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Original Article

Combination of the essential oil constituents citral, eugenol and thymol enhance their inhibitory effect on *Crithidia fasciculata* and *Trypanosoma cruzi* growth

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ABSTRACT

We analyzed the effect of the combination of citral, eugenol and thymol, respectively the main constituents of essential oils of *Cympobogon citratus* (DC) Stapf, Poaceae (lemon grass), *Syzygium aromaticum* (L.) Merr. & L.M. Perry, Myrtaceae (clove) and *Thymus vulgaris* L., Lamiaceae (thyme), on the proliferation of the trypanosomatids *Crithidia fasciculata* and *Trypanosoma cruzi*. The constituents were initially added individually at different concentrations to *C. fasciculata* cultures to estimate the IC_{50}/24h. Concentrations in a triple combination were about 2 times and 16.5 times lower against *C. fasciculata* and *T. cruzi*, respectively, as compared to isolated compounds. Incubation of *C. fasciculata* with the trypanocydal agent benznidazole did not affect parasite growth at concentrations up to 500 µg/ml, but the IC_{50} of this drug against *T. cruzi* was 15.8 µg/ml, a value about 2-5 times higher than that of constituents in the triple combination. Analysis of treated *C. fasciculata* by scanning electron microscopy showed rounding of the cell body. Our data show that combination of essential oil constituents resulted in increased inhibitory activity on growth of both non-pathogenic and pathogenic trypanosomatid species and indicate that the non-patogenic *C. fasciculata* may represent a resistant model for drug screening in trypanosomatids.

Introduction

Protozoa of the Trypanosomatidae family (Euglenozoa, Kinetoplastea) belong to a cosmopolitan group of flagellates, some of which are important parasites of medical, veterinary and agricultural interest. Several members of this family are causal agents of severe diseases in humans (such as *Trypanosoma cruzi*, which causes Chagas disease in the Americas, and *Leishmania* spp. which causes leishmaniasis in the world), animals and plants. This family includes monoxenic trypanosomatids, such as those belonging to the genera *Crithidia* and *Herpetomonas*, which commonly are insect parasites (Maslov et al., 2013; McGhee and Cosgrove, 1980; Podlipaev, 2000; Wallace, 1966), although there are some reports on the isolation of such "lower trypanosomatids" from immunodeficient human hosts (Chicharro and Alvar, 2003; Morio et al., 2008).

Many insect trypanosomatids, such as *Crithidia fasciculata*, are routinely used as well accepted models for biochemical, cell biology and molecular studies regarding biological aspects of the Trypanosomatidae family. They are easily maintained in axenic cultures and contain metabolic pathways and

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homologues to virulence factors similar to those found in pathogenic trypanosomatids, such as *Leishmania* spp. and *Trypanosoma cruzi* (Comini et al., 2005; Grazu et al., 2012; Russell et al., 1984; Santos et al., 2006; Tasanor et al., 2006; Ueda-Nakamura et al., 2006). Due to their physiological similarities with the pathogenic species, the use of non-pathogenic trypanosomatids appears as a model of choice for the screening and evaluation of new trypanocidal compounds.

For the treatment of Chagas disease the most commonly used drug is benznidazole, a nitroimidazole derivative developed in the early 1960s, active in the acute stage of disease, but ineffective in the chronic stage (Santos et al., 2008; Urbina, 2010). This treatment can be highly toxic with a variety of side effects. The difficulties found in the treatment of Chagas disease, along with the increasing resistance of *T. cruzi* to treatment (Coura, 2007; Santos et al., 2008; Teixeira et al., 2006; Urbina, 2010) indicate the need to search new drugs. Greater attention is being given to extracts and biologically active compounds isolated from plants commonly used in folk medicine for the potential development of herbal medicines that could be used as trypanocidal agents.

Essential oils (EO) are complex mixtures of secondary metabolites isolated from various plants. These mixtures are known for their bactericidal, virucidal and fungicidal effects, and are widely used for embalming and for food preservation. The action of essential oils is probably related to their natural protective role in plants (Bakkali et al., 2008). It has been recently shown that several EO, or their constituents, have inhibitory activity against trypanosomatid protozoa (Alviano et al., 2012; Cardoso and Soares, 2010; de Medeiros et al., 2011; Dos Santos et al., 2012; Habila et al., 2010; Misra et al., 2009; Oliveira et al., 2009; Santoro et al., 2007a; 2007b; 2007c; Teixeira et al., 2006; Ueda-Nakamura et al., 2006).

It has been shown that EO of clove [*Syzygium aromaticum* (L.) Merr. & L.M. Perry, Myrtaceae], thyme [*Thymus vulgaris* (L.), Lamiaceae] and lemon grass [*Cympobogon citratus* (DC) Stapf., Poaceae] and their main constituents (eugenol, thymol and citral, respectively) inhibit the growth of *Trypanosoma cruzi* (Santoro et al., 2007a; 2007b; 2007c) and *Leishmania* spp. (Oliveira et al., 2009; Santin et al., 2009). However, in these works EO and their main constituents were tested individually. There are still no data showing that combination of these constituents may result in a synergistic/addictive action on trypanosomatids, increasing the microbicidal activity of these molecules. Therefore, in this study we analyzed the effect of the combination of eugenol, thymol and citral on non-pathogenic (*Crithidia fasciculata*) and pathogenic (*Trypanosoma cruzi*) trypanosomatids.

Materials and methods

Parasites

Crithidia fasciculata and *Trypanosoma cruzi* cultures were maintained at 28°C in LIT (liver infusion-tryptose) medium containing 10% fetal bovine serum, and culture passaging every 24 or 72 h, respectively. Three-day-old *T. cruzi* culture epimastigotes were used for the experiments. *C. fasciculata* stock cultures were stored at 4°C for two weeks maximum and 24 h old cultures (choanomastigote forms) from recent passages were used for the experiments.

Essential oil constituents

Citral, eugenol and thymol were purchased from Sigma (Sigma Chem. Co., St Louis, MO, USA). They are the main constituents of the essential oils of lemon grass (*Cymbopogon citratus*), clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*), respectively. The compounds were diluted in dimethylsulfoxide (DMSO) at an initial concentration of 100 mg/ml (first stock). For use, this first stock was further diluted at 1:100 in LIT medium, thus resulting in a second stock solution at a concentration of 1 mg/ml in culture medium. By this procedure, DMSO was diluted to 1% in the second stock, thus ensuring that when used its final concentration never exceeded 0.5%, a concentration that is not harmful for the parasites. Stocks were stored at 4°C in the dark to prevent degradation (Guimarães et al., 2008). The second stock, used for all experiments, was freshly prepared before use.

Effect of citral, thymol and eugenol on parasite growth

Crithidia fasciculata parasites were added to 1 ml of LIT medium in 24-well plates at a concentration of 1×10^6 cells/ml and then the isolated constituents were added to the wells at different concentrations (20, 50, 100, 200, 300, 400 or 500 µg/ml) in order to estimate the IC_{50}/24 h (growth inhibition by 50% after 24 h of treatment) and the IC_{90}/24 h (growth inhibition by 90%) of isolated compounds. The plates were kept at 28°C and cell counts were performed after 24 h using Neubauer chamber to evaluate the number of living parasites, compared to the growth of untreated control cultures. As a control, untreated parasites were grown in LIT medium containing either 0.5% DMSO or different concentrations (20, 50, 100, 200, 300, 400 or 500 µg/ml) of benznidazole (stock solution: 1 mg/ml in DMSO). All experiments were performed in triplicate.

After determining the IC_{50}/24 h and IC_{90}/24 h of each component individually, experiments were performed with *Crithidia fasciculata* by using combinations (citral+eugenol, citral+thymol, eugenol+thymol, citral+eugenol+thymol) of the IC_{50}/24 h values in LIT medium, to assess possible synergistic/additive effects. Cell counts were then performed after 24 h of incubation using a Neubauer chamber. The data obtained allowed to calculate the percentage of growth inhibition obtained with the different combinations. The triple combination was then further tested at different dilutions (2, 3 and c.) on *T. cruzi*.

Isobologram analysis was performed using CompuSyn software. Results were interpreted using the Combination Index (CI), a quantitative measure of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement (Chou and Talalay, 1981), and the Dose Reduction Index (DRI), a measure of fold reduction of the dose of each drug in a synergistic combination at a given effect level when compared with the doses of each drug alone. All experiments were performed in triplicate.

Microscopy

C. fasciculata parasites incubated for 24 h with the IC_{50}/24 h or IC_{90}/24 h values of the triple combination (citral/eugenol/thymol) were washed in PBS (pH 7.2) and digital images of treated cells were obtained using a Nikon Eclipse E600 light microscope, equipped with differential interference contrast (DIC) objectives.

For scanning electron microscopy (SEM), cells treated for 24 h with the IC_{50}/24 h or IC_{90}/24 h of the triple combination were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and then adhered to glass coverslips pre-coated with 0.1% poly-L-lysine. The samples were fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 15 min, dehydrated in increasing concentrations of acetone (50, 70, 90 and 100%), critical-point dried, mounted on a SEM stub and coated with a 20 nm-thick gold layer. Analysis was performed using a Jeol JSM 6360 scanning electron microscope.

Cytotoxicity

L-929 fibroblasts were cultivated in a 24-wells plate containing 1 ml DMEM (Dulbecco's Modified Eagle`s Medium; Sigma)/10% fetal bovine serum/1% L-glutamine per well. Each well had a round coverslip at the bottom. The plate was incubated at 37°C and 5% CO_2, culture medium was replaced for a new one containing different concentrations (IC_{50}, ½ IC_{50}, ¼ IC_{50} or IC_{90}) of the triple combination after 24 h. After incubation for 24 h at 37°C and 5% CO_2, the medium was removed, the coverslips were washed in PBS buffer (pH 7.2) and cell viability was then calculated after staining with the vital dye Trypan Blue. As a control, cells were grown in medium lacking the constituents. The experiment was performed in triplicate.

Results

A control growth curve showed that *C. fasciculata* axenic cultures reach the exponential phase of growth soon after 24 h, with a stationary phase after 48 h. A similar growth curve was obtained when the cells were cultivated in LIT medium containing 1% DMSO (data not shown), thus demonstrating that this diluent at maximal concentration did not affect cell growth and therefore could not influence the effect of the essential oil constituents on the parasites. Therefore, all experiments were performed with maximal concentration of 0.5% DMSO.

Firstly we established the IC_{50}/24 h and IC_{90}/24 h values for the isolated constituents on *Crithidia fasciculata* (Fig. 1a). The best results were obtained using thymol, followed by citral and eugenol (Table 1). To evaluate a possible synergistic/additive effect with the constituents, thus allowing reduction in concentrations needed to inhibit cell growth, the IC_{50}/24 h values of each constituent were used as a pair combination or as three-constituent combination. The data obtained showed that combinations increased the inhibitory effect up to 90%, with reduction in the drug concentration in the triple combination to nearly half of that needed when the components were used individually (Table 1).

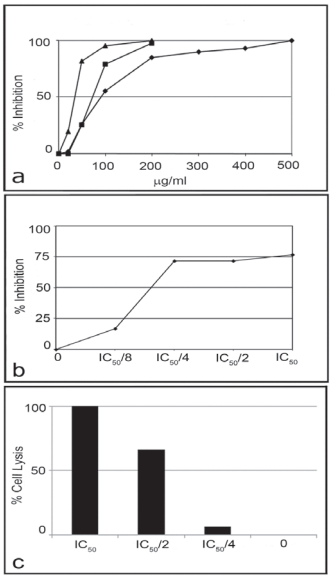

Fig. 1 - Effect of essential oil constituents on trypanosomatids and fibroblasts. A, Effect of thymol (triangle), citral (square) and eugenol (diamond) on *Crithidia fasciculata* growth in LIT medium. B, Effect of different dilutions of the triple combination (from *Crithidia fasciculata* IC_{50}/24 h values, see below) on *Trypanosoma cruzi* growth in LIT medium. C, Cytotoxicity of different concentrations of the triple combination (*Crithidia fasciculata* IC_{50}/24 h values) on L-929 fibroblasts *in vitro*. Final concentration of the triple combination in LIT medium: IC_{50} 48 µg/ml eugenol, 39 µg/ml citral, 17 µg/ml thymol; IC_{50}/2 24 µg/ml eugenol, 19.5 µg/ml citral, 8.5 µg/ml thymol; IC_{50}/4 12 µg/ml eugenol, 9.75 µg/ml citral, 4.25 µg/ml thymol; IC_{50}/8 6 µg/ml eugenol, 4.8 µg/ml citral, 2.13 µg/ml thymol.

Table 1
Concentrations of citral, eugenol and thymol needed to inhibit *C. fasciculata* and *Trypanosoma cruzi* culture growth in 50% or 90% after 24 h of cultivation in LIT medium (IC_{50}/24 h and IC_{90}/24 h, respectively).

Constituent	*Crithidia fasciculata*		*Trypanosoma cruzi*
	IC_{50}/24 h	IC_{90}/24 h	IC_{50}/24 h
Citral	76.3 µg/ml (501.18 µM)	146.0 µg/ml (959.01 µM)	42 µg/ml (275.88 µM)[a]
Eugenol	93.7 µg/ml (570.61 µM)	300 µg/ml (1826.93 µM)	246 µg/ml (1498.08 µM)[b]
Thymol	32.5 µg/ml (216.35 µM)	62.5 µg/ml (416.05 µM)	62 µg/ml (412.73 µM)[c]
Triple Combination: **Citral**	39.1 µg/ml (256.83 µM)	68 µg/ml (446.66 µM)	7.92 µg/ml (52.02 µM)
Triple Combination: **Eugenol**	48.1 µg/ml (292.92 µM)	140 µg/ml (852.57 µM)	9.75 µg/m (59.37 µM)
Triple Combination: **Thymol**	16.6 µg/ml (110.5 µM)	29 µg/ml (193.05µM)	3.45 µg/ml (22.97 µM)
Benznidazol	> 500 µg/ml (1.92 mM)	> 500 µg/ml (1.92 mM)	15.8 µg/ml

[a]Santoro et al., 2007a.
[b]Santoro et al., 2007b.
[c]Santoro et al., 2007c.

The isobologram analysis showed that the increased inhibitory activity observed in *C. fasciculata* with the citral/eugenol combination and with the triple combination was due to synergy (CI < 1). However, the calculated CI for these mixtures was close to 1 and thus could represent an additive effect (Fig. 2; Table 2). The citral/thymol and eugenol/thymol combinations had CI > 1, indicating an antagonist relationship. And concentrations of benznidazole as high as 500 µg/ml (1.92 mM) did not affect *C. fasciculata* growth.

To test whether *C. fasciculata* could represent a model to study the effect of drugs against pathogenic trypanosomatids, different dilutions of the triple combined *C. fasciculata* IC_{50}/24 h value (IC_{50}, IC_{50}/2, IC_{50}/4, IC_{50}/8) were then applied to *Trypanosoma cruzi* cultured epimastigotes. Dilutions up to IC_{50}/4 resulted in about 75% growth inhibition. A 1/6 dilution of the triple *C. fasciculata* IC_{50}/24 h combination was able to inhibit *T. cruzi* epimastigote growth in about 50% (Fig 1b). The resulting recalculated IC_{50}/24 h for citral, eugenol and thymol were 7.92, 9.75 and 3.45 µg/ml, respectively (Table 1). Thus, the individual values for each constituent in the triple IC_{50} combination were about 2-5 times lower (Table 1) than that obtained with benznidazol for *T. cruzi* (15.8 µg/ml).

Isobologram analysis showed that when applied to *T. cruzi* all dilutions of the triple combination resulted in CI < 1

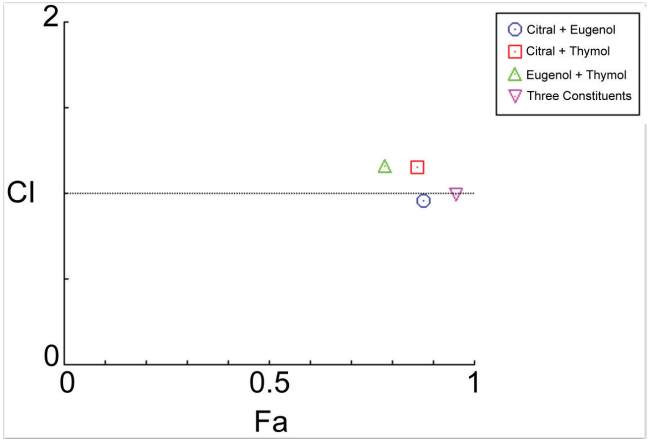

Fig. 2 - Synergy, antagonism and additive effect analysis (isobologram) with *Crithidia fasciculata*. Fa: Effect level; CI: Combination Index. CI < 1: Synergy; CI 1: Additive effect; CI > 1: Antagonism.

Table 2
Composition, concentration (µg/ml in LIT medium) and combination index (CI) of essential oil constituents evaluated on *Crithidia fasciculata*.

Composition	Concentration	Combination Index (CI)
citral+eugenol	76:94	0.96296
citral+thymol	76:33	1.15518
eugenol+thymol	94:33	1.16439
thymol+citral+eugenol	33:76:94	0.99372

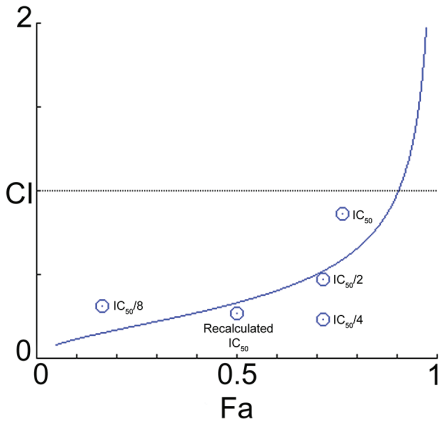

Fig. 3 - Synergy, antagonism and additive effect analysis (isobologram) on *Trypanosoma cruzi*. Fa, Effect level; CI, Combination Index. CI < 1, Synergy; CI 1, Additive effect; CI > 1, Antagonism. Final concentration in LIT medium, IC_{50} 48 µg/ml eugenol, 39 µg/ml citral, 17 µg/ml thymol; IC_{50}/2, 24 µg/ml eugenol, 19.5 µg/ml citral, 8.5 µg/ml thymol; IC_{50}/4, 12 µg/ml eugenol, 9.75 µg/ml citral, 4.25 µg/ml thymol; IC_{50}/8, 6 µg/ml eugenol, 4.8 µg/ml citral, 2.13 µg/ml thymol; Recalculated IC_{50}, 7.92 µg/ml citral, 9.75 µg/ml eugenol, 3.45 µg/ml thymol.

Table 3
Dose, effect level (Fa) and combination index (CI) used for *Trypanosoma cruzi* isobologram.

Dose	Total Dose (µg/ml)	Fa	CI Value
IC_{50}	103.774	0.7657	0.86497
IC_{50}/2	52.02	0.7157	0.47792
IC_{50}/4	26.01	0.7166	0.23856
Recalculated IC_{50}	21.114	0.5	0.27499
IC_{50}/8	13.0356	0.1666	0.31770

IC_{50} 48 µg/ml eugenol, 39 µg/ml citral, 17 µg/ml thymol; IC_{50}/2 24 µg/ml eugenol, 19.5 µg/ml citral, 8.5 µg/ml thymol; IC_{50}/4 12 µg/ml eugenol, 9.75 µg/ml citral, 4.25 µg/ml thymol; IC_{50}/8 6 µg/ml eugenol, 4.8 µg/ml citral, 2.13 µg/ml thymol; Recalculated IC_{50} 7.92 µg/ml citral, 9.75 µg/ml eugenol, 3.45 µg/ml thymol.

(Fig 3; Table 3). According to the Dose Reduction Index (DRI), the concentrations of citral, eugenol and thymol could be reduced 5.75, 26.2 and 15.9 times, respectively, to achieve a Fa (effect level) = 0.5 (50%). Applying these theoretical dilutions to the IC_{50} values of respectively 42, 246 and 62 µg/ml for citral, eugenol and thymol (previously published: see Table 1), the recalculated IC_{50}/24 h values for *T. cruzi* are 7.3, 9.59 and 3.9 µg/ml, respectively. These values are nearly identical as those obtained experimentally (see above).

However, fibroblasts incubated for 24 h with the triple combination IC_{50}/24 h or IC_{90}/24 h values did not survive the treatment: incubation with the ½ IC_{50} value induced death of more than 60% of the cells. A non-toxic dose (less than 8% cell death) was obtained only with the ¼ IC_{50}/24 h values (Fig 1c).

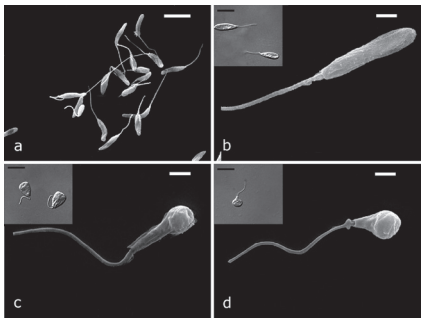

Fig. 4 - Scanning electron microscopy of *C. fasciculata* cells treated for 24 h with the triple IC_{50} or IC_{90} combination. a) control; b) detail of a control cell; c) cell incubated with the IC_{50}; d) cell Incubated with the IC_{90}. Bars, a = 10 µm; b,c,d = 2 µm; Insets, light microscopy images by Differential Interference Contrast (DIC) microscopy; Bar, 10 µm.

After treatment with the triple IC_{50}/24 h or IC_{90}/24 h the *C. fasciculata* parasites presented rounding of the cell body, acquiring a pear-like shape (Fig. 4c, d), in contrast to the elongated body of control cells (Fig. 4a, b). There were no significant morphological differences when comparing treatments with components alone or in combination, except for a more drastic effect for the survivors from the IC_{90}/24 treatment, which presented a more rounded/flattened cell body than those cells treated with the IC_{50}/24 h concentration.

Discussion

Crithidia fasciculata is an insect trypanosomatid that can be useful as a cell biology model for drug screening with potential effect on the pathogenic relatives *Leishmania* spp. and *Trypanosoma cruzi*, the causative agents of *Leishmaniasis* and Chagas disease, respectively (Tasanor et al., 2006). The difficulties found in treatment of both diseases, together with the increasing parasite resistance to treatment (Coura, 2007; Santos et al., 2008; Teixeira et al., 2006) carry the need to search for new drugs. In this sense, the use of non-pathogenic trypanosomatids appears as a model of choice for the screening and evaluation of new compounds, due to their physiological similarities with the pathogenic species.

Plants represent an important source of potential new drugs, with several reports demonstrating the biological activities of essential oils (EO), or their major constituents, on pathogenic (Alviano et al., 2012) or non-pathogenic (Holetz et al., 2003; Pedroso et al., 2006) trypanosomatids. In this work we have tested the inhibitory effects of a combination of the EO constituents eugenol, thymol and citral on the trypanosomatid *C. fasciculata*. When tested separately, the inhibitory effect on culture growth was similar to that observed in previous studies using essential oils/constituents against the trypanosomatids *Crithidia deanei* (Pedroso et al., 2006), *Herpetomonas samuelpessoai* (Holetz et al., 2003), *Leishmania amazonensis* (Ueda-Nakamura et al., 2006), *Leishmania chagasi* (Oliveira et al., 2009) and *Trypanosoma cruzi* (Santoro et al., 2007a; 2007b; 2007c). It has been shown that *C. fasciculata* is more resistant than *Leptomonas* sp. to standard trypanosomatid agents (Bacchi et al., 1974). Our data shows that *C. fasciculata* is highly tolerant to benznidazole (used in Chagas disease treatment), since concentrations as high as 500 µg/ml (1.92 mM) did not affect cell growth. The insect trypanosomatid *Herpetomonas samuelpessoai* is also resistant to benznidazole at concentrations up to 3.84 mM (Holetz et al., 2003). These data indicate that, at least for this reference drug, monoxenic trypanosomatids are more resistant than *T. cruzi* and indicate that the non-patogenic *C. fasciculata* may represent a resistant cell model for drug screening in trypanosomatids.

Different EO components can interact to either reduce or increase antimicrobial efficacy, thus producing four possible effects: 1) Additive, when the combined effect is equal to the sum of individual effects; 2) Antagonis, when the effect of one or both compounds is less when they are applied together than when individually applied; 3) Synergism, when the effect of combined substances is greater than the sum of the individual effects; and 4) Indifference, defined as absence of interactions (Bassole and Juliani, 2012). In this work we have tested for the first time a combination of EO constituents against trypanosomatid protozoa, aiming to observe a possible synergistic/additive effect. Isobologram analysis of the combination of citral, eugenol and thymol showed that on *C. fasciculata* most combinations had an antagonistic/additive effect (CI ≥ 1). However, when different dilutions of the triple combination were applied to *T. cruzi* a synergistic effect (CI < 1) was observed at all dilutions tested, thus indicating that *T. cruzi* is much more susceptible than *C. fasciculata*.

The data obtained so far with pathogenic trypanosomatids indicate a higher sensitivity of these parasites to treatment with individual EO components, as compared to the total oil (Oliveira et al., 2009; Santin et al., 2009; Santoro et al., 2007a; 2007c). Our data indicate that they are also more susceptible to the individual constituents and to the combination than non-pathogenic *C. fasciculata*, at least to the constituents evaluated in this work. Therefore, it is possible that combination of different EO constituents could be more efficient when applied to pathogenic species such as *T. cruzi* and *Leishmania* spp., thus appearing as an important tool in the search for new effective drugs. Also, the use of *C. fasciculata* as a model for study to investigate drug effects may be considered for future experimental designs.

Analysis by scanning electron microscopy (SEM) showed no significant morphological differences after the diverse treatments with *C. fasciculata*, occurring only an increased rounding in cells treated with IC_{90}/24 h concentrations, when compared to cells treated with the IC_{50}/24 h value, and these compared to the control. Previous studies on other trypanosomatids also demonstrated cell rounding (Santoro et al., 2007a; 2007b), besides formation of membrane blebs (Santoro et al., 2007c) or multiseptated cells (Oliveira et al., 2009). Citral incubated *Leishmania amazonensis* showed agglomeration of cells, rounded appearance, rupture of plasma membrane and changes in the flagellar membrane (Santin et al., 2009). The changes in cell morphology can be explained by the properties of the EO constituents. As typically lipophylic molecules, they cross the cell membrane and disrupt the phospholipid layer structure. Cytotoxicity appears to include

such membrane damage, which can lead to the leakage of macromolecules and to lysis. Furthermore, cytotoxicity may be related to depolarization of the mitochondrial membranes by decreasing the membrane potential, besides action in the cytoplasm and nucleus (Bakkali et al., 2008). Our data demonstrate that combining EO constituents increase their inhibitory activity on trypanosomatids, allowing synergistic interactions between them. Thus, the combination of the components activities on different specific cell pathways may also induce high cytotoxicity.

We found a high cytotoxic effect when treating fibroblasts with optimal concentrations that inhibited *C. fasciculata* growth. Previous studies also showed citral toxicity to mammalian cells at concentrations only 2-14 times higher than that considered toxic to *L. amazonensis* and *T. cruzi* (Santin et al., 2009; Santoro et al., 2007a; 2007c). Further studies using combinations of other essential oil constituents may indicate new combinations with lower toxicity to the mammalian cell.

Our data point the need for further studies that could optimize the synergistic effect of the combination of EO constituents, thus allowing the use of lower concentrations of each component in the combination. Finally, the use of non-pathogenic trypanosomatids such as *Crithidia fasciculata* for the screening of new trypanocidal drugs can be effective, since a drug that is active on this parasite may be even more active on *Trypanosoma cruzi* or *Leishmania* spp.

Authorship

CMOA (PhD student) contributed in running the laboratory work, analysis of the data and paper draft. MJS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved for submission.

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