J Parasit Dis (Apr-June 2017) 41(2):345–351
DOI 10.1007/s12639-016-0800-7

ORIGINAL ARTICLE

Trypanocidal action of *Lippia alba* and *Lippia origanoides* essential oils against *Trypanosoma evansi* in vitro and in vivo used mice as experimental model

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Received: 16 June 2015 / Accepted: 26 May 2016 / Published online: 3 June 2016
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Abstract Parasitic diseases have an enormous health and economic impact and are a particular problem in tropical regions of the world. Disease caused by protozoa, such as trypanosomiasis, are the cause of most parasite related morbidity and mortality. Thus, the aim of this study was to verify the trypanocidal effectiveness of *Lippia alba* and *Lippia origanoides* against *Trypanosoma evansi* in vitro and in vivo. *L. alba* and *L. origanoides* were used in vitro on trypomastigotes at different concentrations (0.5, 1.0 and 2.0 %) and exposure times (0, 1, 3, 6 and 9 h). The three concentrations tested showed trypanocidal activity in vitro, completely eliminating the parasites in small concentration after 6 h of assay. In vivo tests were performed using mice as the experimental model. *T. evansi* infected mice were treated with *L. alba* and *L. origanoides* with dose of 1.5 mL kg^{-1} during 5 days. These protocols did not provide curative efficacy, however the mice treated with *L. origanoides* showed a significant increase in the longevity when compared to control group. Active compounds present in essential oils, such as *L. origanoides*, may potentiate the treatment of trypanosomosis when associated with other trypanocidal drugs.

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Keywords "Surra" · Essentials oils · *Lippia* sp. · Trypanosome

Introduction

Trypanosoma evansi is a pathogenic hemoflagelate protozoa belonging to the Salivaria section having a global distribution and affects several animal species (Silva et al. 2002), one of the most common and widespread trypanosomal disease of domestic and wild animals. *Surra* is an important disease in a wide geographic region, and infects horses, donkey's mules, camels, Indian elephants, cattle, buffaloes, sheep, goats, dogs, cats, pigs tapirs, deer, tiger, capybara, foxes, jackals, hyenas, mongoose and bears (Muhammad et al. 2007; Berlin et al. 2009; Habila et al. 2012). The parasite is transmitted mechanically by biting flies such as *Tabanus* and *Stomoxys* (Brun et al. 1998; Herrera et al. 2004). Although this trypanosome can infect the most of the mammals, the camels and horses are the main hosts and represent the greatest economic loss. As a result, the disease is endemic in some regions (Mato Grosso, Pantanal in Brazil), Africa, Asia and South America, and in other regions occurs in outbreaks, hindering the prophylaxis and control (Hoare 1972; Peregrine and Mamman 1993).

Diseases caused by parasitic protozoa cause enormous health and economic impact, in particular to tropical regions of the world, many of which are resource constrained (Andrews et al. 2014). The treatment of trypanosomosis caused by *T. evansi* is based on diminazene aceturate (D.A.), but this drug show to be ineffective for several animals (Peregrine and Mamman 1993). Most of the drugs used for the treatment of the disease do not provide total control of the infection and are associated with recurrence rates and mortality (Da Silva et al. 2008).

The current chemotherapeutic agents are limited in number, usually associated with severe side effects, such as hepatotoxicity and nephrotoxicity (Spinosa et al. 1999), and far from ideal. The severe effects of trypanocidal drugs and relapse of infection after treatment are the major hurdles in control of trypanosomosis. As a result, researchers have tested natural products such as oils of copaiba, andiroba, aroeira, macela and tea tree (Baldissera et al. 2013, 2014; Do Carmo et al. 2015).

The genus *Lippia* has a great number of medicinal species, such as *L. origanoides, L. alnifolia, L. alba* and *L. graveolens*, which are frequently used in folk medicine for the treatment of microbial diseases (Pascual et al. 2001). *Lippia alba* (Verbenaceae) is a bush widely distributed in South America and is popularly known as *cidreira* or *false melissa.* Several studies have reported on the antibactericidal, antifungal and insecticidal activities (Olivero-Verbel et al. 2014; Oliveira et al. 2014; Vera et al. 2014). *Lippia origanoides* (Verbenaceae) is popularly known as *salva-de-Marajó.* The infusions are used as a general antiseptic for the mouth, throat, and wounds, for the treatment of diarrhea, indigestion, flatus, heartburn, nausea, vaginal discharges, menstrual complaints, and fever (Pascual et al. 2001; Oliveira et al. 2007). Study conducted by Molina-Garza et al. (2014) demonstrated the trypanocidal activity of *Lippia graveolens* against *Trypanosoma cruzi,* being able to inhibit by 33 % the growth of epimastigotes forms at a concentration of 150 µg/mL.

Based on the needs of new alternatives for the treatment of this emergent disease and development for curative therapy of *T. evansi*, and on the properties of *L. alba* and *L. origanoides,* and the trypanocidal activity in vitro of some species of *Lippia* mentioned above, in the present study we analyzed for the first time the in vitro and in vivo activity of a *L. alba* and *L. origanoides* against *T. evansi*.

Materials and methods

Plant material

Aerial parts of *Lippia alba* and *Lippia origanoides* were collected in Santarém (Pará, Brazil), coordinates (02°27′25.59″ S and 054°41′05.63″ W) and (02°30′49.9″ S and 054°56′06.8″ W), respectively. The plant was identified by Dr. Salimena Fatima at the Federal University of Juiz de Fora (Minas Gerais, Brazil), registered at the herbarium of the institution under number CESJ 65276 and CESJ 64029, respectively.

Extraction of essential oils

Lippia alba and *Lippia origanoides* essential oils were obtained from aerial parts by hydrodistillation in Clevenger type apparatus (Ming et al. 1996). The extraction time was set at 3 h (time required for best yield).

L. alba and *L. origanoides* characterization

The analysis of the chemical components of *L. alba* and *L. origanoides* essential oils were determined by gas chromatography-mass spectrometry (GC–MS) using a Shimadzu GC-17 AAF, V3, 230 LV apparatus. The samples were diluted in dichloromethane at a concentration of 10 mg/mL when 1 mL was injected in a system equipped with a HP5-MS (30 m × 0.25 mm × 0.25 µm) column; injector split ratio of 1:50. Helium was used as carrier gas at a constant flow of 0.6 mL min^{-1}. The injection port was set at 250 °C and the temperature cycle used was initially 50 °C, ramping at 3 °C/min for 3 min to a final temperature of 250 °C and kept for 15 min with the detector at 280 °C. MS operating parameters: transfer line temperature: 240 °C; electron impact ionization at 70 eV with mass scan range of 40–284 m/z at a sampling rate of 0.03 scan/s; ion source temperature: 200 °C. The components of the essentials oils were identified by comparing their mass spectra with the Wiley 275 and NIST spectral data catalogues with authentic mass spectra and their retention indices (RIs) relative to C8–C20 n-alkane series in a linear temperature- programmed run, following the methods described by Adams (1995). Data were acquired by GCMS Real Time Analysis (GCMS Solutions, Shimadzu Corp.) and processed using GC Image software, ver.2.1 (GC Image, LLC, Lincoln, NE).

Trypanosoma evansi isolate

This study was set up in two consecutive experiments (in vitro and in vivo). The same *T. evansi* isolate (Colpo et al. 2005) was used in both experiments. Two rats (R_1 and R_2) were infected intraperitoneally with trypomastigotes contaminated blood kept cryopreserved in liquid nitrogen. This procedure was performed to obtain a large amount of viable parasites for in vitro tests (R_1), and to infect the experimental groups (R_2).

In vitro tests

The culture medium for *T. evansi* was adapted to Baltz et al. (1985) as previously published by Baldissera et al. (2013). The trypomastigotes were acquired from the infection of one rat (R_1) with a *T. evansi* isolate. Four days post-infection, the rat showed high parasitemia (6.0×10^6 trypanosomes/µL) and it was anesthetized with isoflurane for blood collection by cardiac puncture, and blood was stored in EDTA tubes. For trypanosomes separation, each 200 µL of blood was diluted in complete culture medium

(200 μL), stored in microcentrifuge tubes and centrifuged during 10 min at 400×*g*. The supernatant was removed and resuspended in culture medium and the number of parasites was counted in a *Neubauer* chamber.

The culture medium with the parasites was distributed in microtiter plates (270 μL/well), followed by the addition of 25 μL of *L. alba* and *L. origanoides* (diluted in culture medium) at concentrations of 0.5, 1.0 and 2.0 %. A positive control (D.A. at a dilution of 0.5 %) was also used, at the same volume (25 μL). The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 9 h after the onset of the experiment in *Neubauer* chambers. The microtiter plates were placed in a 5 % CO_2 incubator at 37 °C according to Baltz et al. (1985).

In vivo test

Animal model

Thirty-five, female, conventional, outbred strain, 60-day-old-mice weighing an average of 25 ± 0.9 g were used as the experimental model. They were kept in cages with seven females each, housed on a light/dark cycle of 12 h, in an experimental room with controlled temperature and humidity (22 ± 1 °C; 70 % respectively). They were fed with commercial feed, and water ad libitum. All animals were submitted to a period of 15 days for adaptation.

Experimental design and parasitemia estimation

The mice were divided into five groups (A to E). Group A was consisted of uninfected and untreated mice (negative control); Group B was consisted of infected mice and untreated (positive control); Group C was composed of animals infected and treated with *L. alba* 1.5 mL kg^{-1}; Group D was composed of animals infected and treated with *L. origanoides* 1.5 mL kg^{-1}; Group E composed of animals infected and treated with diminazene aceturate. Infected animals were inoculated intraperitoneally with 0.02 mL of blood from one rat containing 2.5×10^6 trypanosomes. The chosen dose of 1.5 mL kg^{-1} is based on the findings by Baldissera et al. (2013, 2014) using *Achyrocline satureioides* essential oil.

The D.A was administered in a single dose of 3.5 mg kg^{-1}, intramuscularly injection, and 1 h after infection of the animals. *L. alba* and *L. origanoides* are administered orally for 5 days, once a day, starting the 1 h following infection.

The peripheral blood from tail of mice was examined daily for scoring degree of parasitemia. Each slide was prepared with fresh blood and stained by the panoptic method, and visualized at a magnification of 1000× according to Da Silva et al. (2006).

Treatment efficacy

Treatment efficacy was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity and animal mortality were also observed.

Statistical analysis

The data were tested for normality using the Kolmogorov–Smirnov test. Data from in vitro were analyzed by two-way analysis of variance (ANOVA) for repeated measures and comparison of concentrations tested for *L. alba* and *L. origanoides.* Data of the prepatent period and longevity were submitted to two-way analysis of variance followed by Duncan test ($P < 0.05$).

Results

L. alba and *L. origanoides* characterization

Characterization of *L. alba* and *L. origanoides* are shown in Tables 1 and 2, respectively. We identified a total of 70 compounds in *L. alba* essential oil, and 58 compounds in *L. origanoides* essential oil, representing 95.47 and 91.83 % of the total composition, respectively, however show only 5 major constituents. The results indicate that E-citral (29.84 %) was the most abundant compound present in *L. alba* essential oil, and carvacrol (40.73 %) was the most abundant compound present in *L. origanoides* essential oil.

In vitro test

The results showed a trypanocidal effect of *L. alba* and *L. origanoides* on *T. evansi* directly proportional to the concentration used (Fig. 1). Using *L. alba* essential oil, after 1 h, there were no living trypomastigotes in 2 % concentration. A reduction of live trypomastigotes was observed at the concentrations of 0.5 and 1 % when compared with the control group. After 3 h of the assay, there were no

Table 1 Qualitative and quantitative analyses of *L. alba* essential oil

RT (min)	Constituent	Relative percentage (%)	RI cal	RI ref
23.131	E-citral	29.84	1270	1267
22.037	Z-citral	24.42	1241	1238
13.597	Limolene	6.15	1026	1029
30.815	Bicyclogermacrene	3.72	1495	1500
21.599	Z-geraniol	3.56	1229	1230

RT Retention time, *RI calc* calculated Kovats retention index, *RI ref* reference Kovats retention index, *1* Adams (2001), *2* NIST (2002)

Table 2 Qualitative and quantitative analyses of *L. origanoides* essential oil

RT (min)	Constituent	Relative percentage (%)	RI cal	RI ref
24.382	Carvacrol	40.73	1304	1299
13.464	ρ-cimene	13.63	1023	1025
23.973	Thymol	8.19	1293	1290
14.882	γ-terpinene	5.43	1058	1060
16.623	β-linalool	4.19	1100	1099

RT Retention time, *RI calc* calculated Kovats retention index, *RI ref* reference Kovats retention index, *1* Adams (2001), *2* NIST (2002)

living trypomastigotes in 1 % concentration. A reduction of live trypomastigotes was observed at the concentration of 0.5 % when compared with the control group. After 6 h of the assay, there were no living trypomastigotes in 0.5 % concentration and D.A. treatments (Fig. 1a).

The *L. origanoides* results are shown in Fig. 1b. After 1 h of the assay, there were no living trypomastigotes in 1 and 2 % concentrations, and a reduction of live trypomastigotes at the concentration of 0.5 %. After 3 h of the assay, there were no living trypomastigotes forms in 0.5 % concentration. After 6 h of the assay, there were no living trypomastigotes in D.A. treatment. On the contrary, in control tests (not using drugs), the parasites were all alive (Fig. 1), which validates our experiment.

In vivo test

There were no differences between groups regarding the prepatent period (Table 3). Longevity of the group A was

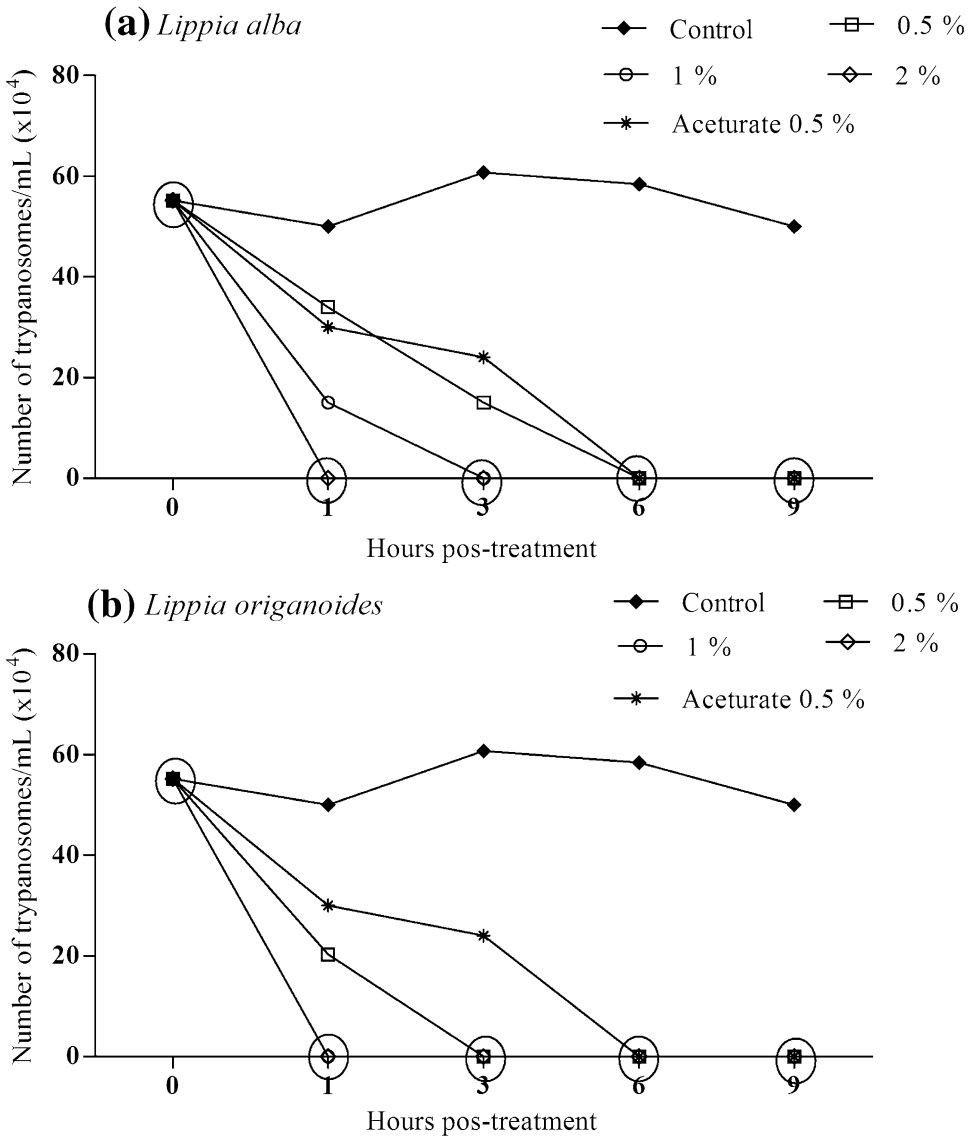

Fig. 1 In vitro, trypanocidal activity in concentrations 0.5, 1.0 and 2.0 % of *L. alba* (**a**) and *L. origanoides* (**b**) against *Trypanosoma evansi* when compared control (untreated) and diminazene aceturate (anti-protozoa drug). The results within a *circle* are not statistically different ($P > 0.05$), at the same time (h)

Table 3 In vivo test—Mean and standard deviation of the prepatent period, longevity and mortality using treatment with *L. alba*, *L. origanoides* and diminazene aceturate (D.A.) in mice experimentally infected by *T. evansi*

Groups (n = 7)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)
A	Negative control	–	15.0^{a} (±0.0)	0/7
B	Positive control	1.0^{a} (±0.0)	3.0^{c} (±0.0)	7/7
C	*Lippia alba* (1.5 mL Kg^{-1})	1.0^{a} (±0.0)	3.0^{c} (±0.0)	7/7
D	*Lippia origanoides* (1.5 mL Kg^{-1})	1.4^{a} (±0.55)	6.5^{b} (±1.01)	7/7
E	D.A. (3.5 mg Kg^{-1})	0.0^{a} (±0.0)	15.0^{a} (±0.0)	0/7

Means followed by same letter in the same column do not differ significantly in the Duncan test. The experiment lasted 15 days post-infection

exactly represented by the days that the experiment lasted (15 days). Longevity in the groups B, C, D and E were 3.0, 3.0, 6.5, and 15 days, respectively. The groups C and D had no curative efficacy, but the group D increased longevity compared to the group B. The animals treated with D.A. showed negative blood smear during the 15 days of the period of our study.

Discussion

This study observed a trypanocidal action in vitro and in vivo of *L. alba* and *L origanoides* essential oils. The results showed a dose-dependent trypanocidal effect of *L. alba* and *L. origanoides* essential oils against *T. evansi* trypomastigotes in vitro. The *L. origanoides* had apparently a faster trypanocidal effect in vitro than the *L. alba*, and even the own diminazene aceturate. Essential oils as well as their compounds have been found to possess a wide spectrum of pharmacological effects including antibacterial, antifungal, antiviral, anthelminthic and antiprotozoal activities (Baldissera et al. 2013, 2014; Macedo et al. 2010; Santos et al. 2010).

The antiprotozoal activity in vitro of diverse essential oils affecting protozoa such as *Leishmania, Trypanosoma brucei* and *Plasmodium* (Mikus et al. 2000; Rosa et al. 2003; Ueda-Nakamura et al. 2006) is well documented. The responsible for biological activity against *T. brucei*, *Leishmania* sp., and *T. cruzi,* being assigned to terpenic, aromatic and aliphatic compounds the biological activities proven.

Genus *Lippia* has been cited by researchers because of the active substances with many therapeutic properties and have a range of biological and pharmacological properties such as antimicrobial, antifungal and anesthesic (Pascual et al. 2001; Toni et al. 2014). In vitro, studies have shown the effect of *L. sidoides, L. origanoides* and *L. graveolens* against different forms of *T. cruzi*. *L. sidoides* showed an IC_{50} of 28.9, 10.3 and 41.7 µg/mL against epimastigotes, trypomastigotes and amastigotes forms of *T. cruzi,* while *L. origanoides* presented an IC_{50} of 26.2, 39.7 and 29.8 µg/mL, respectively (Borges et al. 2012).

Based on the results in vitro, as experiment was designed in vivo, using mice experimentally infected with *T. evansi* as experimental model. However, the therapeutic protocol used with the *L. alba* and *L. origanoides* had no effective curative for all the groups, but in a group's treated with *L. origanoides* (Group D), an increase in longevity of animals was observed. Study conducted by Monzote et al. (2014) demonstrated that carvacrol present in *Chenopodium ambrosioides* essential oil has an IC_{50} of 15.3 and 13.6 µg/mL against promastigote and amastigotes forms of *Leishmania* spp. According Escobar et al. (2010) carvacrol, the major compound found *L. origanoides* showed highest in vitro active of epimastigotes and amastigotes forms of *T. cruzi*, presenting IC_{50} of 3.0 and 27.3 µg/mL, respectively. The mechanism of action of carvacrol is related to mitochondrial membrane. Study suggests that carvacrol damages the mitochondrial membrane potential, leading to changes in the redox ratio, leading to death of the parasite (Monzote et al. 2014). Several natural products, such as alkaloids, terpenes, quinones, flavonoids and saponins with trypanocidal activity have been isolated, such as carvacrol (Wright and Phillipson 1990; Kayser et al. 2003). Considering this findings, we can conclude that the results obtained indicate that the most promising essential oils may result in potential sources of lead compounds for the development of more effective drugs for the treatment of "*Surra*". Based on the result it was concluded that *L. alba* and *L. origanoides* has trypanocidal action against *T. evansi* in culture medium. Nevertheless, the treatment had no curative efficacy in infected mice with *T. evansi,* however *L. origanoides* has increased longevity of animals.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical approval The procedure was approved by the Animal Welfare Committee of Universidade Federal de Santa Maria under number 117/2014.

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