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Trypanocidal and cytotoxic activities of essential oils from medicinal plants of Northeast of Brazil

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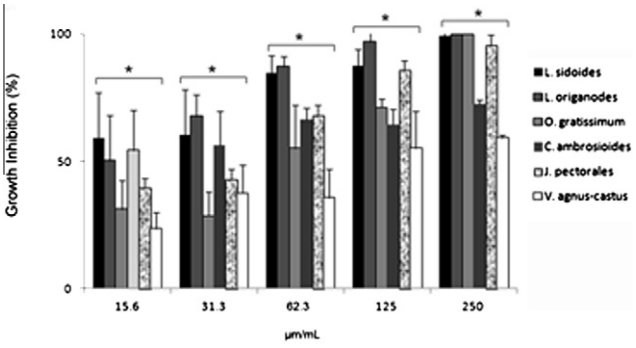
HIGHLIGHTS

- The trypanocidal potential of essential oils from medicinal plants was evaluated.
- The essential oils tested were active against all evolutive forms of *Trypanosoma cruzi*.
- The essential oil from *Lippia sidoides* was the most effective against trypomastigotes.
- *Lippia origanoides* essential oil was the most effective against amastigotes.
- All essential oils were more specific against parasites than mammal cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Chagas disease, caused by *Trypanosoma cruzi*, is an important cause of mortality and morbidity in Latin America. There are no vaccines available, the chemotherapy used to treat this illness has serious side effects and its efficacy on the chronic phase of disease is still a matter of debate. In a search for alternative treatment for Chagas disease, essential oils extracted from traditional medicinal plants *Lippia sidoides*, *Lippia origanoides*, *Chenopodium ambrosioides*, *Ocimum gratissimum*, *Justicia pectorales* and *Vitex agnus-castus* were investigated *in vitro* for trypanocidal and cytotoxic activities. Essential Oils were extracted by hydrodistillation and submitted to chemical analysis by gas chromatography/mass spectrometry. The concentration of essential oils necessary to inhibit 50% of the epimastigotes or amastigotes growth (IC_{50}) and to kill 50% of trypomastigote forms (LC_{50}) was estimated. The most prevalent chemical constituents of these essential oils were monoterpenes and sesquiterpenes. All essential oils tested demonstrated an inhibitory effect on the parasite growth and survival. *L. sidoides* and *L. origanoides* essential oils were the most effective against trypomastigote and amastigote forms respectively. No significant cytotoxic effects were observed in mouse peritoneal macrophages incubated with essential oils which were more selective against the parasites than mammalian cells. Taken together, our results point towards the use of these essential oils as potential chemotherapeutic agent against *T. cruzi*.

1. Introduction

American trypanosomiasis, caused by the protozoa *Trypanosoma cruzi*, is an important public health concern in Latin America

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(Rocha et al., 2007). It is estimated that 18 million people are infected and 100 million people live in areas at risk of infection (WHO, 2008).

The current treatment of Chagas disease is based on the use of nifurtimox (4((5-nitrofurfurylidene)amino)-3-methylthiomorpholine-1,1-dioxide), derived from nitrofuran, and benznidazole (*N*-benzyl-2-nitroimidazole-1-acetamide), a nitroimidazole derivative (Urbina, 2002). These drugs are very toxic to the patients presenting several side effects. Both Nifurtimox and Benznidazole are effective in the acute phase but their effectiveness in the chronic phase of disease is still controversial (Marin-Neto et al., 2009). In addition, the success of treatment also varies according to the geographical area, probably due to differences in drug susceptibility among different *T. cruzi* strains (Castro et al., 2006; Soeiro and De Castro, 2009). For these reasons, the development of new safe and more effective therapeutic agents is still needed.

Plants and their derived products are an interesting source of lead compounds that could be potentially active against protozoa (Croft et al., 2005; Salem and Werbovetz, 2006). Essential oils from aromatic plants have shown many biological activities against various microorganisms (Boyraz and Özcan, 2006; Tagboto and Townson, 2001; Tepe et al., 2006) including *T. cruzi* (Santoro et al., 2007a) and *Leishmania* (De Medeiros et al., 2011; Oliveira et al., 2009). Due to their low density and lipophilic feature they can interact with different intracellular targets (Rocha et al., 2005). Additionally, it has been shown that essential oils constituents also have immune modulatory effects on mammalian host (Cowan, 1999; Rocha et al., 2005). In this context, the present study investigated the *in vitro* activity of essential oils from plants of Northeast of Brazil, used in folk medicine, on epimastigote, trypomastigote and amastigote forms of *T. cruzi*. The cytotoxicity of these essential oils on mammalian cells was also investigated.

2. Materials and methods

2.1. Plant material and essential oil extraction

Lippia sidoides, *Lippia origanoides*, *Chenopodium ambrosioides*, *Ocimum gratissimum*, *Justicia pectorales* and *Vitex agnus-castus* were collected at the Garden of Medicinal and Aromatic Plants of the Universidade Federal do Piaui (UFPI), in Teresina, Piaui, Brazil. Voucher specimens were identified and deposited at the Graziela Barroso Herbarium in UFPI under numbers ICN TEPB18743, TEPB09205, TEPB25418, TEPB25506, TEPB25400 and TEPB18885 respectively. Essential oils were obtained by hydrodistillation, using the modified Clevenger apparatus (Craveiro et al., 1981).

2.2. Essential Oil chemical assay

The analysis of essential oils was performed by gas chromatography/mass spectrometry (GC/MS), with identification of constituents made by comparing the spectra obtained with those of the equipment data bank (Willey Mass Spectral Database 229) and the retention index (RI) calculated for each essential oil constituent (Adams, 2007). The GC/MS analysis was performed with a Shimadzu GC-17A/MS QP5050A-GC/MS system (EI mode 70 eV, source temperature 270 °C, scanned mass ranged 43–350 Daltons). Operating conditions were: capillary DB5 fused silica column (30 m × 0.25 mm; 0.25 mm film thickness); injector temperature 220 °C; column temperature set initially at 40 °C and then programmed at 3 °C/min to 240 °C; carrier gas helium, with linear gas velocity of 1.0 mL/min; split ratio 1:10; injected volume 1.0 mL (1% dilution in dichloromethane); inlet pressure 100.2 kPa. Mass spectra were taken at 70 eV; decomposition speed 1.000; decomposition interval 0.50; fragments from 45 to 450 Daltons were decomposed. A mixture of hydrocarbons (C_9H_{20} to $C_{26}H_{54}$) was injected under these same conditions.

2.3. Parasites

Culture epimastigote forms of *T. cruzi*, strain Dm28c (Contreras et al., 1988), were maintained by weekly passages at 28 °C in LIT medium (Camargo, 1964) supplemented with 10% inactivated fetal bovine serum (FBS). Three-day-old culture forms were used in all experiments. Trypomastigotes, Y strain (Silva and Nussenzweig, 1953), were obtained and harvest from *T. cruzi*-infected Vero cells as previously described (De Souza et al., 2004), cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 UI/mL penicillin and 100 μg/mL streptomycin. The amastigote forms were obtained from infected peritoneal macrophages as detailed bellow.

2.4. Trypanocidal activity

For *in vitro* assay of trypanocidal activity, essential oils were initially dissolved in dimethyl sulphoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA), at a concentration of 50 mg/mL and stored at −20 °C. This stock solution was diluted to obtain a solution at 1 mg/mL in Liver Infusion Tryptose medium (LIT) medium. This solution was once again diluted in the same culture medium at concentrations ranging from 15.6 to 250 μg/mL, so that the final concentration of DMSO never exceeded 0.2%, a concentration which is not toxic for the protozoa. In order to investigate the effects of essential oils on epimastigote growth, the parasites (2×10^6 parasites/mL) were incubated for 72 h at 28 °C in the absence or presence of essential oils and the culture growth was estimated daily by cell counting. Trypomastigote forms (2×10^6 cells/mL) were kept at 37 °C in RPMI medium supplemented with 10% FBS and then incubated for 24 h with essential oils. The IC_{50} (concentration of essential oils that inhibits by 50% the growth of epimastigotes) and LC_{50} (concentration of essential oils that kills 50% of trypomastigotes) were evaluated by cell counting in a Neubauer chamber, after 48 and 24 h of cultivation respectively. The data obtained were analyzed by regression analysis using the SPSS 8.0 software for Windows.

To evaluate the effects of essential oils on intracellular amastigote forms, peritoneal macrophages from Balb/c mice were harvested and seeded at 3×10^5 cells/mL in a 24-well plate, supplemented with 10% inactivated FBS and allowed to adhere for 24 h at 37 °C in 5% CO_2 atmosphere. Adhered macrophages were then infected with culture-derived trypomastigote forms using a ratio 1:10 at 37 °C for 4 h. Afterward, non-interiorized parasites were removed by washing and the infected culture were incubated for 48 h in RPMI 1640 medium or treated with different concentrations of essential oils. The cultures were stained with Giemsa (Sigma–Aldrich, USA) and the parasite infection was determined by counting of total number of intracellular amastigote per 300 cells in duplicate. The concentration that inhibited the intracellular amastigote growth by 50% (IC_{50}) was estimated by regression analysis as described above.

2.5. Cytotoxicity assay

To evaluate the potential cytotoxic effect of essential oils on mammalian cells, peritoneal macrophages from Balb/c mice (5×10^4 cells/well) were seeded in 96-well plates containing RPMI medium, supplemented with 10% inactivated FBS, and incubated for 2 h at 37 °C in 5% CO_2 atmosphere. Non-adherent cells were then removed by washing with PBS and the remaining cells were allowed to growth for additional 48 h in RPMI in the absence or presence of the different concentrations of the essential oils. After

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the incubation period cells were submitted to MTT colorimetric assay as previously described by Mosmann (1983). Briefly, treated and untreated cells were washed and incubated in fresh culture medium containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA) for 3 h at 37 °C. After incubation, the cells were solubilized in DMSO (100 μL/well) and the formazan precipitates derived from MTT reduction was determined spectrophotometrically at 540 nm. Each assay was carried out in triplicate in three independent experiments. The 50% cytotoxic concentration (CC_{50}) was determined by regression analysis. The selectivity index (SI) was determined for trypomastigotes and amastigotes as the ratio of CC_{50} to LC_{50} or IC_{50} values, respectively.

2.6. Ethical standards

All experiments involving the use of experimental animals were performed in accordance to the ethical standards of Fundação Oswaldo Cruz and were approved by the ethics committee (CEUA-FIOCRUZ L-0001/08).

2.7. Statistical analysis

Statistical analysis was performed using ANOVA followed by Dunnetťs test. The data were analyzed by GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

Essential oils can be defined as a volatile complex mixture of constituents obtained from aromatic plants, mainly by hydrodistillation. In this work the chemical characterization of essential oils constituents were performed by comparison of their GC–MS retention data with retention indexes obtained by the combination of the essential oil with eight *n*-alkanes used as internal standards. This procedure greatly improved the identification of essential oil compounds, particularly those with very similar fragmentation patterns (De Lima et al., 2009). The chemical analysis of essential oils used in our experiments is presented in Table 1. The main constituent of *L. sidoides* essential oil was thymol (78.4%), whereas carvacrol (37.3%), thymol (22.4%) and γ-terpinene (10.9%) were the major constituents of *L. origanoides*. The 1,8-cineole was the main component of *V. agnus-castus* (34.3%), followed by α-Terpinyl acetate (10%). *O. gratissimum* presents eugenol (38.4%) as the main constituent followed by 1.8-cineole (21.6%) and (*E*)-ocimene (10%). The terpinolene was the major component of essential oils from *J. pectorales* and *C. ambrosioides* with 86.6% and 69.9% respectively. Other minor essential oil constituents were present at levels below 10%. It is important to note in all the essential oils a predominance of monoterpes ($C_{10}H_{16}$) and sequisterpenes ($C_{15}H_{24}$) hydrocarbons.

All the essential oils tested showed an inhibitory effect on epimastigote growth and caused loss of cell viability of trypomastigote

Table 1
Quantitative and qualitative composition of *L. sidoides*, *L. origanoides*, *C. ambrosioides*, *O. gratissimum*, *J. pectorales* and *V. agnus-castus* essential oils, as determined by GC/MS.

Constituents	Relative amount (%)							
	RI[a]	RI[b]	*L. sidoides*	*L. origanoides*	*O. gratissimum*	*V. agnus-castus*	*J. pectorales*	*C. ambrosioides*
α-Pinene	932	935	–	–	1.9	–	–	–
Sabinene	969	972	–	–	1.8	–	–	–
β-Pinene	974	976	–	–	4.4	–	–	–
Myrcene	988	988	0.4	2.3	1.6	3.4	–	–
α-Phellandrene	1002	989	–	1.5	–	–	–	–
α-Terpinene	1014	1018	0.4			0.8	13.3	
Limonene	1024	1028	0.4	–	–	–	–	–
1,8-Cineole	1026	1033	1.6	–	21.6	34.3	–	–
(*E*)-Ocimene	1044	1049	–	–	10.0	–	–	–
γ-Terpinene	1054	1062	1.1	10.9	–	1.7	–	–
Terpinolene	1086	1084	–	–	–	–	86.6	69.9
Linalool	1095	1091	–	0.3	1.2	–	–	–
trans-Sabinene hydrate	1098	1102	–	0.2	–	–	–	–
Terpinen-4-ol	1174	1177	0.6	0.3	–	–	–	–
α-Terpineol	1186	1189	–	–	1.0	2.9	–	–
(*Z*)-Ocimenone	1226	1228	–	0.3	–	–	–	–
Thymol methyl ether	1232	1235	1.4	0.2	–	–	–	–
Ascaridole	1234	1237	–	–	–	–	–	17.1
Thymol	1289	1298	78.4	22.4	–	–	–	–
Carvacrol	1298	1299	–	37.3	–	–	–	–
Linalool propanoate	1334	1330	–	–	–	0.9	–	–
α-Terpinyl acetate	1346	1347	–	–	–	10.0	–	–
Eugenol	1356	1356	–	–	38.4	–	–	–
α-Copaene	1374	1378	–	0.2	–	–	–	–
β-Elemene	1389	1380	–	–	0.5	–	–	–
(*Z*)-Caryophyllene	1408	1408	–	0.2	–	–	–	–
(*E*)-Caryophyllene	1417	1418	6.2	2.1	5.5	–	–	–
Aromadendrene	1439	1433	0.5	–	–	–	–	–
α-Humulene	1452	1452	0.3	–	–	–	–	–
(*E*) β-Famesene	1454	1458	–	–	–	5.6	–	–
γ-Murolene	1478	1477	–	–	0.6	–	–	–
Germacrene D	1484	1487	–	–	2.3	2.3	–	–
β-Selinene	1489	1490	–	–	5.5	–	–	–
Viridiflorene	1496	1489	0.4	–	–	–	–	–
α-Selinene	1498	1494	–	–	2.0	–	–	–
δ-Cadinene	1522	1537	–	0.2	–	0.9	–	–
Spathulenol	1577	1572	0.4	–	–	–	–	–
Caryophyllene oxide	1582	1581	0.4	0.4	–	–	–	–
Dihydrosclarene	1974	1969	–	–	–	0.7	–	–

[a] Literature Retention Index (Adams, 2007).
[b] Experimental Retention Index.

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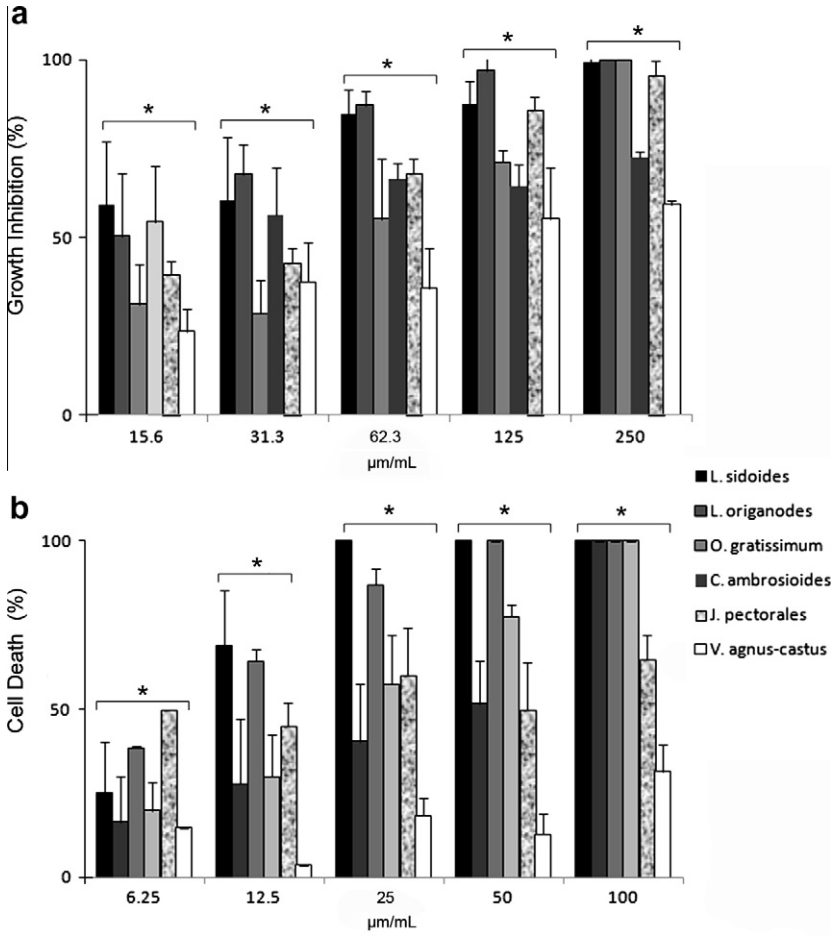

Fig. 1. Effects of *L. sidoides*, *L. origanoides*, *C. ambrosioides*, *O. gratissimum*, *J. pectorales* and *V. agnus-castus* essential oils on (a) epimastigote growth and (b) trypomastigote viability after 48 and 24 h of incubation respectively. The bars represent the mean of three independent experiments in triplicate ± SD. *$p < 0.05$ compared to control.

Table 2
Trypanocidal and cytotoxic effects of essential oils from *L. sidoides*, *L. origanoides*, *J. pectorales*, *O. gratissimum*, *C. ambrosioides* and *V. agnus-castus*.

Essential Oils	Macrophage CC_{50} (µg/mL)	Epimastigote IC_{50} (µg/mL)	Trypomastigote LC_{50} (µg/mL)	Amastigote IC_{50} (µg/mL)	$SI_{(trypo)}$	$SI_{(ama)}$
L. sidoides	192.7 ± 0.3	28.9 ± 4.9	10.3 ± 0.5	41.7 ± 12.3	18.7	4.6
L. origanoides	175.7 ± 0.4	26.2 ± 5.3	39.7 ± 11.4	29.8 ± 3.0	4.4	5.9
J. pectorales	176.9 ± 1.2	56.8 ± 18.9	44.5 ± 10.2	*	3.9	*
O. gratissimum	180.4 ± 1.0	71.1 ± 12.2	11.5 ± 0.3	30.7 ± 2.3	15.7	5.9
C. ambrosioides	275.6 ± 0.7	21.3 ± 6.8	28.1 ± 7.0	50.2 ± 6.0	9.8	5.5
V. agnus-castus	617.9 ± 8.1	157.1 ± 36.5	155.8 ± 5.4	*	3.9	*

* Not determined.

forms in a dose-dependent way. Except for trypamastigotes treated with 12 µg/mL of *V. agnus-castus* essential oil, statistic differences ($p < 0.05$) between treated-cells and control cells were observed for all essential oil treatments (Fig. 1). The essential oils from *C. ambrosioides*, *L. origanoides* and *L. sidoides* were the most effective against epimastigotes, exhibiting IC_{50} values of approximately 21.3, 26.2 and 28.9 µg/mL, respectively, whereas the less effective were the essential oils from *J. pectorales*, *O. gratissimum* and *V. agnus-castus* with IC_{50} values of about 56.8, 71.1 and 157.1 µg/mL, respectively (Table 2). *L. sidoides*, *O. gratissimum*, *C. ambrosioides* and *L. origanoides* were found to be more effective against trypomastigotes with LC_{50}/24 h values of about 10.3, 11.5, 28.1 and 39.7 µg/mL, respectively. *O. gratissimum* essential oil, for example, was about 6 times more toxic for trypomastigote than epimastigote forms. These results are particularly important since the trypomastigotes are the infective forms found in the vertebrate host. On the other hand, essential oils from *J. pectorales* and *V. agnus-castus* presented lower activity against trypomastigotes with a LC_{50}/24 h values of 44.5 and 155.8 µg/mL respectively. Our previous studies have demonstrated that *L. sidoides* and *L. origanoides* were effective against promastigote and amastigote forms of *Leishmania amazonensis* and *O. gratissimum* was effective against *Leishmania chagasi* promastigotes (De Medeiros et al., 2011; Oliveira et al., 2009). Escobar et al. (2010) demonstrated the activity of different Colombian varieties of *L. origanoides* against *T. cruzi* and *L. chagasi*. According to these authors, the qualitative and quantitative differences existing between the *Lippia* essential oils are dependent on the *Lippia* species and on the place of plant collection. These differences have direct implication on the trypanocidal and leishmanicidal activities of these oils.

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Interestingly, a Colombian sample of *L. origanoides*, whose the chemical composition was similar to those found in our *L. origanoides* specimen, also presented similar activities against *T. cruzi*.

Essential oils as well as their components have been found to possess a wide spectrum of pharmacological effects including antibacterial, antifungal, antiviral, antihelminthic and antiprotozoal activities (Macedo et al., 2010; Machado et al., 2011; Ocazionez et al., 2010; Reichling et al., 2009; Santos et al., 2010). They are also known to have important biological activities against trypanosomatids as *Trypanosoma brucei* (Otoguro et al., 2011), *Leishmania* (De Medeiros et al., 2011; Monzote et al., 2007; Oliveira et al., 2009) and *T. cruzi* (Santoro et al., 2007a, 2007b, 2007c). These activities are mainly attributed to the presence terpenic, aromatic and aliphatic constituents (Bakkali et al., 2008; Schelz et al., 2010).

The trypanocidal activity of thymol-rich essential oils has already demonstrated. Santoro et al. (2007c) have showed that treatment of *T. cruzi* with crude essential oil of *Thymus vulgaris* (thyme) caused a dose-dependent growth inhibition of epimastigotes with IC_{50} of 77 μg/mL. Both crude essential oil and thymol, the main constituent of thyme oil, proven to be also effective against trypomastigotes with LC_{50} for thymol of approximately 62 μg/mL Santoro et al. (2007c). The phenolic compound eugenol, the main constituent of *Ocimum gratissimum,* was also found in the essential oil of *Zyzygium aromaticum*, which showed to be effective against *T. cruzi*. However, incubation of eugenol alone was less effective than crude essential oil of *Z. aromaticum* (Santoro et al., 2007b). Although *O. gratissimum* and *Z. aromaticum* essential oils have eugenol as main constituent, the activity of *O. gratissimum,* against *T. cruzi* was higher for both epimastigote and trypomastigote forms as demonstrated in the present study. The contrast of results obtained with *S. aromaticum*/eugenol and *O. gratissimum* essential oil could be explained by synergistic or antagonist effects of other compounds in the oil mixture. Carvacrol, the main constituent of *L. origanoides*, has a broad-spectrum of antimicrobial activity extended to food spoilage or pathogenic fungi, yeast and bacteria as well as human, animal and plant pathogenic microorganisms (Nostro and Papalia, 2012). Furthermore, previous studies have demonstrated that carvacrol bearing essential oils present toxicity against *L. amazonensis* and *T. cruzi*, with no significant toxicity to mammalian cells (Escobar et al., 2010). Taken together these data open perspectives to the use of essential oils and their isolated constituents as potential chemotherapeutic agents for the treatment of parasitic diseases caused by tripanosomatids.

It is usually assumed that terpenic constituents are responsible for the hydrophobic feature of essential oils (Burt et al., 2005) which allows essential oils to freely permeate the cell membranes and kill the parasites by affecting their cytoplasmic metabolic pathways or organelles (Knobloch et al., 1989). On the other hand, essential oils themselves could interact with parasite membrane and cause drastic physiologic changes leading to the loss of membrane permeability which ultimately lead to cell death (Bakkali et al., 2008; Knobloch et al., 1989). However, due to the great number of constituents and the synergistic or antagonistic interactions existing between them it is likely that essential oils have other cellular targets besides the cellular membranes. In fact the interactions of essential oils with lipids and proteins have been reported (Bakkali et al., 2008).

Essential oils and their isolated constituents have been reported to be well tolerated by mammalian cells and some of them present a protective effect against oxidative stress (El-Nekeety et al., 2011). In our work we found that all essential oils presented no toxicity against mammalian cells (>100 μg/mL). The lowest cytotoxic activity was observed in *V. agnus-castus* and *C. ambrosioides*-treated cells with a CC_{50} of 617.9 and 275.6 μg/mL respectively. However, *V. agnus-castus* was also the less effective against trypomastigote forms. *L. sidoides*, *L. origanoides*, *J. pectorales* and *O. gratissimum* showed CC_{50} value of 192.7, 175.7, 176.9 and 180.4 μg/mL, respectively (Table 2). In order to compare the trypanocidal activity and the toxicity of essential oils for mammalian cells, the selectivity index (SI) was estimated. All essential oils showed to be more effective against trypomastigote than mammalian cells with SI equal or superior to 2.0 (Houghton et al., 2007), The SI values for *L. sidoides*, *O. gratissimum* and *C. ambrosioides* were 18.7, 15.7 and 9.8, respectively indicating the high selectivity of these oils towards the parasites. Among all essential oils tested, *L. sidoides* essential oil was both the most selective and effective against trypomastigotes. On the other hand, *V. agnus-castus* and *J. pectorales* presented lower values of SI (3.9) (Table 2).

Since *L. sidoides*, *L. origanoides*, *O. gratissimum* and *C. ambrosioides* showed higher SI values for trypomastigote we have further analyzed the effects of these essential oils against intracellular amastigote forms. Our results showed that except for *L. origanoides*, which presented an IC_{50} of about 29.8 μg/mL, amastigote forms showed to be more resistant to essential oils treatment than trypomastigotes with IC_{50} values of about 30.7, 41.7 and 50.2 μg/mL for *O. gratissimum*, *L. sidoides* and *C. ambrosioides*, respectively. The lower susceptibility of amastigote forms when compared with trypomastigotes can be due to the fact that essential oils must have to cross the host cell membrane and the parasitophorous vacuole membrane in order to gain access to intracellular parasite, whereas the extracellular trypomastigote is directly exposed to essential oils. Furthermore the interaction of essential oils with host cell components and the inner cell environment conditions should be also taken into account. The SI values found for amastigotes showed that all essential oils were more toxic to this form than to mammalian cells.

In conclusion, the low toxicity of essential oils to mammalian cell associated to the significant trypanocidal activity of the essential oils of *L. sidoides*, *L. origanoides*, *C. ambrosioides*, and *O. gratissimum* points these oils as promissory candidates for trypanocidal drugs. Moreover the trypanocidal activity of *L. sidoides* and *J. pectorales* on the three evolutive forms of *T. cruzi* were demonstrated for the first time in this work. Further studies are required to elucidate the mechanisms of parasite death induced by the most promissory essential oils and indentify their putative intracellular targets.

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