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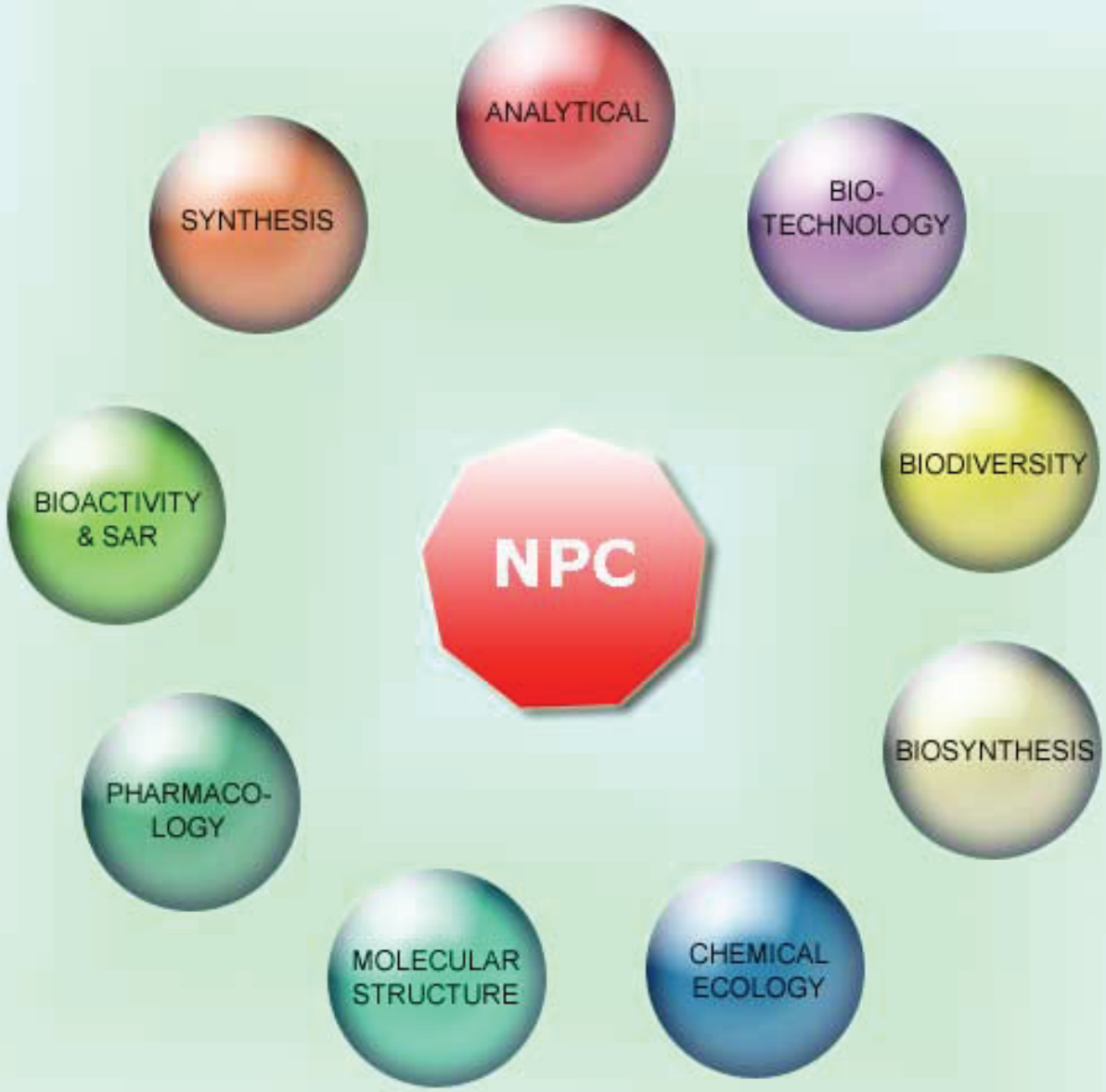

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Chemical Composition and Anti-*Trypanosoma cruzi* Activity of Essential Oils Obtained from Leaves of *Xylopia frutescens* and *X. laevigata* (Annonaceae)

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Essential oils from leaves of *Xylopia frutescens* (XFMJ) and two specimens of *Xylopia laevigata* (XLMC and XLSI) were obtained by hydrodistillation using a Clevenger-type apparatus, and analyzed by GC-MS and GC-FID. Sesquiterpenes dominated the essential oils. The main constituents of XFMJ were (*E*)-caryophyllene (24.8%), bicyclogermacrene (20.8%), germacrene D (17.0%), β-elemene (7.9%), and (*E*)-β-ocimene (6.8%). XLMC contained significant quantities of germacrene D (18.9%), bicyclogermacrene (18.4%), β-elemene (9.5%), δ-selinene (9.2%), (*E*)-caryophyllene (8.5%), germacrene B (5.7%) and γ-muurolene (5.7%), while germacrene D (27.0%), bicyclogermacrene (12.8%), (*E*)-caryophyllene (8.6%), γ-muurolene (8.6%), δ-cadinene (6.8%), and germacrene B (6.0%) were the main components of XLSI. The essential oils had trypanocidal activity against the Y strain of *Trypanosoma cruzi*, with IC_{50} values lower than 30 μg.mL^{-1} and 15 μg.mL^{-1} against epimastigote and trypomastigote forms of *T. cruzi*, respectively, and were also able to reduce the percentage *in vitro* of *T. cruzi*-infected macrophages and the intracellular number of amastigotes at concentrations that were non-cytotoxic to macrophages.

Keywords: *Xylopia frutescens*, *Xylopia laevigata*, Essential oil, Trypanocidal activity.

Xylopia L. (Annonaceae) comprises approximately 157 species of trees and shrubs that are found predominantly in tropical regions [1]. This genus is known for the aromatic fragrance of its flowers and fruits and medicinal purposes [2a,b]. The fruits and seeds of some species of this genus, such as *X. aethiopica*, *X. sericea*, and *X. aromatica*, are also used as either condiments or mixed spices. As a spice, the fruit containing the seeds is usually pounded and used in cooked foods or in the spicing of beverages. As a component of herbal medicine, it is locally used as a carminative, stimulant, and additive to other remedies for the treatment of skin infections, as a digestive, appetizer, and antiemetic agent, and for the management of cough and fever [2a,3].

Recent phytochemical investigations of some species of *Xylopia* have indicated the presence of essential oils (monoterpenes and sesquiterpenes), diterpenes, steroids [4,5,6], alkaloids [7,8], and flavonoids [8], exhibiting several pharmacological activities, such as antifungal [4,6], antioxidant [3,7,8], antileishmanial [9], cytotoxic [10], antinociceptive [11], acaricidal [12], and insecticidal [6].

X. frutescens Aubl. and *X. laevigata* (Mart.) R. E. Fries are two Brazilian species commonly known as 'pindaíba' and 'meiú', respectively, found in the Northeast Region, mainly in Sergipe, Pernambuco, Bahia, and Paraíba States. Previous phytochemical and biological investigations of the stem of *X. laevigata* reported the isolation and identification of sesquiterpenes, steroids, and *ent*-kaurane diterpenoids with antimicrobial and larvicidal activities [6]. Phytochemical and biological investigations of fruits of *X. frutescens* have reported the presence of essential oils (monoterpenes and sesquiterpenes), diterpenes and alkaloids, some of them with antimicrobial and anti-inflammatory activities [13]. Both species are used in traditional medicine. The seeds of *X. frutescens* are used as bladder stimulants and to trigger menstruation, as well as a treatment for rheumatism, halitosis, tooth decay, and intestinal diseases [13], whereas the leaves and flowers of *X. laevigata* are used to treat painful disorders, heart disease, and inflammatory conditions (oral communications received from local woodsmen known as 'mateiros', unpublished data).

In our continuous search for trypanocidal natural products from Annonaceous plants, herein we report the chemical composition of the essential oils from the leaves of *X. frutescens* and *X. laevigata*. There are no previous reports on the chemical composition of the essential oils from the leaves of these species or their trypanocidal action on Chagas' disease.

Hydrodistillation of the leaves of *X. frutescens* from 'Mata do Junco' (XFMJ) and two specimens of *X. laevigata* from 'Mata do Crasto' (XLMC) and 'Serra de Itabaiana' (XLSI) resulted in light yellow crude essential oils with yields of 1.1 ±0.0%, 0.9±0.1% and 1.5±0.0% (w/w), respectively, in relation to the dry weight of the plant material. As shown in Table 1, it was possible to identify 43 compounds: 23 in the essential oil of XFMJ (91.0%); and 33 in both XLMC and XLSI (97.3 and 97.6%, respectively). The essential oils were dominated by sesquiterpenes, with 83.1% in XFMJ, 96.3% in XLMC and 93.5% in XLSI. The major compounds identified in the essential oil of XFMJ were (*E*)-caryophyllene (24.8%), bicyclogermacrene (20.8%), germacrene D (17.0%), β-elemene (7.9%),

Table 1: Essential oil composition of the leaves of *Xylopia frutescens* (XFMJ) and *X. laevigata* (XLMC and XLSI).

Compound				Peak area %		
		RI[a]	RI[b]	XFMJ	XLMC	XLSI
1	α-Pinene	931	932	0.6±0.1	0.3±0.5	1.3±0.4
2	Camphene	947	946	0.3±0.0	0.1±0.1	0.3±0.1
3	β-Pinene	975	974			0.3±0.1
4	Myrcene	988	988	0.2±0.0		
5	Limonene	1028	1024		0.4±0.3	1.7±0.4
6	(*Z*)-β-Ocimene	1036	1032		0.2±0.2	0.4±0.1
7	(*E*)-β-Ocimene	1046	1044	6.8±0.4		0.1±0.1
8	Bicycloelemene	1332	1335	0.2±0.0	0.2±0.0	0.3±0.0
9	δ-Elemene	1335	1335		2.9±0.1	2.7±0.0
10	α-Cubebene	1347	1345	0.3±0.0	0.6±0.1	1.3±0.1
11	α-Ylangene	1370	1373	0.4±0.0	0.3±0.0	0.6±0.1
12	α-Copaene	1376	1374	1.4±0.0	2.5±0.2	4.8±0.4
13	β-Bourbonene	1384	1387		0.1±0.1	0.3±0.0
14	β-Cubebene	1388	1387			1.0±0.0
15	β-Elemene	1390	1389	7.9±0.3	9.5±0.5	
16	(*E*)-Caryophyllene	1421	1417	24.8±0.2	8.5±0.5	8.6±0.3
17	γ-Elemene	1429	1430		1.0±0.0	
18	β-Copaene	1431	1430	0.3±0.0		1.6±0.0
19	α-Guaiene	1435	1437		0.3±0.0	
20	Aromadendrene	1440	1439		1.0±0.1	1.6±0.1
21	*trans*-Muurola-3,5-diene	1450	1451	0.3±0.0		
22	α-Humulene	1457	1452	2.4±0.0	2.0±0.0	1.1±0.0
23	*allo*-Aromadendrene	1461	1458		0.3±0.0	
24	γ-Muurolene	1476	1478	2.1±0.1	5.7±0.3	8.6±0.2
25	Germacrene D	1483	1484	17.0±0.3	18.9±0.4	27.0±0.4
26	δ-Selinene	1488	1492		9.2±0.2	0.2±0.0
27	*trans*-Muurola-4(14),5-diene	1493	1493		0.7±0.0	1.8±0.1
28	Bicyclogermacrene	1497	1500	20.8±0.1	18.4±0.2	12.8±0.4
29	δ-Amorphene	1503	1509		0.6±0.2	0.6±0.1
30	Germacrene A	1508	1509	1.0±0.0	1.1±0.1	
31	γ-Cadinene	1514	1513	0.7±0.0	1.2±0.1	2.5±0.1
32	δ-Cadinene	1519	1522	2.4±0.0	3.3±0.0	6.8±0.2
33	*trans*-Cadina-1,4-diene	1534	1533	0.3±0.0		0.3±0.0
34	α-Cadinene	1538	1537		0.1±0.1	0.7±0.0
35	Selina-3,7(11)-diene	1543	1545			0.3±0.0
36	Germacrene B	1561	1559	0.1±0.1	5.7±0.3	6.0±0.5
37	Spathulenol	1578	1577	0.3±0.1	0.8±0.1	0.9±0.3
38	Caryophyllene oxide	1584	1582		0.4±0.0	0.4±0.1
39	Viridiflorol	1588	1592	0.4±0.0		
40	*epi*-α-Muurolol	1645	1640			0.2±0.3
41	α-Cadinol	1657	1652		0.1±0.2	0.5±0.2
42	*neo*-Intermedeol	1660	1658		0.7±0.3	
43	Abietadiene	2084	2087		0.2±0.2	
	Monoterpenes			7.9	1.0	4.1
	Sesquiterpenes			83.1	96.3	93.5
	Total Identified			91.0	97.3	97.6

RI[a] (calc.), retention indices on DB-5MS column calculated according to ref. [20a]. RI[b] retention indices according to ref. [20b]. Data are expressed as mean ± SD of three analyses.

and (*E*)-β-ocimene (6.8%). Germacrene D (18.9%), bicyclogermacrene (18.4%), β-elemene (9.5%), δ-selinene (9.2%), (*E*)-caryophyllene (8.5%), germacrene B (5.7%), and γ-muurolene (5.7%) were the main compounds in the essential oil of XLMC, whereas germacrene D (27.0%), bicyclogermacrene (12.8%), (*E*)-caryophyllene (8.6%), γ-muurolene (8.6%), δ-cadinene (6.8%), and germacrene B (6.0%) were the major compounds in the essential oil of XLSI (Table 1).

Table 1 shows the chemical composition of the essential oils from the leaves of XFMJ, XLMC and XLSI, which were very similar, differing only in the concentrations of their constituents. These results confirm that *X. laevigata* and *X. frutescens* are typical members of the Annonaceae family, because the chemical constituents identified have been reported in the essential oils of other species of *Xylopia* [5,14]. Although the chemical constituents in the essential oils of *X. frutescens* and *X. laevigata* specimens have been found in other essential oils of *Xylopia* species, recent studies have demonstrated significant variations in the chemical composition of the essential oils of the species of this genus.

Maia *et al.* [5] analyzed the chemical composition of four Amazon *Xylopia* species (*X. aromatica*, *X. caynnensis*, *X. emarginata*, and *X. nitida*) and observed variations in their chemical composition. The main compounds found in the leaf oil of *X. aromatica* were bicyclogermacrene (36.5%), spathulenol (20.5%), and limonene (4.6%); for *X. cayennensis*, α-pinene (29.2%), β-pinene (16.5%), caryophyllene oxide (14.5%), bicyclogermacrene (12.5%), germacrene D (4.7%), and 1,8-cineole (4.5%); for *X. emarginata*, spathulenol (73.0%); and for *X. nitida*, γ-terpinene (44.1%), *p*-cymene (13.7%), α-terpinene (12.6%), and limonene (11.3%). Lago *et al.* [14] analyzed the chemical composition of the leaves of *X. aromatica* from the Savannah region, and verified that the major compounds were α-pinene (26.1%), limonene (22.3%), bicyclogermacrene (20.4%), and β-pinene (19.0%). Tavares *et al.* [15] investigated the chemical constituents from the leaves of *X. langsdorffiana*, a species from northeastern Brazil, and observed that the major compounds were germacrene D (22.9%), *trans*-β-guaiene (22.6%), (*E*)-caryophyllene (15.7%), and α-pinene (7.3%). These variations in the composition of the major constituents, as well as the contents of all components, can be related to soil and climate conditions, water stress, collection place, nutrition, and other abiotic factors.

The difference between the chemical composition of species of *Xylopia* collected in the Amazon and Savannah regions has been investigated to evaluate the influence of the climatic differences [5,14], but for the species of *Xylopia* from northeastern Brazil there have been few investigations. For species of the Amazon region it has been suggested that there is a dominance of oxygenated constituents (mono- and sesquiterpenes) when compared with the species of the Savannah region [5,14]. According to our results and those of Tavares *et al.* [15], the *Xylopia* species from northeastern Brazil are dominated by non-oxygenated constituents (mono- and sesquiterpenes), such as (*E*)-caryophyllene, bicyclogermacrene, and germacrene D.

The *in vitro* trypanocidal activity against epimastigote and trypomastigote forms of *T. cruzi* is presented in Table 2. All essential oils showed significant trypanocidal action with IC_{50} values lower than 30 $\mu g.mL^{-1}$ and 15 $\mu g.mL^{-1}$ against epimastigote and trypomastigote forms, respectively.

In the *in vitro* model of macrophage infection, the essential oils showed trypanocidal activity against intracellular forms of *T. cruzi* at concentrations that were non-toxic to macrophages (10 $\mu g.mL^{-1}$). As shown in Figure 1, the essential oils significantly reduced ($P < 0.05$) the percentage of infected macrophages and the number of intracellular parasites, although less than the reference drug (benznidazole).

Although the oils were less effective than benznidazole, it is important to say that this drug is highly toxic to mammalian cells and its action results in a cure rate of approximately 70–80% in the acute phase and 10–20% in the chronic phase. Even after decades of research there are still no compounds able to cure all Chagas' disease patients, and no substitute for benznidazole has been developed [16].

The trypanocidal activities of the essential oils were considered very closely and could be explained by the similarity in their chemical compositions. In all the essential oils, bicyclogermacrene,

Table 2: IC_{50} values for anti-*T.cruzi* activity.

Essential Oil	IC_{50} µg.mL^{-1}	
	Epimastigote forms	Trypomastigote forms
XFMJ	20.2 (±1.4)	11.9 (±0.6)
XLMC	22.2 (±1.7)	12.7 (±1.9)
XLSI	27.7 (±0.4)	13.4 (±2.1)
Benznidazole[a]	2.8 (±0.7)	2.8 (±0.5)

Data are expressed as mean ± SD of three independent experiments.
[a] Reference drug (positive control).

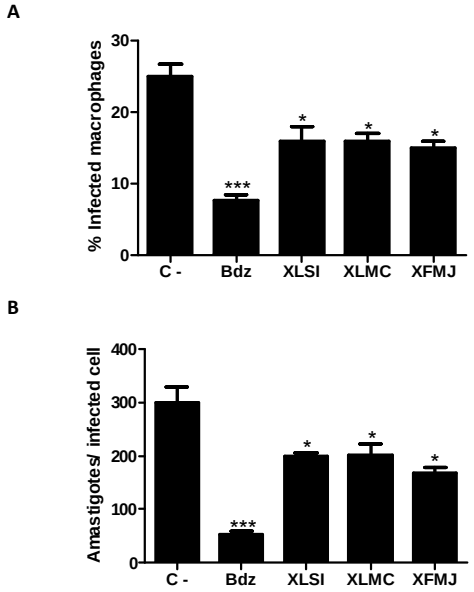

Figure 1: *Xylopia* oils inhibited *T. cruzi* trypomastigote development in macrophages at a concentration of 10 µg.mL^{-1}. Peritoneal macrophages were infected with Y strain trypomastigotes and treated with either benznidazole (10 µg.mL^{-1}) or the essential oils (10 µg.mL^{-1}) or neither (negative control) for 6 hours. The percentage of infected macrophages (A) and the relative number of amastigotes in infected cells (B) were higher in untreated infected controls than in cultures treated the test-inhibitor XLSI, XLMC and XFMJ. Bdz (Benznidazole) was used as a positive control. Standard error of the mean is shown as error bars. ***, $P < 0.001$ vs control; *, $P < 0.05$ vs control.

(*E*)-caryophyllene and germacrene D were detected in high concentration. The significant trypanocidal activity against the different forms of *T. cruzi* could be attributed to the high concentration of these compounds. Recent works have demonstrated that essential oils with high concentration of these compounds possess antiprotozoal properties, in particular against *T. cruzi* [17,18a,b]. The results obtained in this work are considered very promising when compared with other essential oils with trypanocidal activity [17,18a,b,19a,b] and confirm that species of Annonaceae are a natural source of compounds that are biologically active with antiprotozoal properties.

This is the first report of the volatile constituents from the leaves of *X. frutescens* and *X. laevigata* and their trypanocidal action. The significant trypanocidal properties presented by the essential oils suggest that these species are a rich source of biologically active compounds. The presence of (*E*)-caryophyllene, bicyclogermacrene and germacrene D in high concentration in the essential oils could be responsible for the significant trypanocidal activity, because these compounds have been found in other essential oils with trypanocidal action. In addition, this study confirms the importance of chemical and biological investigations of essential oils of Annonaceous species, in particular *Xylopia* spp., in the search for new and safer trypanocidal agents.

Experimental

Plant material: The leaves of *X. laevigata* were collected in January and August 2011 at the 'Parque Nacional Serra de Itabaiana', city of Itabaiana [coordinates: S 10° 44' 53" W 37° 20' 21"] and 'Mata do Crasto' [coordinates: S 11° 24' 05" W 37° 25' 45"], city of Santa Luzia do Itanhy, respectively, whereas the leaves of *X. frutescens* were collected in January 2011 at the 'Mata do Junco, city of Capela [coordinates: S 10° 31' 45" W 37° 03' 32"], all in Sergipe State, Brazil. The identity of the plants was confirmed by Dr Ana Paula do Nascimento Prata, Departamento de Biologia, Universidade Federal de Sergipe (UFS), Brazil, and voucher specimens (24792, 21510, and 19796, respectively) have been deposited in the Herbarium of the Universidade Federal de Sergipe (ASE/UFS).

Hydrodistillation of the essential oils: The essential oils from dried leaves (for 24 h) of *X. laevigata* specimens and *X. frutescens* (200 g each) were obtained by hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oils were dried over anhydrous sodium sulfate and the percentage content was calculated on the basis of the dry weight of plant material. The essential oils were stored in a freezer until analysis. The hydrodistillation was performed in triplicate.

GC-FID and CG-MS analysis of the essential oils: GC-FID analyses were carried out using a Shimadzu GC-17A fitted with a flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a ZB-5MS fused capillary column (30m X 0.25mm X 0.25µm film thickness) coated with 5%-phenyl-arylene-95%-dimethylpolysiloxane. Helium was the carrier gas at 1.0 mL.min^{-1} flow rate. The column temperature program was 40°C/4min, at a rate of 4° C/min to 240°C, then at 10°C/min to 280°C, and at 280°C/2min. The injector and detector temperatures were 250°C and 280°C, respectively. Samples (10 mg.mL^{-1} in CH_2Cl_2) were injected with a 1:50 split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C_8-C_{18}). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without a FID response factor correction. GC-MS analyses were performed on a Shimadzu QP5050A GC-MS system equipped with an AOC-20i auto-injector. A J&W Scientific DB-5MS (coated with 5%-phenyl-95%-dimethylpolysiloxane) fused capillary column (30 m X 0.25 mm X 0.25 µm film thickness) was used as the stationary phase. MS were taken at 70 eV with scan intervals of 0.5s and fragments from 40-550 Da. The other conditions were similar to the GC analysis. Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds run under identical conditions, and by comparison of retention indices [20a,b] and MS [20b] with those in the literature and by comparison of MS with those stored in the NIST and Wiley libraries.

In vitro *trypanocidal activity:* Epimastigotes of *T. cruzi* (Y strain) were maintained at 26°C in LIT medium (Liver Infusion Tryptose), supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 1% hemin (Sigma, Chemical Co., MO, USA), 1% R9 medium (Sigma), and 50 µg.mL-1 of gentamycin (Novafarma, Anápolis, GO, Brazil). Parasites (1 x 10^6 cells.well^{-1}) were cultured in fresh medium in the absence or presence of the essential oils at various concentrations (100 to 1.23 µg.mL^{-1}), in triplicate. Cell growth was determined after culture for 5 days by counting viable forms in a Neubauer chamber. Bloodstream trypomastigote forms of *T. cruzi* were obtained from supernatants of LLC-MK2 cells previously infected and cultured in 96-well plates (4×10^5 cells.well^{-1}) in RPMI (Sigma), supplemented with 10% FBS and 50 µg.mL-1 of gentamycin in the absence or presence of different concentrations of the essential oils, in triplicate. Viable (motile) parasites were counted in a Neubauer chamber 24 h later.

The percentage of inhibition was calculated in relation to untreated cultures. To determine the 50% inhibitory concentration (IC_{50}) of the epimastigote and trypomastigote forms of *T. cruzi*, nonlinear regression on Prism 5.02 GraphPad software was used.

In vitro *macrophage infection and treatment with essential oils:* Peritoneal exudate macrophages (2 x 10^5 cells.well^{-1}) obtained from BALB/c mice were placed in a 24-well plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. Cells were then infected with trypomastigotes at a ratio of 10 parasites per macrophage for 2 h. Free trypomastigotes were removed by successive washes using saline solution. Cultures were incubated in complete medium alone or with the essential oils (10 μg.mL^{-1}) or benznidazole at the same concentration for 6 h. The medium was replaced by a fresh medium and the plate was incubated for 72 h. Cells were fixed in absolute EtOH and the percentage of infected macrophages and the mean number of amastigotes/infected macrophages was determined by manual counting after hematoxylin and eosin staining using an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per macrophage was determined by counting 100 cells per slide. One-way ANOVA and Bonferroni tests were used to determine the statistical significance of the group comparisons.

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Review/Account

Addition/Correction

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