

Original Research Article

Efficacy of some essential oils in mice infected with *Trypanosoma cruzi*

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Abstract

Purpose: To evaluate the efficacy of orally administered *Cymbopogon citratus*, *Zingiber officinale* and *Syzygium aromaticum* essential oils (EOs) in mice infected with *Trypanosoma cruzi*.

Methods: Three experiments were conducted with 48 Swiss mice each. The animals were inoculated with 2×10^6 metacyclic trypomastigotes of *T. cruzi* Y-strain and allocated into the following groups (N = 12): 1) untreated control; 2) treated with benznidazole (BZ); 3) treated with EO 100 mg/kg; and 4) treated with EO 250 mg/kg. The groups were evaluated by fresh blood test, blood culture, conventional polymerase chain reaction (PCR), real-time PCR and cure rate (CR).

Results: All the animals were completely infected with *T. cruzi*. Treatment with *C. citratus* and *Z. officinale* EOs altered some of the parameters derived from the parasitemia curve, but CRs did not differ from BZ. Treatment with *S. aromaticum* EO, on the other hand, not only altered all the parameters derived from the parasitemia curve, similar to BZ, but at the dose of 100 mg/kg, CR was also significantly higher than BZ.

Conclusion: The results indicate that the essential oils tested, especially *S. aromaticum*, exhibited anti-*Trypanosoma cruzi* effect and therefore should be investigated for the treatment of Chagas disease.

Keywords: Chagas disease, *Trypanosoma cruzi*, *Cymbopogon citratus*, *Zingiber officinale*, *Syzygium aromaticum*, Essential oils, Chemotherapy

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INTRODUCTION

Trypanosoma cruzi is the protozoan parasite responsible for the American Trypanosomiasis (also known as Chagas disease), which afflicts about 6 - 7 million people worldwide. Once exclusive to the Americas, where it reached endemic status in 21 Latin American countries, Chagas disease is currently considered a globalized disease [1]. Triatomines are typically the main vectors for the transmission of Chagas disease in endemic countries. In contrast, a new

epidemiological scenario seems to be developing in Brazil as most of the new cases reported (71 %) have been acquired by oral route, especially in non-endemic regions [1,2].

Although it is the parasitic disease with the greatest impact in the Americas, it is often left untreated due to the shortcomings of currently available therapies. Based on only two drugs, benznidazole and nifurtimox, treatment is long and the drugs are highly toxic and poorly

tolerated by patients, and its efficacy is dependent on the phase of infection [1,3,4].

Thus, new drugs for the treatment of Chagas disease with reduced or no side effects are urgently required. Several natural products of plant origin from different structural classes have been shown to be effective against *T. cruzi*. Different essential oils (EOs) have already been tested *in vitro* against *T. Cruzi*, demonstrating biological activity. Among tested EOs, the lemongrass (*Cymbopogon citratus*) EO has been shown to be effective against the amastigote and trypomastigote forms of *T. cruzi*, while the clove (*Syzygium aromaticum*) EO inhibited the growth of different epimastigote forms and blood trypomastigotes [5-7].

These promising results warrant new studies conducted *in vivo* to better ascertain the efficacy of EOs in the treatment of Chagas disease. Therefore, the objective of this animal study was to evaluate the efficacy of *C. citratus*, *Z. officinale* and *S. aromaticum* EOs in mice experimentally infected *T. cruzi*.

EXPERIMENTAL

Ethics statement

This study was approved by the Ethics Committee on the Use of Animals in Experimentation of the State University of Maringá, Brazil (CEUA/UEM, resolution no. 023/2014) and conducted in accordance with the international guidelines for the protection of animals used for scientific purposes [8].

Parasites, animals and infection

The *T. cruzi* Y-strain used in the present study was obtained from the strain bank at the Chagas disease Laboratory at the State University of Maringá. Three experiments, each with 48 female Swiss mice aged 21 to 28 days and weighing between 18 and 20 g, were conducted. The animals were kept in micro-acclimated cages (Alesco®, 20 x 32 x 21 cm) in a 12 h light-dark cycle and rodent food and water *ad libitum*. Each animal was inoculated by gavage with 2×10^6 metacyclic trypomastigotes obtained from the acellular culture suspended in 1 ml of LIT medium according to the protocol described by Dias *et al* [9] and modified by Teston *et al* [10].

Experimental groups, treatment schedules and essential oils

Animals were separated into four groups with 12 animals each, in such a way that mean weight

was similar for all four groups. The following groups were constituted for each experiment: 1) untreated controls (UC); 2) animals treated with benznidazole 100 mg/kg (TBZ); 3) animals treated with one of the experimental EOs 100 mg/kg (TEO100); and 4) animals treated with one of the experimental EOs 250 mg/kg (TEO250). Treatments were initiated on the 5th day after inoculation (day) and both BZ and EOs were administered by gavage in a single daily dose for 20 consecutive days. Lemongrass (*Cymbopogon citratus*; batch 0664), ginger (*Zingiber officinale*; batch 09419), and clove (*Syzygium aromaticum*; batch 09464) EOs were obtained by hydrodistillation of plant material (Quinari Fragrâncias e Cosméticos LTDA, Brazil) and analyzed by gas chromatography coupled to mass spectrometry (GC-MS; Shimadzu QP 2000) under the following conditions: DB-5 column measuring 30 m x 0.25 mm x 0.25 µm; helium 1 ml/min; injector temperature of 240 °C and detector temperature of 230°C under the following operating conditions: column temperature between 50 – 160 °C, at 3 °C/min; volume injected of 1 µl (2 mg of the essential oil in 1 ml of ethyl acetate). The compounds present in the EOs were identified by comparing results to the GC-MS (National Institute of Standards and Technology - Nist.62 Library) and Kovats indices.

Fresh blood test (FBT)

Parasitemia was evaluated daily. Blood was collected from the caudal vein of each animal from the 3rd day according to Brener [11], until zero parasitemia was obtained for three consecutive days. FBT was performed to confirm infection, plot the parasitemia curve, and determine the percentage of animals that were FBT positive (%FBT+). At the end of treatment, parasitemia was also evaluated on alternate days for one week. The following parameters derived from the parasitemia curve were determined: mean patent period (PP), mean of the periods when parasitemia was detected in each mouse; maximum parasitemia peak (Pmax), calculated from the parasitemia peak detected in each mouse in the group; maximum peak day (Dpmax), mean of days each mouse had Pmax detected; total parasitemia (TP), mean of the sum of daily parasitemia of each animal throughout the experiment; and the area under the parasitemia curve (AUC).

Blood culture (BC)

BC was performed in LIT medium 30 days after the end of treatment according to Filardi and Brener [12]. Blood samples were collected from

the retro-orbital venous plexus of treated (TEO100, TEO250 and TBZ) animals and untreated controls (UC) to obtain the percentage of animals that were BC positive (%BC+).

Conventional polymerase chain reaction (cPCR)

cPCR was also performed at 30 days after treatment according to Miyamoto *et al* [13], by adding 200 μ l of blood to an eppendorf tube containing 400 μ l of guanidine 6.0 M/EDTA 0.2 M solution. The primers 121 (5'AGATAATGCATGA-3') and 122 (5'-GGTTCGATTGGGGTTGGT GTAATATA-3') were used to amplify the 330 bp fragment of *T. cruzi* kinetoplast DNA (kDNA) minicircles. Samples that generated these products were considered positive and were observed by electrophoresis in 4.5 % polyacrylamide gel and silver staining. Based on these results, the percentage of mice that were cPCR positive (% cPCR+) was determined.

Qualitative real-time polymerase chain reaction (qPCR)

At 115 dai, the cardiac tissue of three animals from each experimental group was collected *in totum*. Samples (30 mg/animal) were extracted using the chloroform-isopropanol method [14]. The obtained DNA was analyzed using SYBR green qPCR SuperMix UDG/ROX Platinum reagent (Invitrogen, USA) run on the thermocycler Rotor Gene Q (Qiagen, Germany) [15]. PCR was performed using 100 ng of whole genomic DNA (gDNA) and the primers TCZ-F (5=-GCTCTTGCCACAMGGGTGC-3=) and TCZ-R (5=-C CAAGCAGCGGATAGTTTCAGG-3=) [16]. Tissue DNA from healthy and Y-strain infected mice were used as negative and positive controls of extraction, respectively. The mix without the genomic DNA was used as negative control of amplification.

Determination of infectivity and mortality

Infectivity rate (%INF) was calculated based on the number of animals positive for infection in at least one of the tests performed (FBT, BC or cPCR). Animal survival was registered daily, and cumulative mortality rate (%MOR) was calculated for each group.

Evaluation of treatment effectiveness

To evaluate the effectiveness of the different treatments, cure rates (CRs) were obtained from animals treated with the reference drug (BZ) and each of the three tested EOs. Animals presenting negative results in all the tests were considered

cured. CRs were obtained by dividing the number of cured animals by the total number of animals treated, expressed as a percentage.

Statistical analysis

The data were distributed in frequency tables and described in terms of percentages or means. Statistical analyzes were performed using the software, Bioestat 5.3 (Belém, Pará, Brazil), with the significance level set at $p < 0.05$. Proportions were compared using G or Chi-square test. Means were compared using Mann-Whitney or Kruskal-Wallis test while AUC was calculated using GraphPad Prism 5.0 program.

RESULTS

Infectivity rate (INF) was 100 % in all experimental groups, i.e., all animals inoculated with *T. cruzi* Y-strain became infected. Cumulative mortality rate (MOR) was 0 %, i.e., no animal from any of the groups died during the experiments.

Test 1: *Cymbopogon citratus* EO

The chromatographic analysis showed that the lemongrass EO presented in its composition 46.8 % of geranial (α -citral) and 33.5 % of neral (β -citral) as its main constituents. The highest parasitemia levels were observed in UC, followed by TLEO100, TLEO250, and TBZ. Treatment with the reference drug BZ was able to promote zero parasitemia in most animals immediately after the first day of treatment, and in all animals after the third day of treatment. Groups treated with TLEO100 and TLEO250 were positive for parasitemia for almost the entire treatment period. All animals presented negative FBT after the end of treatments (Figure 1).

All parameters derived from the parasitemia curve, except for Dpmax, presented statistically significant differences among groups ($p < 0.001$). TBZ presented the lowest values for all parameters ($p \leq 0.01$). When compared to UC, animals treated with TLEO100 and TLEO250 presented a significant reduction in PP ($p < 0.01$), Pmax ($p < 0.01$), and AUC ($p = 0.0178$). A significant reduction in TP ($p \leq 0.0102$) was also observed but only in those animals treated with TLEO250 (Table 1). The %BC+ varied among groups ($p < 0.03$). TBZ presented the highest %BC+, reaching statistical significance when compared with TLEO100 and TLEO250 ($p = 0.03$).

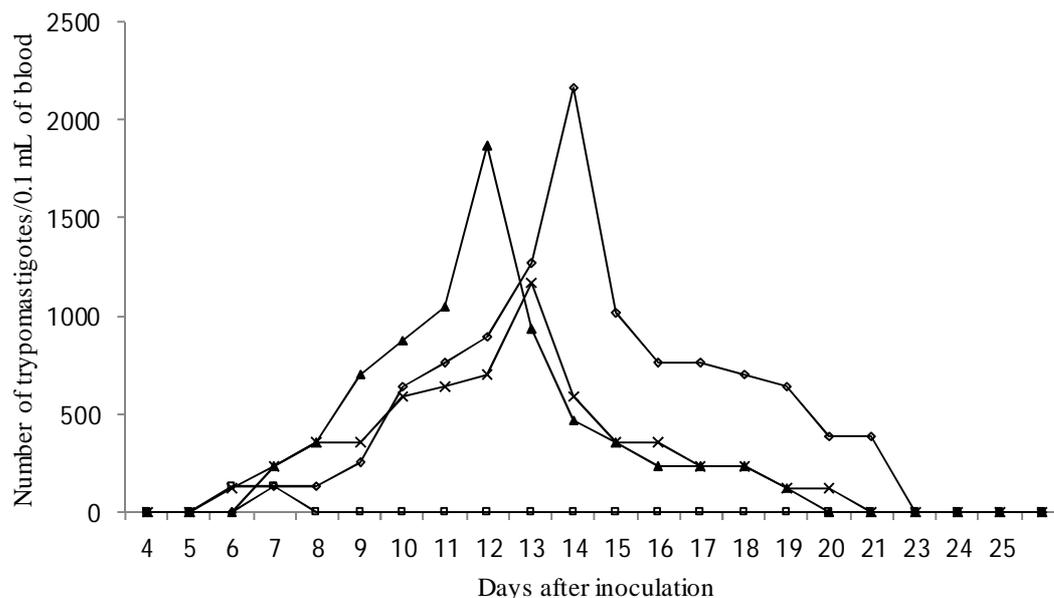


Figure 1: Experiment 1 (treatment started on the 5th day after inoculation). Mean parasitemia curve of mice inoculated with *Trypanosoma cruzi* (Y strain - TcII) treated with benznidazole 100 mg/kg/day (TBZ; □), *Cymbopogon citratus* essential oil at the doses of 100 mg/kg/day (TLEO100; ▲) and 250 mg/kg/day (TLEO250; ×), during 20 consecutive days, and untreated controls (UC; ◇)

Table 1: Statistical comparisons of parasitological and molecular parameters, and cure rates in mice inoculated with *Trypanosoma cruzi* (Y-strain - TcII), treated with benznidazole (TBZ), *Cymbopogon citratus* essential oil at the doses of 100 mg/kg/day (TLEO100) and 250 mg/kg/day (TLEO250), during 20 consecutive days, and untreated controls (UC)

Parameter	UC	TBZ	TLEO100	TLEO250	P-value*
PP	9.8 ± 1.3	0.2 ± 0.4	6.8 ± 2.9	6.1 ± 2.6	<0.0001
Pmax	3181.8 ± 1545.2	254.6 ± 540.0	2450.0 ± 2541.5	1750.0 ± 633.2	<0.0001
Dpmax	14.3 ± 2.3	6.5 ± 0.5	11.6 ± 3.5	13.0 ± 0.6	NS
TP	9672.7 ± 4577.6	233.3 ± 544.9	6883.3 ± 5010.8	5133.3 ± 1823.7	<0.0001
AUC	11136.3 ± 5533.6	233.3 ± 544.9	7991.7 ± 5393.0	5950.0 ± 2203.5	<0.0001
%BC+	50.0 (4/8)	75.0 (6/8)	37.5 (3/8)	22.2 (2/9)	0.03
%cPCR+	100.0 (5/5)	0.0 (0/1)	20.0 (1/5)	85.7 (6/7)	0.02
%qPCR+	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	33.3 (1/3)	NS
Cure rate	-	12.5 (1/8)	50.0 (4/8)	11.1 (1/9)	NS

PP: patent period (in days); Pmax: parasitemia peak (number of trypomastigotes/0.1 ml of blood); Dpmax maximum peak day; TP: total parasitemia; AUC: area under the curve; %BC+: percentage of animals with positive blood culture; %cPCR+: percentage of animals cPCR positive; %qPCR+: percentage of animals with qPCR positive; NS = not significant. * $P \leq 0.05$ was considered significant. G or Chi-square tests were used for the analysis of proportions, while Mann-Whitney or Kruskal-Wallis tests were used for means analysis

cPCR confirmed infection in 4 animals in the UC group, which had previously been FBT+ but were BC-. The %cPCR+ also varied among groups ($p = 0.03$), being smallest in the TBZ group. In TLEO250 group, cPCR+ was observed in 6/7 animals (85.7%). Tissue analysis performed on cardiac samples from three animals of each group, showed qPCR+ only in one mouse in the TLEO250 group, which was also cPCR+. CRs obtained from animals treated with lemongrass EO did not significantly differ from animals treated with BZ. These results demonstrated that

mice orally inoculated with *T. cruzi* Y-strain responded poorly to treatment with both the lemongrass EO (in both dosages) and the reference drug (BZ) (Table 1).

Test 2: *Zingiber officinale* EO

Chromatographic analysis showed that the main constituents of the ginger EO were α -pinene (17.9 %), β -pinene (14.9 %) and zingiberene (14.7 %). FBT was negative for all the animals in the TBZ group. As a result, the animals in the

TBZ group presented null values in all five parameters derived from the parasitemia curve. Significant differences were observed among groups ($p < 0.0001$) in 4/5 of the parasitemia parameters. When compared to UC, animals treated with TGE0100 and TGE0250 demonstrated a significant reduction in PT ($p = 0.02$) and AUC ($p = 0.04$) (Figure 2). BC and cPCR confirmed infectivity in 100 % of the animals tested, including those in the TBZ group. However, no significant differences in %BC+ and

%cPCR+ were observed among the groups. CR for BZ-treated animals was zero, showing once again that the reference drug was not effective in the animals orally inoculated with the *T. cruzi* Y-strain. CRs obtained from animals in the TGE0100 and TGE0250 groups were not significantly different from BZ. Although %qPCR+ was numerically highest in the TGE0250 group and lowest in the TBZ group, no statistical differences were found among groups (Table 2).

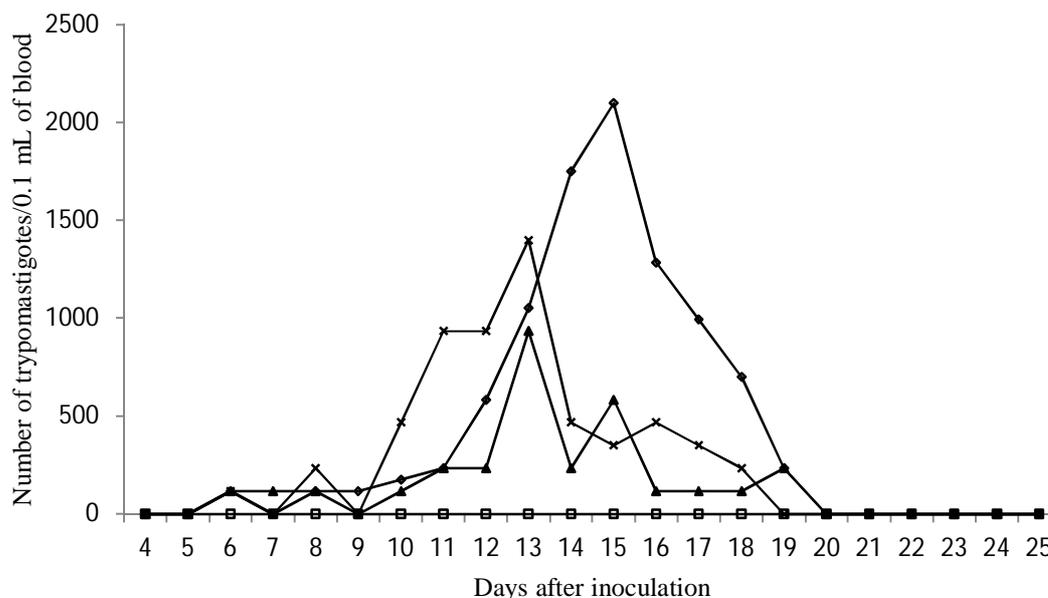


Figure 2: Experiment 2 (treatment started on the 5th day after inoculation). Mean parasitemia curve of mice inoculated with *Trypanosoma cruzi* (Y strain - TcII) treated with benznidazole 100 mg/kg/day (TBZ; □), *Zingiber officinale* essential oil at the doses of 100 mg/kg/day (TGE0100; ▲) and 250 mg/kg/day (TGE0250; ×), during 20 consecutive days, and untreated controls (UC; ◇)

Table 2: Statistical comparisons of parasitological and molecular parameters, and cure rates in mice inoculated with *Trypanosoma cruzi* (Y-strain - TcII), treated with benznidazole 100 mg/kg/day (TBZ), *Zingiber officinale* essential oil at the doses of 100 mg/kg/day (TGE0100) and 250 mg/kg/day (TGE0250), during 20 consecutive days, and untreated controls (UC).

Parameter	UC	TBZ	TGE0100	TGE0250	P-value*
PP	6.4 ± 2.9	0.0	4.3 ± 3.1	4.6 ± 3.4	<0.0001
Pmax	2800 ± 2067.9	0.0	1400.0 ± 596.9	1983.3 ± 1260.5	<0.0001
Dpmax	13.8 ± 2.1	0.0	13.5 ± 1.9	13.4 ± 1.3	NS
TP	8866.7 ± 7146.9	0.0	3266.7 ± 2096.5	5133.3 ± 4235.2	<0.0001
AUC	10208.3 ± 8000.5	0.0	3558.3 ± 2320.8	5425.0 ± 4776.9	<0.0001
%BC+	55.6 (5/9)	75.0 (6/8)	55.6 (5/9)	66.7 (6/9)	NS
%cPCR+	100.0 (4/4)	100.0 (2/2)	50.0 (2/4)	100.0 (2/2)	NS
%qPCR+	33.3 (1/3)	0.0 (0/3)	33.3 (1/3)	66.6 (2/3)	NS
Cure rate	-	0.0 (0/8)	22.2 (2/9)	11.1 (1/9)	NS

PP: patent period (in days); Pmax: parasitemia peak (number of trypanomastigotes/0.1 ml of blood); Dpmax maximum peak day; TP: total parasitemia; AUC: area under the curve; %BC+: percentage of animals with positive blood culture; %cPCR+: percentage of animals cPCR positive; %qPCR+: percentage of animals with qPCR positive; NS = not significant. *Values of $p \leq 0.05$ were considered significant. The G or chi-square tests were used for the analysis of proportions, and the Mann-Whitney or Kruskal-Wallis tests for means analysis

Test 3: *Syzygium aromaticum* EO

The major constituents identified in the clove EO were eugenol and β -caryophyllene at

concentrations of 82.9 % and 13.0 %, respectively. UC group demonstrated the highest parasitemia level ($p < 0.0100$) when compared to the other groups (Figure 3). Only one animal in

the TBZ group presented PP and, as a result, all the parameters derived from the parasitemia curve were significantly lower in this group when compared with the other groups ($p < 0.0001$). When compared to UC, animals in the TCEO100 and TCEO250 groups presented reduced PP, Pmax, Dpmax, TP and AUC values. The %BC+ varied among groups, being highest for the TBZ group and lowest for the TCEO250 group ($p =$

0.01). However, %cPCR+ and %qPCR+ presented no significant differences. None of the animals treated with BZ was cured. CRs demonstrated significant differences among groups ($p = 0.03$). CR induced by the clove EO (TCEO100) was significantly higher than that found for mice in TCEO250 and TBZ groups (Table 3).

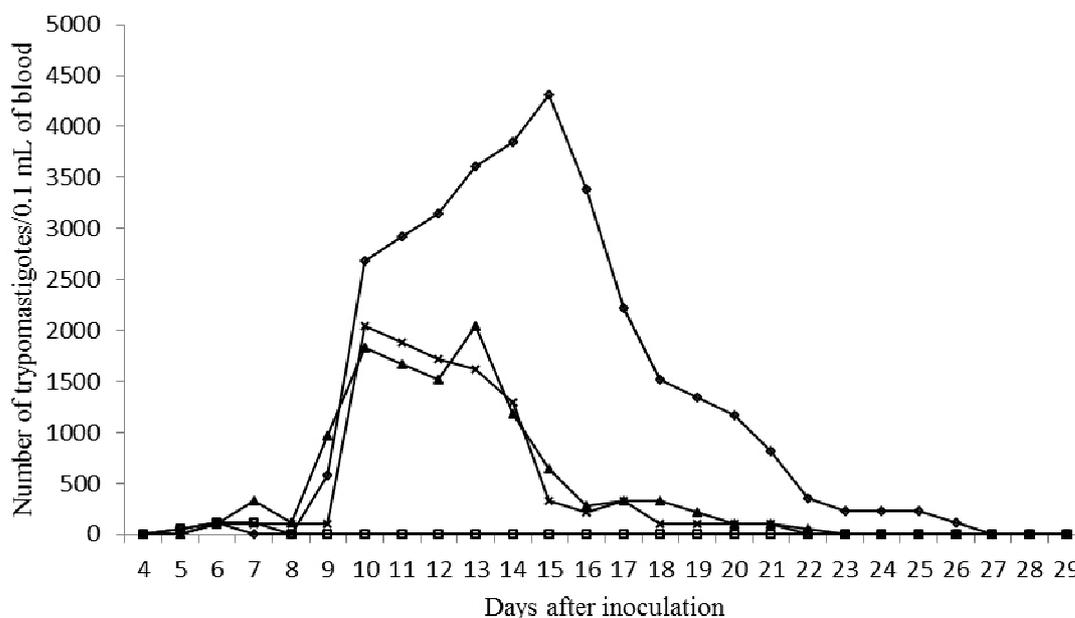


Figure 3: Experiment 3 (treatment started on the 5th day after inoculation). Mean parasitemia curve of mice inoculated with *Trypanosoma cruzi* (Y strain - TcII) treated with benznidazole 100 mg/kg/day (TBZ; □), *Syzygium aromaticum* essential oil at the doses of 100 mg/kg/day (TCEO100; ▲) and 250 mg/kg/day (TCEO250; ×), during 20 consecutive days, and untreated controls (UC; ◇)

Table 3: Statistical comparisons of parasitological and molecular parameters, and cure rates in mice inoculated with *Trypanosoma cruzi* (Y-strain - TcII), treated with benznidazole 100 mg/kg/day (TBZ), *Syzygium aromaticum* essential oil at the doses of 100 mg/kg/day (TCEO100) and 250 mg/kg/day (TCEO250), during 20 consecutive days, and untreated controls (UC)

Parameter	UC	TBZ	TCEO100	TCEO250	P-value*
PP (days)	12.6 ± 2.9	0.1 ± 0.3	6.1 ± 4.3	5.2 ± 3.2	<0.0001
Pmax	5367.0 ± 1872	116.7 ± 386.9	3877.0 ± 2073.0	3446.0 ± 2466.0	<0.0001
Dpmax	12.7 ± 1.4	5.0 ± 0.0	11.2 ± 1.5	11.3 ± 2.0	<0.0001
TP	28233.0 ± 9455	0.0 ± 0.0	8723.0 ± 5516.0	8185.0 ± 6152.0	<0.0001
AUC	32675 ± 11194.7	58.3 ± 202.1	10507.6 ± 6222.7	10146.1 ± 7543.9	<0.0001
%BC+	55.6 (5/9)	77.8 (7/9)	22.2 (2/9)	11.1 (1/9)	0.01
%cPCR+	77.8 (7/9)	77.8 (7/9)	55.6 (5/9)	66.7 (6/9)	NS
%qPCR+	33.3 (1/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	NS
Cure rate	-	0.0 (0/9)	44.4 (4/9)	22.2 (2/9)	0.03

PP: patent period (in days); Pmax: parasitemia peak (number of trypomastigotes/0.1 ml of blood); Dpmax maximum peak day; TP: total parasitemia; AUC: area under the curve; %BC+: percentage of animals with positive blood culture; %cPCR+: percentage of animals cPCR positive; %qPCR+: percentage of animals with qPCR positive; NS = not significant. *Values of $p \leq 0.05$ were considered significant. The G or chi-square tests were used for the analysis of proportions, and the Mann-Whitney or Kruskal-Wallis tests for means analysis

DISCUSSION

Chagas disease is one of 17 neglected tropical infirmities listed by the World Health Organization, with less than 1 % of infected people estimated to receive adequate treatment.

Currently available drugs, benznidazole and nifurtimox, can cure infected patients but are effective only at the beginning of the infection depending on the genetic variation of *T. cruzi*. Moreover, treatment is long with several adverse effects, which results in high rates of

nonadherence [17]. Products derived from medicinal plants may lead the way into the discovery of new drugs, or combination of drugs, for the treatment of Chagas Disease. Indeed, several studies support the therapeutic value of different natural products of plant origin as trypanocidal agents [6,7,18].

In the present study, the effectiveness of three different essential oils (EOs) was evaluated in the treatment of mice orally infected with *T. cruzi*. Three separate tests were conducted following the same design, comparing the results obtained with each EO at the doses of 100 and 250 mg with the results of untreated controls (UC) and animals treated with the reference drug (BZ). Seven parasitological parameters, five of them related to the parasitemia curve (PP, Pmax, Dpmax, TP, and AUC), and two assessing the presence of the parasite in the blood (FBT, and BC); two molecular analysis, one performed in blood (cPCR) and one in the cardiac tissue (qPCR); and the cure rates (CRs) of different treatments were analysed. All inoculated animals became infected and no deaths were recorded during the follow-up period.

The *T. cruzi* Y-strain of TcII DTU [19] is considered highly virulent for albino mice [20]. Apart from being partially resistant to benznidazole and nifurtimox, it presents high infectivity, parasitemia and lethality in mice. As a result, the Y-strain has been widely used in different types of studies, including those screening for new drugs for the treatment of Chagas disease. In the present study, low levels of parasitemia and zero mortality were observed with the oral administration of this strain. This biological behavior was quite different from that previously observed in mice inoculated both intraperitoneally and orally. In a study performed by our group using the same inoculum and inoculation route, mortality rate of 90 %, and Pmax, PT and AUC values approximately 40-fold higher than in the current study were observed [9]. Differences may be due to the prolonged maintenance of the strain in LIT acellular culture medium without blood passages in mice, which may have led to a reduction of the Y-strain virulence in the present study. This is consistent with the low %qPCR+ found in the cardiac tissue of the animals tested. *T. cruzi* Y-strain is biologically heterogeneous and some subpopulations (clones) can be selected after years of laboratory storage [21]. Nonetheless, other biological characteristics of the orally administered Y-strain were maintained, such as PP duration (around 10 days), Dpmax (around day 14), and high infectivity (100 % of inoculated animals became infected) [22].

The three EOs evaluated in this study altered the parasitemia curve profile, demonstrating reduced values of different parameters when compared to the UC group. The use of BZ and TCEO in the two tested doses reduced all the five parameters, indicating a superior suppressive effect on parasitemia than TLOE and TGOE. However, animals treated with BZ presented significantly higher %BC+ (75 %) than animals in the TLEO and TCEO groups. A possible explanation for this result would be that blood trypomastigote forms of the Y-strain are more sensitive to BZ than tissue amastigotes. Moreover, as BC was performed 30 days after the end of the experiment, %BC+ might have started to rise again after the pressure exerted by the drug on parasitemia was ceased. Alternatively, EO penetrating power into tissues might have been greater than BZ, reaching tissue amastigote forms more effectively.

The highest CRs after treatment were 50 % (4/8) obtained with TLEO100 and 44.4 % (4/9) with TCEO100. Lemongrass and clove EOs also promoted the reduction of most parameters evaluated (6/8). Although BZ presented higher suppressive effect on parasitemia, CRs found suggest greater effectiveness of these two EOs, mainly in the elimination of tissue amastigotes. These results place the lemongrass and clove EOs, and their major components, among the potential candidates for the treatment of patients orally infected with *T. cruzi*.

The biological activity of natural products is directly related to their constituents. Lemongrass (*C. citratus*), ginger (*Z. officinale*) and clove (*S. aromaticum*) EOs main constituents are terpenes and terpenoids, which have already been shown to have *in vitro* activity against the protozoan responsible for Chagas disease [7,23]. Promising therapeutic effects of the *C. citratus* EO have already been demonstrated *in vitro* on the genus *Trypanosoma* [24] and *T. cruzi* [5]. Moreover, extracts prepared from ginger have also shown trypanocidal activity against *T. evansi* [25], while ginger and lemongrass oils were effective against four *Leishmania* species [26]. The effect obtained with the use of lemongrass EO on parasitemia in the present study, although inferior to BZ, can be attributed to its major constituents, geranial (α -citral) and neral (β -citral), which have already demonstrated *in vitro* activity against *T. cruzi* epimastigotes and trypomastigotes [27]. In an *in vivo* study, the *Aloysia triphylla* EO also demonstrated activity against *T. cruzi*, and the authors attributed this effect to these same substances found in the lemongrass EO [28].

Among the EOs tested in this study, the clove EO presented the most promising results. The CR found with the use of clove EO (44.4%), while still far from ideal, was higher than that obtained with BZ (0.0%). This result can be attributed to its compounds, eugenol and caryophyllene, which represent almost the totality of the constituents identified. They have already demonstrated antiparasitic effect *in vitro* against promastigotes forms of *Leishmania* and epimastigotes forms of *T. cruzi*, with caryophyllene, in particular, presenting better clinical activity against the two parasites [29].

In this study, the efficacy of the different treatments on the animals orally inoculated with *T. cruzi* was monitored with parasitological (FBT and BC) and molecular techniques (cPCR in blood, and qPCR in cardiac tissue). Moreover, the animals were not subjected to immunosuppression with cyclophosphamide, as currently recommended for cure control of experimental chemotherapy of *T. cruzi* infection. Regarding the molecular analyzes, our results showed that cPCR in blood presented greater detection capacity of *T. cruzi* DNA than qPCR performed in the cardiac tissue. The %qPCR+ results indicate that the capability of Y-strain tested to invade the tissue was low. When applied in blood, the same technique has demonstrated higher detection capability than cPCR in mice orally inoculated with TcIV strains obtained from oral infection outbreaks in the Amazon [10]. These differences may be related to the genetic content of the parasite DTUs, the different targets of the two PCR protocols, and the biological material used in the analyses (blood and cardiac tissue). The amount of parasites and the period over which the tests were performed may also vary. In the present study, blood analyzed with cPCR was collected 55 dai, while cardiac tissue was collected 115 dai.

Mice orally inoculated with the Y-strain did not respond to BZ treatment, with cure rates varying from 0 % to 12.5 % (mean of 4.2 %). This is in contrast with the CRs around 50 % (30-70 %) obtained with mice inoculated intraperitoneally, the classic route used by researchers, with the same strain and inoculum [12,22,30]. This suggests that the Y-strain is partially resistant to BZ *in vivo* when inoculated intraperitoneally, and even less susceptible to the drug when inoculated orally. Thus, the orally inoculated Y-strain in the present study was shown to be resistant to BZ. This is in agreement with results previously obtained by our group, which showed that mice orally infected with *T. cruzi* (TcIV strains), not only presented more severe

infection, but also responded worse to BZ treatment when compared to animals inoculated intraperitoneally [10].

CONCLUSION

Our results showed that the orally inoculated Y strain of *T. cruzi* is resistant to BZ. All the essential oils tested altered the parasitemia curve parameters, but benznidazole had greater suppressive effect. Treatment with *S. aromaticum* EO at the dose of 100 mg proved to be promising in the treatment of *T. cruzi* infection, being more effective than the reference drug. Further studies are required to investigate *S. aromaticum* EO in order to evaluate the toxicity and efficacy of its major components *in vivo*, both alone and in association with other drugs, on the genetic diversity of *T. cruzi*.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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