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Supplementary Material

Antileishmanial Activity and Influence on Mitochondria of the Essential Oil from *Tagetes lucida* Cav. and Its Main Component

Lianet Monzote [1,2,*], Yamilet Gutiérrez [3], Laura Machin [3], Katrin Staniek [4], Ramón Scull [3], Prabodh Satyal [5], Lars Gille [4], and William N. Setzer [2,5,6,*]

S1. Materials and Methods

S1.1. Plants and EO from T. lucida

Aerial parts of *T. lucida* were obtained from Pharmacy and Food Institute, Coronela Locality (23°04′00″N, 82°28′00″ W, 10 m asl), Havana, Cuba, in October 2017. A voucher specimen was deposited in the National Botanic Garden, Havana, Cuba, under number HFC-88671. The fresh plant sample was collected early in the morning, manually crushed and the EO was immediately hydrodistilled using a Clevenger type apparatus for 5 hours. For the biological assays, the EO was dissolved in dimethyl sulfoxide (DMSO; AppliedChem, Panreas, Germany) at 20 mg/mL.

S1.2. Chemical characterization of EO from T. lucida

The EO was analyzed by gas chromatography – mass spectrometry (GC-MS) and identification of the oil components was based on their RI determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the databases [1-4]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

S1.3. Estragole

The pure compound was purchased from Fluka, Switzerland, with a purity of 97%. Estragole was dissolved in DMSO at a concentration of 6 mg/mL to carry out the biological assays.

S1.4. Parasites and culture

LtP strain P10 obtained from Jena Bioscience (Germany) were cultivated in brain heart infusion (BHI; Sigma-Aldrich, St. Louis, MO, USA) medium (37 g/L), supplemented with 5 mg/L hemin (Sigma-Aldrich, St. Louis, MO, USA). A reference strain of LaP (MHOM/77BR/LTB0016), kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil, was used and culture at 26 °C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (HFBS) (Sigma-Aldrich, St. Louis, MO, USA). In both cases, penicillin/streptomycin was used to prevent contamination.

S1.5. Macrophages

Peritoneal macrophage from BALB/c mice (PMM) were obtained from peritoneal cavities of euthanized normal BALB/c mice in ice-cold RPMI 1640 medium (SIGMA, St. Louis, MO, USA), supplemented with same antibiotics. Animals were obtained from the National Center of Laboratory Animals Production (CENPALAB, Mayabeque, Cuba) and used in accordance with the recommendations and guidelines for the care and use of laboratory animals (CEI-IPK 14-12). In parallel, the murine macrophage cell line J774A.1 (ATCC®, TIB-67™, Wesel, Germany) was cultivated

in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented at 10% of HFBS and antibiotics. To maintain cells in suspension, J774 macrophages were maintained on a roller culture apparatus at 5 rpm at 37 °C and 5% CO_2.

S1.6. In vitro antipromastigote activity

The products were assayed against promastigote forms of both parasite strains: LtP and LaP. In the case of LtP, products were assayed in a mixture of yeast extract medium (YEM: consisted of 20.7 g/L yeast extract powder from Solon (Ohio, USA) and 1.2 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 2.9 g/L glucose from Merck (Darmstadt, Germany) at pH 7.4 and phosphate buffered saline (PBS: contained 136 mM NaCl, 1.15 mM KH_2PO_4, 14 mM Na_2HPO_4, 2.7 mM KCl; pH 7.4) at 1:1 (v/v) supplemented with 25,000 U/L penicillin, 25 mg/L streptomycin and 6 μM hemin (SIGMA, St. Louis, MO, USA). Aliquots of 200 μL of parasites suspension (4×10^6 cells/mL) were distributed in 96-well plates with different dilutions of assayed products. Control rows containing YEM:PBS (no activity) and with untreated LtP (100% activity) were also loaded on the plate and incubated at 26 °C for 48 hours. Following, 50 μL of a resazurin (SIGMA, St. Louis, MO, USA) solution in PBS was added to each well giving a final concentration of 20 μM. After additional 4 hours of incubation, the fluorescence was measured at 560 nm excitation and 590 nm emission using a plate reader (Perkin Elmer Enspire, Germany).

Activity of products on LaP was assessed in supplemented Schneider's medium with antibiotics and 10% of HFBS. Briefly, 50 μL of medium was distributed in each well of a 96-well plate. In the last row, 48 μL of additional medium and 2 μL of products were also added. Then, six dilutions 1:2 was carried out taking 50 μL each time and 50 μL of exponentially growing cells at a final concentration of 4×10^6 promastigotes/mL were added to test concentrations of products ranging from 6.25 to 200 μg/mL. Additional control rows containing medium (no activity) and untreated parasites (100% activity) were also included in first and second row, respectively. After 48 hours at 26 °C of incubation, 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; SIGMA, St. Louis, MO, USA) was added, which was prepared at 5 mg/mL in PBS, filtered through a 0.2 μM filter at moment of use. An incubation of additional 4 hours at same conditions was performed, the supernatant was eliminated and the formazan crystals were dissolved with 100 μL of DMSO. Absorbance was then measured using a spectrophotometer (Molecular Devices, USA), at a test wavelength of 560 nm and 620 nm as reference [5,6].

S1.7. Mitochondrial function assessment

To analyze the influence of studied products on mitochondrial function, two assays were used: (i) measurement of oxygen consumption in LtP and (ii) evaluation of variation of membrane mitochondrial potential ($\Delta\psi m$) in LaP.

LtP oxygen consumption was measured using round-bottomed OxoPlates (OP96U, Precision Sensing GmbH, Regensburg, Germany). Thirty milliliters of BHI medium was stirred on a magnetic stirrer for 30 min at 25 °C to saturate it with air (aBHI) in an open Erlenmeyer flask and 2 mL of medium was mixed with approximately 50 mg of the reducing agent sodium dithionite in a tightly closed falcon tube to remove the contained oxygen (dBHI). Aliquots of 200 μL of both calibration media (aBHI and dBHI) were distributed to six wells each of the row A of an OxoPlate. To prevent oxygen exchange with the environment, 70 μL of paraffin oil (SIGMA, St. Louis, MO, USA) were layered on top of each well containing liquid to close the wells. The plate was then incubated in the dark for 15 min at 25 °C for equilibration. Then, the fluorescence of both calibrated media was measured with a microplate reader (Perkin Elmer Enspire, Germany) in a dual kinetic mode using an excitation wavelength of 590 nm and emission wavelengths of 650 nm. The ratio of both intensities was calculated for each well and averaged giving the two calibration constants k0 or k100. Then, the rest of plate was filled with 50 μL of aBHI. The row B was used as respiration control of reference parasites. In the row C, 49 μL of aBHI and 1 μL of test sample were added and serial dilution was performed by transferring 50 μL of the mixture in five subsequent 1:2 dilutions. Then, 150 μL of LtP at 8×10^7 parasites/mL in aBHI were added to each well giving final concentrations between 6.25 and 100 μg/mL of test samples, which were done in triplicate. Immediately, each well was sealed with 70

μL of paraffin oil. The fluorescence intensity of each well was determined in 5-min intervals over 40 min. The oxygen concentration (μM O_2) was calculated using the following equation (PreSens – Precision Sensing GmbH): O_2 (μM) = 100 × (k0/IR – 1) / (k0/k100 – 1) × 2.83. The slope of the change of the oxygen concentrations over time was calculated giving the oxygen consumption rate (μM/min) for each well. The value obtained for aBHI was subtracted to eliminate the influence of the medium. The rate was finally normalized to the oxygen consumption rate of the wells that did contain cells but no test compounds. In parallel, a mixture of rotenone (Sigma-Aldrich, St. Louis, MO, USA) and antimycin A (Sigma-Aldrich, St. Louis, MO, USA) at 4 μM for each were used as positive controls.

In LaP, the Δψm was evaluated using the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) [7]. In this case, the parasites at 10^6 promastigotes/mL were treated with products at 100 μg/mL during 4, 24 and 48 hours in Schneider medium and 26 °C. Negative control was included (treated with 1 μL of DMSO) and positive controls were treated with valinomycin (Sigma-Aldrich, Steinheim, Germany) at 5 μM concentration. At the different incubation periods, 1 μL of 15 μM JC-1 (Biotium, Hayward, USA) was added to each sample and an additional incubation of 10 min were performed. Afterwards, a part of the medium with parasites was observed at 400× using an Olympus fluorescence microscope (Tokyo, Japan) with a U-MWB2 mirror unit (maximum transmittance between 449 nm and 600 nm) and pictures were taken with a Samsung camera (China).

S1.8. In vitro anti-amastigote activity

Activity of products against intracellular amastigotes of *L. amazonensis* (LaA) was also evaluated [8]. PMM at 10^6/mL in 1 mL were plated in 24-well plates with sterile slides and incubated at 37 °C under an atmosphere of 5% CO_2. After 2 hours, non-adherent cells were eliminated and LaP in stationary-phase were added at a 4:1 parasite/macrophage ratio in RPMI medium with antibiotics and 10% of HFBS. The cultures were incubated for 4 hours at same conditions and free parasites were removed. Then, 1995 μL of RPMI completed medium with additional 5 μL of the test samples were added. Five dilutions (1:2) were carried out taking 1000 μL each time, in duplicate cultures and the plate was incubated for 48 hours under the same conditions. Additional control cultures were included, treated with DMSO or reference drug. Then, the supernatant was eliminated and resulting monolayers were fixed with absolute methanol, stained with Giemsa, and examined under light microscopy with immersion oil at 1000×. The number of intracellular amastigotes and the percentage of infected macrophages were determined in 100 macrophages. Infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages and the reduction of the infection rate in comparison to controls was calculated [9]. Pentamidine (Sigma-Aldrich, St. Louis, MO, USA) was used as reference drug.

S1.9. Cytotoxicity assay

Cytotoxicity of products against non-infected macrophages was also determined in parallel, including PMM from BALB/c mice obtained at moment of use in RPMI and murine macrophage cell line J774A.1 (mouse, ATCC, TIB-67™) cultivated in DMEM. First, PMM were collected as previously was described, seeded at 10^6 cells/mL in 96-wells plate and allowed to adhere for 2 hours at 37 °C and 5% CO_2. Then, free cells were removed and 50 μL of RPMI medium supplemented with antibiotics and HFBS was distributed in each well. In the last row, 48 μL of additional medium and 2 μL of products were also added, performing six dilutions 1:2 taking 50 μL each time. Finally, additional 50 μL of medium were added and controls of medium (no activity) or non-treated macrophages (100% viability) were included. The plate was incubated for 48 hours under the same conditions and viable cells were determined using 15 μL of MTT as described above. After 4 hours of incubation at 37 °C and 5% CO_2, formazan crystals were dissolved and optical density was determined using same protocol [5].

In the case of J774A, 200 μL with 10^5 cells/mL were distributed in the wells of the 96-well microplates. The cells were incubated for 24 hours (37 °C, 5% CO_2) to allow attachment, supernatant was discarded to remove non-adherent cells and washed with 200 μL PBS per well. Then, treatment

was carried out as previously was described above and the plate was incubated for additional 24 hours at 37 °C and 5% CO_2. Finally, 50 µL of resazurin was used to determine the cellular viability and the measurement of fluorescence was performed as explained above.

In both cases, pentamidine was also included as positive control

S1.10. Statistical analysis

In promastigote and cytotoxicity assay the median inhibitory concentration (IC_{50}) and median cytotoxic concentration (CC_{50}) was calculated, respectively by lineal regression curves. In all cases, three replicates of each concentration of each product in a plate were included and results were expressed as mean ± standard deviation (SD) of three independent experiments performed in different days. In addition, selectivity indices (SI) were calculated by dividing the CC_{50} for macrophages by the IC_{50} for LaA [10].

Statistical differences between IC_{50} values were determined by Mann-Whitney test using Statistic for Windows Program (Version 10, StatSoft, Inc.), considering statistical differences as $p < 0.05$.

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Table 1. Chemical composition of essential oil from *Tagetes lucida* Cav. from Cuba.

RI_{calc}	RI_{db}	Compound	Area %
848	850	(2*E*)-Hexenal	tr
960	959	Benzaldehyde	tr
988	989	Myrcene	1.1
1004	1005	(3*Z*)-Hexenyl acetate	tr
1029	1030	Limonene	tr
1034	1034	(*Z*)-β-Ocimene	tr
1043	1043	Benzene acetaldehyde	tr
1045	1046	(*E*)-β-Ocimene	0.1
1099	1101	Linalool	0.2
1155	1156	β-Pinene oxide	tr
1205	**1198**	**Methyl chavicol (Estragole)**	**96.9**
1250	1252	Chavicol	0.1
1254	1253	*p*-Anisaldehyde	tr
1286	1285	(*E*)-Anethole	0.1
1350	1356	Eugenol	tr
1366	1370	Chavibetol	0.1
1376	1375	α-Copaene	tr
1378	1380	(*E*)-β-Damascenone	tr
1384	1387	β-Bourbonene	tr
1388	1387	β-Cubebene	tr
1389	1390	β-Elemene	tr
1391	1392	(*Z*)-Jasmone	tr
1398	1398	Methyl eugenol	tr
1420	1417	β-Caryophyllene	0.1
1433	1432	*trans*-α-Bergamotene	tr
1452	1452	(*E*)-β-Farnesene	0.1
1456	1454	α-Humulene	tr
1481	1483	Germacrene D	0.2
1484	1483	*trans*-β-Bergamotene	0.1
1487	1500	Pentadecane	tr
1498	1497	α-Muurolene	tr
1503	1505	(*E*,*E*)-α-Farnesene	0.1
1518	1520	δ-Cadinene	tr
1548	1549	Elemol	0.1
1557	1561	(*E*)-Nerolidol	tr
1563	1561	7-Hydroxyfarnesene	tr
1566	1560	(*E*)-*p*-Methoxycinnamaldehyde	0.4
1582	1587	Caryophyllene oxide	tr
1609	1614	1,10-di-*epi*-Cubenol	tr

1625	1625	(2,7*Z*)-Bisabolandien-4-ol	tr
1632	1633	γ-Eudesmol	tr
1642	1643	*α*-Muurolol (Torreyol)	tr
1644	1644	τ-Muurolol	tr
1655	1655	*α*-Cadinol	tr
1684	1683	Germacra-4(15),5,10(14)-trien-1*α*-ol	tr
1697	1700	Heptadecane	tr
1719	1719	Herniarin	0.1
1836	1836	Neophytadiene	0.1
2106	2106	Phytol	tr
		TOTAL	99.9%

RI_{calc} represents the Kovats retention indices calculated in relation to a series of hydrocarbons. RI_{db} are the retention indices from the databases [12-15]. Area % is the percentage of the respective signals in relation to the total ion current (TIC) of the mass spectrometric (MS) detector. "tr" = trace (< 0.05%).