25].

According to literature, the pharmacological activity of an EO is related to their major components, without ruling out the synergistic contribution that could be made by some components found in relatively inferior abundances. In *C. linearis* oil only one compound (guaiol 7.93%) exceeds 5%. This compound has been reported for its antifungi activity but not antibacterial. The moderate activity observed against *Candida* species of guaiol with MIC range of 500 to 1000 µg/mL was associated with the presence of this compound in the oils of *Callitris neocaledonica* (30.2%) and *C. sulcata* (16.1%) [26]. Therefore, the inactivity of EO tested against fungi, could be related to the fact that no compound is in relatively high abundance to excerpt a relevant activity. On the other hand, the weak antibacterial activity observed against *P. aeruginosa* could be related to the synergic action of some minor components whose antimicrobial activity has been reported: α-terpineol (1.11 %), terpinen-4-ol (0.52%), linalool (0.04%), eucalyptol (2.45 %), bornyl acetate (0.05) and α-humulene (1.49 %) [6].

The EO of *C. linearis* was active against the promastigote forms of *L. amazonensis* (Table 2). The antileishmanial activity has been reported for other oils of the genus. The EO from the leaves of *C. cajucara* and its purified component 7-hydroxycalamenene showed *in vitro* activity againts *L. chagasi* promastigotes with IC_{50} values of 66.7 µg/mL and 11.37 µg/mL, respectively [18]. Additionally, the EO from *C. macrostachyus* fruit showed activity against promastigotes of *L. donovani* and *L. aethiopica* with IC_{50} values of 0.08 and 0.16 µL/mL, respectively [27].

Table 2. Antiparasitic evaluation of the essential oil from *Croton linearis* Jacq. leaves.

Antiparasitic Activity and Cytotoxicity				
BALB/c mice Macrophages	***Leishmania amazonensis***			
	Promastigotes		**Amastigotes**	
CC_{50} (µg/mL)	IC_{50} (µg/mL)	SI	CI_{50} (µg/mL)	SI
89.1 ± 4.3	20.0 ± 4.9	4	13.8 ± 4.3	6.46
L929 cells	***Trypanosoma cruzi***			
	Trypomastigotes			**Amastigotes**
CC_{50} (µg/mL)	IC_{50} (µg/mL) 2 h	IC_{50} (µg/mL) 24 h	SI	% Inhibition infection (10 µg/mL)
306.0 ± 110.0	>300	197.26 ± 8.7	1.55	13.32 ± 4.7

The possible mechanisms of activity of oils against the promastigote forms of *Leishmania* spp. have been described in the literature. Previous studies have suggested that β-caryophyllene (a sesquiterpene hydrocarbon) and nerolidol (an oxygenated sesquiterpene) are examples of terpenic substances with well-characterized anti-*Leishmania* activity, possibly associated with inhibition of cellular isoprenoid biosynthesis [28, 29]. In addition, it has been shown that the lipophilic components of EOs may affect layers of polysaccharides, fatty acids, and phospholipids in plasmatic membranes of promastigotes of *Leishmania* spp. leading to the cell lysis and the release of macromolecules [30]. In the cytoplasm, these substances can disrupt the specific metabolic pathways of lipids and proteins or stimulate depolarization of mitochondrial membranes, which can lead to cell necrosis or apoptosis [31].

None of the major components detected in the EO of *C. linearis* have been reported to be against *Leishmania* spp. in pure conditions, although others in minor concentration have been reported as antileishmanial agents, such as eucalyptol (2.45%) [32] and spathulenol (3.74%) [33]. Likewise, researchers have attributed the activity against different species of *Leishmania* to oxygenated sesquiterpenes, which represent 54.54% of the relative abundance in the *C. linearis* oil. Therefore, a potential synergic action between the oxygenated sesquiterpenes may be one of the causes this leishmanicidal action of *C. linearis* oil [34].

Compared to the amastigote forms of *L. amazonensis*, a more potent activity was observed with an IC_{50} 13.8 µg/mL (Table 2). According to literature, the EO, due to their lipophilic character, diffuse easily through cell membranes, acting directly on the parasite and/or stimulate cellular mechanisms for its elimination. An important pathway that may be involved in the mechanism of anti-*Leishmania* activity could be the stimulation of NO production in the macrophages. Once inside, NO together with reactive oxygen species induce the apoptosis of parasites [35]. However, in order to establish some of these mechanisms other studies are required, since none of the major components has been demonstrated the activity against the intracellular forms of *Leishmania* spp. According to these results, we suggest the activity observed against amastigotes could be related to the synergistic action of several components.

The EO of *C. linearis* showed moderate activity against trypomastigote of *T. cruzi* after 24 hours of treatment. The activity against this parasite has not been previously documented for other *Croton* species. Nevertheless, several oils of other plant species have been active against *T. cruzi*, demonstrating the potential of EO in the search for new therapeutic alternatives to Chagas disease. For example, the EO from *Origanum vulgare* L. inhibited epimastigote growth (IC_{50}/24 h=175 µg/ml) and also induced trypomastigote lysis (IC_{50}/24 h=115 µg/mL) of *T. cruzi*. In the same study, *Thymus vulgaris* L. oil also showed activity with lower IC_{50} values of 77 µg/ml and 38 µg/ml against epimastigotes and trypomastigotes, respectively, after 24 h [36]. The trypanocidal effect of six EO extracted from *Lippia sidoides*, *Lippia origanoides*, *Chenopodium ambrosioides* L., *Ocimum gratissimum*, *Justicia pectorales* and *Vitex agnus-castus* were evaluated. All EOs tested demonstrated an inhibitory effect on the parasite growth of trypomastigote forms, with IC_{50} values in the range of 11.5-155.8 µg/mL [37].

None of the major components of *C. linearis* oil has been reported to show trypanocidal activity, however some of the minor components have been active against *Trypanosoma* spp. such as: allo-aromadendrene (0.50%), linalool (0.04%), α-humulene (1.49%), (*E*)-nerolidol (0.18), sabinene (0.21%), terpinen-4-ol (0.52%) and thymol (0.05%) [9]. These results suggest again that the observed activity could be related to the joint action of these components and not by the major compounds of essential oil. In contrast to the results in the *Leishmania* assay, the EO was not effective against intracellular amastigote form of *T. cruzi* at the concentration of 10 µg/mL, caused a low reduction of infection in L929 cells (Table 2).

Numerous reports the literature suggest regarding the good activity of EO against parasites than against microorganisms. Essential oil obtained from leaves of *Vernonia brasiliana* (L) Druce (germacrene-D 10.19 %) was not active against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), nevertheless it showed activity against trypomastigotes of *T. cruzi* (IC_{50}= 72 µg/mL) and promastigotes of *L. amazonensis* (IC_{50}= 213 µg/mL) [38]. This behavior is very similar to the obtained in our study, which confirms that the biological activity of EO from leaves of *C. linearis* may be related to the synergistic action of all its components. Nevertheless, further studies are required to confirm the antiparasite activity of the pure components, and to establish the possible mechanisms of action.

Due to the need for antiparasitic compounds more selective for the parasite and less toxic to host cells, it was important to evaluated the cytotoxic activity of EO from *C. linearis* on two cell lines, peritoneal macrophages (model used against *Leishmania*) and L929 cells of fibroblasts (model used against *Trypanozoma*), both from mice. The EO showed moderate cytotoxicity against peritoneal macrophages with a IS of 4 and 6 for promastigotes and amastigotes of *Leishmania*, respectively. In contrast, on L929 cells, the oil showed a CC_{50} value of 306.0 µg/mL, however the IS calculated for the trypomastigote of *Trypanosoma* was 1.5, indicating its poor selectivity.

In summary, for first time is described the chemical composition of the essential oil of *C. linearis* leaves, with guaiol the most abundant compound (7.93%). This EO showed activity only against one bacterium (*P. aeruginosa*) and weak activity against *T. cruzi.* In contrast, the oil showed a good activity against promastigotes and amastigotes of *L. amazonensis*, which demonstrate its potential use in the search for a possible candidate for the treatment of leishmaniasis.

Experimental

Plant material: Leaves of *C. linearis* were collected from Siboney-Juticí Ecological Reserve (where is widely distributed), Santiago de Cuba, Cuba in September, 2015. A specimen of the plant was identified and authenticated by the taxonomist Ing. Felix Acosta and a voucher sample was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity BSC "Jorge Sierra Calzado" under number 21 659. The plant was collected on the morning of the same day in which EO was extracted.

Extraction and analysis of essential oil: Fresh leaves from *C. linearis* (200 g) were subjected to hydrodistillation-cohobation extraction method, using a Clevenger equipment for 3 h. Essential oil was dried using anhydrous sodium sulfate and stored under refrigeration at 8 ± 2 ^{0}C, in amber glass bottles until the chemical analysis and bioassays could be conducted. The oil yields were calculated and expressed as a percentage (v:w). Chemical composition of EO was determined by Gas Chromatography-Mass Spectrometry (GC/MS) helped by a Shimadzu (model GCMS-QP500 Ultra) equipment (Unit of Instrumental Analysis, Medical Toxicology Centre, Medical Sciences University/Cuba). The separation conditions were: DB-5MS capillary column (30 m × 0.32 mm × 1 μm), temperature program (30 ^{0}C for 1 min, temperature increase at 4 ^{0}C/min until reaching 250 ^{0}C for 10 min.), and injection temperature (270 ^{0}C). Helium was used as carrier gas at 28.3 cm/second linear velocity and a flow rate of 1 mL/min; Splitless time (1 min); ionic capture detector impact energy: 70 eV. Retention indices (RI) for all the components were determined relative to a series of linear alkanes (C8–C20) injected under the same chromatography conditions. Compounds identification was based on RI comparison and mass spectra computer matching (NIST and FFNSC 1.3. library) and the literature. Compound concentrations (%) were calculated using the GC peak area values.

Antimicrobial activity

Microorganism: The antimicrobial activity of the EO was tested against three bacteria, six yeast and two parasites. Bacterial strains were: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Pseudomona aeruginosa* ATCC 9027. Yeast strains were: *Candida albicans* ATCC B59630 (Azol resistant), *Candida glabrata* ATCC B63155, *Candida kefyr* ATCC B46120, *Candida krusei* ATCC B68404, *Candida parapsilosis* ATCC J941058 and *Candida tropicalis* CDC49. All strains were supplied from culture collection of Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Belgium. Parasite stains were: *Leishmania amazonensis* MHOM/BR/77/LTB0016 supplied by the Institute of Tropical Medicine Pedro Kourí, Habana, Cuba and *Trypanosoma cruzi* (Y and Tulahuen strains) supplied by the Laboratory of Celular Biology, Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, Brazil.

Antibacterial and antifungal activity: *In vitro* antibacterial and antifungal activity were determined by the microdilution method with resazurin (redox indicator) in sterile 96-well microplates [39]. Bacteria were grown in Mueller Hinton Broth and maintained on Tryptone Soy Agar, while the yeast grow up in RPMI-1640 medium. Stock solution of EO was diluted in 100% DMSO at 1 g/mL and serial dilutions were made to obtain three level concentrations: 0.25, 0.5 and 1.0 mg/mL. Each concentration was screened in triplicate. Ampicillin and Miconazol were used as reference drugs. In each well, 10 μL of the watery compound dilutions were added together with 190 μl of bacteria inoculum (5 × 10^5 CFU/mL) and yeast inoculum (5 × 10^3 CFU/mL). In the microplates were included untreated-control wells (100% cell growth) and medium-control wells (0% cell growth). Then, the microplates were incubated at 37^{0}C for 17 hours (for bacteria) and 24 hours (for yeast). After, 20 μL of resazurin (SIGMA, St. Louis, MO, USA) at 50 μg/ml per well were added and the microplates incubated under the same conditions of temperature (bacteria: 30 min and yeast: 4 hours). The microbial growth was determined by fluorimetry method (λ_{ex}= 550 nm, λ_{em}= 590 nm) using a microplate reader (TECAN GENios, Germany). The product was classified as active, when the bacterial growth inhibition (%) was greater than 50%. The results are expressed as % reduction in bacterial growth/viability compared to control wells.

Antileishmanial assay

Anti-promastigote Assay: *S*tock solution of EO was diluted in 100% DMSO at 20 mg/mL. In the first well of 96-well microplate, 2 μL of EO was added to 98 μL of Schneider's medium (SIGMA, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA-Aldrich) and penicillin-streptomycin as antibiotics (SIGMA); while 50 μL of medium were distributed in the other wells. Then, serial dilutions 1:2 were carried out to obtain final concentrations between 12.5 and 200 μg/mL. Afterwards, 50 μL of promastigotes were added at 4 × 10^5 parasite/mL in logarithmic phase. Microplates were sealed with parafilm and incubated at 26^{0}C for 72 hours. After incubation, 20 μL of a solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, SIGMA, St. Louis, MO, USA) at 5 mg/mL was added in each well. An additional incubation of 4 hours was performed, the supernatant was eliminated, and the formazan crystals were dissolved with 100 μL of DMSO. Finally, the microplate was read in an ELISA microplate reader (Sirio S Reader, 2.4-0, Italy) at 540 nm and 620 nm as reference wavelength [40]. The median inhibitory concentration (IC_{50}) was determined by dose-response linear regression analysis. Each experiment was performed in duplicated and the results were expressed as the mean ± standard deviation.

Anti-amastigote Assay: Peritoneal macrophages from BALB/c mice were collected and seeded with a density of 10^6/mL in 24-well plates and incubated at 37^{0}C and 5% CO_2 atmosphere for 2 hours. Non-adherent cells were removed. Cells were infected with promastigotes (in stationary phase) at a ratio of 4:1 parasite/macrophages and incubated for 4 hours under the same conditions. Afterwards, the cells were washed to remove free parasites and 1 990 μL RPMI medium and 10 μL of EO were added in the first well. Then, serial dilutions 1:2 were performed to test concentrations between 12.5 and 100 μg/mL. Plates were incubated for 48 hours under the same conditions above mentioned. Finally, cultures were fixed with absolute methanol, stained with Giemsa and examined under light microscopy (oil immersion lens 1000X). For each sample, the number of intracellular amastigotes and the percentage infected macrophages were determined in 25 macrophages. Results were expressed as the percentage of reduction of the infection rate (which was obtained multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages) in comparison with the negative controls. The IC_{50} was determined by dose-response linear regression analysis. Each experiment was performed in duplicated and the results were expressed as the mean ± standard deviation. In both experiment pentamidine (Richet, Buenos Aires, Argentina) at 10 mg/mL was used as reference drug.

Trypanocidal Activity

Anti-Trypomastigote Assay: Trypomastigote forms (Y strain) were incubated in 96-well microtrite plates, 100 μL of suspension (in RPMI + 5% fetal bovine serum FBS) containing 10^7 parasites/mL added an equal volume of EO (diluted into RPMI + 5% FBS) at twice the desired final concentration (from 9.37 to 300 μg/mL). After 2 and 24 h treatment at 37^{0}C, the number of living parasites was determined by quantifying the light microscopy in a Neubauer chamber. Controls were performed with parasites kept under the same conditions in the absence of treatment. The activity was expressed by calculating the IC_{50} value for 2 and 24 h treatment. IC_{50} values were calculated based on the drug concentration that reduced 50% of parasites by linear regression.

Anti-Amastigotes Assay: In a 96-well plate, 4 × 10^3 L929 cells /well in 80 μL RPMI medium supplemented with 10% FBS, 2% L-

glutamine and phenol red were seeded. The plate was incubated "overnight" in an oven at 37^0C and 5% CO_2 atmosphere. Cells were infected with 40×10^3 trypomastigotes/well, 20 µL diluted in RPMI supplemented with phenol red. The plate was incubated at same conditions and after 2 hours the free parasites were removed with RPMI medium without phenol red supplemented. The plate was incubated under the same conditions for 8 hours until infection was accomplished. Afterwards, the culture medium was exchanged for 180 µL medium supplemented RPMI without phenol red plus 20 µL of EO dilution at 10 µg/mL. The following controls were used per plate: uninfected cells without addition of compounds infected cells, without addition of product, cells infected and treated with Benznidazole and infected cells treated with DMSO 1%. The plate was incubated under the same conditions for 96 hours. Finally, 50 µL/well of chlorophenol red-β-D-galactopyranoside CPRG substrate were added and the plate reading was taken after 16 to 20 h in ELISA reader (VersaMax tunable microplate reader; Molecular Devices, USA) with 570 nm filter [41].

Cytotoxicity assay

Peritoneal macrophages: Resident macrophages from the peritoneal cavity were collected and washed with RPMI 1 640 medium (SIGMA), supplemented with antibiotics (penicillin 200 IU, streptomycin 200 µg/mL). Then, 1-3 × 10^6 macrophages/mL were seeded in 96-well plates (Costar, USA) and incubated for 2 hours at 37 ^{0}C and 5% CO_2 atmosphere. The non-adherent cells were removed by washing with phosphate buffer solution. Subsequently, RPMI medium and EO at the tested concentrations (12.5-200 µg/mL) were added per well. The plates were incubated for 72 hours under the same conditions and macrophages treated with DMSO were included as control. The cellular viability was determined by colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, SIGMA, St. Louis, MO, USA), which 15 µL MTT solution were added per well. The median cytotoxic concentration (CC_{50}) was determined by dose-response linear regression analysis. Each experiment was performed in duplicated and the results were expressed as the mean ± standard deviation.

L929 cells: Monolayers of mice L929 fibroblasts were cultivated (4 × 10^3 cells/well in 96-well microplates) at 37^0C in RPMI 1 640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine (RPMIS). The microplates were incubated for 96 hours at same conditions with different concentrations of EO (up to 1 200 µg) diluted in RPMI (without phenol red). The morphology was evaluated by light microscopy. In each well, 10 µL de resazurin was added and the microplates were incubated for 5 hours. Finally, the cellular viability was determined by the colorimetric bioassay at reference wavelength of 570 and 600 nm [40]. As negative controls, L929 cells in RPMI medium, RPMI medium and RPMI containing each tested compound were included. The results were expressed as the percent differences in the decreases between EO-treated and vehicle-treated cells and the CC_{50} were determined.

The selectivity index (SI) was calculated using the IC_{50} (for protozoan) and CC_{50} (for macrophages and L929 cells) values obtained from each *in-vitro* assay. The SI was used to analyze the toxicity versus activity of EO from *C. linearis* leaves.

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