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ORIGINAL PAPER

Leishmanicidal activity of α-bisabolol from Tunisian chamomile essential oil

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Abstract
According to the World Health Organization, leishmaniasis is considered as a major neglected tropical disease causing an enormous impact on global public health. Available treatments were complicated due to the high resistance, toxicity, and high cost. Therefore, the search for novel sources of anti-*leishmania* agents is an urgent need. In the present study, an in vitro evaluation of the leishmanicidal activity of the essential oil of Tunisian chamomile (*Matricaria recutita* L.) was carried out. Chamomile essential oil exhibits a good activity on promastigotes forms of *L. amazonensis* and *L. infantum* with a low inhibitory concentration at 50% (IC_{50}) (10.8 ± 1.4 and 10.4 ± 0.6 μg/mL, respectively). Bio-guided fractionation was developed and led to the identification of (−)-α-bisabolol as the most active molecule with low IC_{50} (16.0 ± 1.2 and 9.5 ± 0.1 μg/mL for *L. amazonensis* and *L. infantum*, respectively). This isolated sesquiterpene alcohol was studied for its activity on amastigotes forms (IC_{50} = 5.9 ± 1.2 and 4.8 ± 1.3 μg/mL, respectively) and its cytotoxicity (selectivity indexes (SI) were 5.4 and 6.6, respectively). The obtained results showed that (−)-α-bisabolol was able to activate a programmed cell death process in the promastigote stage of the parasite. It causes phosphatidylserine externalization and membrane damage. Moreover, it decreases the mitochondrial membrane potential and total ATP levels. These results highlight the potential use of (−)-α-bisabolol against both *L. amazonensis* and *L. infantum*, and further studies should be undertaken to establish it as novel leishmanicidal therapeutic agents.

Keywords *Leishmania* · Chamomile · (−)-α-Bisabolol · Apoptosis · Mitochondrial membrane potential · ATP levels

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Introduction

Leishmaniasis is a vector-borne disease caused by several species of obligating intramacrophage protozoan parasite (Herwaldt 1999; Shirian et al. 2013). Currently, more than 20 species of *Leishmania* are known to be infectious to humans and are transmitted by the bite of infected phlebotome sand flies. Three types of leishmaniasis have been reported: visceral leishmaniasis (VL), often called kala azar, cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (Desjeux 2004).

CL is the most common form and present a spectrum of clinical manifestations ranging from small skin nodules to massive destruction of the mucous tissues (Handler et al. 2015; López-Arencibia et al. 2015), while VL is the most severe form because parasites migrate to vital organs. It is characterized by prolonged fever, splenomegaly, hypergamma globulinemia,

and pancytopenia (Boelaert et al. 2000; Desjeux 1996; Peters et al. 1990).

Leishmaniasis is considered by the World Health Organization as a major neglected tropical disease causing morbidity throughout the world (WHO 2013). *Leishmania* is endemic in large areas of the tropics, subtropics, and the Mediterranean basin (Herwaldt 1999) and affects mostly the poorest people on earth (Boelaert et al. 2009). It is associated with malnutrition, displacement of the population, poor housing, weak immune systems, and lack of financial resources (Bashaye et al. 2009). In addition, this disease has an enormous impact on global public health, with more than 98 endemic countries and territories. Recently, it has been estimated that approximately 0.2 to 0.4 million new cases of VL and 0.7 to 1.2 million new cases of CL occur each year worldwide (Alvar et al. 2012; WHO 2010). In Tunisia, three *Leishmania* species coexist causing human VL and CL with an annual incidence of 5000 to 10,000 cases (Bousslimi et al. 2014). CL is present in three forms with different causative agents (*L. infantum*, *L. major*, and *L. tropica*) (Aoun and Bouratbine 2014; Kbaier-Hachemi et al. 2008).

Unfortunately, available drugs for the treatment of leishmaniasis (antimoniels pentavalent, amphotericin B, miltefosine, paromomycin, and pentamidine) have several limitations, including the high toxicity (Alvar et al. 2006; Croft and Olliaro 2011; Singh and Sundar 2012; Tempone et al. 2011), the long-term treatment, inefficiency, high cost, and drug resistance (Maltezou 2010; Oryan and Akbari 2016; Reithinger et al. 2007). These limits alarmed an urgent need to search for new natural and effective alternatives. Therefore, increasing attention was accorded to the use of essential oils from medicinal plants as effectives in the treatment on protozoa, especially *Leishmania* (Andrade et al. 2016; de Medeiros et al. 2011). Specifically, essential oils are known by their richness of terpenes; due to their lipophilicity, they can produce many changes in cellular and mitochondrial structure of different pathogens (Morales-Yuste et al. 2010; Rosa et al. 2003).

In this context, our study focuses on one of the most used medicinal plants in the world: *Matricaria recutita* L classified as generally safe (GRAS) without acute toxicity to humans and animals (Bradley 1992; Tolouee et al. 2010). Several studies have reported that chamomile essential oil contains a complex mixture of sesquiterpenes (α-bisabolol, bisabolol-oxides A and B, farnesene) sesquiterpene lactones (chamazulene), and acetylene derivatives (spiroéthers) (Ganzera et al. 2006; McKay and Blumberg 2006). A wide range of biological activities has been recognized for the essential oil such as antifungal, anti-inflammatory, antioxidant, antimicrobial, and sedative (Jamalian et al. 2012; Roby et al. 2013; McKay and Blumberg 2006). Therefore, for the first time, the leishmanicidal activity in vitro of Tunisian chamomile essential oil was studied against both promastigotes and amastigotes forms of *L. amazonensis* and *L. infantum*. Furthermore, a bio-guided isolation and identification of the compound responsible for these effects and the key factors involved in the mechanism of its leishmanicidal action were elucidated. The obtained results highlight a potential use of the active compound (−)-α-bisabolol for the treatment of leishmaniasis in the future.

Material and methods

General methods

^{1}H (600 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker Avance 600 spectrometers; the chemical shifts are given in δ (ppm) with residual $CDCl_3$ (δ_H 7.26, δ_C 77.0) as internal reference and coupling constants in Hz; experiments were carried out with the pulse sequences given by Bruker. Silica gel 60 (particle size 15–40 and 63–200 μm, Macherey-Nagel) and Sephadex LH-20 (Pharmacia Biotech) were used for column chromatography, while silica gel 60 F_{254} (Macherey-Nagel) were used for analytical or preparative thin layer chromatography (TLC). Centrifugal preparative TLC was performed using a Chromatotron (Harrison Research Inc. model 7924T) on 4- or 1-mm silica gel 60 PF_{254} disks with flow rate 2–4 mL min^{-1}. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O-H_2SO_4-$C_2H_4O_2$ (1:4:20). All solvents used were analytical grade from Panreac.

Plant material and essential oil extraction

M. recutita L was collected from the north-west of Tunisia in the region of Beja (alt. 448 m; 36° 81′ N; 9° 05′ E) during spring 2013. Chamomile flowers were separated and thoroughly rinsed in running tap water then air dried for a period of 14 days.

Essential oil was extracted by hydrodistillation for 3 h using Clevenger type apparatus as previously described by Jabri et al. (2016). Briefly, dried flowers were immersed in water and heated to boiling; essential oil was evaporated with water vapor and finally collected in a condenser. The obtained essential oil was dried over anhydrous sodium sulfate, and stored at − 4 °C until analysis.

Bioassay guided fractionation of *M. recutita* L. essential oil

The fractionation of chamomile essential oil was guided by its leishmanicidal activity. Initially, 5 g of essential oil was subjected to silica gel column chromatography eluted with hexanes (1 L), mixtures of hexanes-ethyl acetate (7:3, 6:4, 3:7; 1 L each), ethyl acetate (1 L), and methanol (1 L). Based on their TLC profile, the obtained fractions were combined in six fractions (EO-F1-EO-F6). The most active fractions against

Leishmania spp. was submitted to multiple chromatographic steps, involving Sephadex LH-20, medium-pressure liquid chromatography, and centrifugal preparative TLC to yield two compounds, spathulenol, and α-bisabolol (Fig. 1).

The structures of the compounds were established by spectroscopic and spectrometric methods, including 1D NMR experiments, and comparison with data reported in the literature (Nissanka et al. 2001; Cerceau et al. 2016).

Spathulenol: yellow pale oil; was $[\alpha]^{20}_{D} = +36.4$ (c 0.03, $CHCl_3$); ^1H NMR (CDCl3, 500 MHz), δ 0.50 (1H, *t*, *J* = 12.4 Hz), 0.81 (1H, *m*), 0.90 (1H, *m*), 1.04 (3H, *s*), 1.05 (3H, *s*), 1.28 (3H, *s*), 1.50–1.70 (3H, *m*), 1.78–2.10 (4H, *m*), 2.20 (1H, *m*), 2.42 (1H, *dd*, *J* = 7.1, 13.3 Hz), 4.66 (1H, *s*), 4.68 (1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz), δ 16.3 (q), 20.2 (s), 24.8 (t), 26.0 (q), 26.7 (t), 27.5 (d), 28.6 (q), 29.9 (d), 38.8 (t), 41.7 (t), 53.4 (d), 54.3 (d), 80.9 (s), 106.2 (t), 153.4 (s); HRESIMS *m/z* 220.1790 $[M]^+$ (calcd. for $C_{15}H_{24}O$, 220.1828) (Fig. 2a).

α-Bisabolol: light oil; was $[\alpha]^{20}_{D} = -42.3$ (c 0.2, $CHCl_3$); ^1H NMR (CDCl3, 500 MHz), δ 0.88 (3H, *d*, *J* = 7.2 Hz), 1.28 (2H, *m*), 1.50 (2H, *m*), 1.62 (3H, *s*), 1.65 (3H, *s*), 1.69 (3H, *s*), 1.79 (1H, *t*, *J* = 14.3 Hz), 1.94 (2H, *m*), 2.00 (2H, *m*), 2.06 (2H, *dd*, *J* = 7.6, 15.7 Hz), 5.13 (1H, *t J* = 7.11), 5.37 (1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz), δ 17.7 (q), 22.1 (t), 23.2 (q), 23.3 (q), 23.4 (q), 25.7 (t), 26.9 (t), 31.0 (t), 40.1 (t), 43 (d), 74.3 (s), 120.5 (d), 124.6 (d), 131.7 (s), 134.2 (s); HRESIMS *m/z* 222.1983 $[M]^+$ (calcd. for $C_{15}H_{26}O$, 222.1984) (Fig. 2b).

To study its mechanism of action, (−)-α-bisabolol was purchased from extrasynthese. It exhibits ≥ 90% of purity (GC).

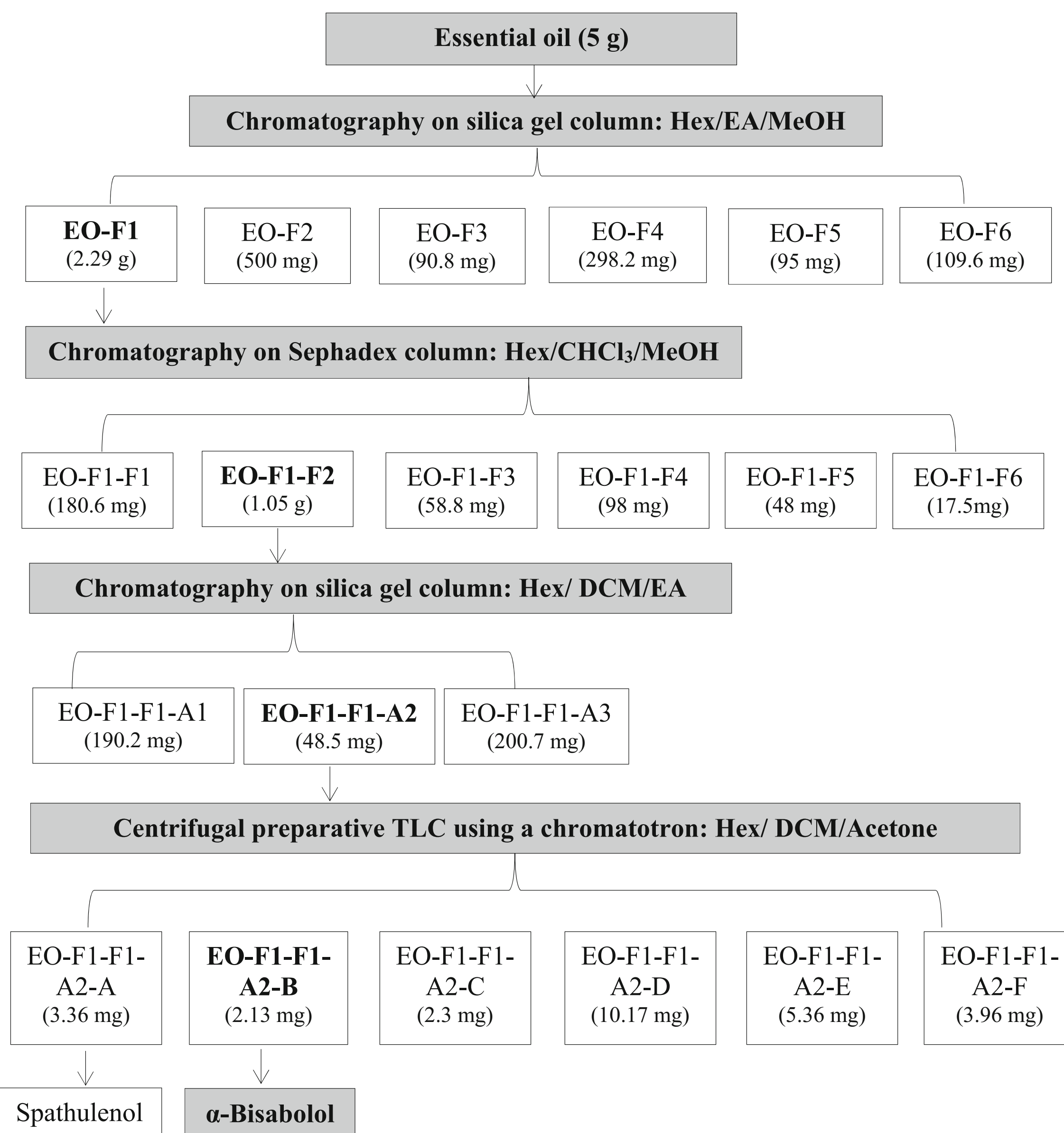

Fig. 1 Scheme of the bio-guided fractionation assay of the essential oil of Tunisian chamomile against *Leishmania* spp. (Hex: hexanes; $CHCl_3$: chloroform; DCM: dichloromethane; EA: ethyl acetate; MeOH: methanol)

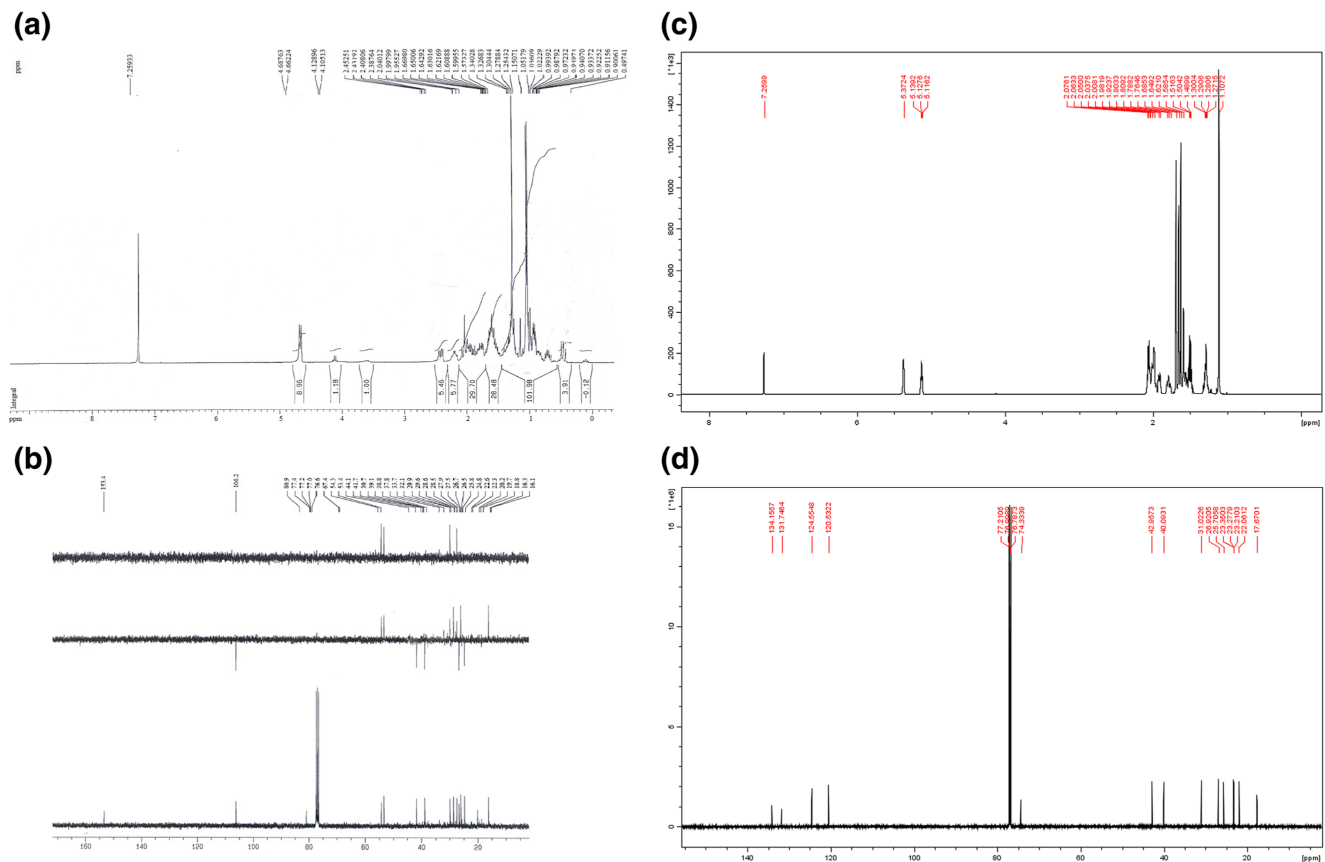

Fig. 2 NMR spectrum of spathulenol (**a**) (^{1}H NMR (**a**), ^{13}C NMR (**b**)) and (−)α-bisabolol (**b**) (^{1}H NMR (**c**), ^{13}C NMR (**d**))

In vitro assays

Parasite strains

The leishmanicidal activity of chamomile essential oil was evaluated against promastigotes of both *L. infantum* (MHOM/ES/1996/BCN-143) and *L. amazonensis* (MHOM/BR/77/LTB0016). Promastigotes were cultured in Schneider's medium (Sigma-Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum at 26 °C and were grown to the log phase as per previous methods. For some of the assays, the parasites were also cultured in RPMI 1640 medium (Gibco), with or without phenol red (López-Arencibia et al. 2017).

In vitro effect on the promastigotes forms of *Leishmania* spp.

Leishmanicidal activities of the assayed fractions and compounds were determined by a colorimetric assay based on Alamar Blue reagent (Invitrogen/Life Technologies, Madrid, Spain) as previously described by Cabrera-Serra et al. (2007). This simple and rapid test is based on oxido/reduction reaction. Briefly, the oxidized, blue, non-fluorescent Alamar Blue is reduced to a pink fluorescent dye in the medium by cell activity. This reaction could be measured either by *colorimetric* or *fluorimetric* (O'Brien et al. 2000).

Essential oil and its fractions were dissolved in dimethyl sulfoxide (DMSO), and further dilutions in RPMI 1640 medium (Gibco/Life Technologies, Madrid, Spain) were made.

Promastigotes were adapted for growth at 26 °C in RPMI 1640 modified medium and supplemented with 10% heat-inactivated fetal bovine serum, in their log phase of growth they were initially counted and diluted (10^{6}/well) and then added to the tested drugs concentration in sterilized 96-well microtiter plates (Corning™) in a final volume 200 μl in each well. As positive controls, Miltefosine was kindly provided by Æterna ZentarisInc. The plates were analyzed after 72 h, on a Microplate Reader Model 680 (Bio-rad, Hercules, CA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentages of inhibition and 50% inhibitory concentrations (IC_{50}) for active compounds were calculated by non-linear regression analysis with 95% confidence limits. All experiments were performed three times each in duplicate and the mean values were also calculated. A paired two-tailed *t* test was used for analysis of the data. Values of $p < 0.05$ were considered significant. The obtained inhibition curve statistical analysis was under taken using the Sigma Plot 12.0 software program (Systat Software Inc.).

Activity assays against intracellular amastigotes of *Leishmania* spp.

For this test, J774A.1 (ATCC TIB-67) murine macrophage cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

Initially, macrophages were plated in 96-well plates (2.5×10^5 cells/mL), infected with metacyclic stage promastigotes of *Leishmania* spp. in a 1:20 ratio, and incubated overnight for internalization. After that non-internalized promastigotes were removed by extensive washing with RPMI 1640 medium, parasitized cells were incubated with medium (untreated control) or with a range of concentrations of tested drugs and incubated for 24 h at 37 °C and 5% CO_2.

For the parasite rescue, controlled lysis of *Leishmania* spp. amastigote-infected macrophages was performed as previously described by Jain et al. (2012). Briefly, the 96-well plate was washed with serum-free RPMI 1640 culture medium, and then the medium was removed and 20 μl of RPMI 1640 (with 0.05% SDS) was added to each well. After that, the plate was shaken for 30 s and 180 μl of RPMI 1640 (with 10% FBS) was added to each well. Finally, 20 μl of Alamar Blue was added into each well and the mixture was incubated at 26 °C for transformation of rescued amastigotes to promastigotes.

After 72 h, the plates were analyzed using an EnSpire multimode plate reader (PerkinElmer, Madrid, Spain) with an excitation wavelength of 570 nm and an emission wavelength of 585 nm. Percentages of inhibition and 50% inhibitory concentrations (IC_{50}) were calculated as using Sigma Plot 12.0 statistical analysis software. All experiments were performed three times each in duplicate, and the mean values were also calculated.

Cytotoxicity assay

A commercial kit was used for the evaluation of drug-induced cytotoxic effects based on the measurement of lactate dehydrogenase (LDH) activity released to the media (LDH cytotoxicity detection kit; Roche Applied Science, Madrid, Spain). The J774A.1 (ATCC TIB-67) murine macrophage cell line was used.

Briefly, the macrophages at concentration of 10^5 cells/mL cultured in RPMI medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 were incubated with different concentrations of α-bisabolol for 24 h in duplicate. After incubation, supernatants were obtained and LDH levels were determined following the manufacturer's instructions. The average absorbance values of the duplicates were calculated and compared with those calculated for the negative and positive controls. Cytotoxicity levels were determined as previously described (Lorenzo-Morales et al. 2010).

Image-based cytometer analysis for apoptosis determination

annexin-V/propidium iodine double (PI) staining assay was performed using the Tali™Apoptosis kit and a Tali™ Image Based Cytometer (Life Technologies Corporation) according to the manufacturer's instructions. Briefly, after treatment with IC_{50} and IC_{90} of α-bisabolol for 24 h, promastigotes were centrifuged (1500 rpm for 10 min) washed twice with the annexin binding buffer (ABB) and incubated with 5 μl of annexin V for 20 min. After that, cells were centrifuged and resuspended in ABB containing 1 μl of PI and incubated for 3 min at room temperature. Finally, 25 μl of the stained cells was loaded into a Tali™ Cellular Analysis Slide and analyzed in the Tali™ Image Based Cytometer. Miltefosine IC_{50} was used as a positive control, while untreated cells were used as negative control. Data were collected using the Tali™ data acquisition and analysis software (Life Technologies Corporation) (López-Arencibia et al. 2015; Sifaoui et al. 2014).

Plasma membrane permeability

The SYTOX® Green assay was performed to detect membrane permeability alterations of parasites. Briefly, 2×10^7 parasites were washed and incubated in PBS (phosphate-buffered saline) with the of SYTOX® Green at a final concentration of 1 μM (Molecular Probes) for 15 min in the dark at room temperature. (−)α-bisabolol was added at the IC_{50} and IC_{90} concentrations. The increase in fluorescence due to binding of the fluorescent marker to the parasitic DNA was measured using an Enspire microplate reader (PerkinElmer, Massachusetts, USA) with excitation filter of 485 nm and emission filter at 520 nm (López-Arencibia et al. 2015; Sifaoui et al. 2014). At the end of the sixth hour, Triton X-100 was added into each well in order, and the results were calculated to represent the percentage relative to Triton X-100 (100% permeabilized cells) (Kulkarni et al. 2009). The data were normalized by subtracting the baseline fluorescence of untreated cells (negative control). In addition, cells were observed in a Leica TSC SPE-confocal microscope equipped with inverted optics at $_{exc} = 482$ nm and $_{em} = 519$ nm.

Analysis of mitochondrial membrane potential

JC-1Mitochondrial Membrane, Potential Assay Kit, Cayman Chemical was used to measure the collapse of electrochemical gradient across the mitochondrial membrane during apoptosis. Promastigotes were initially treated with IC_{50} and IC_{90} concentrations of α-bisabolol and IC_{50} of miltefosine, as a positive control, for 24 h. After that they were centrifuged (1500 rpm × 10 min) and resuspended in JC-1 buffer. One hundred microliters of each treated culture was added to a black 96 well plate (PerkinElmer) then 10 μl of JC-1 was

added and incubated at 26 °C for 30 min. The analysis for mean green and red fluorescence intensity was done using an Enspire microplate reader (PerkinElmer, Massachusetts, USA) for 30 min.

Analysis of ATP levels

ATP level was measured using a Cell Titer-Glo® Luminescent Cell Viability Assay (Promega), which generates a signal proportional to the ATP amount. The effect of the drug on the ATP production was evaluated by the incubation of (10^7 cells/mL) with the previously calculated IC_{50} and IC_{90} of α-bisabolol.

Statistical analysis

Percentages of inhibition and 50% inhibitory concentrations (IC_{50}) were calculated by non-linear regression analysis with 95% confidence limits.

A paired two-tailed *t* test was used for analysis of the data. Values of $p < 0.05$ were considered significant. The obtained inhibition curve statistical analysis was under taken using the Sigma Plot 12.0 software program (Systat Software, Inc.).

Results and discussion

Leishmanicidal activity

In the present study, we evaluated the inhibitory effect of chamomile essential oil, fractions, and isolated molecules against both promastigotes and amastigotes forms of *L. amazonensis* and *L. infantum*. The isolation and the identification of major molecules responsible of this activity were investigated. Our results suggested that the incubation of *Leishmania* spp. promastigotes with tested fractions inhibited both strains growth, and this activity was dose-dependent. Bio-guided fractionation allowed the isolation of the sesquiterpene alcohol (−)-α-bisabolol as the most active molecule as illustrated in Fig. 1 (Cerceau et al. 2016).

The IC_{50} values were low and ranging from (10.8 ± 1.4 and 10.4 ± 0.6 μg/mL, respectively) for the essential oil to (16.0 ± 1.2 and 9.5 ± 0.01 μg/mL) for (−)-α-bisabolol for *L. amasonenzis* and *L. infantum*, respectively (Table 1). Miltefosine showed a higher activity against both parasites than the tested molecule with an IC_{50} (5.3 ± 0.7 and 4.3 ± 1.0) for *L. amazonensis* and *L. infantum*, respectively.

To our knowledge, there are few reports describing the leishmanicidal activity of essential oils containing (−)α-bisabolol. More recently, Andrade et al. (2016) tested the activity of *Matricaria chamomila* against *L. amazonensis*, promastigotes with higher IC_{50}/24 h (60.2 ± 2.2 μg/mL). However, Gawde et al. (2014) tested the same plant but no activity against *L. donovani* was observed. Therefore, our results proved that the pure compound ((−)α-bisabolol) showed a good antileishmanial effect on promastigotes forms of *L. infantum* with lower IC_{50} than observed by Morales-yuste et al. (2010) (85% inhibition at 125 μg/mL). However, Rottini et al. (2015) suggested that the molecule reduces in 50% the survival index of promastigotes of *L. amazonensis* at lower concentration (8.07 μg/mL). Moreover, this sesquiterpene showed also potent activity against *L. tropica* promastigotes (22.6 ± 1.6 μM) (Corpas-López et al. 2016a).

Table 1 Leishmanicidal activity of chamomile essential oil and isolated compounds

Extracts/fractions	*L. amazonensis* IC_{50} (μg/ml) at 96 h	*L. infantum* IC_{50} (μg/ml) at 96 h
Essential oil	10.8 ± 1.4	10.4 ± 0.6
EO-F1	8.8 ± 1.5	9.8 ± 0.9
EO-F2	36.8 ± 1.3	47.2 ± 1.2
EO-F3	28.4 ± 1.7	37.9 ± 1.7
EO-F4	46.7 ± 1.6	33.6 ± 1.5
EO-F5	41.6 ± 1.6	40.7 ± 2.1
EO-F6	153.3 ± 2.2	94.6 ± 3.5
EO-F1-F1	106.6 ± 1.8	113.7 ± 1.9
EO-F1-F2	14.4 ± 0.9	10.1 ± 1.1
EO-F1-F3	18.6 ± 1.1	15.0 ± 0.7
EO-F1-F4	79.3 ± 0.9	68.9 ± 2.7
EO-F1-F5	16.9 ± 0.6	18.3 ± 1.4
EO-F1-F6	51.7 ± 2.2	46.5 ± 3.8
EO-F1-F2-A1	17.8 ± 1.5	18.3 ± 2.6
EO-F1-F2-A2	15.1 ± 1.0	9.3 ± 1.0
EO-F1-F2-A3	16.0 ± 0.6	11.2 ± 1.0
E0-F1-F2-A2-A (spathulenol)	21.6 ± 1.4	16.8 ± 1.7
E01-F2-A2-B ((−)α-bisabolol)	16.0 ± 1.2 IC_{90} = 55.1 ± 0.9	9.5 ± 0.1 IC_{90} = 52.9 ± 2.7
E0-F1-F2-A2-C	37.0 ± 1.0	32.3 ± 1.1
E0-F1-F2-A2-D	27.1 ± 1.1	20.8 ± 2.8
E0-F1-F2-A2-E	25.6 ± 1.3	22.1 ± 2.0
E0-F1-F2-A2-F	38.7 ± 1.4	46.4 ± 2.8
Miltefosine	5.3 ± 0.7 IC_{90} = 42.1 ± 1.0	4.3 ± 1.0 IC_{90} = 10.8 ± 1.0

The data obtained in this study showed that (−)-α-bisabolol exhibited good activity on promastigotes forms of *L. amazonensis* but with higher IC_{50} than the essential oil; this can be explained by a possible synergy between different compounds of the essential oil. This hypothesis was confirmed in several previous studies (Aloui et al. 2016; Sifaoui et al. 2014).

Regarding the amastigotes forms, our results showed a good activity of the sesquiterpene against both *L. amazonensis* and *L. infantum* at lower IC_{50} (5.9 ± 1.2 and 4.8 ± 1.3 μg/mL, respectively) when compared to those for promastigotes

forms. Similarly, many authors suggested that amastigotes were more susceptible to being killed by α-bisabolol treatment. Rottini et al. (2015) suggested that it can reduce in 50% the survival index of *L. amazonensis* amastigotes at 4.29 μg/mL. In the same context, the study carried out by Corpas-López et al. (2015) proved that (−)-α-bisabolol was active against *L. infantum* and *L. donovani*, amastigotes (IC_{50} = 55 and 39 μM, respectively). The authors showed also a good effect of this natural product on *L. tropica* and *L. major* intracellular amastigotes (IC_{50} = 25.2 ± 2.9 and 33.7 ± 8.1 μM, respectively). They suggested that this molecule penetrates the macrophage plasma membrane and the parasitophorous vacuole, reaching the intracellular amastigote (Corpas-López et al. 2016a).

Cytotoxicity assay

Our results showed that the value of 50% cytotoxicity concentration on macrophages was 31.9 μg/mL. The selectivity index was calculated using the IC_{50} for the intracellular amastigotes of both parasites and the CC_{50} (SI = 5.5 and 6.7 for *L. amazonensis* and *L. infantum*, respectively) (Table 2). According to Likhitwitayawuid et al. (1993), molecules used in therapy present an SI higher than 1. Compared to our results, Rottini et al. (2015) found lower CC_{50} (14.8 ± 0.1 μg/mL) for *L. amazonensis* on J774.G8. In their study, Piochon et al. (2009) found that (−)-α-bisabolol CC_{50} was 150 μM. Our results were confirmed by many authors that proved that this compound has very low cytotoxicity on human skin fibroblasts (CC_{50} > 150 μM) (Piochon et al. 2009) and was non-toxic to animals with lethal dose 50% (14 g/kg) by oral administration in rats (Bhatia et al. 2008). It was also described by Corpas-López et al. (2015) as a non-toxic against visceral leishmaniasis in an in vivo murine model orally.

Apoptosis study

Regarding the characteristics of cells apoptosis-like programmed cell death process, many morphological features can be observed including the condensation of the cytoplasm, the significant decrease in the cells volume, the reduction or the loss of the mitochondrial membrane potential, and the condensation of nuclear chromatin and nuclear DNA fragmentation (Das et al. 2001; Roy et al. 2008). Meanwhile, the plasma membrane loses the phospholipidic asymmetry and the phosphatidylserine (PS), and is externalized allowing the recognition of the apoptotic cell by phagocytic cells, such as macrophages. In apoptotic cells, PS is translocated from the inner to the outer surface of the plasma membrane, exposing it to the extracellular environment (Gottlieb 2001; López-Arencibia et al. 2015).

In our study, cell death type was investigated by treatment of *Leishamania* spp. cells with (−)-α-bisabolol IC_{50} and IC_{90} and stained using the Tali™ Apoptosis Kit—Annexin V Alexa Fluor® 488 and propidium iodine based to the high affinity of annexin V for PS. Therefore, apoptotic cells display green fluorescence, dead cells display red and green fluorescence, and live cells show little or no fluorescence (Figs. 3 and 4). Our results suggested an increment of the cellular phosphatidylserine externalization. The percentages of apoptosis induced by the sesquiterpene were 21.66 (IC_{50}) and 40% (IC_{90}) for *L. amazonensis* and 17 (IC_{50}) and 20% (IC_{90}) in the case of *L. infantum* after 24 h of treatment (Fig. 3). The proportions of dead cells were low (1.33% (IC_{50}) and 2% (IC_{90}); 9% (IC_{50}) and 16% (IC_{90}) for *L. amazonensis* and *L. infantum*, respectively) when compared to the death cells in the positive control (16.66%, 61%) (Fig. 3). Here, we clearly report (−)-α-bisabolol as an effective inducer of early apoptosis on studied parasites strains (Fig. 4). Similar results were observed for several antileishmanial drugs, such as miltefosine (Verma et al. 2007). Therefore, the investigation of the cell death of *Leishmania* spp. was illustrated by many authors but they did not suggest the exact mechanism of cell death, such as apoptosis and necrosis (Tiuman et al. 2014). Meanwhile, some natural compounds showed inducing mitochondrial-dependent apoptosis such as Artemisin (Fidalgo and Gille 2011), cleodane (Kathuria et al. 2014), oleanolic acid, and ursolic acid (Yamamoto et al. 2015). Although the most of research evaluated the ultrastructural changes in *Leishmania* spp., few of them are interested to study their exact mechanism of action and to evaluate the apoptosis by PS translocation (Almeida-Souza et al. 2016). The data obtained by Corpas-López et al. (2016b) clearly showed that (−)-α-bisabolol induce apoptosis in *L. tropica*, and its activity was time and dose-dependent PS translocation. Interestingly, previous reports found that this sesquiterpene has a good cytotoxic effect on human and rat malignant glioma cell lines and through the mitochondrial pathway (Cavalieri et al. 2004) and also could induce apoptosis in several human cancer, such as the human liver carcinoma cell line Hep G2 (Chen et al. 2010).

Table 2 In vitro effects of (−)α-bisabolol on amastigote stage of *Leishmania* spp.

Drug substance	*L. amazonensis* IC_{50} (μg/ml)	*L. infantum* IC_{50} (μg/ml)	J774A.1 CC50
(−)α-Bisabolol	5.9 ± 1.2	4.8 ± 1.3	31.9
Miltefosine	2.4 ± 1.0	0.4 ± 0.2	29.4

Plasma membrane permeabilization

In order to evaluate the effect of (−)-α-bisabolol on the membrane of the parasites tested, the SYTOX Green fluorescent probe was used because it is impermeable to cells

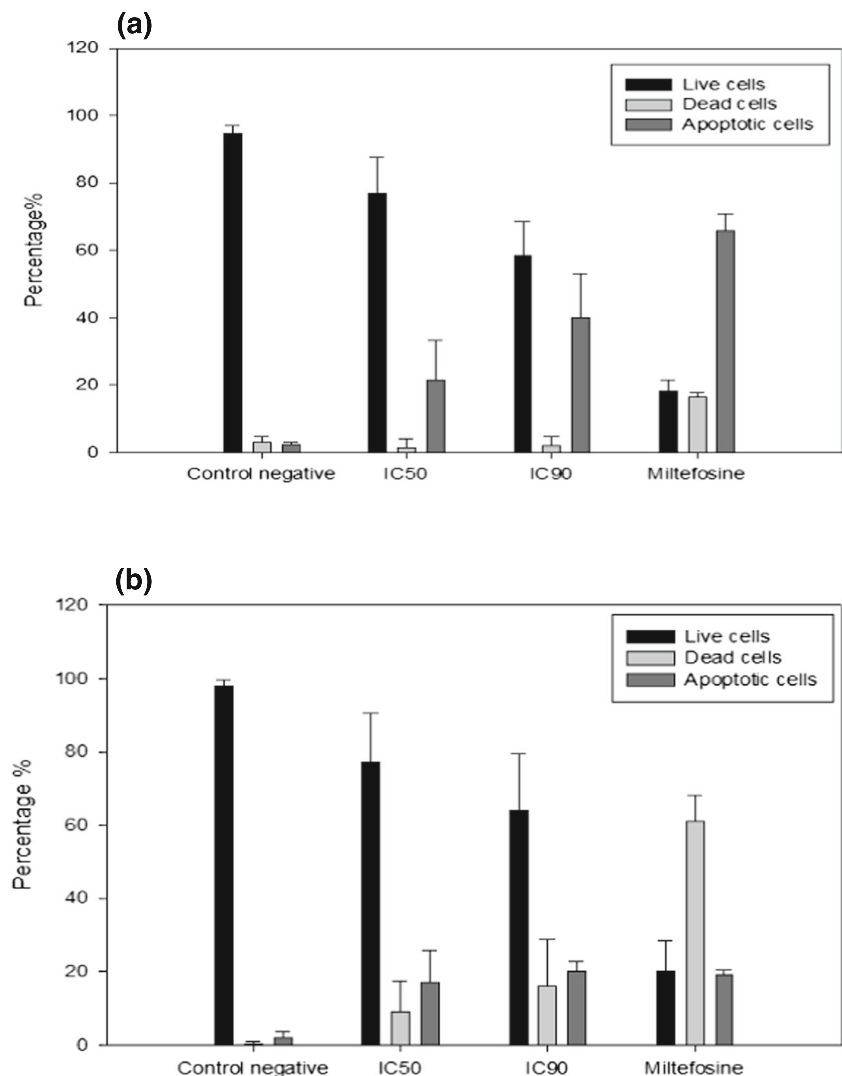

Fig. 3 Results of the phosphatidylserine exposure after 24 h of incubation of *L. amazonensis* (**a**) and *L. infantum* (**b**) promastigotes with the IC_{50} and IC_{90} of (−)-α-bisabolol for apoptosis determination. Cell viability and apoptosis were evaluated with the Tali™ image-based cytometer using the Tali™ apoptosis kit. As a negative control, we used untreated cells. IC_{50} of miltefosine was used as a positive control. Results are represented in percentages

with intact membranes. Therefore, it can diffuse through permeabilized membranes and exhibit a 500-fold increase in fluorescence upon binding to nucleic acids (Rasmussen et al. 2016). Obtained results suggest that the tested molecule clearly affects the permeability of the plasma membrane without causing necrotic effects (Figs. 5 and 6). Many authors have suggested that (−)-α-bisabolol may affect the plasma membrane of the parasite. However, they suggested that the effect could be explained by inhibiting the synthesis of ergosterol, which is an important component of cell membrane (Salomao et al. 2013). Other research groups were based on the hypothesis that the lipophilicity of terpenes can facilitate their penetration into the lipid bilayer of cell membranes. Consequently, they can produce major changes in the membrane structures of cells and mitochondria of different pathogens (Sikkema et al. 1995). Rosa et al. (2003) suggest that cell death may be due to this interaction with cell membranes in the case of some species of *Leishmania*.

More recently, Rigo and Vinante (2016) investigated the effect of α-bisabolol on CML-T1, Jurkat, and HeLa cell lines. It was showed able to induce membrane pores in both mitochondria and lysosomes, activating caspase-dependent or -independent death pathways and triggering both autophagy and apoptosis.

Mitochondrial transmembrane potential ($\Delta\psi_m$)

According to Nuydens et al. (1999), JC-1 staining was considered an important determinant of cell function and health and considered as an indicator of an activated mitochondrial state (Inacio et al. 2014). It is a cationic lipophilic mitochondrial vital dye that concentrates in the mitochondria in response to the mitochondrial transmembrane potential ($\Delta\psi_m$). Initially, this dye exists as a monomer at low concentrations where the emission is at 530 nm accompanied with green fluorescence. At higher concentration, it forms aggregates *J* when it is

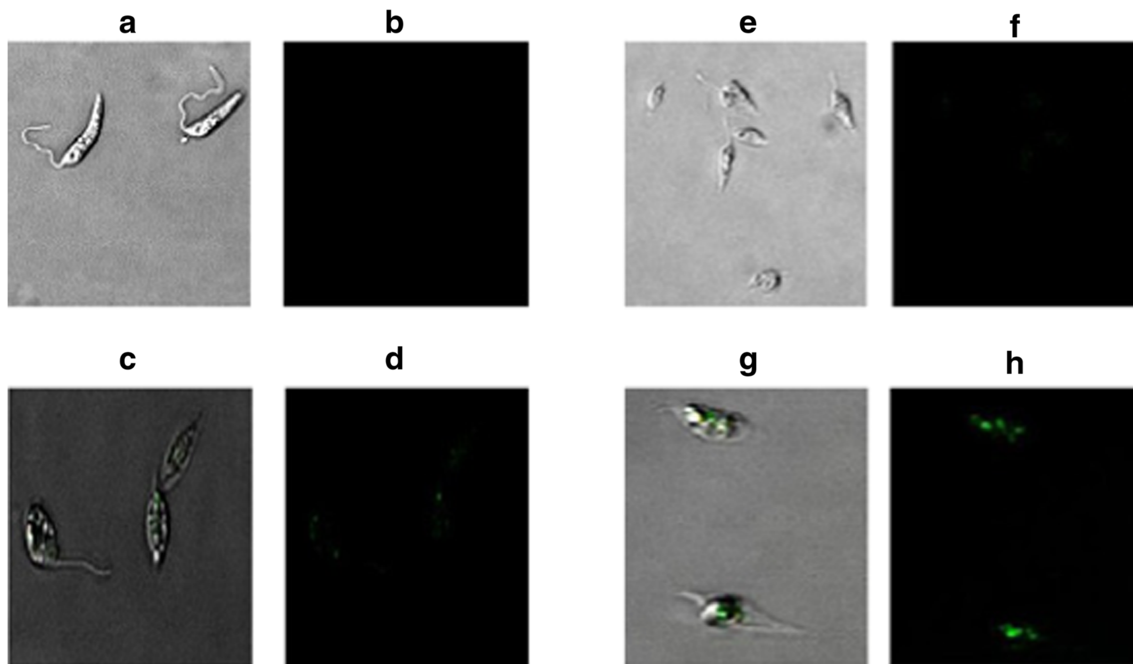

Fig. 4 Confocal microscopy for apoptosis determination. Cells were observed in a Leica TSC SPE-confocal microscope equipped with inverted optics. *L. amazonensis* and *L. infantum* were treated with IC_{90} of (−)-α-bisabolol (**c** and **d**; **g** and **h**, respectively). **a** and **b** were *L. amazonensis* negative control; **e** and **f** were *L. infantum* negative control. As a negative control, untreated cells were used

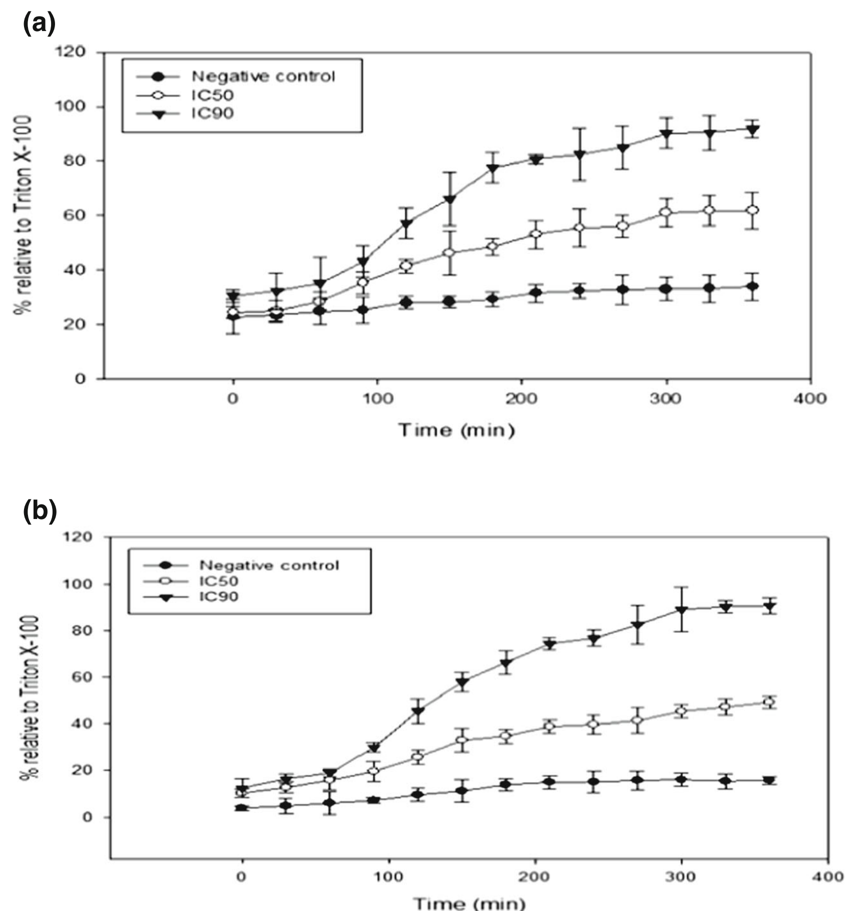

Fig. 5 Permeation of *L. amazonensis* (**a**) and *L. infantum* (**b**) to the vital dye SYTOX® green caused by addition of IC_{50} and IC_{90} (−)α-bisabolol. As a negative control, untreated cells were used. The maximum permeability values were obtained by adding of 0.1% Triton X-100 at the end of the sixth hour. Results were represented as the percentage relative to Triton X-100 (100% permeabilized cells). Error bars represent the standard deviations (SD). Each data point indicates the mean of the results of three measurements

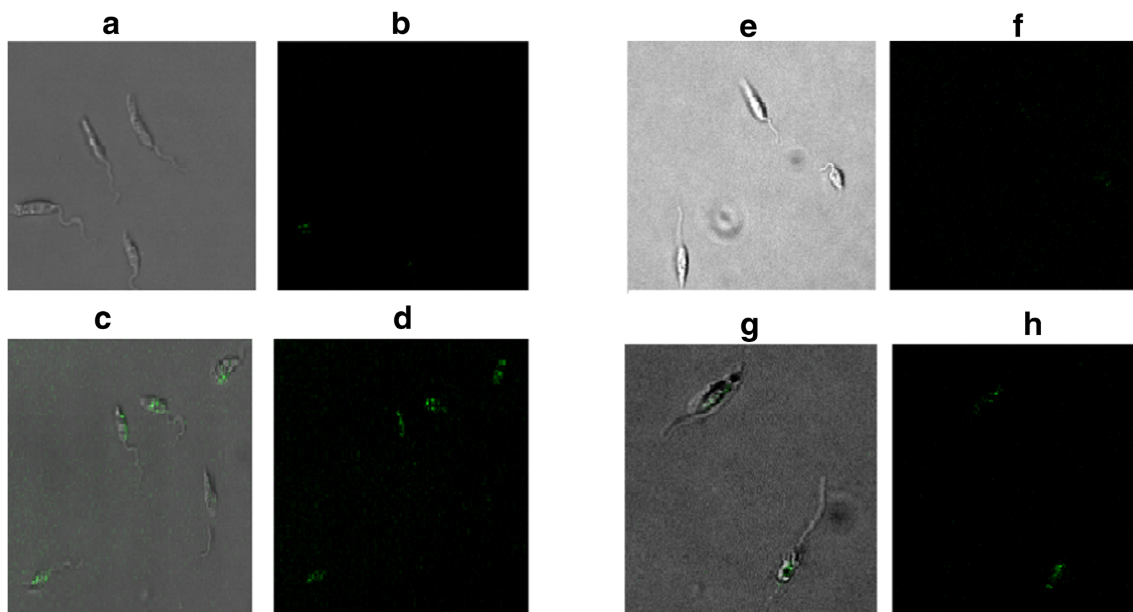

Fig. 6 Permeation of *L. amazonensis* (**a**) and *L. infantum* (**b**) to the vital dye SYTOX®green caused by addition of IC_{90} of (−)α-bisabolol (**c** and **d**; **g** and **h**, respectively). **a** and **b** were *L. amazonensis* negative control. **e** and **f** were *L. infantum* negative control. Cells were observed in a Leica TSC SPE confocal microscope equipped with inverted optics (λ_{exc} = 482 nm and λ_{em} = 519 nm)

accumulated in the mitochondria and the emission is at 590 nm (red fluorescence) (López-Arencibia et al. 2015; Sifaoui et al. 2014).

Obtained results show that (−)-α-bisabolol causes a significant decrease in the potential of mitochondrial membrane of the two tested parasites (Fig. 7). Compared with the negative control, the molecule reduces the membrane potential to 74.24% (IC_{50}) and 52.1% (IC_{90}) in the case of *L. amazonensis* as well as *L. infantum*, the reduction in membrane potential range from 72.9 (IC_{50}) to 39.027% (IC_{90}).

Although, many authors demonstrated that drug-induced variations in $\Delta\Psi_m$ are associated with cell survival in *Leishmania* spp., such as *L. donovani* and *L. amazonensis* (Mehta and Shaha 2006).

ATP levels

In eukaryotes, mitochondria are considered responsible for respiration and oxidative phosphorylation (Roy et al. 2008). Indeed, the intracellular ATP content was considered as a direct marker of the health of the mitochondria of cells (Kathuria et al. 2014). A decrease in $\Delta\Psi_m$ may suggest the increased permeability of the proton through the internal mitochondrial membrane because it decreases the synthesis of ATP and thus ends by parasite death (Gottlieb 2001). In our study, we demonstrated that IC_{50} and IC_{90} of (−)-α-bisabolol significantly decreased the ATP levels of the two parasites after 24 h of treatment. Compared to control (100% of ATP), the sesquiterpene has dose-dependent decreased level of ATP in *L. amazonensis* to 9.13 (IC_{50}) and 4.40% (IC_{90}) and also to

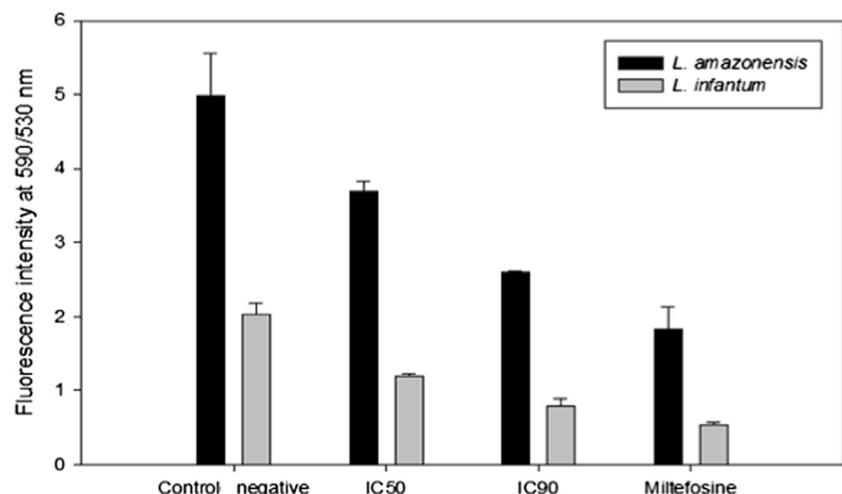

Fig. 7 Fluorescence intensity at 590/530 nm after 24 h of treatment with IC_{50} and IC_{90} of (−)α-bisabolol. IC_{50} of miltefosine was used as a positive control,while untreated cells were used as a negative control. Error bars represent the standard deviations (SD). Each data point indicates the mean of the results of three measurements

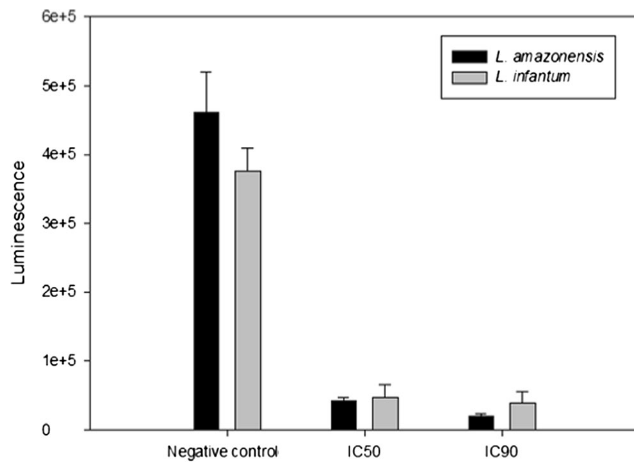

Fig. 8 ATP levels of *L. amazonensis* and *L. infantum* after 24 h of incubation with IC_{50} and IC_{90} of (−)α-bisabolol. Error bars represent the standard deviations (SD). Each data point indicates the mean of the results of three measurements

12.39 (IC_{50}) and 10.19% (IC_{90}) in the case of *L. infantum* (Fig. 8). Our results were confirmed by similar research, which revealed that the ATP is a key molecule for chromatin condensation, nuclear fragmentation, and regulation and maintenance of ion homeostasis during the apoptosis (Roy et al. 2008).

Conclusion

In summary, our study investigated the potent leishmanicidal activity of Tunisian chamomile essential oil against both *L. amazonensis* and *L. infantum*. More importantly, the isolation and the identification of (−)-α-bisabolol as the bioactive compound was elucidate by bio-guided fractionation. The sesquiterpene mechanism of action was investigated and it was proved as an inducer of apoptosis. Its effects on the mitochondrial activity were observed including the strong increase of both mitochondrial potential and ATP levels. These promising results suggested that the non-toxic (−)-α-bisabolol can be a natural potential alternative to the available drugs. Further studies are required to confirm the characteristics of apoptosis, such as the DNA fragmentation, the chromatin condensation, and the ROS generation; also, it is necessary to validate its activity in vivo.

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