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Activity of essential oils on the growth of *Leishmania infantum* promastigotes†

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ABSTRACT: In order to contribute to the search for new therapeutic agents for leishmaniasis, we report the effect of several essential oils on the growth of *Leishmania infantum* promastigotes. Eight of the tested essential oils revealed activity below 150 μg/ml, the most active being *Cymbopogon citratus*, *Juniperus oxycedrus* berries and *Thymus capitelatus* oils, with 50% effective concentration values in the range 16–51 μg/ml. The results support the concept that several essential oils or some of their constituents can become useful in the research of new therapeutic agents for leishmaniasis and in the clinical management of this parasitic disease. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: *Leishmania infantum*; essential oils; activity assay; human parasitoses; natural products

Introduction

Protozoan parasites of the genus *Leishmania* cause visceral, cutaneous and mucosal diseases in humans, which are collectively referred as leishmaniasis, affecting more than 12 million people worldwide and being responsible for high rates of mortality in tropical and subtropical countries.[1,2] At least 13 identified *Leishmania* species are able to infect humans, each one with its unique epidemic pattern and clinical manifestations of infection. Nevertheless, from a generic point of view, there are three main typical manifestations of leishmaniasis: visceral leishmaniasis (*L. donovani*, *L. infantum*); cutaneous leishmaniasis (*L. major*, *L. tropica*); and mucocutaneous leishmaniasis.[3] In all cases, the leishmanial infection is transmitted to the vertebrate hosts by the bite of a sandfly.

The number and efficacy of drugs available for the treatment of human and animal leishmaniasis are limited. The treatment of choice, in spite of its toxicity, is still based on the use of pentavalent antimonial drugs or, alternatively, pentamidine and amphotericin B.[3,5] Considering the toxicity, side-effects, rate of relapse, cost, length of the treatment and the resistance that the parasites show to these drugs, more attention should be given to the search for new chemotherapeutics. One of the major obstacles to develop a good anti-*Leishmania* drug is the protection of parasites inside macrophages, requiring compounds effective on the parasite but preserving the host cell.[4,6,7]

Screening plant extracts is a valuable research option for the search of anti-*Leishmania* leads and drugs.[8] Plant extracts offer a huge diversity of compositions and constituents, most of them commercially unavailable and structurally difficult to synthesize; several plant extracts have already been tested for anti-*Leishmania* activity, although most of them are aqueous or alcoholic extracts and so are exclusively composed of polar molecules.[9] However, other kind of extracts, prepared by distillation (essential oils), composed of a huge diversity of small (<300 Da) hydrophobic molecules, most of them accomplishing theoretical criteria of drug-likeness prediction,[10] offer peculiar advantages and expectations. Such molecules easily diffuse across cell membranes and consequently gain advantage with regard to interactions with intracellular targets.[11] Our previous research showed the potential of essential oils as natural antimicrobial agents, particularly against yeasts, filamentous fungi and also flagellated protozoa, as the intestinal parasite *Giardia lamblia*.[12–25]

Rosa *et al*.[26] and Ueda-Nakamura *et al*.[27] reported the effects of the essential oils from *Croton cajucara* and *Ocimum gratissimum* on *L. amazonensis*. Monzote *et al*.[28–31] revealed the activity of *Chenopodium ambrosioides* against *Leishmania amazonensis* and *L. donovani*. The effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham. and *Ocimum gratissimum* L. on *Leishmania chagasi* were investigated by Oliveira *et al*.[32] As there are few reports on the activity of essential oils on endemic Old World *Leishmania* species, in the present work we focused on the screening of a set of 19 essential oils for their effects on *L. infantum* promastigotes, of one of those Old World species.

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Materials and Methods

Essential Oils

Essential oils from the aerial parts of *Crithmum maritimum* L., *Cymbopogum citratus* (DC) Stapf., *Distichoselinum tenuifolium* (Lag.) García Martín & Silvestre, *Eryngium maritimum* L., *Lavandula viridis* L'Hér., *Lippia graveolens* H.B.K., *Mentha cervina* L., *M.* × *piperita* L., *Origanum virens* Hoffmanns. & Link, *Rosmarinus officinalis* L., *Seseli tortuosum* L., *Thymbra capitata* (L.) Cav., *Thymus capitelatus* Hoffmanns. & Link, *T. mastichina* L. and *T. zygis* Loefl. Ex L. subsp. *sylvestris* (Hoffmanns & Link) Brot. ex Coutinho (two chemotypes), *Lavandula viridis* L'Hérand from the leaves, and from the berries of *Juniperus oxycedrus* L. were prepared at our laboratory (CEF, Coimbra) by water distillation, using a Clevenger-type apparatus and following the procedure described in the *European Pharmacopoeia* (1997).[33] The oil from the buds of *Syzygium aromaticum* (L.) Merr. & Perry was acquired from Segredo da Planta (Portugal).

Essential oil analysis. Analysis was carried out by gas chromatography (GC) and 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 (Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm); oven temperature programme, 70–220°C at 3°C/min, then held at 220°C for 15 min; injector temperature, 250°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; detector temperature, 250°C. GC–MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies), operated by HP Enhanced ChemStation software, version A.03.00. GC parameters were as described above; interface temperature, 250°C; MS source temperature, 230°C; MS quadrupole temperature, 150°C; ionization energy, 70 eV; ionization current, 60 μA; scan range, 35–350 units; scans/s, 4.51.

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} *n*-alkanes, were compared with those of reference samples included in our laboratory database. Acquired mass spectra were compared with reference spectra from our own database, Wiley/NIST database[34] and literature data.[35,36] Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

Parasites and Cultures

Promastigote forms of *L. infantum* Nicolle (zymodeme MON-1) were maintained at 26°C by weekly transfers in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated fetal bovine serum (FBS).

Growth Inhibition Assays

Essential oils were dispersed in dimethyl sulphoxide (DMSO; Sigma Chemical) at 100 mg/ml and then diluted to 1 mg/ml with culture medium (RPMI 1640). Appropriated volumes of these solutions were incorporated afterwards to assays to give final oil concentrations in the range 10–400 μg/ml. The DMSO concentration never exceeded 0.4%, which was proved to be not toxic for the protozoa. Promastigotes of *L. infantum* (10^6 cells/ml) in the log phase of growth were incubated in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated fetal bovine serum (FBS) in the absence or presence of different concentrations of essential oils and DMSO at 26°C.

The inhibitory effect on cell growth was then estimated by the tetrazolium-dye (MTT) colorimetric method.[37] The concentration that inhibited culture growth by 50% (IC_{50}) was determined after 24 h by dose–response regression analysis, plotted by GraphPad Prism 5.

Cytotoxicity Assay

The cytotoxic effects on mammalian cells of the most active essential oils were assessed with bovine aortic endothelial cells (primary culture), incubated under microaerophilic conditions in the presence of the essential oil at *Leishmania*'s inhibitory concentration. The tetrazolium-dye colorimetric assay[37] was used to detect living cells which have the ability to reduce yellow 3-(4,5-dimethylthiazole-2-yl)-2,5 (MTT) to a blue formazan product. Briefly, the cells were cultivated in 24-well tissue culture plates (500 μl, containing 10^6 cells/ml in RPMI 1640 medium/DMEM medium, supplemented with 10% FBS) at 37°C and 5% CO_2 atmosphere. When the macrophages reached log phase (3–4 days), the medium was removed and the cells were then incubated with fresh medium added of essential oil, or with fresh medium as the control. After further incubation for 14 h, control and treated cells were washed three times with PBS, pH 7.2. 50 μl MTT solution (5 mg/ml in PBS) and 450 μl PBS were added to each well. After 1 h incubation at 37°C, the cells were then washed three times with PBS. 500 μl DMSO was added to the wells and the optical density was measured at 530 nm. Cell viability was determined using the following formula: $[100 - (L2/L1) \times 100]$, where *L1* is the percentage of viable control cells and *L2* is the percentage of viable treated cells, as previously described.

Statistical Analysis

All experiments were performed in triplicate. The mean and standard deviation (SD) of at least six independent assays were determined. Statistical analysis between mean values obtained for the experimental groups was done by Student's *t*-test. $p \leq 0.05$ was considered significant.

Results and Discussion

Chemical diversity and compositional data of the 19 tested essential oils are summarized in Table 1. The compositions were extensively elucidated, attaining identification rates > 90%, except for *Eryngium maritimum* oil, for which only 71.8% of the composition was elucidated. In general, compositions are analogous to those typical of the oils from each source species and type. In this set of 19 essential oils, >100 different compounds were identified, representing the major chemical families reported for this kind of plant extract – monoterpene

Table 1. Global composition and major constituents of the tested essential oils. (Information merely indicative)

	Monoterpene hydrocarbons	Oxygen–containing monoterpenes	Sesquiterpene hydrocarbons	Oxygen–containing sequiterpenes	Phenylpropanoids	Aliphatic compounds	Diterpenoids	*Identified: compounds (n)*/total (%)	Major compounds (>2.0%)
Crithmum maritimum	74.2	7.4	2.0	0.3	13.8	0.6	–	(31)/98.3	α-Pinene (2.1%), *p*-cymene (7.1%), β-phellandrene (8.0%), *Z*-β-ocimene (5.2%), γ-terpinene (48.7%), thymyl-methyl oxide (6.3%), dillapiole (13.8%)
Cymbopogon citratus	6.7	88.6	t	t	–	1.9	–	(16)/97.0	Myrcene (6.4%), neral (32.6%), geranial (48.4%)
Distichoselinum tenuifolium	91.2	0.5	0.5	0.5	0.1	–	–	(26)/92.7	Myrcene (84.6%), limonene (2.2%)
Eryngium maritimum	5.25	t	53.7	12.9	–	–	–	(21)/71.8	α-Pinene (3.6%), germacrene D (41.1%), bicyclogermacrene (2.1%), Germacrene B (2.4%), δ-cadinene (2.1%)
Juniperus oxycedrus (berries oil)	66.9	5.7	11.3	5.5	–	t	2.6	(44)/92.0	α-Pinene (56.4%), myrcene (4.0%), γ-cadinene (2.3%), manoyl oxide (2.2%)
Juniperus oxycedrus (leaves oil)	87.3	6.5	0.9	2.0	–	–	0.5	(40)/97.7	α-Pinene (76.4 %), δ-3-carene (2.7%)
Lavandula viridis	17.3	58.3	18.6	–	–	1.9	–	(38)/96.1	α-Pinene (9.2%), camphene (2.7%), 1,8-cineole (29.7%), linalool (9.0%), camphor (10.0%), borneol (2.7%), (*Z*)-α-bisabolene (6.3%), selina-3,7(11)-diene (6.6%)
Lippia graveolens	28.9	41.0	10.3	10.8	–	–	–	(46)/90.3	Myrcene (3.4%), δ-3-carene (4.3%), *p*-cymene (16.9%), 1,8-cineole (6.6%), *trans*-sabinene hydrate (2.3%), linalool (5.4%), α-terpineol (3.6%), thymol (19.8%), (*E*)-caryophyllene (2.4%), caryophyllene oxide (5.7%)
Mentha cervina	8.5	89.7	0.3	–	–	2.0	–	(16) 99.5	Limonene (5.4%), isomenthone (10.6%), pulegone (74.8%)
Mentha × *piperita*	5.6	90.5	1.9	0.3	–	0.3		(29) 98.6	1,8-Cineole (5.8%), menthone (9.8%), menthofuran (10.9%), *neo*-menthol (4.0%), menthol (44.0%), *neo*-isomenthol (2.9%), pulegone (2.4%), menthyl acetate (7.8%)
Origanum virens	22.5	74.5	1.3	0.5	–	0.5	–	(28) 99.3	Myrcene (2.4%), *p*-cymene (7.4%), γ-terpinene (7.9%), thymol (2.1%), carvacrol (68.2%)
Rosmarinus officinalis	64.3	33.7	1.2	0.2	–	0.4	–	(31) 99.8	α-Pinene (11.1%), camphene (3.4%), myrcene (32.0%), *p*-cymene (3.8%), 1,8-cineole (13.7%), limonene (6.6%), linalool (2.1%), camphor (11.9%)
Seseli tortuosum	84.3	1.4	2.4	2.3	–	–	–	(27) 90.3	α-Pinene (27.4%), camphene (2.1%), sabinene (2.0%), β-pinene (16.0%), myrcene (3.0%), limonene (10.0%), (*Z*)-β-ocimene (8.0%), γ-terpinene (9.3%)
Syzygium aromaticum	0.8	1.6	9.5	0.4	85.3	–	–	(20) 97.3	Eugenol (85.3%), α-humulene (6.8%)
Thymbra capitata	12.5	79.7	4.7	0.8	–	0.3	–	(28) 98.1	*p*-Cymene (5.5%), γ-terpinene (3.6%), linalool (2.8%), carvacrol (74.6%), (*E*)-caryophyllene (3.9%)
Thymus capitelatus	18.2	78.8	0.5	0.5	–	–	–	(28) 97.9	α-Pinene (4.5%), camphene (6.5%), sabinene (3.0%), β-pinene (2.0%), 1,8-cineole (58.6%), borneol (10.0%)
Thymus mastichina	20.6	74.5	0.3	2.3	–	–	–	(24) 97.7	α-Pinene (3.6%), sabinene (3.7%), β-pinene (5.3%), myrcene (2.0%), 1,8-cineole (64.6%), limonene (5.0%), α-terpinyl acetate (5.0%)
Thymus zygis subsp. *sylvestris* (chemotype geraniol)	1.8	91.0	0.9	1.5	–	1.2	–	(27) 96.3	Camphor (3.9%), geraniol (33.1%), geranyl acetate (44.5%)
Thymus zygis subsp. *sylvestris* (chemotype thymol)	71.0	22.1	1.6	–	–	1.0		(31) 95.7	α-Thujene (2.6%), myrcene (3.0%), α-terpinene (2.7%), *p*-cymene (36.6); γ-terpinene (21.0%), linalool (2.9%), thymol (15.2%)

t, trace (<0.05%).

Table 2. Inhibitory concentration (IC_{50}) of the active essential oils against *L. infantum*

Essential oil	IC_{50} (μg/ml) (range of mean values) 95% Confidence intervals
Thymbra capitata	130 (119–142)
Origanum virens	196 (189–202)
Syzygium aromaticum	220 (204–238)
Thymus zygis subsp. *sylvestris* (chemotype thymol)	293 (261–334)
Lippia graveolens	171 (151–193)
Cymbopogon citratus	25 (20–31)
Mentha × *piperita*	198 (180–217)
Thymus zygis subsp. *sylvestris* (chemotype geraniol)	162 (151–174)
Lavandula viridis	263 (248–279)
Crithmum maritimum	122 (109–136)
Distichoselinum tenuifolium	295 (272–321)
Eryngium maritimum	205 (199–211)
Juniperus oxycedrus (berries oil)	51 (46–56)
Juniperus oxycedrus (leaves oil)	127 (107–150)
Mentha cervina	178 (152–209)
Seseli tortuosum	133 (115–153)
Thymus capitelatus	37 (30–46)
Thymus mastichina	133 (111–159)

hydrocarbons, sesquiterpene hydrocarbons, oxygen-containing mono- and sesquiterpenoids, diterpenoids, phenylpropanoids and volatile aliphatic compounds. Forty-nine of these compounds were found in concentrations >2.0% in at least one essential oil. Six monoterpene hydrocarbons (α-pinene, β-pinene, myrcene, limonene, *p*-cymene and γ-terpinene), 13 oxygen-containing monoterpenes (1,8-cineole, camphor, borneol, menthofuran, isomenthone, pulegone, neral, geranial, menthol, thymol, carvacrol, geraniol and geranyl acetate), the sesquiterpene germacrene D and the phenylpropanoids eugenol and dillapiole exceeded 10.0% of the total composition of at least one essential oil.

Table 2 summarizes the results of the inhibitory concentrations (IC_{50}) of the essential oils on the growth of *L. infantum*. *Cymbopogon citratus*, *Juniperus oxycedrus* (berries oil), and *Thymus capitelatus* oils were generally the most effective to inhibit *Leishmania* growth (IC_{50} values of 16–51 μg/ml). The oils from *Thymbra capitata*, *Crithmum maritimum*, *Juniperus oxycedrus* (leaves oil), *Seseli tortuosum* and *Thymus mastichina* exhibited activity of <150 μg/ml, and *Origanum virens*, *Lippia graveolens*, *Mentha cervina*, *Mentha* × *piperita*, *Thymus zygis* subsp. *sylvestris* (chemotype geraniol) (IC_{50} values of 150–200 μg/ml), as well as *Syzygium aromaticum*, *Thymus zygis* subsp. *sylvestris* (chemotype thymol), *Lavandula viridis*, *Distichoselinum tenuifolium* and *Eryngium maritimum* (IC_{50} values > 200 μg/ml) inhibit *Leishmania* growing at higher concentrations. Only *Rosmarinus officinalis* essential oil did not show any activity.

These *in vitro* results showed the potential of essential oils (*C. citratus*, *T. capitelatus* and *J. oxycedrus* berries) as hopeful sources for lead or active molecules against *Leishmania*. The ability of most of essential oil compounds, to easily diffuse through cell membranes and interact with intracellular targets, can minimize the problem on diffusing inside macrophages to inhibit intracellular promastigotes and differentiation into the amastigote form. Additionally, the most active essential oils tested did not show toxicity to mammalian endothelial cells at IC_{50} for *Leishmania*.

Considering that *L. infantum* may express visceral and cutaneous disease in humans and animals, we believe that the most active essential oils, particularly that of *C. citratus*, can be a hopeful alternative as an inhibitor of *Leishmania* growth to be used in human and animal disease.

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