



Essential oil from leaves of *Lantana camara*: a potential source of medicine against leishmaniasis

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Abstract: Leishmaniasis is an infection of viscera or tegument caused by protozoa *Leishmania* sp. The extensive period required for the treatment, which involves the use of toxic medicines, leads patients to drop treatment increasing the development of resistant forms of *Leishmania* sp. *Lantana camara* L., Verbenaceae, is a tropical plant native from America. Folk uses have been described for treatment of tumors, tetanus, rheumatism and malaria. This study evaluates the leishmanicidal activity of the essential oil of leaves from *L. camara* on promastigote forms of *Leishmania chagasi* and *L. amazonensis* and its toxic effects on *Artemia salina* (brine shrimp test), macrophage cultures and BALB/c mice. The chemical composition was evaluated using the gas chromatography coupled with mass spectrometer (GC-MS). Thirty substances, mostly mono and sesquiterpenes were identified. The most representative constituents were: germacrene D (24.90%), farnesene derivatives (22%) and (*E*)-cariophyllene (14.31%). Bioassays revealed a significant leishmanicidal activity of essential oil against *L. amazonensis* (IC₅₀ 0.25 µg/mL) and a potential toxic effect on *Brine shrimp* (LC₅₀ 10 µg/mL) and macrophage assays (CC₅₀ 4 µg/mL), while there was no toxic manifestation on mice. The data show the relevant potential of *L. camara* as a source of medicine for leishmaniasis treatment.

Introduction

Leishmaniasis is endemic in 88 countries of Tropical and Sub-tropical regions, affecting more than 12 million people (WHO, 2010). Protozoan parasites of the *Leishmania* genus are the causative agents of human leishmaniasis, which has a spectrum of diseases ranging from self-healing ulcers to fatal visceral infection. In Brazil, among the fourteen species of *Leishmania* identified, ten are known to infect human beings (Cruz et al., 2009). In humans, visceral, cutaneous, and mucosal leishmaniasis result from infection of macrophages throughout the mononuclear-phagocyte system, in the skin, and in the naso-oropharyngeal mucosa, respectively (Murray et al., 2005).

Until now, no vaccines have been available for any form of leishmaniasis, and the chemotherapy is still inadequate since drugs currently used (Amphotericin B, Pentavalent antimony, Pentamidine isethionate, Paramomycin sulfate) exhibits high levels of toxicity (Clem, 2010). In this context, natural substances in particular natural products from plants against leishmaniasis, can be an interesting source of drugs against leishmaniasis (Salem & Werbovetz, 2006). Numerous studies about the activity of plant extracts against both forms of *Leishmania* sp., the promastigote form (infecting form) and the amastigote form (pathogenic form), have been performed (Bezerra et al., 2006; Cammerer et al., 2007; Mishra et al., 2007).

Lantana camara L., Verbenaceae, a native species

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of tropical America, was introduced in several countries as a hedge and an ornamental shrub (Raju, 2000). All parts of the plant are known by having toxic characteristics due to the hepatotoxic triterpenes (Sharma et al., 2007). Its toxicity was also showed in ruminant grazing animals (Tokarnia et al., 1999). On the other hand, therapeutic characteristics of this plant should be considered once the population uses it to treat several health disorders as respiratory diseases, rheumatism, malaria, dysenteries, headache, hypertension and cancer (Ghisalberti, 2000; Deena & Thoppil, 2000).

The literature has shown that the essential oil from leaves of *L. camara* (EOLC) was able to play as an anti-inflammatory agent *in vivo* and has a potential antimicrobial action *in vitro* (Deena & Thoppil, 2000). Braga et al. (2007) showed that the methanol extract from leaves of *L. camara* had a potential activity *in vitro* against promastigote forms of *Leishmania amazonensis*.

In view of the fact that none evaluation about the potential of the EOLC against *Leishmania* sp. are known and the compounds that are the major responsible for the high levels of toxicity (triterpenes), are not present in essential oil, the present work aim to evaluate the potential of EOLC against *L. amazonensis* and *L. chagasi*. This evaluation is done *in vitro* with the promastigote form of *Leishmania*. Further the toxicity of EOLC is evaluated *in vitro* with brine shrimp and macrophages and *in vivo* with BALB/c mice.

Materials and Methods

Plant material

Leaves of *Lantana camara* L., Verbenaceae, were collected in Juiz de Fora-MG, Brazil, in February 2007. The plant material was identified by Dr. Fátima Regina Gonçalves Salimena (Departamento de Botânica, Universidade Federal de Juiz de Fora), and a voucher specimen (CESJ 46090) was deposited at the Herbarium Leopoldo Krieger (Universidade Federal de Juiz de Fora). Before obtaining the essential oil, air dried leaves were weighted to calculate the yield percentage of essential oil. Essential oil was obtained by means of hydro-distillation using a Clevenger apparatus and a glass balloon of 3 L. The procedure of essential oil extraction took 2 h after boiling had started. The essential oil was maintained in an amber glass recipient well closed, weighted and kept at -20°C.

Chemical analysis of compounds from essential oil

The EOLC was analyzed by means of GC-MS (QP5000 - Shimadzu). The chromatography apparatus was equipped of capillary column ZB-5mM, Helium as carrier (32cm/s); injector temperature was 260 °C;

temperature programmed 60-240 °C (2 °C/min); mass spectrum of 70 eV. The components of the essential oil were identified based on the basis of their retention indices. The confirmation of its components was done by comparison of their mass spectra with published spectra (Adams, 2001) and those of reference compound from the library of National Institute of Standard and Technology (NIST) database.

Leishmanicidal assays

Promastigote forms of *Leishmania amazonensis* (MHOM/Br/75/Josefa isolated from patient with diffuse cutaneous form) were cultured in Warren's medium (brain heart infusion, Himédia/Mumbai, Índia, hemin and folic acid) and *Leishmania chagasi* promastigote forms (MHOM/Br/74/PP75 isolated from patient with visceral form) were cultured in 199 medium, both supplemented with 10% fetal bovine serum at 24 °C. The antileishmanial activity was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method based on tetrazolium salt reduction by mitochondrial dehydrogenases (Mossmann, 1983). Log phase promastigotes of two *Leishmania* species, were seeded in 96-well tissue culture plates (2.0 x 10⁶ cells/mL for *L. amazonensis* and 3.0 x 10⁶ cells/mL for *L. chagasi*). After 1 h at 24 °C, the parasites were exposed to increasing dilution of EOLC previously diluted with dimethylsulfoxide (DMSO). The EOLC concentrations were obtained by performing serial dilutions (1:2 v/v) from 250 µg/mL to 3.91 µg/mL and from 25 µg/mL to 0.05 µg/mL. Each concentration was performed in triplicate. Two independent experiments were performed for each species during 72 h incubation period. The DMSO concentration in wells was not higher than 0.01%. Controls with DMSO and without EOLC were also performed. The readouts were done using a spectrophotometer (Spectramax 190, Molecular Devices) at 570 nm and the results were expressed as the concentrations inhibiting parasite growth by 50% (IC₅₀) after 72 h incubation period. Amphotericin B was used as the reference standard drug with a 72 h incubation period. For data analysis: IC₅₀ values were obtained using the adjusted straight lines in figure 1.

In vitro and in vivo toxic assays

Macrophages

To evaluate the *in vitro* cytotoxic effect of EOLC on live cells, macrophages from J774A.1 lineage was used. 10⁶ cells/well were added in 96 well plates which were kept for 24 h in CO₂ 5% incubator at 37 °C, allowing macrophages adherence. After 24 h, each well was washed with PBS and received RPMI-1640 medium with 10% of fetal bovine serum. Then, the solutions of

essential oil containing DMSO and the DMSO solutions which were at the same concentrations used in the Leishmanicidal assays, were added to the wells. The plates were kept in the CO₂ 5%, incubator at 37 °C, for 48 or 72 h. Macrophages viability was determined by the MTT assay, as described above, and was confirmed by comparison of its morphology with the cells from the control group via light microscopy. Dose response curves were plotted taking the values expressed as percentage of control optical density and CC50 values (50% cytotoxicity concentration) were obtained using *GraFit* Version 5 software (Erithacus Software Ltd., Horley, U.K).

Hatching of brine shrimp larvae

Artificial saline water was used to permit the hatch of brine shrimp larvae. It was prepared dissolving mineral enriched sea salt (SERA, Germany) in de-ionized water at a concentration of 35 g/L. Brine shrimp eggs (Tropfish) were sprinkled into a 100 mL becker of containing 50 mL of saline solution and were kept during 48h under constant oxygenate at room temperature (26 °C). After this period, the eggs had hatched and second instar nauplii (larvae) were observed to be swimming near the light source. Sample preparation: EOLC was diluted in DMSO and saline solution water at a standard concentration of 20 mg/mL using shaking. The final percentage of DMSO in standard solution was 1.0%. Brine shrimp test using *Artemia salina*: triplicates for each concentration of EOLC and controls were used. Three first assay 5 mL tubes were pre-filled with 1250 µL of saline solution. Next, 2750 µL of essential oil solution were added to the three first tubes. Another set of tubes received 2000 µL of saline solution. A serial dilution was performed transferring 2000.0 µL of solution from three first tubes (initial concentration = 1.375 mg/mL) to following tubes. The final concentration of the last tubes was 0.001328 mg/mL). Finally, ten second instar larvae in a minimum of saline solution were added to all tubes. Positive and negative controls were performed using artificial saline water and timol. Timol and DMSO (the vehicle) were tested at the same concentrations used for EOLC. The tubes were kept at room temperature and mortality was evaluated after 24 h (Fabri et al., 2011). Statistical analysis to adjust death percentage was performed using Wilcoxon and Lichfield method through Probit analysis (Finney, 1971), permitting the determination of LC50 (50% lethal concentration of nauplii).

In vivo toxicity test with BALB/c mice

In order to evaluate the *in vivo* toxicity of EOLC ten BALB/c mice from Centro de Biologia da Reprodução, Universidade Federal de Juiz de Fora, were used. This methodology to investigate the acute toxicity

is in accordance to the Animal Ethic Committee (AEC Number 063/2007-CCEA), which uses the principle of “3Rs” (NC3Rs, 2012) by the reduced number of animals used in experiments (Ministério da Saúde, 2004). The animals weightening approximately 28 g were separated in two groups of five animals each (test group: EOLC and control group: DMSO). During the first day, and only during that day, the test group received 0.1 mL of EOLC solution in DMSO and sterile H₂O with essential oil concentration of 84 mg/mL. This corresponds to a mean dose of 300 mg/kg. The control group received the same amount of DMSO solution at 0.1% in sterile H₂O, which corresponds to the same amount of DMSO administrated to the test group. The solutions were administered by gavage. Following a protocol suggested by Peters (1948), during four consecutive days the body masses of the animals were measured and analyzed using ANOVA test statistics.

Results and Discussion

The EOLC yielded 0.33% (w/w) providing approximately 100 µL of essential oil from each extraction using 300 g of dried leaves. This value is in total agreement with the yield value of 0.2% (w/w) reported in the recent work of Chowdhury et al. (2007). About the composition of EOLC, thirty constituents representing 86.79% of the EOLC were identified. The major constituents were: germacrene-D (24.90%), (*E*)-cariophyllene (14.31%), (*E,E*)-farnesene (11.58%), α -cariophyllene (8.34%), (*Z,E*)- α -farnesene (5.03%) (*E*)- β -farnesene (4.04%), β -sesquiphelandrene (3.35%), β -elemene (2.27%), 2-pentadecan-1-ol (1.38%) and (*E*)-diidro-apofarnesol (1.13%). The chemical compounds of the EOLC evaluated by comparison of identified peaks by GC/MS analysis are shown in the Table 1.

The constitution of EOLC as reported in literature varies greatly depending on the localization and period of collection. Da Silva et al. (1999) found large variations of EOLC composition within samples from several places in the Amazon region of North Brazil.

The germacrene-D was present in some EOLC analyzed (Sundufu & Shoushan, 2003; Da Silva et al., 1999; Khan et al., 2002) and the cariophyllene groups were observed to be the only versatile components present in every EOLC analyzed by different authors (Ngassoum et al., 1999; Sefidkon, 2002; Rana, 2005), which is confirmed by the present work.

On the other hand our data shows, for the very first time that the farnesene derivatives to be representing more than 20% of the EOLC (*E,E*)-farnesene (11.58%), (*Z,E*)- α -farnesene 5.03%, (*E*)- β -farnesene (4.04%), (*E*)-diidro-apofarnesol (1.13%), (*Z*)- β -farnesene (0.49%), in contrast with other works, which had shown small amounts of these derivates, or only one kind of them in an

EOLC (Benites et al., 2009; Padalia et al., 2010).

Table 1. Chemical constituents of essential oil from leaves of *Lantana camara* from Brazil.

Peak number	Constituents	Kovats Index	%
1	α -pinene	938	0.23
2	β -pinene	978	0.25
3	mircene	982	0.27
4	heptan-2,2,4,6,6-pentametil	989	0.25
5	linalool	1035	0.67
6	α -terpineol	1202	0.73
7	1,5-heptadiene, 2,5-dimethyl-3-methylene	1332	0.39
8	1,5-heptadiene, 2,5-dimethyl-3-methylene	1336	0.64
9	longiciclene	1374	0.39
10	β -cubebene	1387	0.41
11	β -elemene	1390	2.27
12	(Z)-cariophyllene	1405	0.83
13	(E)-cariophyllene	1420	14.31
14	cis-thujopsene	1425	0.47
15	γ -elemene	1431	0.48
16	(Z)- β -farnesene	1444	0.49
17	α -cariophyllene	1456	8.34
18	(E)- β -farnesene	1459	4.04
19	germacrene-D	1484	24.90
20	Not identified	1487	5.78
21	(Z,E)- α -farnesene	1496	5.03
22	(E,E)-farnesene	1500	11.58
23	(Z)- γ -bisabolene	1510	0.42
24	β -sesquiphelandrene	1514	3.35
25	(E)- γ -bisabolene	1520	0.78
26	spatulanol	1526	0.45
27	2-pentadecin-1-ol	1558	1.38
28	davanone	1579	0.77
29	(E)-diidro-apofarnesol	1581	1.13
30	eudesmol	1610	0.89
31	diidromircene,1,6-diol-Z	1620	0.65

The potential of EOLC against promastigote forms of *L. chagasi* and *L. amazonensis* was evaluated *in vitro*. The toxic aspects of the EOLC were analyzed through the *in vitro* Brine shrimp test, as well as by the macrophage culture and measuring the masses of BALB/c mice that received EOLC or DMSO solution. The data obtained from EOLC bioassays on promastigote forms of *Leishmania* revealed a growth inhibition activity of EOLC at low concentrations for the both species of *Leishmania* sp used in this study, Figure 1.

It is worth to highlight that the EOLC was able to inhibit 100% *L. amazonensis* proliferation

with concentrations above 3 μ g/mL. For *L. chagasi* an inhibition of about 90% was found with a concentration of 250 μ g/mL. Extrapolating the straight line of *L. chagasi* data of Figure 1 one may estimate that total inhibition would be obtained with 1 mg/mL of the EOLC. Below the saturation values, 3 μ g/mL and 1 mg/mL, the inhibition shows a smooth increase with the logarithm of concentration, which permit the determination of IC50 values (Table 2).

The species *L. amazonensis* was found to be much more susceptible to the EOLC than the *L. chagasi*, which can be noted from the IC50 values (*L. amazonensis* with 0.25 μ g/mL and *L. chagasi* with 18 μ g/mL). The lower susceptibility of *L. chagasi* is frequently found also with respect to other drugs (Morais-Teixeira et al., 2008; Braga et al., 2007) including the standard drug Amphotericin B (Table 2).

Table 2. Effect of essential oil of *Lantana camara* on promastigote forms from *Leishmania* observed in 72 h culture period.

Sample	<i>L. amazonensis</i> IC50 (μ g/mL) \pm SD	<i>L. chagasi</i> IC50 (μ g/mL) \pm SD
EOLC	0.25 \pm 0.08	18 \pm 4
Amphotericin B	0.90 \pm 0.05	1.91 \pm 0.02

The mechanism of action by which EOLC inhibits the parasite growth is still unknown, however some authors (Wink, 2008; Cabral et al., 2009; Laouer et al., 2009) have shown the biological activity of the major compounds present in this EOLC against other eukaryotes parasites and prokaryote microorganisms, which are distant from *Leishmania* genus, as punctuated before, this work is the first one that demonstrates the activity of EOLC on promastigote culture of *Leishmania*, so it is relevant to consider some previous studies which enables the construction of few hypothesis to contribute for explanation of EOLC activity on this protozoa culture.

According to Kasali et al. (2004) germacrene-D was able to inhibit the growth of some bacteria and fungi during the *in vitro* assays. The presence of germacrene-D in EOLC composition used in *Leishmania* assays developed in the present work, could be considered as a responsible by the inhibitory growth effect on promastigote culture. This hypothesis is based on the Amphotericin B activity, which is able to act as an antifungal and an antileishmanial drug.

For the first time, farnesene derivatives (*E*)-diidro-apofarnesol, (*E*)- β -farnesene, (*Z*)- β -farnesene, (*E,E*)-farnesene, (*Z,E*)- α -farnesene) were found as one of the major compounds groups representing 22.27% of the total percentage in the EOLC used in this study. The real role of farnesene derivatives on the growth of promastigote forms of *Leishmania* is still unclear and

needs further studies, although it is pertinent to notice that farnesol, a derivative alcohol from farnesenes, was relevant in increasing susceptibility of the *Staphylococcus aureus* culture (Kuroda et al., 2007) and against the fungi *Candida albicans* in culture (Sato et al., 2004). Consequently the action of farnesene derivatives on *Leishmania* culture, could be related with the enhance of promastigote susceptibility as it was cited in the case of other microorganisms, independent of the evolutionary distance, once there is a drug example with this possible action, which was also cited, the Amphotericin B.

Once the cariophyllene derivatives present in EOLC of this work did not inhibit growth of other protozoan species in culture, such as the amoebic and giardial ones according Calzada et al. (2001), it might be that this molecule doesn't have any inhibitory action on promastigote forms growth. Another possible suggestion as to the active components of the EOLC may be obtained from studies of other active essential oils on *Leishmania*. Costa et al. (2009a) identified the compounds of essential oil from leaves of *Annona foetida* (Annonaceae) and tested antileishmanial action with promastigotes of *L. amazonensis* (IC₅₀ 16 µg/mL) and *L. chagasi* (IC₅₀ 27 µg/mL). The substances β-cubebene, β-elemene, (*E*)-cariophyllene, (*E*)-γ-bisabolene and spathulenol are present in both essential oils and the active components may be from this group.

The Brine shrimp used to evaluate the toxicity of EOLC revealed on lethality assay a LC₅₀ 12±3 µg/mL, and it is still in accordance with Costa et al. (2009b), that reported a LC₅₀ value of 14 µg/mL for the same EOLC. The essential oil activity on *Artemia salina* revealed a 48-fold higher LC₅₀ than the IC₅₀ from *L. amazonensis*, and even the saturating concentration of 3 µg/mL, which inhibits *L. amazonensis* completely, is still four times smaller than LC₅₀. This is an interesting relation between inhibitory doses of promastigote growth and a multi-cellular organism. However, its toxicity has to be considered as extremely high, since its LC₅₀ is significantly lower than 1000 µg/mL, the pattern limit of toxicity (Costa et al., 2009).

Unfortunately, in the case of *L. chagasi* the results are less promising. The fact that *L. chagasi* is more resistant than *L. amazonensis* is known in literature. Braga et al. (2007) found a IC₅₀ value of the methanol extract of leaves from *L. camara* of 14 µg/mL for *L. amazonensis* and of 250 µg/mL for *L. chagasi* (Braga et al., 2007). Even though the IC₅₀ (*L. chagasi*) of the EOLC is lower than that of vegetal extracts of twenty different species of plants tested against the same strain of promastigote forms used in the present work (Braga et al., 2007). A similar situation was found with respect to cytotoxicity of EOLC. The investigation about the cytotoxicity of EOLC was realized with the macrophage culture and it revealed low values of CC₅₀ for both different culture times evaluated (48 h, 6.2 µg/mL and 72 h, 4.7 µg/mL). These values are almost

nineteen times bigger than the IC₅₀ of *L. amazonensis* and they are still bigger than the saturating concentration of 3 µg/mL, which inhibits *L. amazonensis* completely. Nevertheless, the values 48 h, 6.2 µg/mL and 72 h, 4.7 µg/mL represent the presence of a high cytotoxicity (Costa et al., 2009b). In the case of *L. chagasi* the comparison of IC₅₀ and CC₅₀ is less favorable. But this toxic effect is not due to hepatotoxic triterpenes, once these compounds are not present in the EOLC composition. Consequently, new studies are necessary to explain the high toxicity exhibited by the EOLC used in the present study.

According to Santos et al. (2008) the toxic effects on bovines occur approximately 2 h after the ingestion of some parts of the plant and after 24-48h the animals reveal an appetite inhibition leading to reduction of body mass. In the present study, 300 mg/mL per kg of body mass of EOLC were administered to BALB/c mice and did not reveal significant alterations related to their masses through the 4-day analysis according to the ANOVA test (*p* value 0.6472). Since results of toxicity test *in vivo* with BALB/c mice did not reveal a significant difference in masses of mice (*p*>0.05), it suggests absence of acute manifestation of toxicity in mice caused by EOLC.

According to Sanchez-Brunete et al. (2004), Amphotericin B caused behavior alterations and death in hamsters that received it at doses that ranged from 10 to 20 mg/mL per kg of body weight along a 48 h period. Comparing the doses used in this study to those used in the mentioned one, despite different species, it is possible to suggest a remarkably higher toxicity of Amphotericin B than EOLC, which revealed significant inhibitory activity of promastigote proliferation *in vitro*.

Additionally, it is possible to suggest that its toxic effect observed in brine shrimp test and mammals cells *in vitro* is not related to the presence of germacrene-D, because it was evaluated in the same models and no toxic effect was observed according to Biavatti et al. (2001).

Moreover, regardless of the high toxicity shown for oil through the brine shrimp test and macrophages, it is important to consider that the amphotericin B, second-line drug for the treatment of cutaneous mucosa leishmaniasis, also has high toxicity to humans, even causing kidney failure (Lopez-Berestein et al., 1987; 1989). However, according to Sampaio & Marsden (1997) the association of liposomes with amphotericin B increases its tolerability by patients because this association interacts directly with the parasite ergosterol and reacts less with the cholesterol of the host. And it also enhances the ability of macrophage to phagocyte the parasite. Similar association techniques may also work with EOLC.

Thus, our work has confirmed the diversity of EOLC composition, once, for the first time, the farnesene derivatives were identified. In addition, the presence of potential activity of EOLC against promastigote forms was revealed. Despite the high toxic results obtained

for the macrophage culture and brine shrimp test, the absence of its toxicity on BALB/c mice model encourage investment in new researches. In conclusion, the results obtained suggesting that the EOLC may be a potential source of medicine for leishmaniasis treatment. Further studies are necessary to determine the active components in the EOLC and whether the leishmanicidal and citotoxic components are distinct chemical species.

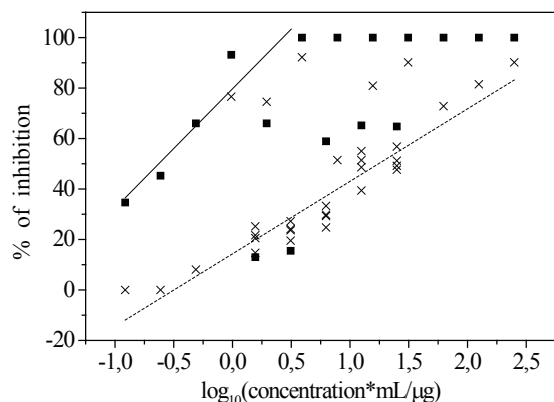


Figure 1. Effect of essential oil from leaves of *Lantana camara* on promastigote culture of *L. amazonensis* (squares) and *L. chagasi* (crosses). The straight lines indicate the general behaviors up to the saturation concentrations. They were obtained by fitting reduced sets of data points that ignored dubious results and the saturation region.

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