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Monoterpenic aldehydes as potential anti-*Leishmania* agents: Activity of *Cymbopogon citratus* and citral on *L. infantum*, *L. tropica* and *L. major*

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ABSTRACT

In order to contribute for the search of new drugs for leishmaniasis, we study the susceptibility of *Leishmania infantum*, *Leishmania tropica* and *Leishmania major* to *Cymbopogon citratus* essential oil and major compounds, mrycene and citral. *C. citratus* and citral were the most active inhibiting *L. infantum*, *L. tropica* and *L. major* growth at IC_{50} concentrations ranging from 25 to 52 μg/ml and from 34 to 42 μg/ml, respectively. *L. infantum* promastigotes exposed to essential oil and citral underwent considerable ultrastructural alterations, namely mitochondrial and kinetoplast swelling, autophagosomal structures, disruption of nuclear membrane and nuclear chromatin condensation. *C. citratus* essential oil and citral promoted the leishmanicidal effect by triggering a programmed cell death. In fact, the leishmanicidal activity was mediated via apoptosis as evidenced by externalization of phosphatidylserine, loss of mitochondrial membrane potential, and cell-cycle arrest at the G(0)/G(1) phase. Taken together, ours findings lead us to propose that citral was responsible for anti-*Leishmania* activity of the *C. citratus* and both may represent a valuable source for therapeutic control of leishmaniasis.

1. Introduction

Leishmania, a unicellular trypanosomatid protozoan parasite, is the causative organism of leishmaniasis, which comprises a wide disease spectrum ranging from localized, self-healing, cutaneous lesions to disfiguring mucocutaneous leishmaniasis and the visceral form, which can be fatal if neglected (Murray et al., 2005; WHO, 2009; Postigo, 2010). In the past decade, unresponsiveness to antimonials, the first line of treatment, has increased substantially in visceral leishmaniasis, mainly at endemic areas like India (Croft et al., 2006; Natera et al., 2007). Amphotericin B, pentamidine and miltefosine have been used as alternative drugs. Current treatments are limited, have the potential to develop resistance, are expensive, are long length and possess unacceptable toxicity (Leandro and Campino, 2003).

In the ongoing search for better leishmanicidal compounds, plant-derived products are gaining ground (Anthony et al., 2005; Sen et al., 2010). Essential oils, plant extracts prepared by distillation, are composed by a huge diversity of small hydrophobic molecules, most of them accomplishing theoretical criteria's of druglikeness prediction (Lipinski et al., 1997). Such molecules easily diffuse across cell membranes and consequently gain advantage in what concerns to interactions with intracellular targets, being a valuable research option for the search of anti-*Leishmania* leads and drugs (Edris, 2007).

C. citratus (DC) *Stapf*, Family *Poaceae*, is a widely used herb in tropical countries namely on Southeast Asia, African and South America countries and is also known as a source of ethnomedicines. *C. citratus* is commonly used in folk medicine in Angola for the treatment of gastrointestinal disturbances, and as an antispasmodic, anti-inflammatory, anti-pyretic, and diuretic. Some studies have demonstrated its antimicrobial activity, namely antibacterial, antifungal, and antiprotozoa properties (Santoro et al., 2007a,b;

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Mayaud et al., 2008; Irkin and Korukluoglu, 2009; Oliveira et al., 2009).

However, there are few reports on the activity of essential oils on endemic *Leishmania* species responsible for cutaneous and visceral leishmaniasis of the old world. So, the present work we focused on the leishmanicidal activity of *C. citratus* and major compounds, myrcene and citral, on three old world *Leishmania* species, namely*Leishmania infantum, Leishmania tropica* and *Leishmania major*. Additionally, we undertake other essays to demonstrate the safety of the essential oil and compounds and elucidate the mechanisms that contribute to leishmanicidal activity.

2. Material and methods

2.1. Plant material

2.1.1. Origin

Plant material from *C. citratus* was obtained from a local market in Luanda, Angola. The plants were identified by a taxonomist (Dr. Jorge Paiva, University of Coimbra), and voucher specimens (Cabo S. Vicente COI00033066; Arrifana COI00033067) were deposited at the Herbarium of the Department of Botany of the University of Coimbra (COI).

2.1.2. Essential oil

The essential oil from the aerial parts *C. citratus* (DC) Stapf was isolated by water distillation for 3 h from air dried material, using a Clevenger-type apparatus, following the procedure described in the European Pharmacopoeia (Council of Europe, 1997).

2.1.3. Essentials oils analysis

Analysis was carried out by gas chromatography (GC) and by gas chromatography-mass spectroscopy (GC/MS). Analytical GC was carried out in a Hewlett–Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detection (FID) systems. A graphpak divider (Agilent Technologies, part No. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 and SupelcoWax-10. GC–MS was carried out in a Hewlett–Packard 6890 gas chromatograph fitted with a HP1 fused silica column, interfaced with an Hewlett–Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. Components of each essential oil were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C_8–C_{23} of *n*-alkanes, were compared with those of authentic samples included in our own laboratory database. Acquired mass spectra were compared with reference spectra from our own database; Wiley/NIST database (Wiley, 2007) and literature data (Joulain and Konig, 1998; Adams, 2004). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

2.2. Parasites and cultures

Promastigote forms of *L. infantum* Nicolle (zymodeme MON-1), *L. tropica* (ATCC 50129) and *L. major* BCN were maintained at 26 °C by weekly transfers in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated fetal bovine serum (FBS). These cells were used to study the effects of essential oils on *Leishmania* promastigotes growth.

2.3. Viability assays

Essential oil and major compounds (citral and myrcene) were initially diluted in dimethyl sulfoxide (DMSO; Sigma Chemical) at 100 mg mL^{-1} and then in culture medium in order to get a range of concentrations from 10 to 400 μg mL^{-1}. Log phase promastigotes of *L. infantum*, *L. tropica* and *L. major* (10^6 cells/ml) were incubated in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated FBS in the presence of different concentrations of essential oil and compounds or DMSO (vehicle control) at 26 °C. Effects on viability were estimated by tetrazolium-dye (MTT) colorimetric method (Monzote et al., 2007).The concentration that inhibited viability by 50% (IC_{50}) was determined after 24 h for *L. infantum* and *L. tropica* and after 48 h for *L. major*, through dose–response regression analysis, plotted by GraphPad Prism 5.

2.4. Transmission and scanning electron microscopy

L. infantum promastigotes were exposed to essential oil and citral at concentrations that inhibit viability by 50% (IC_{50}) and the morphological alterations were investigated by electronic microscopy. For ultrastructural studies with transmission electronic microscopy, the samples were treated as reported previously (Sousa et al., 2001). Briefly, cell were fixed with glutaraldehyde in sodium cacodylate buffer, post fixed in osmium tetroxide and uranyl acetate, dehydrated in ethanol and in propylene oxide and embedded in Epon 812 (TAAB 812 resin). Ultrathin sections were stained with lead citrate and uranyl acetate. For Scanning electronic microscopy, the samples were fixed and postfixed as described for transmission, dehydrated in ethanol, critical point dried using CO_2 and sputter-coat with gold. The specimens were examined in JEOL JEM-100 SX transmission electron microscopy (TEM) at 80 kV and in JEOL JSM-5400 scanning electron microscope (SEM) at 15 kV.

2.5. Flow cytometry

2.5.1. Cell cycle analysis

For flow cytometry analysis of DNA content, exponentially grown *L. infantum* promastigote cells (10^6) were treated with *C. citratus* essential oil and citral at IC_{50} concentrations for 3h, 5h, 7h, and 24 h at 26 °C. Promastigote suspension was then fixed in 200 μl of 70% ethanol for 30 min. at 4 °C. Next, cells were washed in PBS, and resuspended in 500 μl of PI solution (PI/Rnase, Immunostep) for 15 min. at room temperature (Darzynkiewicz et al., 2001). Cells were then analyzed by flow cytometry (Facs Calibur–Beckton–Dickinson). Results were treated using ModFit LT V 2.0 programme.

2.5.2. Analysis of phosphatidylserine externalization

Double staining for annexin V-FITC and propidium iodide (PI) was performed as described previously (Vermes et al., 1995). Briefly, *L. infantum* promastigotes (10^6 cells) were exposed to essential oil and citral at IC_{50} concentrations for 3 h, 5 h, 7 h, and 24 h at 26 °C. Cells were then washed with PBS and ressuspended in binding buffer (10 mM HEPES–NaOH, pH 7.4, 140 NaC1, 2.5 mM $CaCl_2$). To 100 μl of this suspension were added 5 μl of Annexin V FITC and 5 μl of PI (AnnexinV-FITC Apoptosis detection Kit, Immunostep). After 15 min incubation in the dark at room temperature, it was added 400 μl binding buffer and cells were then analyzed by flow cytometry (Facs Calibur–Beckton–Dickinson). Data analysis was carried out using the program Paint-a-gate, and values are expressed as a percentage of positive cells for a given marker, relatively to the number of cells analyzed.