ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/270218602

Chemical composition of essential oils from Annona vepretorum Mart. and Annona squamosa L. (Annonaceae) leaves and their antimalarial and trypanocidal activities

Article *in* Journal of Essential Oil Research · March 2015

Impact Factor: 0.79 · DOI: 10.1080/10412905.2014.982876

CITATION
1

READS
60

7 authors, including:

Emmanoel Vilaca Costa
Federal University of Amazonas
72 PUBLICATIONS **554** CITATIONS
SEE PROFILE

Cássio Santana Meira
Fundação Oswaldo Cruz
17 PUBLICATIONS **70** CITATIONS
SEE PROFILE

Leociley Menezes
Universidade Federal do Paraná
9 PUBLICATIONS **30** CITATIONS
SEE PROFILE

Milena Soares
Fundação Oswaldo Cruz
198 PUBLICATIONS **3,422** CITATIONS
SEE PROFILE

Available from: Emmanoel Vilaca Costa
Retrieved on: 11 May 2016

This article was downloaded by: [152.245.207.5]
On: 01 March 2015, At: 15:09
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

CrossMark
Click for updates

Journal of Essential Oil Research
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tjeo20

Chemical composition of essential oils from Annona vepretorum Mart. and Annona squamosa L. (Annonaceae) leaves and their antimalarial and trypanocidal activities

Cássio S. Meira[a], Elisalva T. Guimarães[ab], Taís S. Macedo[a], Thanany B. da Silva[c], Leociley R.A. Menezes[c], Emmanoel V. Costa[d] & Milena B.P. Soares[ae]

[a] Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA, Brazil
[b] Departamento de Ciências da Vida, Universidade do Estado da Bahia, Salvador, BA, Brazil
[c] Departamento de Química, Universidade Federal de Sergipe, São Cristovão, SE, Brazil
[d] Departamento de Química, Universidade Federal de Sergipe, Itabaiana, SE, Brazil
[e] Centro de Biotecnologia e Terapia Celular, Hospital São Rafael, Salvador, BA, Brazil

Published online: 25 Nov 2014.

To cite this article: Cássio S. Meira, Elisalva T. Guimarães, Taís S. Macedo, Thanany B. da Silva, Leociley R.A. Menezes, Emmanoel V. Costa & Milena B.P. Soares (2015) Chemical composition of essential oils from Annona vepretorum Mart. and Annona squamosa L. (Annonaceae) leaves and their antimalarial and trypanocidal activities, Journal of Essential Oil Research, 27:2, 160-168, DOI: 10.1080/10412905.2014.982876

To link to this article: http://dx.doi.org/10.1080/10412905.2014.982876

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at http://www.tandfonline.com/page/terms-and-conditions

Journal of Essential Oil Research, 2015
Vol. 27, No. 2, 160–168, http://dx.doi.org/10.1080/10412905.2014.982876

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Chemical composition of essential oils from *Annona vepretorum* Mart. and *Annona squamosa* L. (Annonaceae) leaves and their antimalarial and trypanocidal activities

Cássio S. Meira[a], Elisalva T. Guimarães[a,b], Taís S. Macedo[a], Thanany B. da Silva[c], Leociley R.A. Menezes[c], Emmanoel V. Costa[d] and Milena B.P. Soares[a,e]*

[a]*Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA, Brazil;* [b]*Departamento de Ciências da Vida, Universidade do Estado da Bahia, Salvador, BA, Brazil;* [c]*Departamento de Química, Universidade Federal de Sergipe, São Cristovão, SE, Brazil;* [d]*Departamento de Química, Universidade Federal de Sergipe, Itabaiana, SE, Brazil;* [e]*Centro de Biotecnologia e Terapia Celular, Hospital São Rafael, Salvador, BA, Brazil*

(*Received 21 October 2013; accepted 27 October 2014*)

Here, we describe the trypanocidal and antimalarial activities from essential oils extracted from *Annona vepretorum* (AVOE) and *Annona squamosa* (ASOE) (Annonaceae) leaves. The essential oils were obtained by hydrodistillation and analyzed by gas chromatography–mass spectrometry (GC–MS) and GC–flame ionization detection (GC–FID). A total of twenty-one compounds were identified in AVOE and twenty-three in ASOE. The sesquiterpenes are more abundant in the both essential oils. ASOE contained significant quantities of (*E*)-caryophyllene (27.4%), germacrene D (17.1%) and bicyclogermacrene (10.8%). The major compounds in AVOE were bicyclogermacrene (39.0%), spathulenol (14.0%) and α-phellandrene (11.5%). The essential oils demonstrated potent trypanocidal and antimalarial activities with values of IC_{50} lower than 20 μg/mL, and a strong inhibition of the proliferation of amastigotes, the clinically relevant forms of *Trypanosoma cruzi*. In addition, through ultrastructural studies and flow cytometry analysis with trypomastigotes of *T. cruzi*, we identified significant ultrastructural alterations induced by the essential oils, especially in the cell membrane and mitochondria, which ultimately results in necrotic parasite death.

Keywords: *Annona squamosa*; *Annona vepretorum*; essential oil; antimalarial activity; trypanocidal activity

Downloaded by [152.245.207.5] at 15:09 01 March 2015

Introduction

Tropical diseases caused by single-celled parasites, such as malaria and Chagas's disease, affect about one billion people worldwide, with a high prevalence in tropical regions (1). Quality pharmaceutical treatment of both diseases is dramatically limited to a few classes of drugs, which, in many cases, are associated with high toxicity, variable efficacy and resistance, constituting a public health problem in developing countries (2, 3). Therefore, the development of new therapies for the treatment of parasitic diseases is necessary.

Annona L. (Annonaceae) comprises approximately 162 species of trees and shrubs that are found predominantly in lowland tropical regions (4). Previous chemical and pharmacological investigations of some species from this genus have indicated the presence of important bioactive compounds (alkaloids, acetogenins and terpenes) that exhibit various pharmacological activities, including cytotoxicity against tumor cell lines, antimicrobial and particularly trypanocidal properties (5–7).

To date, only *Annona squamosa* (ASOE) has been used in folk medicine. Its leaves are reported to posses stimulant, antispasmodic, sudorific, anthelmintic and insecticidal properties. A poultice of the leaves is used as a cataplasm over boils and ulcers to induce suppuration (8). The search for trypanocidal and antiplasmodial compounds from annonaceous plants continues and here we report on the chemical composition of essential oils from leaves of ASOE and *Annona vepretorum* Mart. (AVOE) as well as their antiplasmodial and trypanocidal activities.

Experimental

Plant material

ASOE leaves were collected in September 2012 at the 'Campus Universitário' of the Universidade Federal de Sergipe, city of São Cristóvão, Sergipe, Brazil, coordinates: [10°55'26" S, 37°06'05"W], while AVOE leaves were collected in March 2012 at the city of Poço Redondo, Sergipe, Brazil, coordinates: [09°47'44"S, 37°40'35"W]. Plant identification was confirmed by Dr. Ana Paula do Nascimento Prata, Department of Biology, Universidade Federal de Sergipe (UFS), Brazil, and voucher specimens (19001 and 23158, respectively) have been deposited in the Herbarium of the Universidade Federal de Sergipe (ASE/UFS).

*Corresponding author. Email: milena@bahia.fiocruz.br

© 2014 Taylor & Francis

Downloaded by [152.245.207.5] at 15:09 01 March 2015

Hydrodistilation of the essential oils

The essential oils from dried leaves of ASOE and AVOE (200 g each) were stove dried with circulating air at 40°C for 24 hours and submitted to hydrodistillation for 3 hours 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 (−20°C) until analyzed. The hydrodistillation was performed in triplicate.

GC-FID and CG-MS analysis of the essential oils

Gas chromatography–flame ionization detection (GC–FID) analyses were carried out using a Shimadzu GC-17A fitted with an FID and an electronic integrator. Separation of the compounds was achieved employing a ZB-5MS fused capillary column (30 m × 0.25 mm × 0.25 μm film thickness) coated with 5%-phenyl-arylene–95%-dimethylpolysiloxane. Helium was the carrier gas at 1.0 mL/minute flow rate. The column temperature program was 40°C for 4 minutes, at a rate of 4°C/minute to 240°C, then at 10°C/minute to 280°C, and at 280°C for 2 minutes. The injector and detector temperatures were 250°C and 280°C, respectively. Samples (10 mg/mL in CH_2Cl_2) were injected with a 1:50 split ratio. Retention indices (RIs) were generated with a standard solution of *n*-alkanes (C_8–C_{20}). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without an FID response factor correction. GC–mass spectrometry (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 × 0.25 mm × 0.25 μm film thickness) was used as the stationary phase. MS were taken at 70 eV with scan intervals of 0.5 seconds and fragments from 40 to 550 Da. The other conditions were similar to the GC analysis.

Identification of oil constituents

The essential oil components were identified by comparison of (i) their retention times (t_R) with those of some standard compounds [α- and β-pinenes, (*E*)-caryophyllene, limonene and spathulenol] analyzed under identical conditions; (ii) their RIs (determined on a DB-5MS column relative to the t_R of a series of *n*-alkanes (C_8–C_{20}), according to (9) with those published in the literature (9); and (iii) their mass spectra with those listed in the NIST (05, 05s, 21 and 107) and Wiley 8 mass spectral libraries, and those published in the literature (10).

Animals and parasites

BALB/c female mice (eight to ten weeks old) were obtained from Centro de Pesquisas Gonçalo Moniz (Fundação Oswaldo Cruz, Bahia, Brazil) and maintained in sterilized cages under a controlled environment, receiving a balanced rodent diet and water *ad libitum*. All animal experiments and procedures were approved by the institution's committee on the ethical handling of laboratory animals. Epimastigotes of *Trypanosoma 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 of gentamycin (Novafarma, Anápolis, GO, Brazil) (11). Tissue culture trypomastigotes (Y strain) were obtained from the supernatants of five- to six-day-old infected LLC-MK2 cells maintained in RPMI-1640 medium supplemented with 10% FBS and 50 μg/mL of gentamycin at 37°C in a 5% humidified CO_2 atmosphere (12). W2 strain *Plasmodium falciparum* (chloroquine-resistant) was maintained in continuous culture of human erythrocytes (blood group O^+) using the RPMI 1640 medium supplemented with 10% human plasma (13).

Cytotoxicity to mammalian cells

To determine the cytotoxicity of the essential oils, peritoneal exudate macrophages obtained from BALB/c mice were placed into ninety-six-well plates at a cell density 1×10^5 cells/well in RPMI-1640 medium without phenol red supplemented with 10% FBS and 50 μg/mL of gentamycin and incubated for 24 hours at 37°C and 5% CO_2. After that time, each compound were added at five concentrations ranging from 1.23 to 100 μg/mL in triplicate and incubated for 72 hours. The cytotoxic effect was measured by the AlamarBlue assay as described by Meira et al. (14) Gentian violet was used as positive control, at concentrations ranging from 0.04 to 10 μg/mL.

Antimalarial activity

Parasites grown at 1–2% parasitemia and 2.5% hematocrit were distributed onto ninety-six-wells culture plate and incubated with the compounds at five concentrations ranging from 1.23 to 100 μg/mL in culture medium (RPMI 1640) without hypoxanthine. After 24 hours, [^{3}H]-hypoxanthine was added, the plate were incubated again and parasites were harvested using a cell harvester to quantify the [^{3}H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with

[^{3}H]-hypoxanthine uptake in treated versus untreated parasite (15). Non-linear regression was used to determine the inhibitory concentration 50% (IC_{50}) for *P. falciparum* on Prism 5.02 GraphPad software. Mefloquine (Farmaguinhos, Rio de Janeiro, Brazil) was used as standard drug.

Trypanocidal activity

Epimastigotes (1×10^6 cells/well) were placed in fresh medium in LIT medium in the absence or presence of the essential oils at various concentrations (100–1.23 μg/mL) in triplicate. Cell growth was determined after culture for five days by counting viable forms in a Neubauer chamber (16). Bloodstream trypomastigotes forms of *T. cruzi* were obtained from supernatants of previously infected LLC-MK2 cells placed in ninety-six-well plates (4×10^5 cell/well) in RPMI medium supplemented with 10% FBS and 50 μg/mL of gentamycin in the absence or presence of different concentrations of the essential oils, in triplicate. Viable parasites were counted in a Neubauer chamber 24 hours later (16). The percentage of inhibition was calculated in relation to untreated cultures. To determine the inhibitory concentration 50% (IC_{50}) for epimastigote and trypomastigote forms of *T. cruzi*, we used non-linear regression on Prism 5.02 GraphPad software. Benznidazole (LAFEPE, Recife, Brazil) was used as the positive control.

In vitro *macrophage infection and treatment with essential oils*

Peritoneal macrophages (2×10^5 cells/well) obtained from BALB/c mice were cultured in a twenty-four-well-plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 hours. Cells were infected with trypomastigotes at a ratio of ten parasites per macrophage for 2 hours. Free trypomastigotes were removed by successive washes using saline solution and the cells were incubated for 24 hours to allow full internalization and differentiation of trypomastigotes to amastigotes. Next, cultures were incubated in complete medium alone or with the essential oils or benznidazole (10 μg/mL) for 72 hours. Cells were fixed in absolute alcohol and the percentage of infected macrophages and the relative number of amastigotes per 100 macrophages was determined by manual counting following hematoxylin and eosin staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per 100 macrophages was determined by counting 100 cells per slide. The one-way analysis of variance (ANOVA) and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons.

Electron microscopy analysis

Trypomastigotes (4×10^7 cells/well) of Y strain *T. cruzi* were incubated for 24 hours at 37°C in the absence or presence of AVOE (11.2 or 22.4 μg/mL) or ASOE (12.7 or 25.4 μg/mL). After incubation, the parasites were fixed for 1 hour at room temperature with 2% formaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 hour at room temperature. After fixation, parasites were processed for transmission electron microscopy (TEM) as previously described (14). Images were observed under a Zeiss 109 transmission electron microscope.

Propidium iodide and annexin V staining

Trypomastigotes (1×10^7) were incubated for 72 hours at 37°C in the absence or presence of AVOE (11.2 or 22.4 μg/mL) or ASOE (12.7 or 25.4 μg/mL). After incubation, the parasites were labeled for propidium iodide (PI) and annexin V using the annexin V-FITC apoptosis detection kit (Sigma, MO), according to the manufacturer's instructions. Acquisition and analyses was performed using a FACS Calibur flow cytometer (Becton Dickinson, CA), with FlowJo software (Tree Star, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to trypomastigotes forms of *T. cruzi*.

Results and discussion

Hydrodistillation of ASOE and AVOE leaves produced red and light yellow crude essential oils, with a yield of 0.34±0.10% and 0.76±0.02% (w/w), respectively, in relation to the dry weight of the plant material. The sesquiterpenes are more abundant in the essential oils, with 95.5% in ASOE and 65.1% in AVOE (Table 1). The major compounds identified in AVOE essential oil were bicyclogermacrene (39.0%), spathulenol (14.0%), α-phellandrene (11.5%), (*E*)-β-ocimene (8.6%), *o*-cymene (6.0%), α-pinene (6.0%) and germacrene D (5.5%). The major compounds identified in ASOE essential oil were (*E*)-caryophyllene (27.4%), germacrene D (17.1%), bicyclogermacrene (10.8%), (*Z*)-caryophyllene (7.3%), β-elemene (6.2%), α-humulene (5.7%), *epi*-α-cadinol (4.3%), γ-cadinene (4.2%) and δ-elemene (4.1%) (Table 1). Recently, Costa et al. (6) described the chemical composition of the essential oil from the leaves of AVOE, identifying eighteen compounds less than this study. These variations, or detector sensitivity or sample dilution 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 (16). Although

Downloaded by [152.245.207.5] at 15:09 01 March 2015

Table 1. Essential oil composition from leaves of *Annona squamosa* (ASOE) and *Annona vepretorum* (AVOE).

Compound		RI[a]	RI[b]	Peak area %	
				ASOE	AVOE
1	α-Pinene	931	932	0.6 ± 0.0	6.0 ± 0.1
2	Camphene	935	946	0.8 ± 0.0	–
3	β-Pinene	975	974	–	0.3 ± 0.0
4	Myrcene	988	988	–	0.6 ± 0.0
5	α-Phellandrene	1004	1002	–	11.5 ± 0.0
6	*o*-Cymene	1023	1022	–	6.0 ± 0.4
7	Limonene	1027	1024	0.6 ± 0.0	0.5 ± 0.0
8	(*Z*)-β-Ocimene	1035	1032	–	0.3 ± 0.0
9	(*E*)-β-Ocimene	1046	1044	–	8.6 ± 0.4
10	Linalool	1099	1095	–	0.2 ± 0.0
11	δ-Elemene	1332	1335	4.1 ± 0.0	0.6 ± 0.0
12	α-Copaene	1376	1374	0.8 ± 0.0	0.3 ± 0.0
13	β-Bourbonene	1385	1387	0.8 ± 0.0	–
14	β-Elemene	1389	1389	6.2 ± 0.0	1.2 ± 0.1
15	(*Z*)-Caryophyllene	1406	1408	7.3 ± 0.0	–
16	α-Gurjenene	1408	1409	–	0.1 ± 0.2
17	(*E*)-Caryophyllene	1421	1417	27.4 ± 0.6	1.1 ± 0.0
18	Aromadendrene	1439	1439	–	0.4 ± 0.0
19	*allo*-Aromadendrene	1453	1458	–	0.3 ± 0.0
20	*dehydro*-Aromadendrene	1461	1460	–	1.4 ± 0.1
21	α-Humulene	1463	1452	5.7 ± 0.0	–
22	Germacrene D	1482	1484	17.1 ± 0.2	5.5 ± 0.0
23	Viridiflorene	1492	1496	0.5 ± 0.6	0.3 ± 0.0
24	Bicyclogermacrene	1498	1500	10.8 ± 0.1	39.0 ± 0.3
25	Germacrene A	1517	1508	0.6 ± 0.0	–
26	δ-Amorfene	1518	1511	–	0.4 ± 0.0
27	γ-Cadinene	1521	1513	4.2 ± 0.0	–
28	δ- Cadinene	1525	1522	2.2 ± 0.0	–
29	Germacrene B	1564	1559	1.7 ± 0.0	–
30	Spathulenol	1579	1577	0.2 ± 0.3	14.0 ± 1.0
31	Caryophyllene Oxide	1586	1582	0.9 ± 0.0	–
32	Globulol	1588	1592	–	0.5 ± 0.0
33	*epi*-α-Cadinol	1643	1638	4.3 ± 0.1	–
34	α-Cadinol	1657	1652	0.7 ± 0.0	–
	Monoterpene identified			2.0	34.0
	Sesquiterpene identified			95.5	65.1
	Total			97.5	99.1

Note: RI[a] (calc.), retention indices on DB-5MS column calculated according to (9). RI[b] retention indices according to (10).

Table 2. Cytotoxicity against macrophages, effect on erythrocitic stages of *Plasmodium falciparum*, and trypanocidal action against trypomastigote and epimastigote forms of *Trypanosoma cruzi* of the essential oils of *Annona vepretorum* (AVOE) and *Annona squamosa* (ASOE).

	LC_{50} (μg/mL)	IC_{50} (μg/mL) Pla.	IC_{50} (μg/mL) Try.	IC_{50} (μg/mL) Epi.
AVOE	39.7 (± 0.6)	9.9 (± 0.7)	11.2 (± 0.5)	16.2 (± 1.2)
ASOE	28.8 (± 0.9)	14.7 (± 2.9)	12.7 (± 0.5)	14 9 (± 0;7)
Benznidazole[a]	–	–	2.7 (± 0.5)	2.7 (± 0.7)
Mefloquine[b]	–	0.02 (± 0.0)	–	–
Gentian violet[c]	0.19 (± 0.0)	–	–	–

Note: Values are means (± SD) of two independent experiments. [a]Positive control against epimastigote (Epi.) and trypomastigote (Try.) forms of *T. cruzi.* [b]Positive control against *P. falciparum* (Pla.). [c]Positive control against mammalian cells.

several of the constituents present in this study are in agreement with Costa et al. (6), some compounds are described for the first time, such as *o*-cymene, limonene, (*Z*)-β-ocimene, linalool, δ-elemene, α-copaene, α-gurjenene, and *dehydro*-aromadendrene that contributes to the chemical knowledge of the essential

Downloaded by [152.245.207.5] at 15:09 01 March 2015

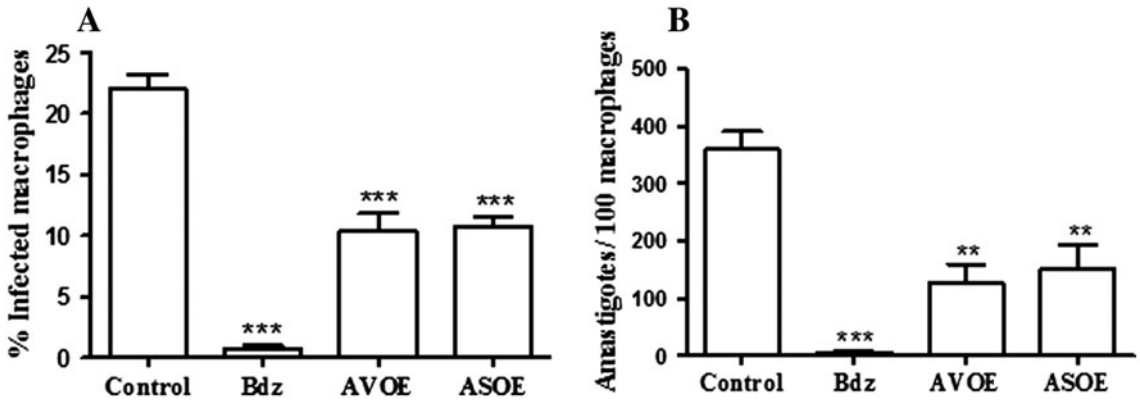

Figure 1. *Annona* oils inhibited *Trypanosoma cruzi* amastigotes proliferation in macrophages at a concentration of 10 μg/mL. Mouse peritoneal macrophages were infected with Y strain trypomastigotes for 2 hours and treated with the essential oils *Annona vepretorum* (AVOE) and *Annona squamosa* (ASOE) (10 μg/mL) or benznidazole (10 μg/mL), a standard drug. Cells were stained with hematoxylin and eosin and analyzed by optical microscopy. The percentage of infected macrophages (A) and the relative number of amastigotes per 100 macrophages (B) are higher in untreated infected controls than in cultures treated with the essential oils AVOE and ASOE. Bdz, benznidazole. Values represent the mean ± SEM of triplicates. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the control group.

Downloaded by [152.245.207.5] at 15:09 01 March 2015

oil of AVOE. Recently, Garg and Gupta (17) reported the chemical composition of the ASOE leaf oil from the northern India plains and observed that the major constituents were (*E*)-caryophyllene (22.9%), germacrene D (21.3%), bicyclogermacrene (8.5%), β-elemene (7.8%), γ-cadinene (6.7%), α-muurolol (5.7%), and aromadendrene (4.8%). The results from our present study were very similar to those reported by Garg and Gupta (17) except for the presence of the other major compounds (*Z*)-caryophyllene (7.3%), α-humulene (5.7%), *epi*-α-cadinol (4.3%) and δ-elemene (4.1%) that can be explained by the same reason observed in AVOE. However, all constituents identified in ASOE and AVOE are commonly found in Annonaceae species. The presence of the compounds bicyclogermacrene, (*E*)-caryophyllene and germacrene D in high concentrations has been observed in almost all *Annona* essential oils investigated and could be considered chemotaxonomic markers (6, 7).

As the chemical composition of the essential oils AVOE and ASOE was elucidated, their antimalarial and trypanocidal activities were evaluated. The essential oils displayed potent antimalarial activity against erythrocitic stages of *P. falciparum* and trypanocidal action against *T. cruzi* epimastigotes and trypomastigotes. As shown in Table 2, AVOE and ASOE demonstrated IC_{50} values of 9.9 and 14.7 μg/mL, respectively, against *P. falciparum*, IC_{50} values of 16.2 and 14.9 μg/mL, respectively, against epimastigotes forms of *T. cruzi* and IC_{50} values of 11.2 and 12.7 μg/mL, respectively, against trypomastigotes forms of *T. cruzi*.

Next, essential oils cytotoxicity to mouse macrophages was analyzed. AVOE and ASOE exhibited LC_{50} values of 39.7 and 28.8 μg/mL respectively (Table 2), therefore being several times less cytotoxic in comparison with gentian violet (LC_{50} = 0.19 μg/mL), the reference drug in this assay, and presenting a selective cytotoxicity against parasites. Given the selectivity of AVOE and ASOE against extracellular forms of *T. cruzi,* we assayed an *in vitro* model of mouse macrophages infected with Y strain trypomastigotes to evaluate the ability of these essential oils against intracellular amastigotes, the chronic clinically relevant forms of *T. cruzi*. In this assay, cells previously infected were incubated with 10 μg/mL of AVOE or ASOE or benznidazole for 72 hours. After treatment, cells were stained with hematoxylin and eosin, and observed by optical microscopy in order to evaluate the number of infected macrophages and the relative number of amastigotes per 100 macrophages. As shown in Figure 1, both essential oils significantly reduced the number of infected macrophages ($p < 0.001$) and the relative number of amastigotes per 100 macrophages ($p < 0.01$), showing a strong inhibition of the proliferation of amastigotes. Benznidazole was more effective than the essential oils, nearly eradicating the proliferation of amastigotes. Although the essential oils were less effective than benznidazole or mefloquine in the biological assays, it is important to say that these reference drugs are highly toxic to hosts and showing a variable efficacy in several strains that have some degree of resistance to these drugs (18, 19). Furthermore, results presented in this study are considered very promising when compared with other results of Annonaceae essential oils with trypanocidal and antimalarial activity (6, 16, 20).

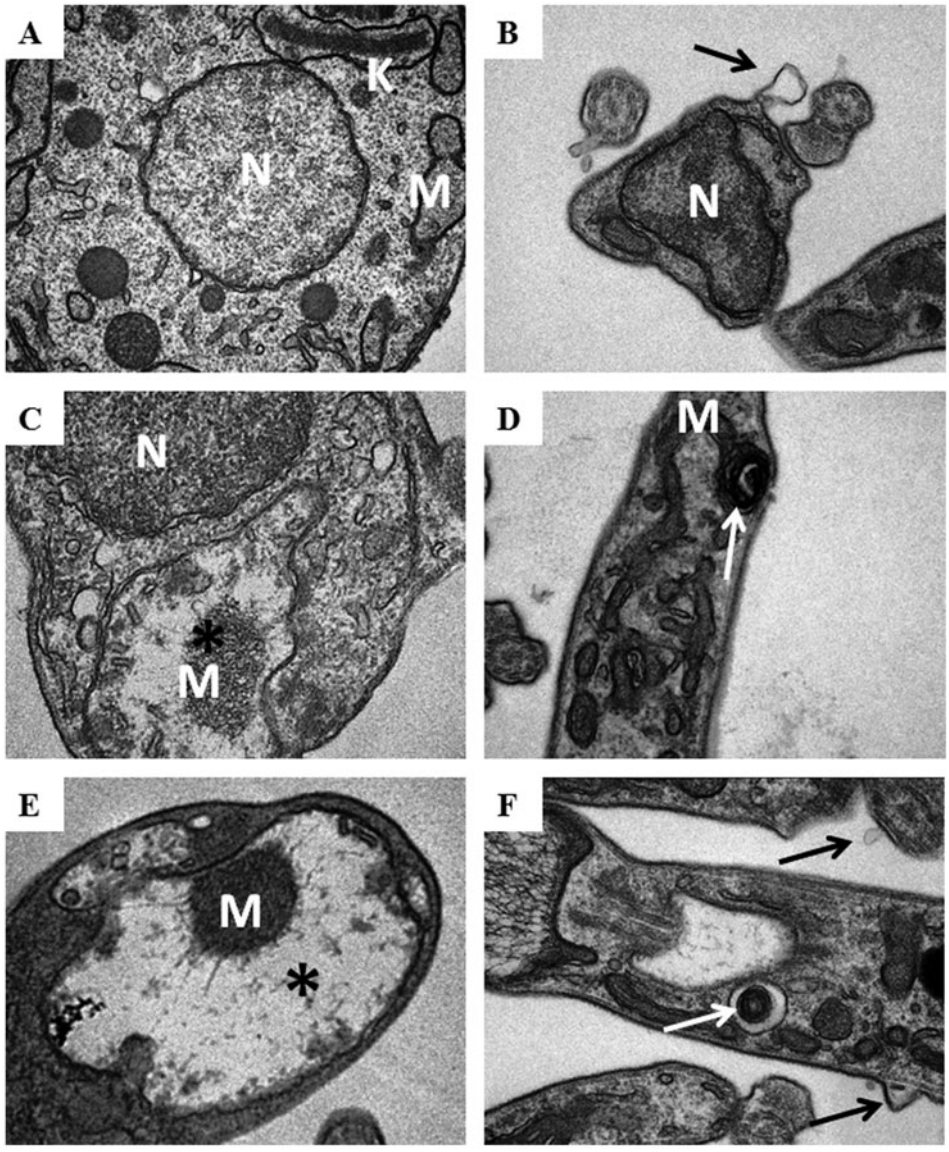

Figure 2. Transmission electron microscopy (TEM) of trypomastigotes treated with *Annona vepretorum* (AVOE) and *Annona squamosa* (ASOE) for 24 hours. (A) Untreated trypomastigotes, kinetoplast (K), mitochondrion (M) and nucleous (N); (B, C) trypomastigotes treated with AVOE (11.2 μg/mL) showing alterations in parasite plasma membrane (black arrow) and intense swelling mitochondrial (asterisk) accompanied by a large loss of density (D) trypomastigotes treated with AVOE (22.4 μg/mL) showing myelin-figures near mitochondria (white arrow); (E) trypomastigotes treated with ASOE (12.7 μg/mL) showing intense swelling mitochondrial (asterisk) accompanied by a large loss of density; (F) trypomastigotes treated with ASOE (25.4 μg/mL) showing myelin-figures near mitochondria (white arrow) and alterations in parasite plasma membrane (black arrow). Bars = 0.5 μm.

After confirming that AVOE and ASOE were able to kill parasites, our next step was to understand how they affect parasite cells through assays with trypomastigotes forms of *T. cruzi*, once the trypanocidal activity of species of Annonaceae is better documented than the antimalarial activity. For this purpose, we used TEM to examine the ultrastructural morphology of trypomastigotes (Y strain) treated with AVOE or ASOE for 24 hours. As we can see in Figure 2, thin sections of untreated trypomastigotes observed by TEM revealed normal appearance of organelles, intact plasma membrane and parasite cytoplasm without alterations (Figure 2A). However, the treatment with AVOE (11.2 or 22.4 μg/mL) or ASOE (12.7 or 25.4 μg/mL) caused plasma membrane alterations (Figures 2B and 2F) and intense swelling mitochondrial accompanied by a large loss of electron density of its matrix (Figures 2C and 2E). We also observed the presence of myelin-like figures near mitochondria (Figure 2D and 2F). Interestingly, similar alterations in the mitochondria were

Downloaded by [152.245.207.5] at 15:09 01 March 2015

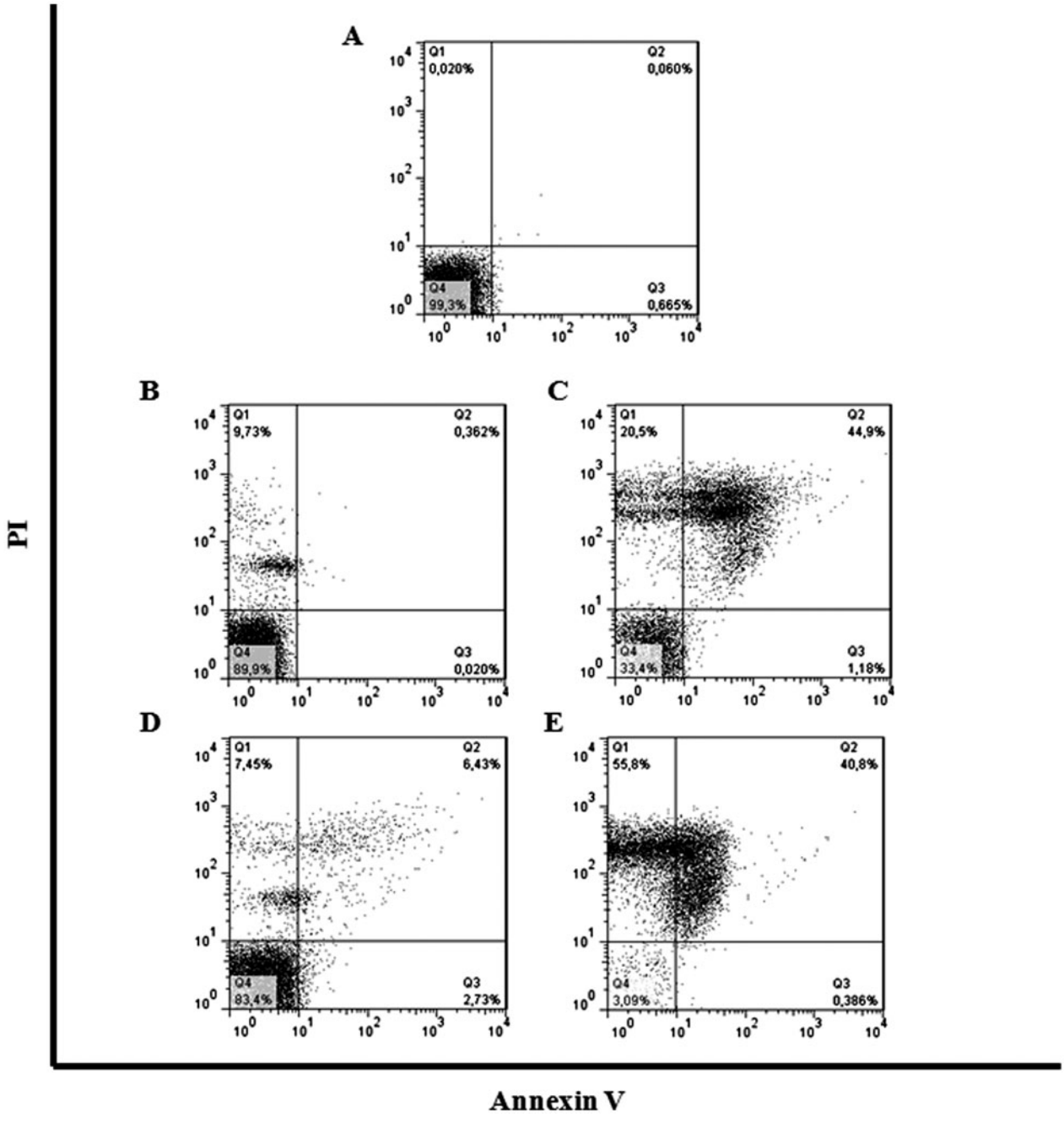

Figure 3. Flow cytometry analysis of trypomastigotes treated with *Annona vepretorum* (AVOE) and *Annona squamosa* (ASOE) and incubated with propidium iodide (PI) and annexin V. (A) Untreated trypomastigotes; (B) trypomastigotes treated with AVOE (11.2 μg/mL); (C) trypomastigotes treated with AVOE (22.4 μg/mL); (D) trypomastigotes treated with ASOE (12.7 μg/mL); (E) trypomastigotes treated with ASOE (25.4 μg/mL).

found when *T. cruzi* were incubated with IC-195,739 or 22,26-azasterol, which are know sterol biosynthesis inhibitors (21, 22). Alterations in cell membrane and the appearance of concentric membranous structures have also been previously reported after treatment with sterol biosynthesis inhibitors (23, 24). This might be an indicative that the trypanocidal activity of these essential oils, in part, can be assigned to an interference in the lipid metabolism. Finally, to understand the mechanism by which AVOE and ASOE cause parasite death, a double staining with annexin V and PI was performed for flow cytometry analysis. Eventual phosphatidylserine flipping in the membrane surface is an alteration observed in the apoptotic process and a

Downloaded by [152.245.207.5] at 15:09 01 March 2015

collapse of the cell, making it permeable to PI, is an alteration observed in the process of necrosis (25). As shown in Figure 3, in untreated cultures, most parasites were negative for annexin V and PI staining, demonstrating cell viability. In comparison with untreated parasites, a significant increase in the number of PI-positive parasites was observed under treatment with AVOE (11.2 or 22.4 μg/mL) or ASOE (12.7 or 25.4 μg/mL). Parasites treated with AVOE (22.4 μg/mL) for 72 hours showed 20.5% and 44.9% of parasites positively stained for PI alone and PI + annexin V, respectively; whereas 1.18% parasites cells were stained only for annexin V. Therefore, the treatment with these essential oils increases the number of PI staining, which is characteristic of parasite cell death through necrosis induction.

In conclusion, our results show that the essential oils AVOE and ASOE are potent agents against *P. falciparum* and *T. cruzi*, exhibiting significant selectivity for these parasites. In addition, through ultrastructural studies and flow cytometry analysis with trypomastigotes of *T. cruzi* (Y strain), we identified significant ultrastructural alterations induced by the essential oils, especially in the cell membrane and mitochondria, which ultimately results in necrotic parasite death.

Downloaded by [152.245.207.5] at 15:09 01 March 2015

Acknowledgements

The authors are grateful to FAPITEC/SE (Editais N° 07/2009 and 10/2009), FAPESB, CNPq and CAPES for financial support, and Professor Dr. Ana Paula do Nascimento Prata of the DBI/UFS for the botanical identification.

References

1. F. Scala, E. Fattorusso, M. Menna, O. Taglialatela-Scafati, M. Tierney, M. Kaiser and D. Tasdemir, *Bromopyrrole alkanoids as lead compounds against protozoan parasites*. Marine Drugs, **8**, 2162–2174 (2010).
2. M. Moreno, D.A. D'avoça, M.N. Silva, L.M. Galvão, A.M. Macedo, E. Chiari, E.D. Gontijo and B. Zingales, *Trypanosoma cruzi benznidazole susceptibilty in vitro does not predict the therapeutic outcome of human Chagas disease*. Mem. Inst. Oswaldo Cruz, **105**, 918–24 (2010).
3. World Health Organization, Malaria Global Programme. http://www.who.int/mediacentre/factsheers/fs094/en/index.html (30 March 2011).
4. L.W. Chatrou, M.D. Pirie, R.H.J. Erkens, T.L.P. Couvreur, K.M. Neubig, J.R. Abbott, J.B. Mols, J.W. Maas, R.M.K. Saunders and M.W. Chase, *A new subfamilial and tribal classification of the pantropical flowering plant family Annonaceae informed by molecular phylogenetics*. Bot. J. Linnean Soc., **69**, 5–40 (2012).
5. F.Q. Alali, X.X. Liu and J.L. Mclaughlin, *Annonaceous acetogenins: Recent progress*. J. Nat. Prod., **62**, 504–540 (1999).
6. E.V. Costa, L.M. Dutra, P.C.L. Nogueira, V.R.S. Moraes, M.J. Salvador, L.H.G. Ribeiro and F.R. Gadelha, *Essential oil from the leaves of Annona vepretorum: Chemical composition and bioactivity*. Nat. Prod. Commun., **7**, 265–266 (2012).
7. E.V. Costa, L.M. Dutra, M.J. Salvador, L.H.G. Ribeiro, F.R. Gadelha and J.E. Carvalho, *Chemical composition of the essential oils of Annona pickelii and Annona salzmannii (Annonaceae), and their antitumour and trypanocidal activities*. Nat. Prod. Res., **27**, 997–1001 (2013).
8. M.P. Corrêa, *Dicionário das plantas úteis do Brasil e das exóticas cultivadas*. IBDF, Rio de Janeiro (1984).
9. H. Van Den Dool and P.D. Kratz, *A generalization of the retention index system including linear temperature programmed gas–liquid partition chromatography*. J. Chromatogr., **11**, 463–471 (1963).
10. R.P. Adams, *Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. Allured Publishing Corp., Carol Stream, IL (2007).
11. E.P. Camargo, *Growth and differentiation in* Trypanosoma cruzi*. Origin of metacyclic trypanosomes in liquid media*. Rev. Inst. Med. Trop. São Paulo, **6**, 93–100 (1964).
12. M.N. Meirelles, T.C. Araújo Jorge and W. Souza, *Interaction of* Trypanosoma cruzi *with macrophages* in vitro*: Dissociation of the attachment and internalization pahses by low temperature and cytochalasin B*. Z. Parasit., **68**, 6–14 (1982).
13. W. Trager and J.B. Jensen, *Human malaria parasites in continuous culture. 1976*. J. Parasitol., **91**, 484–486 (2005).
14. C.S. Meira, E.T. Guimarães, T.M. Bastos, D.R. Moreira, T.C. Tomassini, I.M. Ribeiro, R.R. Santos and M.B.P. Soares, *Physalins B and F, seco-steroids isolated from Physalis angulata L., strongly inhibit proliferation, ultrastructure and infectivity of* Trypanosoma cruzi. Parasitology, **140**, 1811–1821 (2013).
15. R. Desjardins, C. Canfield, J. Haynes and J. Chulay, *Quantitative assessment of antimalarial activity* in vitro *by a semiautomated microdiluition technique*. Antimicrob. Agents Chemother., **16**, 710–718 (1979).
16. T.B. Silva, L.R.A. Menezes, M.F.C. Sampaio, C.S. Meira, E.T. Guimarães, M.B.P. Soares, A.P.N. Prata, P.C.L. Nogueira and E.V. Costa, *Chemical composition and anti-*Trypanosoma cruzi *activity of essential oils obtained from leaves of* Xylopia frutescens *and* X. laevigata *(Annonaceae)*. Nat. Prod. Commun., **8**, 403–406 (2013).
17. S.N. Garg and D. Gupta, *Composition of the leaf oil of Annona squamosa L. from the North Indian plains*. J. Essent. Oil Res., **17**, 257–258 (2005).
18. M. Schlitzer, *Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics and drugs in clinical development*. ChemMedChem, **7**, 944–986 (2007).
19. E. Izumi, T. Ueda-Nakamura and B.P. Dias Filho, V.F. Veiga Júnior and C.V. Nakamura, *Natural products and Chagas' disease: A review of plant compounds studied for activity against* Trypanosoma cruzi. Nat. Prod. Rep., **28**, 809–823 (2011).
20. F.F. Boyom, V. Ngouana, P.H.A. Zollo, C. Menut, J.M. Bessiere, J. Gut and P.J. Rosenthal, *Composition and anti-plasmodial activities of essential oils from some Cameroonian medicinal plants*. Phytochemistry, **64**, 1269–1275 (2003).
21. K. Lazardi, J.A. Urbina and W. De Souza, *Ultrastructural alterations induced by ICI 195,739, a bis-triazole derivative with strong antiproliferative action against* Trypanosoma (Schizotrypanum) cruzi. Antimicrob. Agents Chemother., **35**, 736–40 (1991).

22. J. Vivas, J.A. Urbina and W. De Souza, *Ultrastructural alterations in* Trypanosoma (Schizotrypanum) cruzi *induced by D24(25)-sterol methyltransferase inhibitors and their combinations with ketoconazole*. Int. J. Antimicrob. Chemother., **7**, 235–40 (1996).
23. M.V. Braga, J.A. Urbina and W. De Souza, *Effects of squalene synthase inhibitors on the growth and ultrastructure of* Trypanosoma cruzi. Int. J. Antimicrob. Agents, **24**, 72–78 (2004).
24. R.M. Santa-Rita, R. Lira, H.S. Barbosa, J.A. Urbina and S.L. Castro, *Anti-proliferative synergy of lysophospholipid analogues and ketoconazole against* Trypanosoma cruzi *(kinetoplastida: Trypanosomatidae): Cellular and ultrastructural analysis*. J. Antimicrob. Chemother., **55**, 780–784 (2005).
25. A.L. Matsuo, L.S. Silva, A.C. Torrecilhas, B.S. Pascoalino, T.C. Ramos, E.G. Rodrigues, S. Schenkaman, A.C. Caires and L.R. Travassos, In vitro *and* in vivo *trypanocidal effects of the cyclopalladated compound 7a, a drug candidate for treatment of Chagas disease*. Antimicrob. Agents Chemother., **54**, 3318–3325 (2010).

Downloaded by [152.245.207.5] at 15:09 01 March 2015