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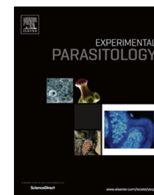

Essential oil from *Chenopodium ambrosioides* and main components: Activity against *Leishmania*, their mitochondria and other microorganisms

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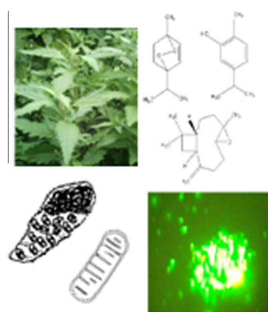
HIGHLIGHTS

- *Chenopodium ambrosioides* oil showed potential antileishmanial activity.
- *Chenopodium* oil caused better selectivity compared with its major components.
- Products cause a breakdown of mitochondrial membrane potential and redox indexes.
- Oil showed effect against *Plasmodium falciparum* and *Trypanosoma brucei*.

GRAPHICAL ABSTRACT



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ABSTRACT

Chenopodium ambrosioides is an aromatic herb used by native people to treat parasitic diseases. The aim of this work is to compare the *in vitro* anti-leishmanial activity of the essential oil (EO) from *C. ambrosioides* and its major components (ascaridole, carvacrol and caryophyllene oxide) and study their mechanism of action and activity against a panel of microorganism. Antileishmanial activity and cytotoxicity of the EO and major components was study. In addition, experiments to elucidate the mechanism of action were perform and activities against other microorganisms (bacteria, fungi and protozoa) were evaluate. All products were active against promastigote and amastigote forms of *Leishmania*. Ascaridole exhibited the better antileishmanial activity and the EO the highest selectivity index. The exploration of

Abbreviations: $\Delta\psi m$, mitochondrial membrane potential; Asc, ascaridole; Carv, carvacrol; Caryo, caryophyllene oxide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EO, essential oil; FCSi, inactivated fetal calf serum; FIC, fractional inhibitory concentration; HFBS, heat-inactivated fetal bovine serum; IC_{50}, concentration at which inhibition of the activity was 50%; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; MCF, mitochondrial crude fraction; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; O–O, bound oxygen–oxygen; PP, peroxidation potential; SHT, sulfhydryl groups; TDR, Tropical Diseases Research; WHO, World Health Organization.

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Ascaridole
Mitochondria
Antimicrobial activity

the mechanism suggests that the products cause a breakdown of mitochondrial membrane potential and a modification of redox indexes. Only EO showed antiprotozoal effect against *Plasmodium falciparum* and *Trypanosoma brucei*; while no activity against bacteria and fungi was observed. Our results demonstrate the potentialities of EO in cellular and molecular system, which could be consider in future studies to develop new antileishmanial drugs with a wide anti-parasitic spectrum.

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1. Introduction

Chenopodium ambrosioides L. (*Chenopodiaceae*), an aromatic plant abundant in tropics and adjacent warmer regions of America and Africa, has been used by native people to treat parasitic diseases (Cruz et al., 2007). Beginning in the 19th century, *C. ambrosioides* was steam distilled to produce the essential oil (EO), a potent anti-helmintic agent. However, years later, the commercial use of the oil declined in favour of modern medications. Nevertheless, endemic population continues to treat parasitic diseases in humans with apparent success (França et al., 1996; Quinlan et al., 2002). Based on increased research efforts on natural medications from *C. ambrosioides* (Monzote, 2007) several pharmacological activities have been reporting, such as antitumor (Nascimento et al., 2006), antibacterial (Lall and Meyer, 1999), antifungi (Kishore et al., 1993) and antiprotozoal (Kiuchi et al., 2002; Monzote et al., 2004; Guerra et al., 2001).

In previous studies, we focused our attention on the pharmacology of EO from *C. ambrosioides.* The potential antileishmanial effect of EO was demonstrate against promastigotes and intracellular amastigotes, as well as against experimental cutaneous leishmaniasis in BALB/c mice (Monzote et al., 2006). The major components identified in the *Chenopodium*-oil were ascaridole (Asc), carvacrol (Carv) and caryophyllene oxide (Caryo) with 22%, 62% and 5%, respectively (Monzote et al., 2006). Already, in Smillie and Pessoa (1924) showed that the anti-helmintic properties of EO were due to the compound Asc. Although the anti-helmintic activity of EO is long known, its action against *Leishmania* was only recently reported (Monzote et al., 2006) and the role of the major components (Asc, Carv, Caryo) is still unknown.

The aim of this work is to compare the *in vitro* antileishmanial activity (cytotoxicity/selectivity) of the EO from *C. ambrosioides* and its major components, as well as to study their mechanism of action and activity of EO and main components against a wide panel of microorganism (bacteria, fungi and protozoa).

2. Materials and methods

2.1. Parasite and cell cultures

The following microorganisms were used in this study: *Leishmania amazonensis* MHOM/77BR/LTB0016, *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538, *Trichophyton rubrum* B68183, *Candida albicans* B59630, chloroquine-susceptible *Plasmodium falciparum* Ghana, suramin-sensitive *Trypanosoma brucei* Squib-427, nifurtimox-sensitive *Trypanosoma cruzi* Tulahuen CL2 and *L. infantum* MHOM/MA(BE)/67.

2.2. EO of C. ambrosioides and major components

C. ambrosioides was collected and the EO was extracted as previously described Halliwell and Whiteman (2004). A voucher specimen (No. ROIG4639) was deposited at the Experimental Station of Medicinal Plants "Dr. Juan Tomás Roig", Cuba. The Asc (Fig. 1; Compound 1) was obtain by chemical synthesis as previously published (Monzote et al., 2009). Carv (Fig. 1; Compound 2) and Caryo (Fig. 1; Compound 3) were obtained from Sigma Aldrich (Vienna, Austria). All products were dilute in dimethyl sulfoxide (DMSO).

2.3. Reference drugs

Erythromycin and chloramphenicol were purchased from Sigma–Aldrich (Bornem, Belgium); while miconazole and flucytosine were kindly supply by Janssen Pharmaceuticals (Beerse, Belgium). Benznidazol, chloroquine, miltefosine and suramin were kindly provided by Tropical Diseases Research (TDR) Program from World Health Organization (WHO).

2.4. Animals

Female BALB/c mice, with a body weight of approximately 20–22 g, were obtained from The National Centre of Laboratory Animals Production (CENPALAB, Cuba) and maintained according to "Guideline on the Care and Use of Laboratory Animals". Protocol to animal use was approval by Ethic Committee from Institute of Tropical Medicine Pedro Kouri, Havana, Cuba (CEI-IPK 13-10).

2.5. Antileishmanial and cytotoxic activities of essential oil from C. ambrosioides and its major components

Eleven concentrations of the EO and its major components were assayed against promastigotes in Schneider's medium (SIGMA, St. Louis, MO, USA), 10% heat-inactivated fetal bovine serum (HFBS) (SIGMA, St. Louis, MO, USA), 100 μg of streptomycin/mL, and 100 U penicillin/mL. Exponentially growing cells (10^5 promastigotes/mL, 199 μL) were distributed in 96-well plates. One microliter of products dissolved in DMSO or 1 μL of DMSO as control was added and incubated at 26 °C. After 3 days, the parasites were incubated for 3 h with p-nitrophenol phosphate (20 mg/mL) dissolved in 1 M sodium acetate buffer (BDH, Poole, England), pH 5.5, with 1% Triton X-100 (BDH, Poole, England) at 37 °C. The absorbance was determined in an EMS Reader MF Version 2.4-0, at a wavelength of 405 nm. The half inhibitory concentration (IC_{50}) was obtained by fitting a sigmoidal E_{max} model to dose–response curves (Bodley et al., 1995).

The activity against intracellular amastigotes was evaluated as described previously (Caio et al., 1999). The peritoneal macrophages were harvested and plated in 24-Well Lab-Tek (Costar®, USA) and incubated at 37 °C under an atmosphere of 5% CO_2 for 2 h. Non-adherent cells were removed and stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio. The cultures were incubating for further 4 h and free parasites were removed. Then, 990 μL of RPMI completed medium and 10 μL of the different products dissolved in DMSO were add in duplicate for further 48 h. The cultures were then fix in absolute methanol, stained with Giemsa, and examined under a light microscope. The number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages per each sample and percentage of infected macrophages. The results were express as percent of reduction of the infection rate in comparison to that of the controls, where the infection rates were obtain by multiplying the percentage of infected macrophages by the number of

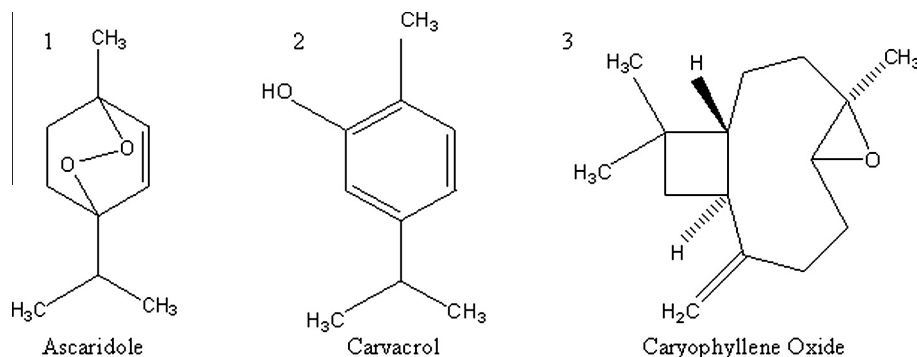

Fig. 1. Chemical structure of main compounds of EO from *Chenopodium ambrosioides*.

amastigotes per infected macrophages. The IC_{50} value was determined from the linear regression of concentration–response curves.

The IC_{50} of the EO and its major components for the viability of mouse peritoneal macrophages (host cells for the amastigote form of the parasite) was determined (Sladowski et al., 1993). Macrophages were collect and incubated as described previously. Then, 1 μL of products were added to 200 μL medium containing 10% HFBS and antibiotics. Macrophages treated with 1 μL DMSO were included as controls. The cytotoxicity was determined using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA). MTT solutions were prepared at 5 mg/mL in saline solution, filtered and sterilized at moment of use, and 15 μL was added to each well. After incubation for an additional 3 h the formazan crystals were dissolved by addition of 100 μL DMSO. The optical density was determined using an EMS Reader MF Version 2.4-0, at a test wavelength of 560 nm and a reference wavelength of 630 nm. The IC_{50} was obtained by fitting a sigmoidal E_{max} model to dose–response curves. Selectivity indexes were calculated by dividing the IC_{50} for peritoneal macrophage of BALB/c mice by the IC_{50} for *L. amazonensis* amastigotes.

2.6. Antileishmanial activity of essential oil from C. ambrosioides and Asc in combination with chelating agents

The analysis of the role of low molecular iron in the activity of EO and Asc against promastigotes of *L. amazonensis* was performed using chelating agents. The activity of the EO and Asc was measured in presence of desferioxamine (125 μg/mL) (SIGMA, St. Louis, MO, USA) or ethylenediaminetetraacetic acid (187 μg/mL) (EDTA; BDH, Poole, England). The IC_{50} was measured with the same protocol as described above and the experiments were carrying out in triplicate. Results were express as mean ± standard deviation. Posterior, the fractional inhibitory concentration (FIC) index was calculated, which is assess as follows: $FIC = [A]/IC_{50}A + [B]/IC_{50}B$, where $IC_{50}A$ and $IC_{50}B$ are the IC_{50} of each compound alone and [A] and [B] are the IC_{50} of the essential oil and the other compound when both are used in combination. A FIC index less than or equal to 0.5 indicates synergy; while an index greater than 4 indicates antagonism. When the FIC index is greater than 0.5 and less than 4, this indicates indifference (Johnson et al., 2004).

2.7. Inhibition of NADH- and succinate-dependent cytochrome c reduction

Subcultures of 1 L of *L. amazonensis* were harvest at logarithmic phase (10^7 cells/mL) and washed twice in Hanks buffer. In parallel, the liver of BALB/c mice was isolated, cut in piece in cold saline solution and homogenized in a Potter–Elvehjem homogenizer. Suspension of *Leishmania* parasites or BALB/c mice liver was dissolved in hypo-osmotic 5 mM Tris–HCl (pH = 7.4) for 10 min at 25 °C. The suspension resulted was homogenized again in a Potter–Elvehjem homogenizer on ice and then centrifuged at 1000*g* for 10 min. The supernatant was next centrifuge for 20 min at 13,000*g*. The pellet, containing the mitochondrial crude fraction (MCF), was resuspended in isotonic phosphate saline buffer (50 mM NaH_2PO_4, 90 mM NaCl, 5 mM KCl; pH = 7.2).

Reduction of cytochrome c in the presence of NADH or succinate was measured as changes of the 560 nm absorption after 1 h of incubation. The solution contained 0.5 mg/mL of mitochondrial fraction, 20 μM cytochrome c^{3+}, 0.2 mM NADH or 5 mM succinate (SIGMA, St. Louis, MO, USA) and 1 μL of different concentrations of the compounds or their vehicle, in a volume of 100 μL. For each concentration of a drug measurements were performed in duplicate and the IC_{50} value was determined from the linear regression of concentration–response curves. Three evaluations were carried out and the results are expresses as mean ± standard deviation (Luque-Ortega et al., 2004).

2.8. Variation of mitochondrial membrane potential in L. amazonensis

The variation of mitochondrial membrane potential ($\Delta\psi m$) was monitor by using JC-1 dye as probe (Dey and Moraes, 2000). JC-1 is a cationic mitochondrial vital dye that becomes concentrated in the mitochondria in proportion to $\Delta\psi m$ and ATP-generating capacity (Sudhandiran and Shaha, 2003). Briefly, promastigotes of *L. amazonensis* were collected after treatment (72 h) with EO, Asc, Carv and Caryo at 10 μg/mL, valinomycin at 5 μM and 1 μL of DMSO, subsequently incubated for 10 min with 10 μM JC-1 at 37 °C, washed and resuspended in medium. The fluorescence was observe under a Leitz Wetzler (Germany) fluorescence microscope at 400×.

2.9. Evaluation of REDOX status of promastigotes of L. amazonensis treated with the EO from C. ambrosioides and their major components

Subcultures of 1 mL of *L. amazonensis* at logarithmic phase (10^5 promastigotes/mL) were treated with 10 μg/mL of EO, Asc, Carv, Caryo or DMSO as control. After an incubation of 72 h at 28 °C, the peroxidation potential (PP) and sulfhydryl group (SHT) concentrations were measure.

For the determination of the susceptibility to lipid peroxidation, samples were incubated with a solution of cupric sulphate (final concentration of 2 mM) at 37 °C for 24 h.

Malondialdehyde (MDA) concentrations were analyzed with the LPO-586 kit obtained from Calbiochem (La Jolla, C.A., USA). In this assay, stable chromophore production was measurement after

Table 1
Anti-leishmanial and cytotoxic activity of EO from *C. ambrosioides* and their main pure compounds.

Products	IC_{50}[a] ± SD[b] (μg/mL)			Selectivity index[c]
	Promastigotes	Amastigotes	Macrophages	
Chenopodium-oil	3.7 ± 0.6	4.6 ± 0.7	58.2 ± 0.05	13
Ascaridole	0.1 ± 0.01	0.3 ± 0.05	1.1 ± 0.04	4
Carvacrol	15.3 ± 4.6	13.6 ± 1.8	32.3 ± 1.9	2
Caryophyllene oxide	4.9 ± 2.3	4.4 ± 0.4	4.5 ± 0.04	1

[a] IC_{50}: concentration of drug that caused 50% of mortality.
[b] SD: standard deviation.
[c] Selectivity index: IC_{50} of macrophage/IC_{50} of amastigotes.

40 min of incubation at 45 °C using a wavelength of 586 nm by Pharmacia Spectrophotometer. Freshly prepared solutions of MDA bis[dimethylacetal] (Sigma, St. Louis, MO, USA) assayed under identical conditions were used as reference standards (Erdelmeier et al., 1998; Esterbaver and Cheeseman, 1990). The PP was calculated by subtracting the MDA concentrations at time 0 from the one obtained at 24 h (Ozdemirler et al., 1995). Protein sulfhydryl groups were analyzed with the method described by Sedlak and Lindsay (1968). SHT (Glutathione) (Sigma, St. Louis, MO, USA) was used to generate standard curves.

2.10. Integrated antimicrobial screening

Test plate production was performed in 96-well plates (Greiner, Germany) at fourfold dilutions in a dose-titration range of 64–0.25 μg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). All tests were running in duplicate. The integrated panel of microbial screens for the present study and the standard screening methods were adopted as have been described previously (Cos et al., 2006). Activity of the products against protozoa *P. falciparum* (Makler et al., 1993), trypomastigotes of *T. b. brucei* (Hirumi and Hirumi, 1989), intracellular amastigotes of *T. cruzi* (Buckner et al., 1996) and *L. infantum* were perform. In parallel, inhibition on *E. coli*, *S. aureus*, *T. rubrum* and *C. albicans* was assay. The IC_{50} value was determined from the linear regression of the concentration–response curves and the results were express as the mean ± standard deviation of at least two independent experiments.

3. Results and discussion

Medicinal plants are some of our oldest medicines. Their increasing use in recent years is a clear evidence of public interest in alternatives to conventional drugs. However, there is a limited amount data to confirm their efficacy and little or not scientific reports regarding possible mechanism involved. Therefore, we studied the anti-parasitic activities of *Chenopodium*-oil (EO) and its major components *in vitro*.

The antileishmanial activity against promastigotes and amastigotes of *L. amazonensis*, the cytotoxicity and selectivity index of *Chenopodium*-oil, Asc, Carv and Caryo are show in Table 1. All products were active against both forms of *Leishmania* parasites. The Asc showed the better antileishmanial activity; while the *Chenopodium*-oil showed the highest selectivity index.

In *in vitro* models with promastigotes and amastigotes of *L. amazonensis*, Asc showed a greater inhibitory effect than EO, Carv and Caryo, with an IC_{50} of 0.1 μg/mL for promastigote and 1.1 μg/mL for amastigote forms. Other reports demonstrated that Asc was effective against *T. cruzi* (Kiuchi et al., 2002) and *P. falciparum* (Pollack et al., 1990). In addition, our results support the hypothesis that Asc is the main anti-parasitic agent in *Chenopodium*-oil.

In parallel, the cytotoxicity assay showed that Asc was slightly selective against parasites in comparison with mammalian cells, with a selectivity index of 4. Carv and Caryo exhibited an unspecific activity, with a selectivity index of 2 and 1, respectively. For a safe application of antileishmanial drugs or natural formulation, a selectivity index greater than 10 is desired (Pink et al., 2005). For that reason, the EO as a natural mixture appears to be a more promising drug than pure Asc, due to selectivity index of for EO of 13 vs. for Asc of 4. A similar result was obtained for the EO from *Cymbopogon citratus*, which was significantly better than the pure citral, the major component of this oil (Santin et al., 2009).

Asc have been considered the main active principle of *C. ambrosioides*. This compounds is an endoperoxide with an internal oxygen–oxygen (O–O) bond, which plays possibly a key role in many of its bioactivities (Dembitsky et al., 2008). An important example has been well document: the potent antimalarial activity of artemisinin derivatives, which lead to formation of toxic radical intermediates that cause death of plasmodial parasites (O'Neil and Posner, 2004). In previous studies, we demonstrated by electron spin resonance techniques that Asc, as well as EO, form a carbon-centered radical intermediates (Monzote et al., 2009). This raised the question if the available radical is the key for antileishmanial activity of Asc and EO. Then, a comparison of antileishmanial activity of EO and Asc alone and in presence of chelating agents was performing. Two different chelators was use: EDTA, which is able to sequester metal ions, and desferioxamine, which sequesters Fe ions.

An increased IC_{50} value was obtain after treatment of promastigotes of *L. amazonensis* with the EO or Asc in the presence of desferrioxamine or EDTA (Table 2). An antagonistic effect between both tested products and chelating agents was demonstrated by the FIC index, which was higher than 4 in all cases. This result demonstrated that peroxide reactivity and carbon radical formation triggered by iron are the key factors for the pharmacological action, as have been reported for artemisinin (O'Neil and Posner, 2004).

Concerning to antileishmanial mechanism of action of *Chenopodium*-oil so far no information was available. Recently, the possible toxic mechanisms in mammals involving the inhibition of mitochondrial functions have been reporting (Monzote et al., 2009). NADH- and succinate-dependent cytochrome c reduction was inhibited in MCF of *L. amazonensis* or liver mitochondria from BALB/c mice in presence of EO from *C. ambrosioides* and major pure compounds (Table 3). The EO showed lower IC_{50} values in *Leishmania* MCF compared with mammalian mitochondria in

Table 2
Effect of EO from *C. ambrosioides* and Asc in presence of chelators agents against promastigotes of *L. amazonensis*.

Products	Essential oil from *C. ambrosioides*		Ascaridole	
	IC_{50}[a] ± SD[b] (μg/mL)	FIC[c] Index	IC_{50}[a] ± SD[b] (μg/mL)	FIC[c] index
Products alone	3.7 ± 0.6	–	0.1 ± 0.01	–
Combined with desferrioxamine[d]	36.3 ± 1.6	14	33.8 ± 1.0	339
Combined with EDTA[e]	37.4 ± 0.1	13	30.9 ± 2.3	309

[a] IC_{50}: concentration of drug that caused 50% of inhibitory growth.
[b] SD: standard deviation.
[c] FIC index: fractional inhibitory concentration (FIC) index.
[d] IC_{50} of desferrioxamine = 13.0 ± 0.4 μg/mL.
[e] IC_{50} of EDTA = 65.9 ± 4.0 μg/mL.

Table 3
Effect of EO from *C. ambrosioides* and major pure components on MCF from promastigotes of *L. amazonensis* and liver of BALB/c mice.

Products	IC_{50}[a] ± SD[b] (μg/mL)			
	NADH-triggered cytochrome c reduction		Succinate-triggered cytochrome c reduction	
	MCF-La[c]	MCF-BM[d]	MCF-La	MCF-BM
Chenopodium-oil	67.8 ± 0.1[*]	199.7 ± 9.0	59.1 ± 5.8[*]	254.7 ± 11.0
Ascaridole	147.5 ± 11.2	127.8 ± 4.3	209.0 ± 1.8	75.9 ± 1.1
Carvacrol	170.3 ± 8.3	141.0 ± 2.6	180.7 ± 10.3	116.1 ± 8.5
Caryophyllene oxide	98.7 ± 8.5	52.2 ± 3.8	128.7 ± 9.8	91.0 ± 4.7

[a] IC_{50}: concentration of drug that caused 50% of inhibitory.
[b] SD: standard deviation.
[c] MCF-La: mitochondrial crude fraction from *L. amazonensis*.
[d] MCF-BM: mitochondrial crude fraction from BALB/c mice.
[*] Statistical differences between MCF-La and MCF-BM ($p < 0.05$).

NADH- and succinate-dependent cytochrome c reduction ($p < 0.05$); while the pure major compounds showed similar activity or displayed a higher toxicity in mammalian mitochondria. The selective activity showed by EO against MCF from *Leishmania*, is in concordance with the results obtained in cellular model of *Leishmania* parasites. A possible explanation for these results could be that the major (and minor) components of EO interact with each other or with related cellular signaling pathways causing synergistic effects.

Nevertheless, the data obtained for cellular and mitochondrial models differ quantitatively. The ratio of activity observed for *Chenopodium*-oil in the cellular system was 13; while in experiments with MCF systems the selectivity index was only 4 for both NADH and succinate as substrates. The finding suggests that in these experiments the NADH- and succinate-triggered cytochrome c reduction is not specifically sensitive to EO in *Leishmania* mitochondria. However, does not exclude that there are other more sensitive and more selective targets (such as $\Delta\psi m$) for EO in leishmanial mitochondria. Alternatively, others damages in the parasite caused by EO, such as free radical-triggered DNA or protein-alterations, or parasite-specific transporters, such as the P2 amino-purine transporter (De Koning, 2001), could contribute to specific killing of *Leishmania* by facility the intracellular accumulation of EO.

The measurement of the $\Delta\psi m$ after incubation of *L. amazonensis* promastigotes with EO and major compounds suggests that

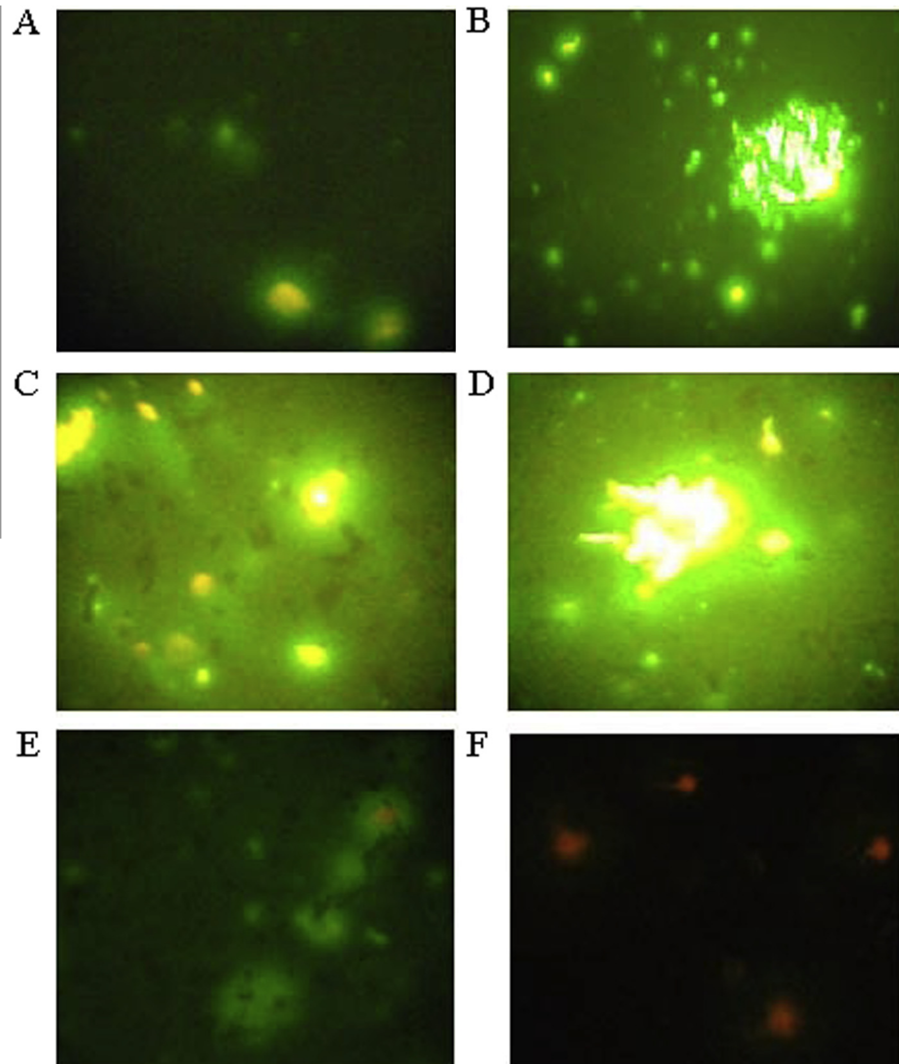

Fig. 2. Effect of EO from *C. ambrosioides* and pure compounds on mitochondrial membrane potential of *L. amazonensis* promastigotes. Green color corresponds to JC-1 monomers indicating low membrane potential and orange color indicates JC-1 aggregates, which are formed in mitochondria with high membrane potential. (A): cells incubated with EO; (B): cells incubated with Ascaridole; (C): cells incubated with Carvacrol; (D): cells incubated with Caryophyllene oxide; (E): cells incubated with valinomycin; (F): control cells treated with vehicle (DMSO). (For interpretation of color in this Figure, the reader is referred to the web version of this article.)

mitochondrial dysfunction is involved in the antileishmanial effects of EO. The parasites treated with the EO and its major components exhibited a green fluorescence after incubation with JC-1 dye (Fig. 2), as well as the cultures treated with the inonophore valinomycin, a mitochondrial uncoupler (Soltoff and Mandel, 1968). The absence of JC-1 aggregates (orange fluorescence) demonstrated a low mitochondrial membrane potential. However, promastigotes treated with the vehicle only showed an additional orange fluorescence, which correspond to a high mitochondrial membrane potential. The changes in fluorescence suggest a breakdown of mitochondrial $\Delta\psi m$ in promastigotes treated with the evaluated products. Similar results have been report by other natural's products such as epigallocatechin-3-gallate (Inacio et al., 2012) and quercetin (Fonseca-Silva et al., 2011) on *L. amazonensis* promastigotes. In the control cells, a normal $\Delta\psi m$ is visible and emit orange fluorescence.

Maintenance of proper $\Delta\psi m$ is essential for the survival of cells (Gottlieb, 2003). In *Leishmania*, a single cell organism with a single mitochondrion, total loss of potential would result in immediate death. $\Delta\psi m$ depends on various factors, such as mitochondrial substrate supply, mitochondrial electron transfer, physico-chemical properties of the inner mitochondrial membrane preventing H^+ back diffusion, activity of uncoupling proteins, pore formation, oxidation state of lipids and protein thiols as well as other processes. Therefore, our present data on this problem do not allow deciding whether loss of $\Delta\psi m$ is a primary effect of EO (influencing directly mitochondrial functions) or arises subsequent to other cellular effects of EO triggering apoptosis via mitochondria (Gottlieb, 2003).

Based on the role of Asc as precursor of oxygen radicals it was obvious to elucidate the SHT and the PP as possible reasons for the mitochondrial dysfunction. Therefore, we studied the redox status of *Leishmania* promastigotes after treatment with EO and its major components. The quantification of redox indexes showed differences between untreated promastigotes of *L. amazonensis* incubated during 72 h and cultures treated with EO or pure compounds (Fig. 3). In the experiments of PP, an significant increase ($p < 0.05$) was observed in treated cultures with Car respect to control; while a statistical decrease ($p < 0.05$) results with Caryo incubation. In parallel, significant reduction ($p < 0.05$) of SHT was caused in parasites by EO, Asc and Car.

The PP index is inversely proportional to antioxidant capacity; while-SH or thiol groups is important in maintaining molecules structure and functions including proteins, regulating thiol-disulfide status of the cell, and detoxifying foreign compounds and free radicals. –SH groups react with hydrogen peroxide (H_2O_2) and the hydroxyl ($OH^{\cdot}$) radical and may prevent tissue damage (Hensley et al., 2000; Halliwell and Whiteman, 2004). In case of Car the results could suggest a state of oxidative stress with the inability of antioxidant systems on both lipid and soluble layers in the parasites. In addition, EO and Asc caused on decreased of –SH groups, which also suggest inability of soluble antioxidant systems. The alteration of redox indexes detected in the treated cell could be a consequence of ROS augmented generation related to mitochondrial dysfunction and its loss of proper $\Delta\psi m$, with subsequent death of parasites.

Follow, as anti-parasitic properties of *C. ambrosioides* have been document, the evaluation of EO and its major compounds in a number of microorganisms was performed by an integrated screening experiment. EO inhibited the growth of all protozoa parasites studied. A potential activity against *T. b. brucei* and *P. falciparum* was observed (Table 4). Against bacteria and fungi EO was not active ($IC_{50} > 64$ μg/mL), except against *T. rubrum* for which an IC_{50} value of 6.1 ± 0.5 μg/mL was obtained. The main pure compounds were also evaluated, which showed no activity or unspecific effects against all microorganism evaluated.

The results corroborate the advantage of EO over the pure compounds based on their activity and selectivity. For EO only antiparasitic activity and no activity against bacteria and fungi were observed. A similar IC_{50} for EO in different *Leishmania* species (*L. infantum*, IC_{50} = 6.4 ± 0.6 μg/mL and *L. amazonensis*, IC_{50} = 4.5 ± 0.7 μg/mL) suggests that EO could be also effective against different forms of leishmaniasis including cutaneous and visceral clinical manifestations. In addition, EO exhibited also activity against other protozoa pathogens, including *T. cruzi*, *T. brucei* and *P. falciparum*. Likewise, the activity of *C. ambrosioides* cultivated in Cuba against extracellular protozoa, including *Trichomonas vaginalis* (Monzote et al., 2004) and *Giardia lamblia* (Guerra et al., 2001), has been report.

Recently, one of the strategies in control programs to treat neglected tropical diseases, which include *Leishmania* and *Trypanosoma*, have been basing on the simultaneous targeting of the most highly prevalent parasites (Hotez et al., 2008). EO from *C. ambrosioides* demonstrates their wide spectrum as anti-parasitic agent, which will be useful to treat patients with multiple-parasite species, particularly in endemic areas of various parasite infections

Table 4
Activity of EO from *C. ambrosioides* against protozoa parasites.

Products	IC_{50}[a] ± SD[b] (μg/mL)			
	P. falciparum	*T. cruzi*	*T. brucei*	*L. infantum*
Essential oil	0.2 ± 0.2	1.9 ± 0.3	0.2 ± 0.07	6.4 ± 0.6
Reference drugs	0.3 ± 0.1[c]	2.2 ± 0.5[d]	0.05 ± 0.05[e]	7.7 ± 4.5[f]

[a] IC_{50}: concentration of compounds that caused 50% of inhibition growth.
[b] SD: standard deviation.
[c] Chloroquine.
[d] Benznidazol.
[e] Suramine.
[f] Miltefosine.

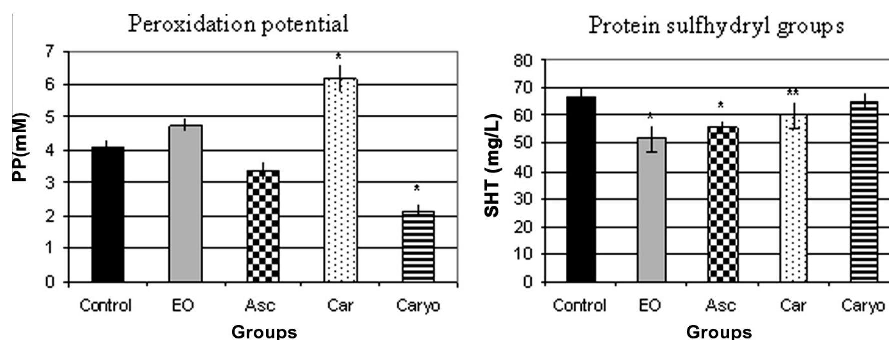

Fig. 3. Redox indexes in promastigotes of *L. amazonensis* treated with EO of *C. ambrosioides* and its major pure components. EO: Essential oil from *C. ambrosioides*; Asc: Ascaridole; Car: Carvacrol; Caryo: Caryophyllene oxide; *: Statistical differences with $p < 0.05$ compared with control group, different number of asterisks correspond to statistical differences between groups with different treatments.

where the differential diagnostics is difficult. Further studies of EO in animal models of trypanosomiasis and malaria should corroborate the *in vitro* data obtained, as well as if the effect could also relate with the inhibition of mitochondrial functions.

4. Conclusion

In conclusion, our results demonstrated that the EO from *C. ambrosioides* showed a better activity/selectivity in molecular and cellular systems, in comparison with its pure major compounds. Standardization of EO as a natural medication or formulation of an artificial mixture of pure compounds deserves consideration in future studies to develop new drugs with a wide anti-parasitic spectrum.

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