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Original article

Activity, toxicity and analysis of resistance of essential oil from *Chenopodium ambrosioides* after intraperitoneal, oral and intralesional administration in BALB/c mice infected with *Leishmania amazonensis*: A preliminary study

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Abstract

The World Health Organization has classified the leishmaniasis as a major tropical disease. Current therapy is toxic, expensive and cause several adverse effects. The majority of people in endemic areas of leishmaniasis depend of natural and traditional medicine. This study was developed to examine the activity of the essential oil from *Chenopodium ambrosioides* in BALB/c mice infected with *Leishmania amazonensis*. The infected animals received two cycle of treatment by different routes (intraperitoneal, oral or intralesional route). The intraperitoneal administration of the essential oil at dose of 30 mg/Kg prevented lesion development and decrease the parasite burden. Oral administration retarded the infection in the experimental model compared with untreated mice, although it was less effective that the intraperitoneal route. The administration by intralesional route did not show activity. Intraperitoneal and oral treatment at 30 mg/Kg with the essential oil had better antileishmanial effect that treatment with the reference drug, amphotericin B at 1 mg/Kg. Preliminarily, we examined the toxicity and the resistance after treatment. Signs of toxicity were evident only in the animals treated by intraperitoneal route. No resistance was detected in *L. amazonensis* isolates obtained from treated mice. These data clearly demonstrated that this natural product could be an alternative for the development of a new drug against cutaneous leishmaniasis based in the ethnomedical information.

Keywords: *Chenopodium ambrosioides*; *Leishmania amazonensis*; Balb/c; Essential oil; Treatment

1. Introduction

Leishmaniasis is a disease resulting from infection by protozoan parasites of the genus *Leishmania* (family *Trypanosomatidae*), which can affect man and several species of mammals [1]. Two million new human cases arise every year, and at least 350 million people are exposed to the risk of parasite infections [2]. Infections by *Leishmania* parasite can assume different clinical forms, depending both infecting species and on the host factors that determine the immunological response to the infectious agent [1,2].

The control of leishmaniasis remains a serious problem. As a zoonotic infection transmission is difficult to interrupt, although some attempts to reduce vector and mammalian reservoir populations have been successful. An effective vaccine against leishmaniasis is not available; chemotherapy is the only effective way to treat all forms of disease [3].

Pentavalent antimonials are still the first choice among drugs used for the treatment of leishmaniasis. Alternatively, pentamidine, amphotericin B (AmB) and paromomycin can be used. In general, these compounds are toxic and expensive, and require long-term use during treatment [4], which has compelled the search for new antileishmanial agents.

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Due to limited availability of effective pharmaceutical products, most people in areas where leishmaniasis is endemic depend largely on popular treatments and traditional medicine to alleviate the symptoms. In addition to the various physical methods, the treatment of leishmaniasis following traditional medical practices of different cultures depends heavily on the use of native plants [5].

Chenopodium ambrosioides has been reported for its potential antiparasitic properties, including antiprotozoal activity against *Trypanosoma cruzi* [6] and *Plasmodium falciparum* [7]. Antihelmintic effect against *Ancilostoma duodenale, Trichuris trichuria* and *Ascaris lumbricoides* [8] has been reported also.

In a previous work, we demonstrated that the essential oil from this plant was effective against promastigotes and intracellular amastigotes of *Leishmania amazonensis*. In this study, the efficacy against the experimental cutaneous leishmaniasis caused by the same specie of *Leishmania* was demonstrated also [9].

In the traditional medicine, the treatment of leishmaniasis usually consists in the oral administration or topical preparations of plants extracts. In the present work, we study the pharmacological activity, toxicity and resistance of the essential oil, after their administration by intraperitoneal, oral and intralesional routes in a model of cutaneous leishmaniasis in BALB/c mice, caused by *L. amazonensis.*

2. Materials and methods

2.1. Essential oil from C. ambrosioides

Chenopodium ambrosioides L. (*Chenopodiaceae*) was collected and the essential oil was extracted as previously described [9]. Miglyol 810 (Hüls Aktiengesellschaft) was used as vehicle to dilute the essential oil to carrying out the experiments with infected mice.

2.2. Parasite cultures

L. amazonensis (MHOM/77BR/LTB0016) was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26 °C in Schneider's medium (SIGMA, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (SIGMA, St. Louis, MO, USA), 100 μg of streptomycin/ml, and 100 U of penicillin/mL. The parasites were used no later than at the fifth *in vitro* passage.

2.3. Animals

Female BALB/c mice, with a body weight of approximately 20 to 22 g, were obtained from The National Center of Laboratory Animals Production (CENPALAB) and maintained according to ''Guideline on the Care and Used of Laboratory Animals''. The temperature and humidity were controlled, with 12 h light/dark cycle and given water and food ''ad libitum'' for all animals.

2.4. Biological evaluation

The experiments were carried out as showed in Fig. 1. On day 0, the mice received subcutaneous injections in the right hind footpad of 5×10^6 stationary-phase *L. amazonensis* promastigotes. Four weeks post-infection (p.i.) the animals were randomly divided into eight groups of 12 mice each one and the treatment was initiated. The essential oil was administered using the following routes and doses: intraperitoneal: 30 mg/Kg/0.1 mL; oral: 30 mg/Kg/0.1 mL, intralesional: 3%/0.02 mL. Another three groups received 0.1 mL of Miglyol by intraperitoneal and oral routes, or 0.02 mL intralesionally. Alternatively, 1 mg/Kg of AmB (Imefa, Ciudad de la Habana, Cuba) was injected intraperitoneally in 0.1 mL of distilled water in other group. Finally, untreated mice was also included. All the treatments were administered daily from day 30 p.i. to the day 45 p.i. Sixteen days p.i. the mice received a second cycle of treatment up to day 75 p.i. On day 90 p.i. the experiment finished.

Disease progression was monitored weekly by measuring footpads swelling of the lesion diameter between 30 and 90 days p.i., using a calliper. Average lesion size was calculated as the differences obtained between infected and uninfected footpads.

On day 45 and 75 p.i., three animals of each group were killed by cervical dislocation and parasite burden determined, using the culture microtitration method [10]. A sample of the lesion was excised, weighted and homogenised in 4 ml of Schneider's medium with 10% of fetal bovine serum and antibiotics. Under sterile conditions, serial fourfold dilution was prepared in plates of 96-well containing 225 μl of culture medium. After 7 days of incubation at 26 °C, plates were examined with an inverted microscope. The presence or absence of mobile promastigotes was recorded in each well. The final titre was defined as the last dilutions for which the well contained at least one parasite. The parasite burden was calculated as follows: parasite burden = (geometric mean of reciprocal titres from each duplicate/weight of homogenised cross section) × 400.

2.5. Evaluation of the toxicity after treatment

On day 45 and 75 p.i., we search for gross-pathological changes in the thoracic and abdominal cavity, in order to determining the possible toxicity produced by the essential oil through the examine damages caused to the peritoneum, spleen, pancreas, stomach, liver, kidney, diaphragmatic faces, heart and lungs.

2.6. Analysis of resistance level of the parasite after treatment

We evaluated the susceptibility *in vitro* of promastigotes isolate from infected mice after two cycle of treatment with the essential oil administered intraperitoneal and orally. The

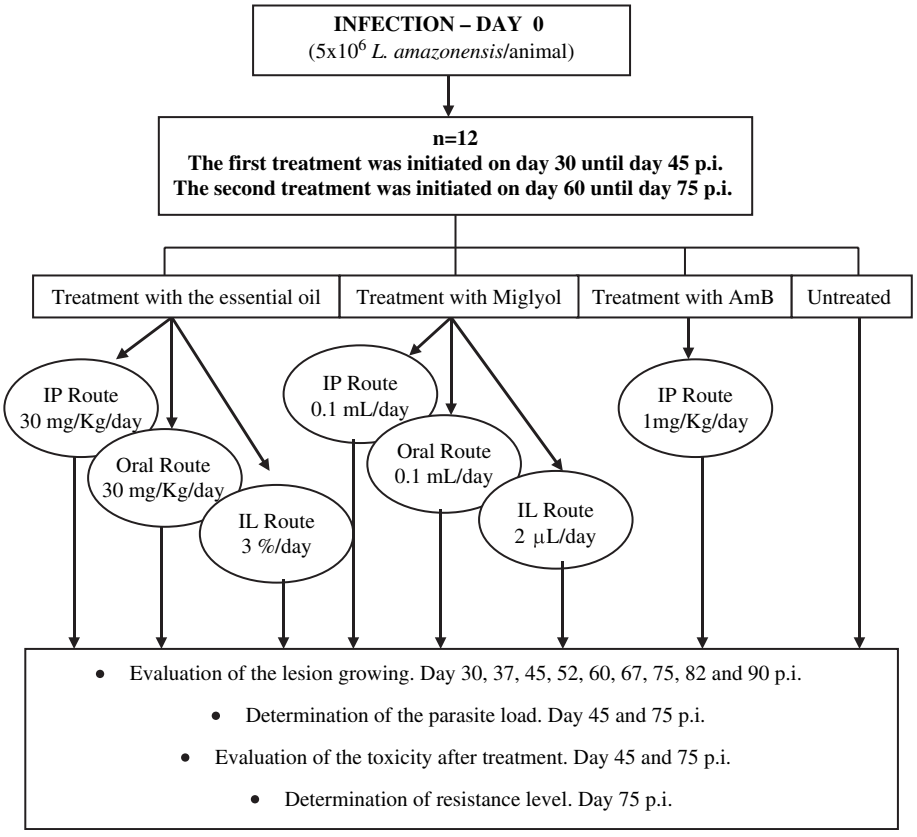

Fig. 1. Experiment protocol with the essential oil from *C. ambrosioides* on the *in vivo* activity, toxicity and resistance. IP: intraperitoneal; IL: intralesional; p.i.: post-infection.

parasites were isolated from cutaneous lesion at day 75 p.i. and cultured in Schneider's medium at 26 °C for 72 h.

Log phase promastigotes (initial density of 1×10^5/mL) were exposed either to 0.5% dimethylsulfoxide (DMSO) or at different concentrations of the essential oil (between 500 to 0.5 μg/mL) at 26 °C, in a 96-well plate. After 72 h of incubation, motile parasites were detected by direct observation in an inverted microscope and the minimal inhibitory concentration (MIC) was determined. The MIC value was defined as the minimum concentration at which no motile parasite was observed microscopically.

Then, parasites were incubated for another 3 h with *p*-nitrophenol phosphate (20 mg/ml) dissolved in buffer of sodium acetate 1 M (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 50% inhibitory concentration (IC_{50}) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal E_{max} model [11].

Resistance indexes were determined as IC_{50} ratio between the new isolates after treatment and wild type line [12].

2.7. Statistical analysis

Lesion progression and parasite burden were analysed for statistical significance by analysis of variance test (ANOVA-MANOVA), using the STATISTICAL for Windows Program (Release 4.5, StatSoft., Inc. 1993).

3. Results

3.1. Biological evaluation

The effect of treatment with the essential oil from *C. ambrosioides* to BALB/c mice infected with *L. amazonensis* is shown in Fig. 2. No significant differences ($P > 0.05$) were observed between BALB/c mice on day 30 p.i., when treatment started.

Fig. 2A shows that intraperitoneal administration of the essential oil at a dose of 30 mg/Kg prevented lesion development compared with the animals treated with the vehicle, as well as untreated mice ($P < 0.05$). Fig. 2B shows that mice treated with the essential oil by the oral route developed significantly smaller footpad lesions compared with animals treated orally with Miglyol and untreated mice ($P < 0.05$). Fig. 2C shows that the intralesional treatment did not decrease the lesion size ($P > 0.05$) respecting untreated animals, although the lesion size was significantly lower ($P < 0.05$) than the animals treated with the solvent by the same route from 45 day p.i. to the end of the experiment.

The efficacy of the essential oil administered by different routes was compared with respect to reference antileishmanial drug AmB (Fig. 3). The lesion size in animals treated orally

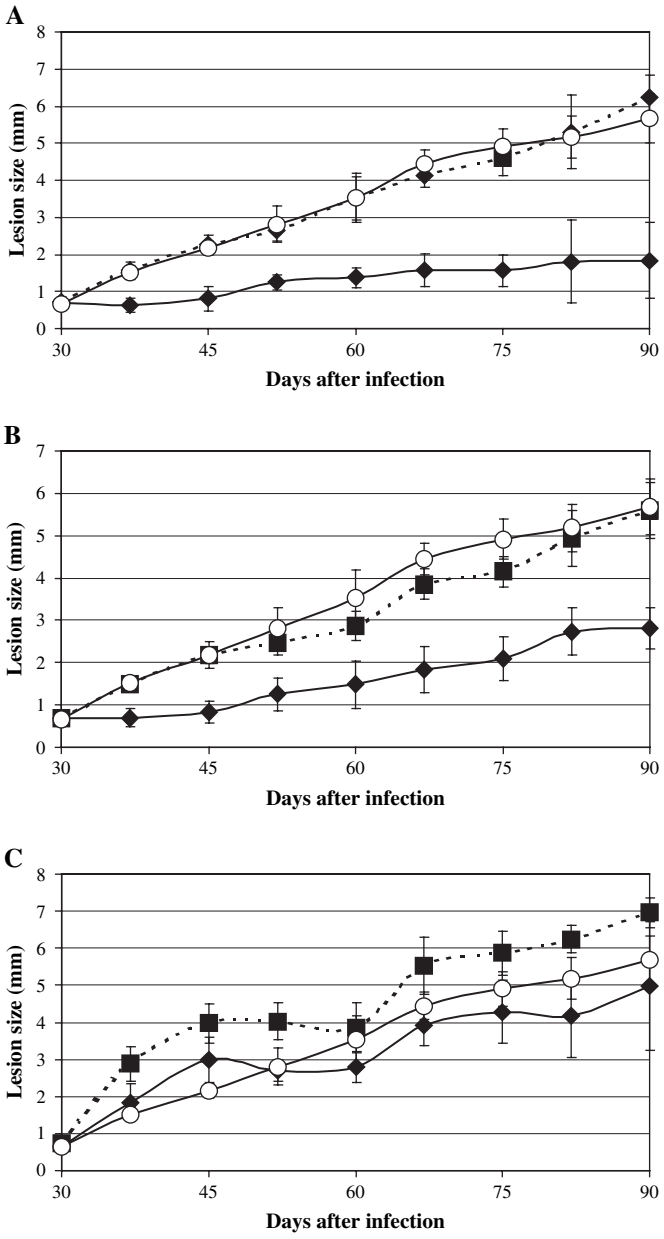

Fig. 2. Effect of the essential oil from *C. ambrosioides* on lesion growth using different routes of administration. BALB/c mice were infected in the footpad by subcutaneous infection with 5×10^6 promastigotes of *L. amazonensis*. A: Intraperitoneal administration; B: Oral administration; C: Intralesional administration. Untreated animals (○); animals treated with the essential oil (◆); animals treated with Miglyol (■). Lesion sizes were measured at the indicated times post-infection (mean ± standard deviation). The treatment was performed at 30 mg/kg.

with the essential oil was similar to that in mice treated by the intraperitoneal route, although from day 75 p.i. we observe an increase of the lesion size ($P < 0.01$). The intralesional treatment with the essential oil as well as treatment with AmB were less active ($P > 0.05$).

Fig. 4 shows the parasite burden in the footpad, determined by culture microtitration method on day 45 and 75 p.i. No parasites were detected in the cutaneous lesion from animals treated with the essential oil, after the first cycle of treatment (day 45 p.i.); including those treated by intralesional route.

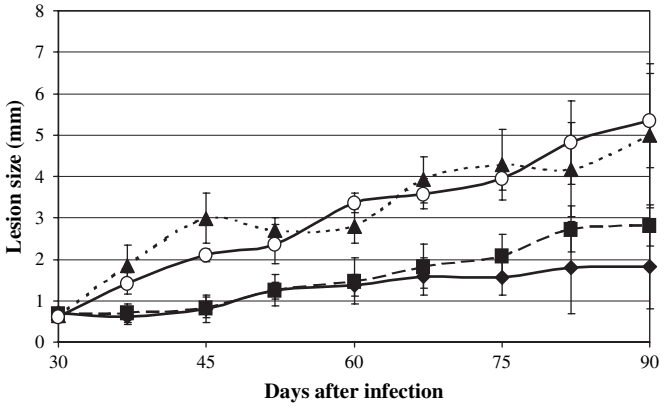

Fig. 3. Effect of essential oil from *C. ambrosioides* on lesion growth using different routes of administration and compared with amphotericin B treatment. Animals treated with the essential oil: 30 mg/Kg by intraperitoneal route (◆); 30 mg/Kg by oral route (■); 3% by intralesional route (▲) and animals treated with 1 mg/Kg of AmB (○). Lesion size was measured at the indicated times (mean ± standard deviation).

After the second cycle of treatment (day 75 p.i.) a considerable smaller number of parasites ($P < 0.05$) were quantified in mice treated with the essential oil by intraperitoneal route respecting untreated animals. Oral treatment reduced the parasite load compared with untreated mice ($P < 0.05$), but a significant difference ($P < 0.05$) was also observed between BALB/c mice treated by intraperitoneal and oral route. However, the parasite burden quantified from lesion of mice treated by intralesional route was similar to untreated animals ($P > 0.05$).

3.2. Evaluation of the toxicity after treatment

Preliminary studies indicated that intraperitoneal administration of the essential oil caused some small abcess in peritoneal cavity and death of two animals after 25 injections (Day 72 p.i.). Oral and intralesional administration of the essential oil did not show signs of toxicity.

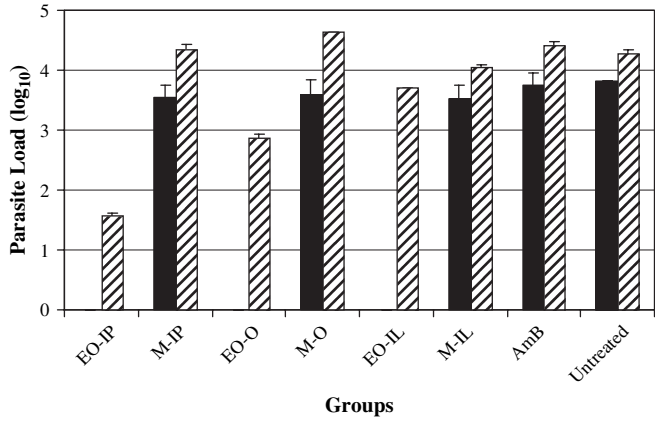

Fig. 4. Parasite load (mean ± standard deviation) after treatment with the essential oil from *C. ambrosioides* in BALB/c mice infected with 5×10^6 promastigotes. Effect after of the first cycle of treatment, day 45 p.i. (■); effect after the second cycle of treatment (▨). EO: essential oil; M: Miglyol (solvent); IP: intraperitoneal administration; O: oral administration; IL: intralesional administration.

3.3. Analysis of resistance level of the parasite after treatment

L. amazonensis isolates from BALB/c mice treated with the essential oil by intraperitoneal and oral route showed a similar susceptibility compared with the wild type strains (Table 1), with resistance index of 1.8 and 1.5, respectively. The growing of promastigotes isolated from BALB/c mice treated with the essential oil by intralesional route was unsuccessful.

4. Discussion

We have demonstrated in previous work that intraperitoneal administration of the essential oil from *C. ambrosioides* at dose of 30 mg/Kg per day, prevented lesion development in mice infected with *L. amazonensis* [9]. Here we present data about the efficacy, toxicity and analysis of resistance to the parasite after the intraperitoneal, oral or intralesional administration of this product.

Among the administration routes tested, the intraperitoneal was the most effective in controlling the disease after its establishment, which is over one month after inoculation of the parasites. The effectiveness of the treatment was confirmed by the reduction in lesion size and decreases the parasite load.

Oral administration of the essential oil produces the same effect that mice treatment by intraperitoneal route, except for a slight transient recrudescence in lesion size between 8 and 12 week p.i. The effectiveness of the oral route results in a good absorption of the essential oil through the gastrointestinal tract. For that reason, it must also be assumed that the principal active was metabolised at low levels and are transported via the systemic circulation from intestinal mucosa to the infected tissue. However, a partial loss of the drug may occur due to exchange interactions through of the different compartment such as the blood, the liver and others. This assumption is logical, since the superior in the lesion size.

A number of studies of oral chemotherapy from medicinal plants against the cutaneous leishmaniasis have been carried out during the past decades.

The treatment with 2-phenylquinoline from *Galipea longiflora*, a Bolivian medicinal plant, have been examined in *L. venezuelensis*-infected BALB/c mice [13]. When given orally at 50 mg/Kg twice daily for 15 days, they observed a decrease of the lesion weight and the parasite burden; but the complete cure did not occur after the treatment. Oral administration of aqueous extract from *Kalanchoe pinnata* was effective in controlling the lesion growth in BALB/c mice infected with *L. amazonensis* at 4 mg/Kg [14]. In this case, the effect of the extract has no a direct inhibitory action on the parasite, possibly, the protection against murine leishmaniasis is due to its effect on the immune system.

In contrast, our study showed that intralesional administration of the evaluated oil had modest activity; inferior to treatment by intraperitoneal and oral routes, but substantially better than the group treated with the vehicle by the same route. One explanation may be due to the inability of the footpad to absorb the solvents (Miglyol) that in consequence, cause a deposit in the footpad and an apparent swelling. Studies to find appropriate solvents should be done. Similar results have been described in studies of the activity of the licochalcone A against the cutaneous leishmaniasis in mice infected with *L. major* [15].

We use AmB as standard antileishmanial drug. AmB is one of the most active antileishmanial agents. However, drawbacks to AmB include the requirement for infusions, length of therapy, adverse reactions, close laboratory monitoring for potential toxicity and, to some extent cost [16]. In our study the AmB did not prevent the progression of the cutaneous disease, probably due to a reduced distribution of the drug to the skin and subcutaneous tissue or the affinity by lipoproteins present in the blood, which would compromise efficacy [17,18].

Preliminary experiments were carried out to examine the potential toxicity of the essential oil *in vivo*. Oral and intralesional administration of essential oil in BALB/c mice did not exhibit any observable signs of toxicity in these animals. In mice treated by intraperitoneal routes with the essential oil, we observed some small abcess in the peritoneal cavity, as well as in the mice treated with the solvent, by the same route. Miglyol is a non-toxic carrier for studies in experimental animals. In this case, we think that the damage might be result of the intraperitoneal injection. On the other hand, intraperitoneal injection of the essential oil at a concentration of 30 mg/Kg caused the death of two animals on day 72 p.i., possibly by accumulation of the compound after repeated doses. We cannot explain this toxicity because the mode of action and the kinetics in the animals (distribution and accumulation) of the essential oil are still unknown.

L. amazonensis has particular importance since it is one the most frequent species causing human disease and is associated with anergic diffuse leishmaniasis, a disfiguring cutaneous disease which is very difficult to cure [19]. The cutaneous disease expression depends on complex interactions between host factors and the biodiversity of the parasite itself [20]. *Leishmania* parasites are evolutionarily successful organism, and they must develop highly sophisticated actions to combat the host's killing mechanism [21], that include the immune response of the host and the chemotherapy. The development of drug resistance in the parasites is another major impediment in the successful treatment of the conventional drugs [22].

In others studies, the IC_{50} in *L. infantum* strains from untreated dogs was lower than five strain isolated after dogs

Table 1
Influence of the treatment with the essential oil on sensitivity of *Leishmania* promastigote strains

Leishmania Strains	MIC[a] (μg/mL)	IC_{50}[b] (μg/mL)	Resistance Index[c]
Wild Type	27.82	3.74	–
After IP Treatment[d]	30.12	6.71	1.8
After O Treatment[e]	27.81	5.55	1.5

[a] MIC; concentration of the essential oil that caused 100% of mortality.
[b] IC_{50}; concentration of the essential oil that caused 50% of mortality.
[c] Resistance Index; IC_{50} of the isolated line/IC_{50} of wild type line.
[d] *Leishmania* strain after intraperitoneal treatment with the essential oil.
[e] *Leishmania* strain after oral treatment with the essential oil.

have been treated with meglumine antimoniate (20.4 mg Sb^V/Kg/12 h/10 days) [23].

In our work the resistance index was less twelve compared with the wild type strains. We can thus assume that the drug pressure received by strains was very low to develop the expression of other phenotypes like drug resistance. As part of a series of studies on the antileishmanial activity of some compounds (miltefosine, atovaquone), promastigotes line resistant have been selected by stepwise increases in drug pressure *in vitro* [12,24]. In the mentioned studies, selection of resistant lines *in vitro* had showed a high level of resistance, but this induction had been found after fifth or more treatments [12,24].

It was not possible to determine the resistance in parasite isolates from mice treated with the essential oil by intralesional route because the growing rate of the promastigotes in Schneider's culture media was unsuccessful. The growth capacity *in vitro* of *Leishmania* varies of one isolate to another that are initially difficult to grow adapt to culture after repeated passages probably due to selection of phenotypes with lower growing rate [23].

The complete cure of the animals treated with the essential oil did not occur. However, while untreated animals develop the inexorable disease, the mice treated with the essential oil by intraperitoneal and oral routes, had small lesions and low parasite burden. The model of cutaneous leishmaniasis due to *L. amazonensis* is not a perfect model, because is a highly virulent strain and cause a disseminating, ''non-cure'' and fatal diseases in BALB/c mice [13].

The efficacy and the reduced toxicity of the essential oil when it is administered by oral route will facilitate long term treatment, in order to produce a consistent protection against the cutaneous leishmaniasis.

Our previous results [9] together with the data presented here are promising results to continue the study of the essential oil from *C. ambrosioides* as antileishmanial drug. On the other hands, the easy extraction of the oil and the cost-effectiveness comparison with other drugs are important considerations to take into account people in developing countries. Future experiments should be development to study the pharmacokinetic and toxicological properties of this natural product.

References

[1] Mauël J. Vaccination against *Leishmania* infections. Current Opinion in Drug Targets -Immunology Endocrinology and Metabolic Disorders 2002;2:201–26.

[2] Desjeux P. The increase in risk factors for leishmaniasis worldwide. Transaction Royal Society of Tropical Medicine and Hygiene 2001;95:239–43.

[3] Croft SL, Yardley V. Chemotherapy of Leishmaniasis. Current Opinions in Pharmaceutical Desing 2002;8:319–42.

[4] Berman JD. Human Leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clinical Infection Diseases 1997;24:684–703.

[5] Chan MJ, Peña LM. Plant natural products with leishmanicidal activity. Natural Product Report 2001;18:674–88.

[6] Kiuchi F, Itano Y, Uchiyama N, Honda G, Tsubouchi A, Nakajima-Shimada J, et al. Monoterpene hidroperoxides with trypanocidal activity from *Chenopodium ambrosioides*. Journal Natural Products 2002;65: 509–12.

[7] Pollack Y, Segal R, Golenser J. The effect of ascaridole on the in vitro development of *Plasmodium falciparum*. Parasitology Research 1990;76:570–2.

[8] Giove NRA. Tradicional medicine in the treatment of enteroparasitosis. Revista de Gastroenterologia de Peru 1996;16:197–202.

[9] Monzote L, Montalvo AM, Almanonni AS, Scull R, Miranda M, Abreu J. Activity of the essential oil from *Chenopodium ambrosioides* grown in Cuba against Leishmania amazonensis. Chemotherapy 2006; 52:130–6.

[10] Buffet PA, Sulahian A, Garin YJF, Nassar N, Derouin F. Culture Microtitration a sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. Antimicrobial Agents and Chemotherapy 1995;39:2167–8.

[11] Bodley AL, McGarry MW, Shapiro TA. Drug cytotoxicity assay for African Trypanosomes and *Leishmania* Species. Journal Infection Diseases 1995;172:1157–9.

[12] Cauchetier E, Loiseau PM, Lehman J, Rivollet D, Fleury J, Astier A, et al. Characterisation of atovaquone resistance in *Leishmania infantum* promastigotes. International Journal of Parasitology 2002;32: 1043–51.

[13] Fournet A, Ferreira ME, Rojas de Arias A, Torres de Ortiz S, Fuentes S, Nakayama H. In vivo efficacy of oral and intralesional administration of 2-substituted quinolines in experimental treatment of New World cutaneous leishmaniasis caused by *Leishmania amazonensis.* Antimicrobial Agents and Chemotherapy 1996;40:2447–51.

[14] Da Silva SAG, Costa SS, Mendoça SCF, Silva EM, Moraes VLG, Rossi-Bergmann B. Therapeutic effect of oral *Kalanchoe pinnata* leaf extract in murine leishmaniasis. Acta Tropica 1995;60:201–10.

[15] Chen M, Brogger S, Theander TG, Kharazmi A. Antileishmanial activity of Licochalcone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania donovani*. Antimicrobial Agents and Chemotherapy 1994;38:1339–44.

[16] Dardari Z, Lemrani M, Bahloul A, Sebban A, Hassar M, Kitane S. Antileishmanial activity of a new 8-hydroxyquinoline derivative designed 7-[5′-(3′-phenylisoxazolino)methyl]-8-hydroxyquinoline: preliminary study. Farmaco 2004;59:195–9.

[17] Mullen AB, Carter KC, Baillie AJ. Comparison of the efficacies of various formulations of amphotericin B against murine visceral leishmaniasis. Antimicrobial Agents and Chemotherapy 1997;41: 2089–92.

[18] Al-Abdely HM, Graybill JR, Loebenberg D, Melby PC. Efficacy of the triazole SCH56592 against *Leishmania amazonensis* and *Leishmania donovani* in experimental murine cutaneous and visceral leishmaniases. Antimicrobial Agents and Chemotherapy 1999;43:2910–4.

[19] Garcez LM, Goto H, Ramos PK, Briguido MC, Gomes PAF, Souza RA. *Leishmania (Leishmania) amazonensis*-induced cutaneous leishmaniasis in the primate *Cebus apella*: a model for vaccine trials. International Journal of Parasitology 2002;32:1755–64.

[20] Garin YJF, Meneceur P, Sulahian A, Derouin F. Microplate method for obtaining *Leishmania* clonal populations. Journal of Parasitology 2002;88:803–4.

[21] Genestra M, Echevarria A, Cysne-Finkelstein L, Vignólio-Alves l, Leon LL. Effect of amidine derivatives on nitric oxide production by *Leishmania amazonensis* promastigotes and axenic amastigotes. Nitric Oxide 2003;8:1–6.

[22] Singh TR, Sundar S. Identification of a gene linked to drug resistance in field isolates of *Leshmania donovani*. Annals of Tropical Medicine and Parasitology 2002;96:839–41.

[23] Carrió J, Portús M. In vitro susceptibility to pentavalent antimony in *Leishmania infantum* strains is not modified during in vitro or in vivo passages but is modified after host treatment with meglumine antimoniate. Pharmacology 2002;2:11–5.

[24] Seifert K, Matu S, Pérez-Victoria FJ, Castanys S, Gamarro F, Croft SL. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). International Journal of Antimicrobial Agents 2003;22:380–7.