Biomedicine & Pharmacotherapy 133 (2021) 111025

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Vernonia brasiliana (L.) Druce induces ultrastructural changes and apoptosis-like death of *Leishmania infantum* promastigotes

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ARTICLE INFO

Keywords:
β-caryophyllene
DH82 cells
Flow cytometry
Isobologram
Leishmaniasis
Transmission electron microscopy

ABSTRACT

The present study aimed to evaluate the antileishmanial effect, the mechanisms of action and the association with miltefosine of *Vernonia brasiliana* essential oil against *Leishmania infantum* promastigotes. This essential oil was obtained by hydrodistillation and its chemical composition was determined by gas chromatography-mass spectrometry (GC–MS). The antileishmanial activity against *L. infantum* promastigotes and cytotoxicity on DH82 cells were evaluated by MTT colorimetric assay. Ultrastructural alterations were evaluated by transmission electron microscopy. Changes in mitochondrial membrane potential, in the production of reactive oxygen species, and analysis of apoptotic events were determined by flow cytometry. The association between the essential oil and miltefosine was evaluated using the modified isobologram method. The most abundant component of the essential oil was β-caryophyllene (21.47 %). Anti-*Leishmania* assays indicated an IC_{50} of 39.01 ± 1.080 μg/mL for promastigote forms after 72 h of treatment. The cytotoxic concentration for DH82 cells was 63.13 ± 1.211 μg/mL after 24 h of treatment. The effect against *L. infantum* was proven through the ultrastructural changes caused by the oil, such as kinetoplast and mitochondrial swelling, vesicles in the flagellar pocket, discontinuity of the nuclear membrane, nuclear fragmentation and condensation, and loss of organelles. It was observed that the oil leads to a decrease in the mitochondrial membrane potential (35.10 %, $p = 0.0031$), increased reactive oxygen species production, and cell death by late apoptosis (17.60 %, $p = 0.020$). The combination of the essential oil and miltefosine exhibited an antagonistic effect. This study evidences the antileishmanial action of *V. brasiliana* essential oil against *L. infantum* promastigotes.

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https://doi.org/10.1016/j.biopha.2020.111025
Received 6 October 2020; Received in revised form 8 November 2020; Accepted 15 November 2020

1. Introduction

Leishmaniasis is a neglected zoonosis that affects mainly the impoverished population and those living under a situation of social and economic vulnerability [1]. It is an infectious, non-contagious, and chronic disease caused by *Leishmania* protozoa and spread by the bite of an infected female sandfly [2].

Dogs are the most important domestic reservoir of *L. infantum*, the causative agent of canine leishmaniasis (CanL), and also the main source of infection of the vector. Thus, their presence in endemic areas represents a risk factor for the development of the human disease [3]. The canine disease presents numerous clinical signs, including cutaneous and visceral signs, but the majority of animals can remain asymptomatic for years [4].

The treatment of leishmaniasis is still a challenge, especially for CanL. The development of the first chemotherapeutic drugs occurred several decades ago. The so-called first-line drugs are now antique and disadvantageous but still are used. When sick animals no longer respond to therapy with pentavalent antimonials, there are other options available, such as allopurinol, amphotericin B, pentamidine, paromomycin, domperidone, and miltefosine. Among the mentioned drugs, miltefosine stands out as the first oral drug approved for treating CanL [4]. Even so, there are barriers to a proper treatment, as the cost of medications, adverse effects, unsatisfactory clinical responses, and the emergence of resistant parasite strains. Therefore, there is a need to look for alternative therapeutic strategies that are cheaper, more effective, and accessible to the population [5,6].

One of the pathways for the formulation of new drugs is the use of plants as sources of new bioactive compounds [7]. Crude extracts and essential oils have chemical constituents with a variety of biological activities, which can be attributed to the presence of secondary metabolites, such as alkaloids, phenolic compounds, and terpenes [8]. Among the plants used for medicinal purposes, we highlight those belonging to the genus *Vernonia*.

The *Vernonia* genus belongs to the Asteraceae family, also known as Compositae, and to the Vernoniae tribe. Numerically, it is one of the largest genera of the family, with approximately 1000 species [9]. It comprises shrubs, lianas, and trees, distributed mainly in tropical regions, and routinely used as part of traditional medicine [10]. The traditional use of *Vernonia* is mostly widespread in Africa, although Brazil has the largest number of species. They are used in the treatment of amebiasis, malaria, sexually transmitted diseases, in addition to being useful in combating stress and as an aphrodisiac [11,12]. Several of its pharmacological properties have been proved, as the antiparasitic effect against obligate intracellular parasites, as *Trypanosoma brucei rhodesiense* [13], *Toxoplasma gondii* [14], *Plasmodium* spp. [15,16], and *Leishmania* spp. [17–23].

Concerning antileishmanial action, extracts [17,18], fractions [19], isolated compounds [20,21], and essential oils [22,23] of four species of *Vernonia* were tested and showed activity against *Leishmania* spp. However, except for one study, the biological effect was evident only against species responsible for cutaneous leishmaniasis. Only Moreira et al. [23] points out the leishmanicidal action against *L. infantum*. In this study, the essential oil of *V. polyanthes* was testes and displayed a significant activity against *L. infantum* promastigote forms, with IC_{50} value of 19.4 μg/mL.

V. brasiliana is typical of the Brazilian territory and is used in popular medicine, especially by indigenous peoples, quilombolas, and riverside populations [24]. When compared to other specimens of the genus, its biological properties are not as explored. The number of reports in the literature is reduced and restricted to antimicrobial [25], insecticidal and trypanocidal [26], antiplasmodial [27–29], and anti-*Leishmania amazonensis* actions [22,30]. Both studies with *L. amazonensis* assessed the essential oil from the leaves of *V. brasiliana* against promastigote forms. Moreover, Cortez de Sá also evaluated the ultrastructural changes caused by the essential oil and evidenced that it led to damages that compromise parasite viability [30].

Due to the clinical and epidemiological relevance of CanL, there is a need to search for bioactive compounds with properties against *L. infantum* to be used as a therapeutic alternative. Thus, this study aimed to evaluate the antileishmanial effect of *V. brasiliana* essential oil (VBEO) in *L. infantum*, describe its mechanism of action and investigate the interaction of VBEO and miltefosine in promastigote forms.

2. Material and methods

2.1. Reagents

The reagents 2, 7-dichlorodihydro-fluorescein (H_2DCFDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco′s modified Eagle′s medium (DMEM), dimethyl sulfoxide (DMSO), EPON 812 resin, glutaraldehyde, hydrogen peroxide, miltefosine, osmium tetroxide, Schneider's insect medium, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, and penicillin were acquired from Gibco (Gaithersburg, MD, USA). FITC Annexin V/Dead Cell Apoptosis Kit was obtained from Invitrogen™ (Carlsbad, CA, USA) and tetramethylrhodamine ethyl ester (TMRE) from Molecular Probes (Carlsbad, CA, USA).

2.2. Plant material

V. brasiliana leaves were collected in the city of Santa Luzia, Maranhão State, Brazil (4°13′43.6″S 45°59′51.4″W). The plant material was identified at the Herbarium "Prisco Bezerra" of the Universidade Federal do Ceará, and a voucher specimen was deposited under number 55227.

2.3. Essential oil extraction

The leaves were dried and submitted to hydrodistillation for the obtention of the essential oil [31]. An aliquot was sent to the Instituto de Tecnologia em Fármacos (FIOCRUZ/RJ) for chemical identification through gas chromatography-mass spectrometry (GC–MS).

2.4. Gas chromatography–mass spectrometry analyses (CG–MS)

The essential oil from *V. brasiliana* leaves was dissolved in ethyl acetate (1 mg/mL) and analyzed on a Shimadzu QP 5000 gas chromatograph, with ZB–5 ms capillary column (5 % phenyl arylene 95 % dimethylpolysiloxane) coupled at 70 eV (40–500 Da) HP 5MS mass selective detector of electronic impact, with a transference temperature of 280 °C. Chromatographic analyses were carried out under the following conditions: volume injection of 0.3 μL of ethyl acetate, helium as carrier gas (99.99 %), injector temperature of 280 °C, split mode (1:10), the initial temperature of 40 °C (5 min) and final temperature of 300 °C (7.5 min) [32]. Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction. The identification of essential oil components was achieved based on similarity with data from Wiley Registry of Mass Spectral Data, 7th Edition (Wiley Interscience, New York).

2.5. Parasites

Promastigote forms of *L. infantum* (MCAN/BR/2014/21BAÇO) were cultured at 26 °C in Schneider's Insect Medium (Sigma, St. Louis, MO, USA), supplemented with 20 % fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA), 100 U/mL of penicillin (Gibco, Gaithersburg, MD, USA), 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA) and 2% sterile male human urine.

2.6. Cell culture

Canine macrophage cell line DH82 (ATCC® CRL-10389™) was cultured in DMEM medium (Sigma, St. Louis, MO, USA), supplemented with 15 % FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and 2 mM of L-glutamine (Gibco, Gaithersburg, MD, USA), at 37 °C and 5% CO_2.

2.7. Activity against promastigote forms

L. infantum promastigotes (10^6 parasites/mL) were plated into 96-well plates and treated with different concentrations obtained by serial dilutions 1:2 of VBEO (100–3.125 μg/mL), with a final volume of 100 μL/well. After 72 h incubation, the viability of the parasites was measured by the modified colorimetric method with tetrazolium-dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [33]. 10 μL of MTT (5 mg/mL) was added in each well and after four hours, 80 μL of DMSO was added to dissolve the formazan crystals. The absorbance was analyzed on a spectrophotometer at a wavelength of 570 nm. Data were normalized using the formula:

% survival = sample OD − blank OD/control OD − blank OD x 100

The results were used to calculate the IC_{50} (inhibitory concentration for 50 % of parasites). Miltefosine (40–1.25 μg/mL) was used as a reference drug.

2.8. Cytotoxicity assay

DH82 cells were seeded in 96-well plates (5×10^5 cells/mL) and treated with different concentrations obtained by serial dilutions 1:2 of the essential oil (250–1.95 μg/mL). After 24 h, cell viability was measured by MTT colorimetric assay. 10 μL of MTT was added in each well and after four hours, the medium was discarded and then 50 μL of DMSO was added. Data were normalized using the aforementioned formula, for CC_{50} (cytotoxic concentration for 50 % of cells) calculation. Miltefosine was used as a control (100–3.125 μg/mL).

2.9. Transmission electron microscopy

Promastigote forms of *L. infantum* were treated with the essential oil at the IC_{50} concentration for 72 h. The parasites were then fixed with 2.5 % glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7,4) overnight, post-fixed in a 1 % osmium tetroxide solution, washed in 0.1 M sodium-cacodylate buffer, dehydrated in acetone series, and embedded in EPON 812 resin. Ultrathin sections were obtained from 100 nm cuts in Sorvall MT 2-B (Porter Blum) ultramicrotone (Sorvall, Newtown, CT, USA) stained with 5 % uranyl acetate aqueous solution and lead citrate (1.33 % lead nitrate and 1.76 % sodium citrate), and examined in a transmission electron microscope JEM-1011 (JEOL, Tokyo, Japan) operating at 80 kV [34]. Images were recorded with a Gatan 785 ES1000W Erlangshen camera.

2.10. Determination of mitochondrial membrane potential (MMP)($\Delta\Psi m$)

L. infantum promastigotes (2×10^6 parasites/mL) were treated with VBEO for 72 h, at the IC_{50} concentration. Parasites were centrifuged at 1500 rpm for 5 min at room temperature, washed in PBS, incubated with 300 μL of tetramethylrhodamine ethyl ester (TMRE, 50 nM) in the dark for 15 min, at room temperature, and submitted to flow cytometry. Parasites killed by heat (60 °C, 1 h) and parasites treated with 1% DMSO were used as positive and negative controls, respectively.

2.11. Detection of reactive oxygen species (ROS) production

ROS production was evaluated using fluorescent indicator 2, 7-dichlorodihydro-fluorescein (H_2DCFDA). L. infantum promastigotes (2×10^6 parasites/mL) were treated with the essential oil for 72 h at 26 °C, at the IC_{50} concentration. Then, the parasites were centrifuged, washed with PBS, and incubated with 150 μL of H_2DCFDA (5 μM) for 30 min in the dark, at room temperature. The H_2DCFDA-fluorescence intensity was measured by flow cytometry and hydrogen peroxide (50 μM) and parasites treated with 1% DMSO were used as positive and negative controls, respectively.

2.12. Detection of L. infantum apoptosis by flow cytometry Infantum apoptosis by flow cytometry

For analysis of parasite apoptosis (phosphatidylserine (PS) externalization) and necrosis (plasma membrane integrity), we used the FITC Annexin V-FITC and propidium iodide (PI)/ Dead Cell Apoptosis Kit (Invitrogen™), according to the manufacturer's instructions, followed by flow cytometry analysis. Briefly, promastigote forms of L. infantum (2×10^6 parasites/mL) were incubated with the essential oil of *V. brasiliana* at the IC_{50} concentration for 72 h at 26 °C. Parasites were centrifuged at 1500 rpm for 5 min at room temperature, washed in PBS, resuspended in 100 μL of 1X annexin-binding buffer, 5 μL of annexin V and 1 μL of PI (100 μg/mL). After 15 min of incubation, protected from light, at room temperature, 400 μL of 1X annexin-binding buffer was added in each sample. From analysis, promastigotes were classified according to their staining as apoptotic parasites (annexin V^+; PI^{neg}), late apoptotic/necrotic parasites (annexin V^+; PI^+), and viable parasites (annexin V^{neg}; PI^{neg}). Miltefosine (50 μM) was used as an antileishmanial reference drug and untreated parasites as control.

2.13. Flow cytometry

Twenty thousand-event acquisitions were performed on Beckman Coulter CytoFlex flow cytometer. The limits for the quadrant markers in dot plots and histograms were set based on non-staining controls. The flow cytometric analyses to determine the MMP, ROS and apoptosis was performed using CytoExpert software (Beckman Coulter, Inc., Brea, CA, USA). TMRE, H_2DCFDA, Annexin V-FITC and PI fluorescence were excited by 488nm-blue laser and their fluorescence were collected at 585/42; 525/40; 525/40; and 610/20 bandpass filters, respectively.

2.14. Determination of drug interactions

The interaction between VBEO and miltefosine in *L. infantum* promastigotes was evaluated using the modified isobologram method [35]. The IC_{50} of both compounds, previously obtained, were used to establish the maximum concentration of each drug in the combination. The highest concentrations of the solutions were prepared in proportions of 4:1, 3:2, 2:3 and 1:4 of VBEO and miltefosine, as follows:

Association 1 (4:1): 80 % of VBEO (240 μg/mL) + 20 % of miltefosine (20 μg/mL).

Association 2 (3:2): 60 % of VBEO (180 μg/mL) + 40 % of miltefosine (40 μg/mL).

Association 3 (2:3): 40 % of VBEO (120 μg/mL) + 60 % of miltefosine (60 μg/mL).

Association 4 (1:4): 20 % of VBEO (60 μg/mL) + 80 % of miltefosine (80 μg/mL).

The experiments were performed in the same way as for the determination of antileishmanial activity, described in item 2.7. After 72 h of incubation, parasite viability was measured by MTT colorimetric method and the absorbance was analyzed on a spectrophotometer, at a wavelength of 570 nm. After data normalization, fractional inhibitory concentrations (FIC) at the IC_{50} level were calculated for both drugs, as follows: $FIC_{50} = IC_{50}$ drugs in combination/IC_{50} drug alone. FIC_{50}s of each drug ratio (1:4, 2:3, 3:2 and 4:1) were used to build the isobologram. Later, the sum of the FIC_{50}s for each ratio was determined ($\sum FIC_{50} = FIC_{50}$ VBEO + FIC_{50} miltefosine) and then, the fractional inhibitory concentration index (FICI) was calculated, as an overall mean of $\sum FIC_{50}$s. The value obtained was used to classify the nature of the interaction as synergistic ($FICI < 0.5$), additive ($0.5 < FICI < 4$) or antagonist ($FICI > 4$) [36]. Five independent assays were performed, each one in octuplicate.

2.15. Statistical analysis

Values were expressed as mean ± standard deviation. The results were analyzed by the Kruskal–Wallis test, followed by Dunn's multiple comparison test. Statistical analyses were performed with GraphPad Prism 7.00 software (San Diego, CA, USA) and differences were considered significant when $p < 0.05$.

3. Results

3.1. Chemical composition

To identify and quantify the chemical constituents present in the essential oil extracted from *V. brasiliana*, a chromatographic analysis was done, and the compounds found were listed according to retention time. Gas chromatography-mass spectrometry (GC–MS) revealed the presence of 28 compounds, of which the main constituents detected were β-caryophyllene (peak 7, 21.47 %), followed by germacrene-D (peak 15, 14.57 %), caryophyllene oxide (peak 23, 10.28 %), α-humulene (peak 10, 8.85 %), and α-copaene (peak 4, 5.15 %) as presented at Table 1, Fig. 1 and Supplementary material (Fig. S1-S5). VBEO was mostly composed of sesquiterpenes but presents a small fraction of monoterpenes (terpinen-4-ol) and norisoprenoids (β-ionone).

Table 1
Chemical composition of *Vernonia brasiliana* essential oil.

Peaks	*Vernonia brasiliana* essential oil		
	Compounds	Rt (min)	Content (Peak Area %)
1	terpinen-4-ol	7.18	0.1
2	α-cubenene	9.617	0.11
3	Ciclosativene	9.981	0.2
4	α-copaene	10.062	5.15
5	β-bourbonene	10.196	2.15
6	α-gurjunene	10.523	0.43
7	β-caryophyllene	10.746	21.47
8	β-cubenene	10.857	0.87
9	Isoledene	10.894	0.12
10	α-humulene	11.236	8.85
11	Alloaromadendrene	11.295	3.03
12	Aromadendrene	11.295	3.03
13	α-amorphene	11.466	0.99
14	β-ionone	11.526	0.55
15	Germacrene-D	11.593	14.57
16	α-elemene	11.704	0.32
17	α-muurolene	11.778	2.95
18	γ-cadinene	12.001	0.46
19	δ-cadinene	12.046	2.71
20	cis-calamenene	12.113	0.55
21	α-calacorene	12.677	0.34
22	Spathulenol	12.9	2.35
23	Caryophyllene oxide	12.982	10.28
24	salvial-4(14)-en-1-one	13.108	0.74
25	Viridiflorene	13.264	0.71
26	(3E,5E,8Z)-3,7,11-Trimetildodeca-1,3,5,8,10-pentaene	13.383	0.68
27	τ-muurulol	13.747	1.81
28	τ-cadinol	13.91	1.13

Rt: retention time (minutes) of the compounds in column. Peak Area %: percentage of the normalized area which indicates the relative distribution of the compounds in the sample.

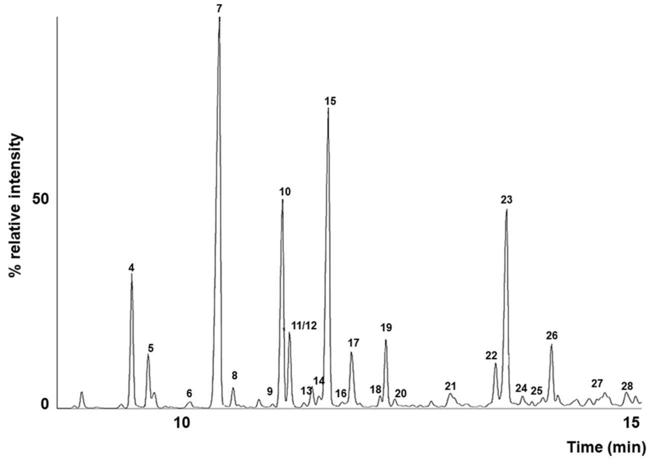

Fig. 1. Total ion chromatogram registered by GC–MS of *Vernonia brasiliana* essential oil showing 28 peaks of which stand out the major compounds: β-caryophyllene **(7)** (Rt=10.74 min; 21.47 %), germacrene-D **(15)** (Rt=11.59 min; 14.57 %), caryophyllene oxide **(23)** (Rt=12.98 min; 10.28 %), α-humulene **(10)** (Rt=11.23 min; 8.85 %), and α-copaene **(4)** (Rt=10.06 min; 5.15 %). The mass spectra of majority compounds identified are presented at supplementary material (S1-S5).

3.2. Anti-Leishmania activity and cytotoxicity

Antileishmanial activity assays were performed to evaluate the effect of the essential oil in promastigote forms of L. infantum. The results showed that VBEO had a concentration-dependent activity, with an IC_{50} value of 39.01 ± 1.080 μg/mL. In DH82 cells, the oil was cytotoxic at a concentration of 63.13 ± 1.211 μg/mL (Fig. 2), indicating that VBEO was more toxic to the parasites than the cells. The reference drug miltefosine showed antileishmanial activity and cytotoxicity as expected (Table 2).

3.3. Ultrastructural changes

Transmission electron microscopy was used to investigate the effects of *V. brasiliana* essential oil at the IC_{50} concentration (39.01 μg/mL) for 72 h on *L. infantum* promastigotes ultrastructure and morphology.

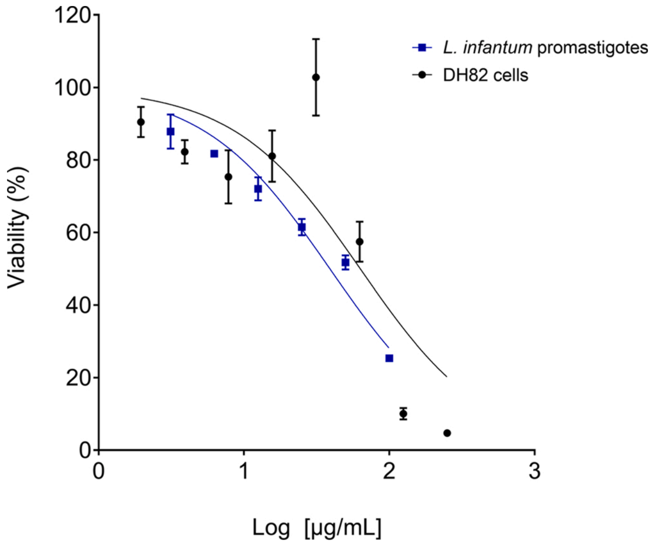

Fig. 2. Dose-response curve of the effects of *Vernonia brasiliana* essential oil on the viability of *Leishmania infantum* promastigote forms and DH82 macrophages. Data represents the mean ± standard error of five independent experiments carried out in triplicate.

Table 2

Antileishmanial activity, cytotoxicity, and selectivity index of *Vernonia brasiliana* essential oil for 72 h of treatment.

Compounds	Cytotoxicity (DH82 cells)	L. infantum promastigotes	
	CC_{50} (µg/mL)	IC_{50} (µg/mL)	SI
VBEO	63.13 ± 1.211	39.01 ± 1.080	1.61
Miltefosine	2.541 ± 1.164	4.608 ± 1.110	0.55

Data represent mean ± SD. VBEO: *Vernonia brasiliana* essential oil; CC_{50}: cytotoxic concentration for 50 % of cells; IC_{50}: inhibitory concentration for 50 % of parasites. SI: Selectivity index.

Untreated promastigotes showed preserved ultrastructure (Fig. 3A), while parasites treated with VBEO presented a variation in the morphology of the parasites to a more rounded shape. Swelling in the mitochondria (Fig. 3B) and kinetoplast (Fig. 3C and D), loss of mitochondrial cristae (Fig. 3B, star) and kinetoplast (Fig. 3C and D, asterisk), and formation of membranous and vesicular structures was noted in the flagellar pocket in *L. infantum* promastigotes (Fig. 3B and C, thin arrows). The nucleus showed a decondensation of chromatin surrounding the nuclear membrane (Fig. 3A), discontinuity of chromatin (Fig. 3B and C, thick arrows), absence of nucleolus, and dilatation of the nuclear membrane (Fig. 3D, thick arrows). Prominent nuclear changes were observed, such as chromatin fragmentation and dispersion (Fig. 4A, white asterisk), irregular chromatin condensation (Fig. 4B and C), and nuclear pyknosis (Fig. 4D, arrowhead). Loss of cytoplasmic organelles (Fig. 4A, B, C, D), severe cytoplasm damage, and rupture of the plasma membrane were also observed (Fig. 4E).

3.4. Mitochondrial membrane potential (Δψm)

Flow cytometry analyses were performed to evaluate the mitochondrial transmembrane potential and the results showed that VBEO leads to a significant decrease in the Δψm in L. infantum promastigotes. Untreated parasites showed 81.99 % of cells stained with TMRE. The treatment with VBEO at 39.01 µg/mL for 72 h caused mitochondrial damage and reduced the Δψm to 35.10 % ($p = 0.0031$) (Fig. 5).

3.5. ROS production

To investigate whether VBEO leads to ROS production, ROS levels were measured using the cell-permeable dye H_2DCFDA. The generation of ROS was enhanced to 65.93 ± 0.852 % in L. infantum promastigotes treated with VBEO at 39.01 µg/mL for 72 h ($p = 0.011$), compared to untreated parasites. Hydrogen peroxide (H_2O_2) was used as a control and it induced ROS formation to 72.78 ± 6.504 % ($p = 0.012$) (Fig. 6).

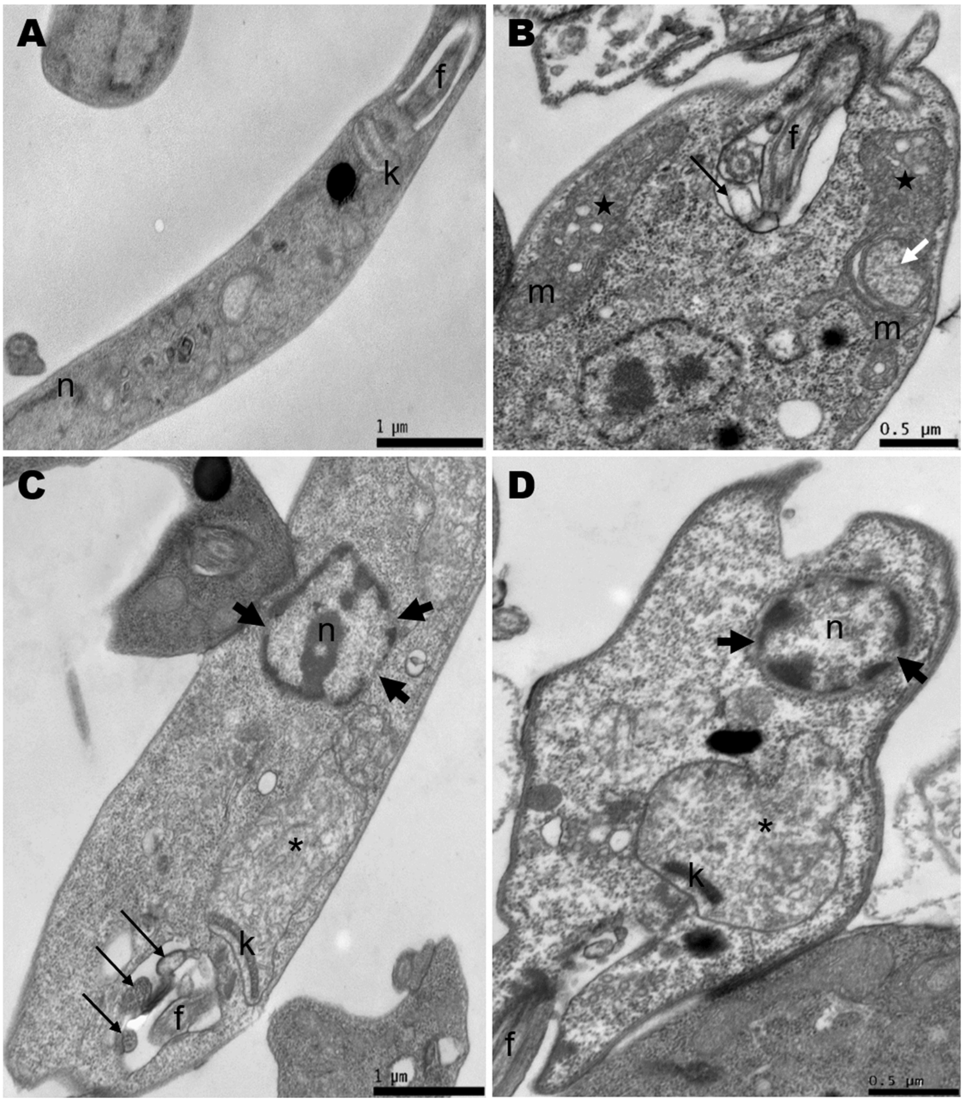

Fig. 3. Transmission electron microscopy of *Leishmania infantum* promastigotes treated with *Vernonia brasiliana* (39.01 µg/mL) for 72 h. (A) Untreated control, with normal characteristics of the protozoan. (B) Mitochondrial swelling, with breakdown of mitochondrial cristae (star) and presence of a circular and granular material inside the organelle (white arrow); membranous and vesicular structures in the flagellar pocket (B and C, thin arrow), kinetoplast swelling (C and D, asterisk), and nucleus membrane discontinuity (C and D, block arrow). Dilation of nuclear membrane and absence of nucleolus (D, block arrow). f: flagellum; k: kinetoplast; m: mitochondria; n: nucleus.

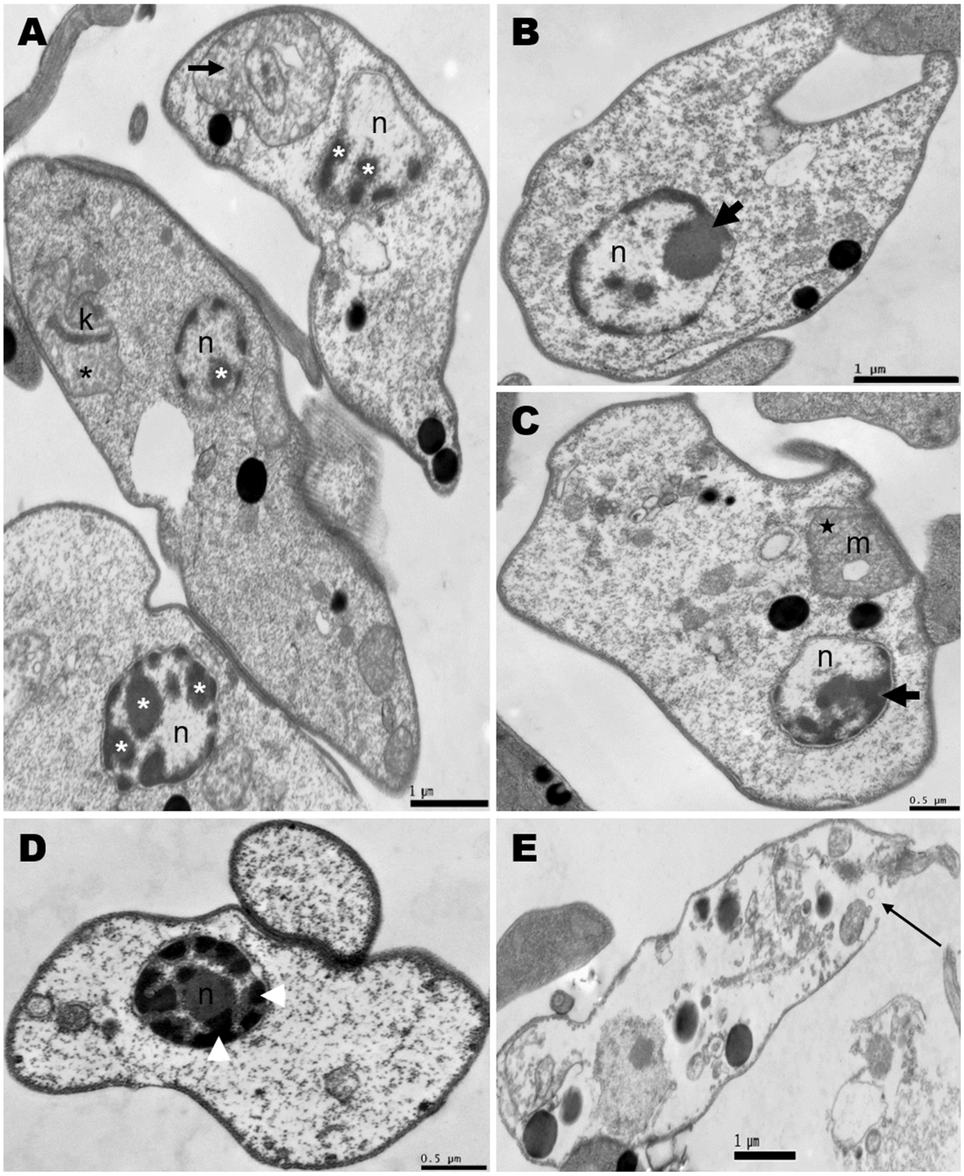

Fig. 4. Ultrastructural effects of *Vernonia brasiliana* essential oil on *Leishmania infantum* promastigotes treated for 72 h. Marked changes in parasite morphology are observed. (A) Fragmentation and dispersion of nuclear chromatin (white asterisk), presence of an autophagosomal structure (arrow), and kinetoplast swelling (asterisk). (B-C) Loss of cytoplasmic organelles, and chromatin condensation (block arrow). In C, mitochondrial swelling and loss of cristae are also present (star). (D) Nuclear pyknosis (arrowhead) and rounding of the parasite. (E) Severe damage of the cytoplasm, rupture of the plasma membrane (thin arrow). k: kinetoplast; m: mitochondria; n: nucleus.

3.6. Evaluation of phosphatidylserine exposure and cell membrane integrity

To determine the mechanism of cell death triggered by VBEO, whether apoptosis or necrosis, the externalization of phosphatidylserine and cell membrane integrity were evaluated using Annexin V-FITC and PI staining. After treatment with VBEO for 72 h, the number of viable parasites decreased from 95.67 % to 68.82 %. The percentage of the necrotic population increased to 7.41 % ($p = 0.028$) and the apoptotic population increased to 6.17 % ($p = 0.022$), compared to untreated parasites. The intensity of Annexin-V and PI fluorescence was increased up to 17.60 % ($p = 0.020$) compared to the intensity of untreated parasites, suggesting a late apoptotic process. There is a statistically significant difference in the percentage of necrotic, late apoptotic, and apoptotic parasites (Fig. 7D-F) between the groups treated with VBEO and the reference drug miltefosine compared to the untreated group. These data demonstrate that the treatment with VBEO induces late apoptosis in L. infantum promastigotes.

3.7. Antileishmanial association of VBEO-miltefosine

The IC_{50} values for both drugs, alone and in each association, the fractional inhibitory concentrations and the fractional inhibitory concentration index are shown in Table 3. The interaction of VBEO and miltefosine was classified as antagonist, with a FIC index of 4.779.

In the first combination (4:1), no leishmanicidal activity was observed, since IC_{50} values obtained for the essential oil and miltefosine were higher than the top concentrations used in the experiments. It was also observed that IC_{50} values for the essential oil decreased at each combination rate (Fig. 8A). IC_{50} values increased for miltefosine, except in a 1:4 ratio (Fig. 8B). An isobologram was constructed based on the FIC_{50} values of each association (Fig. 8C). Points corresponding to the FIC_{50} values of VBEO and miltefosine were connected by a tendency line. All points were located above the theoretical additive line, thus characterizing antagonism.

4. Discussion

In this study, we assessed the chemical composition of the essential oil extracted from the leaves of *V. brasiliana*, its cytotoxicity against

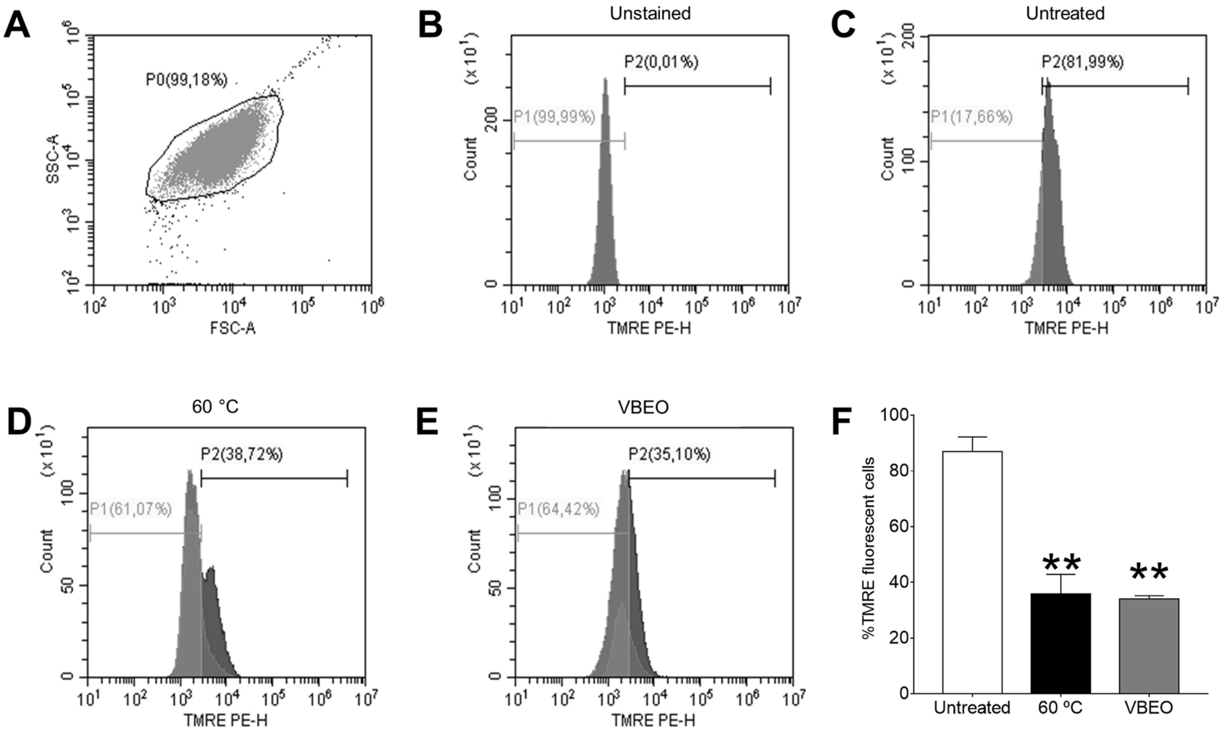

Fig. 5. Changes in the mitochondrial membrane potential in Leishmania infantum promastigotes incubated for 72 h with Vernonia brasiliana essential oil. (A) Promastigotes captured in the gated region and representative histogram. (B) Unstained parasites. (C) Untreated parasites. (D) Promastigote forms of L. infantum killed by heat, standard protocol for assessing mitochondrial viability. (E) Statistically significant differences are observed in the percentage of cells marked with TMRE between the untreated group and the groups treated with VBEO and parasites killed by heat. (**) $p < 0.01$ when compared to untreated parasites by Mann-Whitney test.

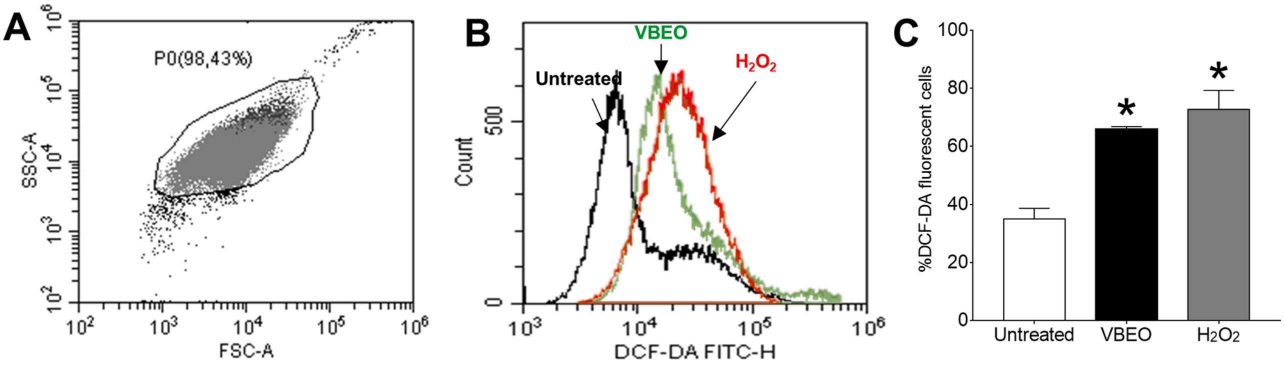

Fig. 6. Evaluation of intracellular ROS levels in Leishmania infantum promastigotes after incubation with Vernonia brasiliana essential oil for 72 h. (A) Promastigotes captured in the gated region and representative histogram. (B) The green line shows increased ROS production in parasites treated with the essential oil when compared to control parasites (black line). The same was observed in the group treated with H_2O_2 (red line), a natural ROS inducer. (C) Statistically significant differences are observed in the percentage of parasites marked with H_2DCFDA between the untreated group and the groups treated with VBEO and H_2O_2. (*) $p < 0.05$ when compared to untreated parasites by Mann-Whitney test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

DH82 cells, its anti-leishmanial action on *L. infantum* promastigotes, the alterations it induces in promastigote forms, and the effect of the combination of the essential oil with the reference drug miltefosine.

Our chromatographic analysis revealed that VBEO is rich in sesquiterpenes, which comprise 26 of the 28 compounds found. The first report of the chemical characterization of the essential oil from *V. brasiliana* leaves was made by Maia et al. [25] that identified 8 substances, of which 7 were sesquiterpenes. Similarly, Cortez de Sá [30] found 7 compounds, all classified as sesquiterpenes. When comparing the results obtained by Martins et al. [22], they observed the presence of 53 chemical constituents, 17 belonging to the sesquiterpene class.

It is noticed that there is a variation in the number of compounds found in each essential oil analyzed, which can be explained due to environmental variations (seasonality, rainfall rate, the incidence of UV rays, soil nutrients, plant age and stage of development, the time of day the plant was collected, etc.), that influences in the total content of secondary metabolites in plants [37]. However, despite quantitative differences, most of the components found in the cited studies belong to the same chemical family (terpenes). In addition, the major compounds in all studies mentioned, including ours, were the same (β-caryophyllene or germacrene-D), which may indicate that the essential oils of *V. brasiliana* leaves follow a pattern in its chemical composition.

Except for salvial-4(14)-en-1-one and (3E,5E,8Z)-3,7,11-trimethyldodeca-1,3,5,8,10-pentaene), all compounds found in VBEO have

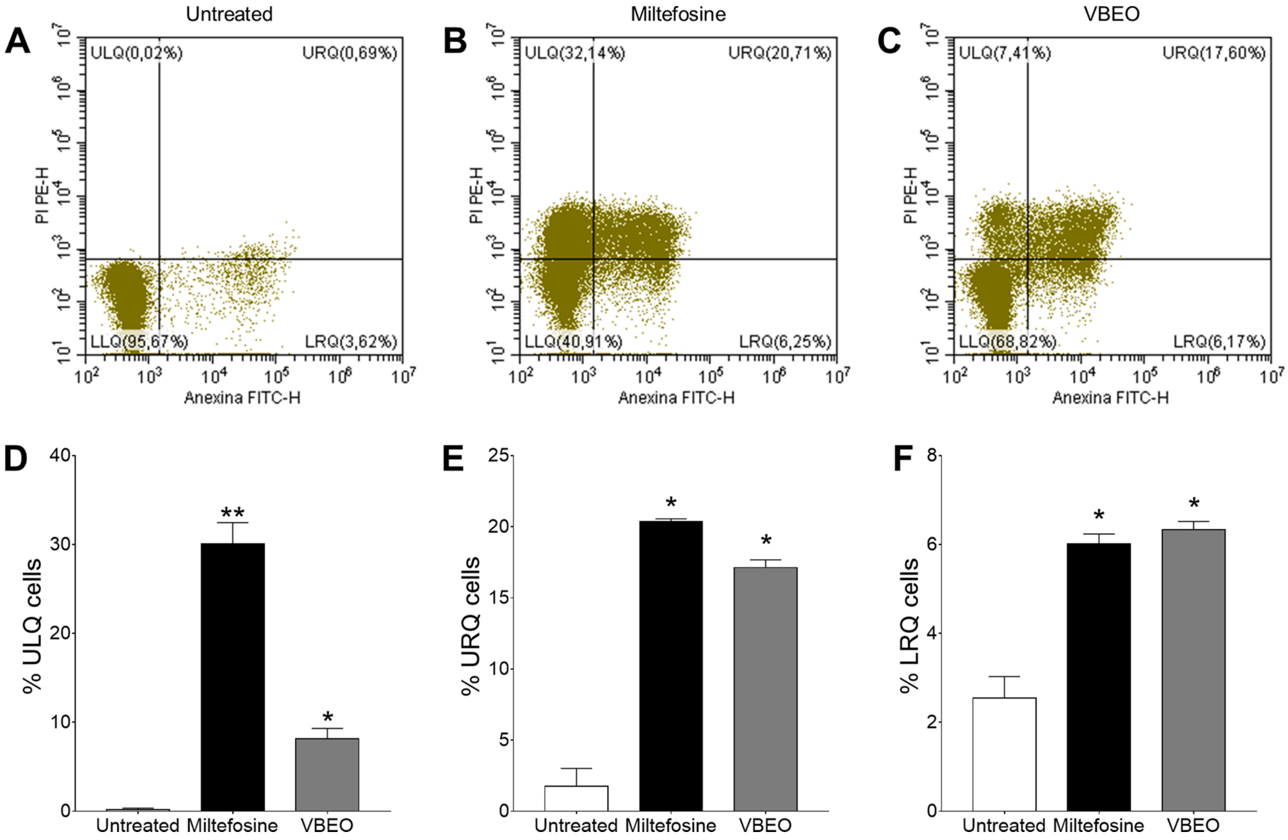

Fig. 7. Changes in phosphatidylserine exposure and plasma membrane integrity in Leishmania infantum promatigotes treated with IC_{50} of Vernonia brasiliana essential oil for 72 h. Parasites were labelled with Annexin V-FITC and PI. (A) Untreated promastigotes used as control. (B) Parasites treated with miltefosine, an apoptosis-inducing drug, at a concentration of 50 μM. (C) Promastigotes treated with Vernonia brasiliana essential oil, at the IC_{50} concentration (39.01 μg/mL). In D, E and F, statistical differences between the percentage of necrotic, apoptotic and late apoptotic cells, respectively, are shown. In all, the results obtained in the groups treated with the essential oil and miltefosine were statistically different when compared to untreated parasites. (*) $p < 0.05$; (**) $p < 0.01$ when compared to untreated parasites by Mann-Whitney test. ULQ: upper left quadrant; URQ: upper right quadrant; LRQ: low right quadrant.

Table 3
IC_{50}, FIC_{50} and ΣFIC_{50} of *Vernonia brasiliana* essential oil-miltefosine combination against *Leishmania infantum* promastigotes.

Combination rate		Combined drugs					
		IC_{50} (μg/mL)		FIC_{50}		$\sum FIC_{50}$	FICI
VBEO	Miltefosine	VBEO	Miltefosine	VBEO	Miltefosine		
5	0	39.01 ± 1.080	–	–	–	–	
4	1	126.7 ± 1.095	10.56 ± 1.095	3.247	2.291	5.538	
3	2	70.87 ± 1.056	15.75 ± 1.056	1.816	3.417	5.233	4.779
2	3	36.34 ± 1.084	18.17 ± 1.084	0.931	3.943	4.874	
1	4	11.03 ± 1.056	14.7 ± 1.056	0.282	3.190	3.472	
0	5	–	4.608 ± 1.110	–	–	–	

Data expressed as mean ± SD. VBEO: *Vernonia brasiliana* essential oil; IC_{50}: inhibitory concentration for 50 % of parasites. FIC_{50}: fractional inhibitory concentrations; $\sum FIC_{50}$: sum of fractional inhibitory concentrations; FICI: fractional inhibitory concentration index.

been described in the literature as active components of essential oils or plant extracts with leishmanicidal activity [22,38–47], which would be already an indicator of its biological effect.

Regarding the anti-*Leishmania* activity of VBEO, there are few reports in the literature, all with *L. amazonensis*. In the published studies, there was a variation in the IC_{50} values of the essential oil, from 213 μg/mL after 48 h of treatment [22] to 1.73 μg/mL at 72 h of treatment [30]. This shows that there are factors that can directly interfere with the biological activity of the oil, such as its chemical composition, the treatment time, the *Leishmania* strain used in the experiments (intrinsic sensitivity of the strain to the drug), among others.

In the light of our knowledge, this is the first description of the effect of VBEO on *L. infantum* promastigotes, which showed that the oil is active, with an IC_{50} of 39.01 μg/mL, considered promising for further evaluation. Promastigote forms are widely used in screening tests to search for new drugs, due to the ease of performing the experiments, but it is necessary to evaluate the potential drug action in amastigote forms [48,49]. Our study shows a satisfactory result of the essential oil in promastigote forms of *L. infantum*, and additional tests will confirm whether we are facing a possible leishmanicidal drug.

For cytotoxicity experiments, we used the DH82 cell line and obtained a CC_{50} of 63.13 μg/mL. As these cells are canine macrophages, they are a model closer to that found in the domestic reservoir. This is the first report of VBEO toxicity in DH82 macrophages. Cytotoxic effects of the essential oil were also evaluated in mice peritoneal macrophages, RAW 264.7 and Vero cells. Vero and RAW 264.7 cells showed CC_{50}

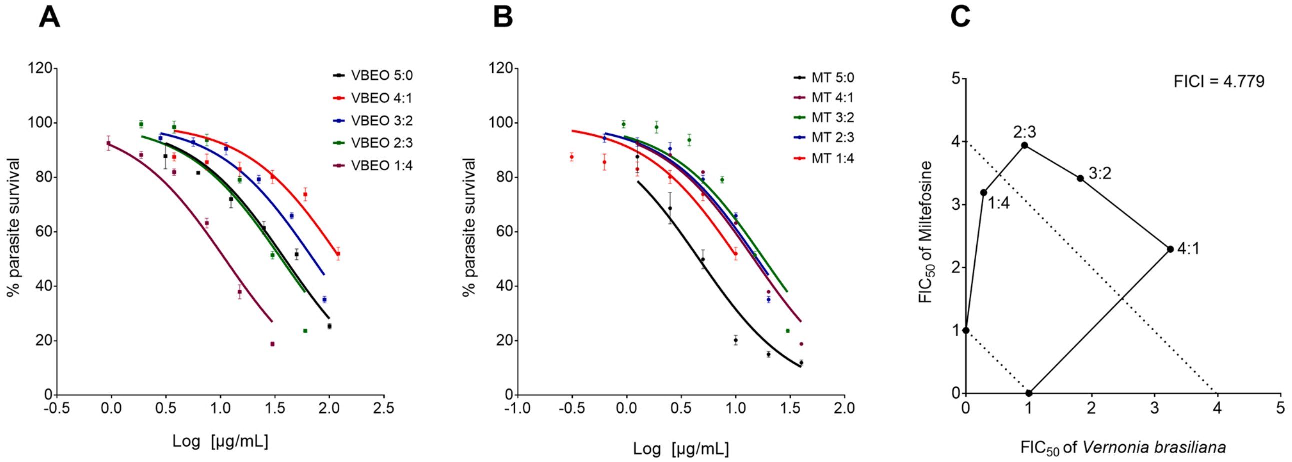

Fig. 8. Antagonistic interaction between *Vernonia brasiliana* essential oil and miltefosine on *Leishmania infantum* promastigotes. In A and B, the dose-effect curves of different combinations of the essential oil and miltefosine are observed. In C, a representative isobologram of the *in vitro* interactions between the respective drugs. The dotted line corresponds to the predicted positions of the experimental points for a simple additive effect. The points corresponding to the FIC values were connected by a tendency line. VBEO: *Vernonia brasiliana* essential oil; MT: miltefosine.

values of 151 and 198 µg/mL, respectively [22], while peritoneal macrophages were cytotoxic at a concentration of 88.35 µg/mL [30], closer to that obtained in our study. These data allow us to infer that VBEO is safe for several cell lines.

The antileishmanial activity of the essential oil of *V. brasiliana* against promastigote forms of *L. infantum* was confirmed by the ultrastructural changes found in the treated parasites. Kinetoplast and mitochondrial swelling are indicators of damage in this organelle and were the most reported structural finding in the literature for *L. infantum* promastigotes, treated with essential oils from *Thymus capitellatus* [50], *Cymbopogon citratus* [51], *Croton cajucara* [52], *Lavandula luisieri* and *L. viridis* [53]. Other cellular alterations found in our study, such as the presence of vesicular formations in the flagellar pocket, disruption of nuclear membrane and nuclear chromatin condensation, resembling the nucleus of apoptotic cells, have also been described for *L. infantum* promastigotes treated with *T. capitellatus* [50], *C. citratus* [51] and *C. cajucara* [52] essential oils.

Transmission electron microscopy showed that VBEO induced changes in *L. infantum* mitochondria. Usually, these are associated with alteration of mitochondrial membrane potential, as already observed in the literature [38,51]. To confirm mitochondrial dysfunction, the ΔΨm was evaluated by flow cytometry using TMRE, a positively charged permeable dye that can detect negative charge across a healthy mitochondrion of viable cells [54]. The measurement of the membrane potential of *L. infantum* promastigotes after incubation with VBEO suggests that the loss of ΔΨm is involved in its antileishmanial effect.

Some authors have demonstrated similar findings in parasites treated with essential oils. Rottini et al. [38] showed that the essential oil of *Endlicheria bracteolate*, at the concentration of 7.93 µg/mL, leads to mitochondrial damage in *L. amazonensis* promastigotes, independently of the treatment time. In experiments conducted by Machado et al. [51], *C. citratus* essential oil (25 µg/mL) caused depolarization of mitochondrial membrane potential in promastigotes of *L. infantum*. The same authors evaluated the effect of *T. capitelatus* essential oil (37 µg/mL), also against *L. infantum* promastigotes, and observed that it induced a decrease on ΔΨm [50].

In cells, it is observed that the depolarization of mitochondrial membrane potential is linked to increased levels of ROS, and subsequent cell death [55,56]. Thus, since VBEO led to mitochondrial membrane depolarization, it is plausible that ROS generation had increased in *L. infantum* promastigotes. Similar observations were made with other essential oils: the eugenol-rich oil of *Syzygium aromaticum* (EROSA) led to a significant increase in ROS levels in promastigotes of *L. donovani* [57], in the same way as the essential oil from *Artemisia annua* leaves, for the same parasites [58].

Previous studies have demonstrated that oxidative stress is involved in apoptotic-like cell death in *Leishmania* [59,60]. To evaluate cell death induction, parasites treated with VBEO were double-stained with Annexin-V and PI, and it was observed that the essential oil induced late apoptosis in *L. infantum* promastigotes.

There is still no agreement on the most appropriate terminology to refer to the cell death process in *Leishmania*. According to the review published by Basmaciyan and Casanovas [61], the authors claim that the terms “apoptosis-like cell death” or “programmed cell death” are not correct and that the term “apoptosis” is proper. In contrast, Menna-Barreto [62] says the opposite, that “apoptosis-like cell death” is more suitable. Here we use the terminology found most frequently in the literature.

An apoptosis-like process has been proposed as a cell death mechanism for *Leishmania* parasites [63]. The essential oils from both *A. herba-alba* and *A. campestris* killed *L. infantum* promastigotes by triggering apoptosis, in a dose and time-dependent manner [64]. *C. citratus* essential oil and its major compound citral induce apoptosis in *L. infantum*, at its IC_{50} values (25 and 42 µg/mL, respectively) [51]. An increment of the cellular phosphatidylserine externalization, characteristic of apoptosis, was observed in promastigote forms of *L. donovani* treated with the *A. annua* essential oil [58]. Also, in *L. donovani* promastigotes, treatment with EROSA led to a significant percentage of parasites stained positive for both Annexin-V and PI (55.60 %), indicating late apoptotic phase [57], as well as in our study.

Among the compounds identified in VBEO, eight are admittedly apoptosis inducers: β-caryophyllene, caryophyllene oxide, α-humulene, terpinen-4-ol, β-bourbonene, isoledene, β-ionone, and δ−cadinene. The apoptotic effect of these isolated substances, as well as their effects on ROS intracellular production and mitochondrial membrane potential, have already been described in the literature for several cell lines, as MG-63 [65,66], OVCAR3 [67,68], HCT116 [69–71], PC-3 [72–74], MCF-7 [72,75], L-929 fibroblasts [75], HL-60 cells [76], and others. There are also reports that caryophyllene oxide induces late apoptosis in *Trypanosoma cruzi* epimastigotes [77], a kinetoplastid as well as *Leishmania infantum*.

As cell death mechanisms are similar in cells and protozoa, the broad description of the apoptotic effects for the isolated compounds was sufficient to base our results and to suggest that the late apoptosis cell death of *L. infantum* promastigotes caused by VBEO is related to the presence of these active compounds.

To improve the antileishmanial effect of VBEO, the effect of its association with miltefosine, a standard drug for the treatment of visceral leishmaniasis, was evaluated. However, the results obtained showed that the combination had an antagonistic effect.

To date, few studies reported the association between synthetic drugs and natural compounds, or between substances isolated from plants, in the treatment of leishmaniasis. An additive effect between oxiranes and meglumine antimoniate (Glucantime®) against *L. amazonensis* amastigotes were observed by Gonçalves-Oliveira et al. [78], that may lead to an increment of global antileishmanial potential both in vitro and in vivo. When evaluating the effect between combinations of the major compounds of the essential oil of *Chenopodium ambrosioides* (ascaridole, carvacrol, and caryophyllene oxide) against *L. amazonensis* promastigotes, Pastor et al. [79] observed a synergistic effect of ascaridol-carvacrol combination and additive interaction for ascaridol-caryophyllene oxide and carvacrol-caryophyllene oxide. In general, the studies reported additive and synergistic relationship between the tested compounds. Our results, by contrast, showed an antagonistic interaction between a known leishmanicidal drug (miltefosine) and an essential oil with anti-*Leishmania* properties. This is important to emphasize that, even when combining two substances with recognized antiprotozoal activity, the result of the association will not necessarily be the same.

The present work demonstrates that the essential oil from *V. brasiliana* leaves holds antileishmanial activity against *L. infantum* promastigotes, and it is more toxic to the parasites than to DH82 cells. In contrast, its association with miltefosine did not show the same biological effect. The antiparasitic effect of VBEO was confirmed by the ultrastructural changes, decreased mitochondrial membrane potential, and increased ROS production, which together induced cell death by late apoptosis. Therefore, our overall results strongly suggest VBEO has active molecules that can be explored as sources for new antileishmanial drugs.

Funding

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES) [Finance Code 001], and by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ [grant number E-26/010.001759/2019]. The APC was funded by the Instituto Oswaldo Cruz – IOC. Dr. Fernando Almeida-Souza is a postdoctoral researcher fellow of CAPES [grant number 88887.363006/2019-00]. Dra. Ana Lucia Abreu-Silva is a research productivity fellow of National Scientific and Technological Development Council (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq) [grant number 309885/2017-5].

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank the Flow Cytometry Core Facility, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil for all flow cytometry analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.111025.

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