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Original article

Sesquiterpenoids and flavonoids from *Inula viscosa* induce programmed cell death in kinetoplastids

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ABSTRACT

Neglected tropical diseases such as leishmaniasis and American trypanosomiasis represent an increasing health problem. Current treatments are not satisfactory which remains an urgent need for novel, cheap and safe chemotherapies. In the course of our ongoing search for new potential anti-protozoal agents, this study aimed to perform a bio-guided fractionation of *Inula viscosa* (Asteraceae) using *in vitro* assays against three strains of *Leishmania* and *Trypanosma* genus. Eight known compounds were identified from the ethanolic extract of leaves, sesquiterpenoids (**3** and **4**) and flavonoids (**5** and **6**) were characterized as the main bioactive constituents. Sesquiterpene lactones **3** and **4** (IC_{50} values between 4.99 and 14.26 μM) showed promising antiparasitic activity against promastigotes of *L. donovani*, *L. amazonensis* and epimastigotes of *T. cruzi*. Their structures were successfully characterized by spectroscopic techniques including 1D and 2D NMR experiments. Furthermore, the main bioactive compounds **4**, **5** and **6** displayed higher potency (IC_{50} values between 0.64 and 2.13 μM) against amastigotes of *L. amazonensis* than miltefosine (IC_{50} 3.11 μM), and a low toxicity on macrophages cell line (SI > 45). The analysis of structure-activity relationship (SAR) of the anti-protozoal activity revealed that lactonization or oxidation enhanced the biological profile, suggesting that the hydrophobic moiety was presumably involved in the activity by increasing the affinity and/or cell membrane permeability. In order to get an insight into the mechanism of action of these compounds, programmed cell death (PCD) experiments were performed, and the obtained results suggest that the reported compounds induced PCD in the treated parasites. These results highlight that sesquiterpenoids and flavonoids from *I. viscosa* could constitute an interesting scaffold for the development of novel antikinetoplastid agents.

1. Introduction

Parasitic diseases caused by kinetoplastids such as *Leishmania* and *Trypanosoma* species, known as neglected tropical diseases (NTDs), are responsible for high mortality, disability and morbidity rates [1]. According to World Health Organization (WHO), American trypanosomiasis, also known as Chagas disease, is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi* with approximately more than 10 000 deaths per year, and more than 25 million people are at a higher risk of acquiring this disease [2]. Furthermore, leishmaniasis is a complex infectious disease with a varied spectrum of clinical manifestations, which range from self-healing cutaneous ulceration to progressive and lethal visceral infection [3]. It is prevalent in more than 98 endemic countries in the world with an

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estimated annual incidence of almost 0.2 to 0.4 million new cases of visceral manifestation and 0.7–1.2 million new cases of cutaneous form [4]. The emergence of resistance, toxicity and high cost of the current treatment reveal the urgent need of alternative chemotherapeutic agents. In this context, more interest has been given to the development of new efficient drugs using science advances such as nanotechnology [5], chemical synthesis [6] and natural products [7]. Among these approaches, plants present a rich source of bioactive compounds [8]. For instance, Asteraceae family has mainly presented promising antiparasitic activities against *Leishmania* and *Trypanosoma* genus [9]. Belonging to this family, *Inula viscosa* also known as *Dittrichia viscosa* is a medicinal perennial herb native to the Mediterranean basin and has been widely used in traditional medicine to treat different diseases [10].Previous chemical investigations have characterized various phytochemicals in *I. viscosa* such as flavonoids, sesquiterpenes lactones, acids and glycolipids with a wide range of therapeutic indications [11–13]. Indeed, flavonoids and sesquiterpene lactones are a group of natural products widely described as potent antiparasitic agents [14,15]. Recently, quercetin related compounds have targeted the arginase in *Leishmania amazonensis* showing a potent activity [16], and artemisinin which is an illustrative example for sesquiterpene lactones, has displayed antiparasitic potency [17] through the induction of apoptotic-like cell death [18,19].

Indeed, cell death of different developmental stages of kinetoplastids from *Trypanosoma* and *Leishmania* genus have been coupled to the occurrence of apoptotic markers such as DNA fragmentation, cell shrinkage, chromatin condensation, loss of mitochondrial membrane potential $\Delta\Psi$ and membrane blebbing [20–22].

Hence, in the present work we report a bio-guided fractionation of *I. viscosa* ethanolic extract in order to characterize leishmanicidal and trypanosomal compounds, to elucidate their mechanism of action targeting the PCD, and to analyze the structure-activity relationship (SAR) involved in their anti-protozoal activity.

2. Materials and methods

2.1. General procedure, chemicals and reagents

Optical rotations were determined on a Perkin-Elmer 241 automatic polarimeter. NMR spectra, ROESY (spin lock field 2500 Hz), HSQC and HMBC (optimized for J = 7.7 Hz) were performed on a Bruker Avance 500 and 600 spectrometers at 300°K. Silica gel 60 (particle size 15–40 and 63–200 µm, Macherey-Nagel) was used for column chromatography, while silica gel 60 F254 (Macherey-Nagel) was used for analytical or preparative TLC and Sephadex LH-20 (Sigma-Aldrich) were used for the column chromatographic (CC) separation. Centrifugal preparative TLC was performed using a Chromatotron (Harrison Research Inc. model 7924 T) on 4- or 1-mm silica gel 60 PF254 disks with flow rate $2-4$ mL min^{-1}. The spots were visualized by UV light and heating silica gel plates sprayed with $H_2OH_2SO_4$-AcOH (1:4:20). All the used solvents were analytical grade from Panreac. Reagents, deuterated solvents were purchased from Sigma-Aldrich, benznidazole from Aldrich and miltefosine from Æterna Zentaris. For biological tests, Schneider's medium (Sigma-Aldrich), RPMI 1640 and LIT media (Gibco®), alamarBlue® reagent (Invitrogen, Life Technologies), EnSpire® Multimode Plate Reader (Perkin Elmer), and Leika DMIL inverted microscope (Leika, Wetzlar, Germany) were used.

2.2. Plant material identification and extraction

In the present work, the studied plants have been selected from an ethnopharmacological study undertaken in the central north of Morocco. The used parts of the most recommended plants as described by herbalists have been collected in the Atlas Mountains, Imouzzer region-Morocco in July 2017, then identified by botanists.

In order to obtain the crude extracts, cleaned used parts of the different plants were shade dried at room temperature and subsequently milled with a simple commercial electric grinder. Powdered material was extracted by maceration in ethanol (1:10 w/v) for 6 h at room temperature with continuous stirring at 500 rpm (revolutions per minute). The resulting mixture was filtered using Whatman filter n°1 and the solvent was concentrated under vacuum in a rotary evaporator at 45 °C, dried extracts were stored in a refrigerator at 4 °C until further use. Amongst the sixteen tested plants, *I. viscosa* extract (5 g, 5%) was the most active against the three evaluated parasites; therefore, it was selected for further bioassays. The geographical coordinates for this species are 33°55′ 37 N, 5°2′50 W and a voucher specimen (RAB107342) has been deposited in the Herbarium of the Scientific Institute of Rabat, Morocco.

2.3. Bio-guided fractionation procedure

The prepared EtOH extracts were assayed for their antiprotozoal activity against promastigotes of *L. amazonensis* and *L. donovani* and epimastigotes of *T. cruzi*. After that, a bio-guided fractionation methodology was used to identify antiprotozoal compounds in the ethanolic extract of *I. viscosa* as the most active plant. Thus, the active EtOH extract (5 g) was subjected to CC on silica gel eluted with mixtures of hexane-EtOAc (100:0 to 0:100, 0.5 L each one) of increasing polarity to afford twelve fractions that were combined into seven fractions (F1-F7) based on their TLC profile. The antiprotozoal activity was focused on the active fractions F2 (670.6 mg), F3 (871.5 mg) and F4 (1.7155 g), which were subjected to column chromatography on sephadex LH-20 column, using a $MeOH-CHCl_3$ system eluent (1:1, 2 L) to afford between forty and fifty six sub-fractions which were combined again based on their TLC profiles (F2/1 to F2/5), (F3/1 to F3/6) and (F4/1 to F4/12), respectively. Indeed, the sub-fractions F3/1 (638.84 mg) and F3/2 (103.56 mg) showed a promising activity against the three tested strains and were subjected to multiple chromatographic steps on silica gel, involving medium-pressure liquid chromatography, centrifugal planar chromatography and preparative TLC using mixtures of hexane-EtOAc, hexane-Et_2O, CH_2Cl_2-EtOAc and CH_2Cl_2-acetone as eluent to yield compounds **3** (1.78 mg), **4** (38.5 mg), **5** (3.6 mg) and **7** (1.95 mg). Following the same purification procedures, fraction F2 yielded compounds **1** (3.3 mg) and **3**, while fraction F4 afforded compounds **2** (8.5 mg), **6** (28.5 mg) and **8** (5.4 mg).

2.4. Leishmanicidal and trypanocidal assays

2.4.1. Parasite strains

The antiparasitic activity of crude extract, fractions and pure compounds of *I. viscosa* was evaluated against the promastigote stage of *Leishmania donovani* (MHOM/IN/90/GE1F8R), promastigote and amastigote forms of *Leishmania amazonensis* (MHOM/BR/77/LTB0016) and epimastigote form of *Trypanosoma cruzi* (Y strain).

2.4.2. In vitro antileishmanial evaluation

2.4.2.1. Anti-promastigotes assay. The bioassay was performed using the alamarBlue® method as previously described [23]. Promastigotes of *L. donovani* and *L. amazonensis* were grown at 26 °C in RPMI 1640 modified medium (Gibco) and supplemented with 10 % heat-inactivated foetal bovine serum. Cultures in the logarithmic phase were seeded in sterilized 96-well microtiter plates (Corning™) (10^6 parasites/mL) containing the samples dissolved in dimethyl sulfoxide 1% (DMSO) at the suitable concentration to be tested in serial dilutions using *Leishmania* medium (RPMI 1640) to get a final volume of 200 µL per well, then 20 µL of alamarBlue® were added to the entire plate. After an incubation of 72 h, the plate was checked up visually using an inverted microscope, then analyzed by an EnSpire multimode plate reader (PerkinElmer, MA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Leishmanicidal activity was expressed as IC_{50} values (the concentration of a sample which caused

a 50 % reduction in parasite viability). Those values were calculated by linear regression analysis with 95 % confidence limits.

2.4.2.2. Anti-amastigotes assay. The active pure compounds from *I. viscosa* were tested against the intra-macrophagic stage of *L. amazonensis* as previously described in literature [24]. In a 96-well flat bottom plate, the J774A.1 cell line macrophages were cultured at a density of 2×10^5 cells/mL in RPMI 1640 medium supplemented with 10 % heat-inactivated foetal bovine serum and incubated for one hour at 37 °C in a 5 % CO_2 atmosphere in order to allow almost complete attachment of the cells. After that, macrophages were infected with *L. amazonensis* promastigotes in the stationary phase (7 days old culture) with a ratio of 1:10 (macrophage/ parasite) at a concentration of 2×10^6 cells/mL, then incubated at 37 °C in 5% CO_2 for 24 h. Wells were washed with medium to remove non-phagocytosed promastigotes. Thereafter, the infected macrophages were treated with the pure compounds (dissolved in DMSO at the desired concentration) for 24 h. The medium was removed carefully to be replaced by 30 µL of 0.05 % SDS and the plate was shaken for 30 s. Subsequently, 170 µL of Schneider's medium were added to each well to give a final volume of 200 µL and 20 µL of alamarBlue® were added to the plate and incubated at 26 °C for 72 h in order to give enough time to the transformation from promastigotes to amastigotes. After 72 h of incubation, plates were analyzed using the same protocol of promastigotes test.

2.4.3. In vitro evaluation on T. cruzi epimastigotes

The extract, fractions and pure molecules were tested against the epimastigotes of *T. cruzi*. Briefly, in 96-well plates samples dissolved in DMSO were serially diluted in 100 µL of LIT medium supplemented with 10 % heat-inactivated fetal bovine serum to obtain the desire concentrations selected from the first screening. In all tests, 1 % DMSO was used to dissolve the highest dose of the compounds without inducing any effects on the parasites. After that, epimastigotes in logarithmic growth phase were counted, adjusted to 5×10^5 cells/mL, distributed in the previous 96-well plate and incubated at 27 °C for 72 h. The plate was observed under an inverted microscope after 72 h of incubation and analyzed statistically as described in the leishmanicidal test.

2.5. Assessment of cytotoxicity on macrophages

The cytotoxicity assay of the pure active compounds was performed as described in literature [25]. Briefly, 2×10^5 of macrophages culture were placed in a 96-well plate for 2 h in a 5 % CO_2 incubator at 37 °C, then serial dilutions of the 4 active molecules were prepared in deep well, then transmitted to the plate containing the culture of macrophages giving a final volume of 100 µL. Finally, the plate was incubated in 5 % CO_2 atmosphere for 24 h. The percentage of cell viability was evaluated using the alamarBlue® assay (10 %). Dose response curves were plotted and the CC_{50} values were calculated.

2.6. Mechanisms of cell death

In order to analyze the programmed cell death pathway induced in the studied parasites, a common step between the four kits was performed. Briefly, parasites were treated with the tested compounds at their IC_{90} for 24 h. After that, cells were centrifuged (1500 rpm for 10 min), washed twice with PBS (phosphate buffered saline) and incubated with the suitable reagent of each marker as described below. For the four assays, an untreated control was used. After incubations, an EVOS FL Cell Imaging System AMF4300, Life Technologies, Madrid, Spain was used to observe and analyze the obtained results in order to score and to count cells.

2.6.1. Chromatin condensation determination

To analyze the compacted state of chromatin in apoptotic cells, a double-stain apoptosis detection kit (Hoechst 33342/PI) (Life Technologies) was used as recommended by the manufacturer. The strains were incubated with Hoechst 33342 at 5 µg/mL and PI at 1 µg/mL. After 15 min of incubation at 26 °C, samples were analyzed.

Table 1
Leishmanicidal and trypanosomal activity against promastigotes of *L. amazonensis* and *L. donovani* and epimastigotes of *T. cruzi* of the extract, fractions and sub-fractions from leaves of *I. viscosa*.

Samples	*L. amazonensis* IC_{50} (µg/mL)	*L. donovani* IC_{50} (µg/mL)	*T. cruzi* IC_{50} (µg/mL)
Crude extract	18.10 ± 2.08	10.87 ± 2.11	12.18 ± 0.50
F2	35.66 ± 1.78	31.07 ± 2.17	8.67 ± 0.45
F2/4	15.95 ± 0.80	52.95 ± 3.18	13.74 ± 0.96
F3	3.80 ± 0.23	4.48 ± 0.15	6.61 ± 0.87
F3/1	6.44 ± 0.02	7.71 ± 2.46	9.04 ± 1.86
F3/2	12.72 ± 1.27	10.66 ± 2.35	23.93 ± 1.91
F4	3.63 ± 0.14	27.12 ± 01.27	27.00 ± 0.87
F4/3	15.46 ± 0.75	12.52 ± 0.42	11.18 ± 1.13
F4/4	17.85 ± 0.85	6.6 ± 1.22	10.76 ± 2.12
F4/5	12.77 ± 0.05	9.08 ± 0.66	8.59 ± 1.58
F4/6	19.60 ± 2.65	18.01 ± 0.90	9.89 ± 0.08
F4/7−9	13.03 ± 1.11	10.76 ± 2.90	5.41 ± 1.38
F4/10	32.17 ± 1.93	36.62 ± 1.46	22.13 ± 1.90
F4/10/1	7.64 ± 0.86	7.93 ± 0.15	2.20 ± 0.49
Miltefosine	2.64 ± 0.10	1.35 ± 0.11	–
Benznidazole	–	–	1.81 ± 0.50

IC_{50}: Inhibitory Concentration that inhibits 50 % of the growth of the tested parasite.
IC_{50}: Means ± Standard deviation.
Fractions and sub-fractions that showed $IC_{50} > 100$ µg/mL were excluded.

2.6.2. Analysis of mitochondrial membrane potential

The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical) was used to measure the collapse of an electrochemical gradient across the mitochondrial membrane. Treated promastigotes re-suspended in JC-1 buffer were incubated with JC-1 reagent (1:10 v/v) and then incubated at 26 °C for 30 min. Analysis for mean green and red fluorescence intensity and depolarization of the mitochondrial membrane potential was analyzed.

2.6.3. Determination of ATP level

ATP level was measured using a Cell Titer-Glo® Luminescent Cell Viability Assay (Promega). The effect of the compounds on the ATP production was evaluated by incubating parasitic strains (10^6cells/mL) with the tested molecules at their IC_{90} for 24 h.

2.6.4. Plasma membrane permeability

To detect the plasma membrane permeability of parasites, the SYTOX® Green assay was performed. The treated parasites were incubated with SYTOX® Green at a final concentration of 1 µM (Molecular Probes) for 15 min in the dark at room temperature. The increase in fluorescence due to binding of the fluorescent marker to the parasitic DNA was observed.

2.6.5. Oxidative stress

CellRox Deep Red Oxidative Stress Reagent (Thermo Fisher Scientific) was used to measure the generation of Reactive Oxygen Species (ROS) in cells exhibiting strong fluorogenic signal under oxidative state. The assay involves the incubation of the treated parasites with 5 µM of CellRox Reagent for 30 min at 26 °C, then the centrifugation of cells to be re-suspended in buffer. H_2O_2 at 600 µM for 30 min was used as positive control. The signal for Deep Red is localized in the cytoplasm.

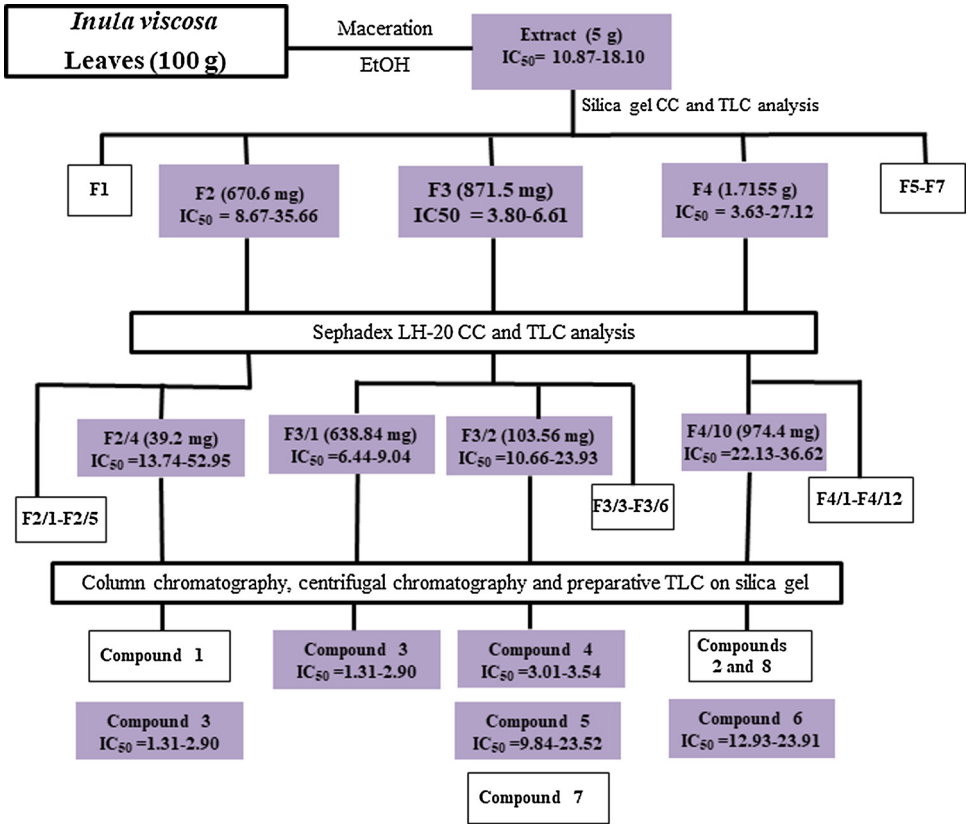

Fig. 1. Flowchart of antiprotozoal bio-guided fractionation of *Inula viscosa* leaves against promastigotes of *L. amazonensis* and *L. donovani* and epimastigotes of *T. cruzi* (*Tc*). IC_{50}: Inhibitory concentration that inhibits 50 % of the growth of the tested parasite. IC_{50} values in μg/mL.

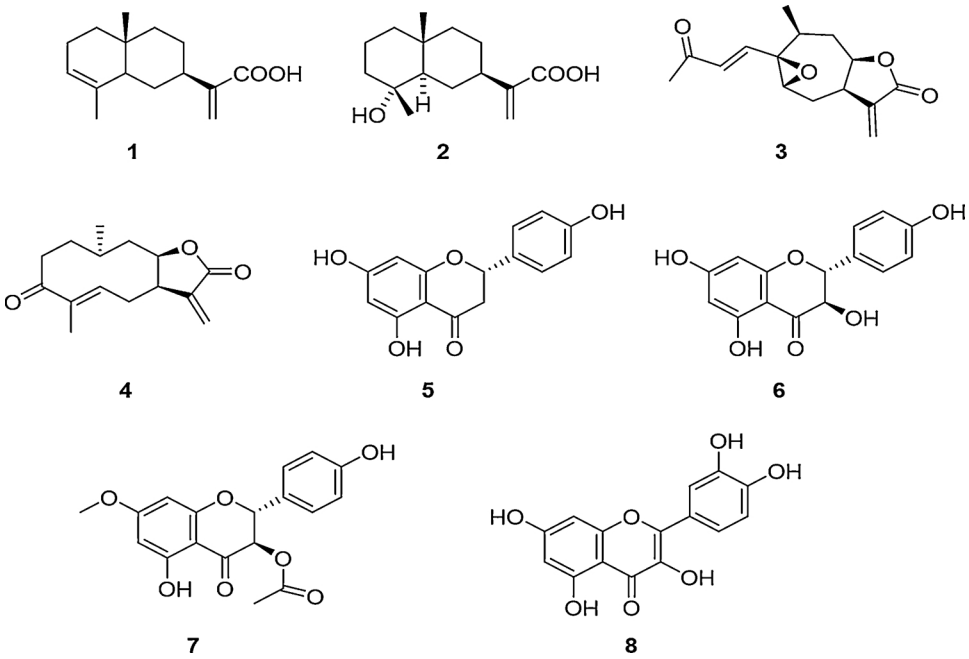

Fig. 2. Chemical structure of natural compounds **1**-**8** isolated from leaves of *Inula viscosa*.

2.7. Statistical analysis

All assays were carried out in triplicate. The results were defined as the mean values of three experiments. The obtained inhibition curves was performed using the Sigma Plot 12.0 software program (Systat Software Inc.). Statistical analyses were performed using the GraphPad Prism 8.0.2. Fluorescence intensity (RFU) was performed using ImageJ software program as the means values of three repetitions. Differences between the values were assessed using a one-way analysis of variance (ANOVA). Data are presented as means ± SD and $p < 0.05$ was considered statistically significant.

Table 2
Leishmanicidal, trypanocidal, cytotoxic activity and selectivity index against promastigotes of *Leismania* spp., epimastigotes of *T. cruzi* and murine macrophages of the active sesquiterpenoids and flavonoids isolated from *I. viscosa.*

Compounds	*L. amazonensis*		*L. donovani*		*T. cruzi*		Murine macrophages
	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI	CC_{50} (μM)
3	9.53 ± 2.44	1.92	11.06 ± 2.33	1.66	4.99 ± 0.04	3.67	18.44 ± 1.30
4	12.12 ± 0.64	3.50	14.26 ± 1.69	2.97	12.52 ± 0.16	3.38	42.36 ± 2.54
5	81.08 ± 3.24	1.18	86.45 ± 2.60	1.11	36.17 ± 2.17	2.65	95.93 ± 0.50
6	44.86 ± 2.95	> 7.73	54.46 ± 2.36	> 6.3	82.95 ± 1.65	> 4.18	> 100
Miltefosine	6.48 ± 0.24	11.14	3.31 ± 0.27	21.79	–	–	72.18 ± 1.25
Benznidazole	–	–	–	–	6.95 ± 1.92	57.51	399.91 ± 1.04

IC_{50}: Inhibitory Concentration that inhibits 50 % of the growth of the tested parasite.
IC_{50}: Means ± Standard deviation.
CC_{50}: Cytotoxic Concentration that reduces 50 % of the murine macrophages' viability.
SI: Selectivity Index (CC_{50} /IC_{50}).
Compounds that showed IC_{50} > 100 μM were excluded.

Table 3
Leishmanicidal activity and selectivity index of select compounds against amastigote stage of *L. amazonensis.*

Compounds	*L. amazonensis* IC_{50} (μM)	SI^a
3	6.98 ± 0.42	2.63
4	0.64 ± 0.08	65.75
5	2.13 ± 1.21	45.00
6	1.91 ± 0.83	> 181.81
Miltefosine	3.11 ± 0.29	23.16

IC_{50}: Inhibitory Concentration that inhibits 50 % of the growth of the tested parasite.
IC_{50}: Means ± Standard deviation.
CC_{50}: Cytotoxic Concentration that reduces 50 % of the murine macrophages' viability.
SI^a: Selectivity Index (amastigotes) (CC_{50} /IC_{50}).

Table 4
Leishmanicidal and trypanocidal activity of the selected compounds for study of mechanism of action.

Compounds	IC_{90} (μM)		
	L. donovani	*L. amazonensis*	*T. cruzi*
3	76.25 ± 3.05	12.85 ± 1.07	17.00 ± 0.33
4	94.23 ± 0.57	48.53 ± 0.18	22.11 ± 0.30
5	107.66 ± 1.76	322.42 ± 0.97	191.68 ± 0.77
6	135.57 ± 0.20	158.23 ± 3.37	173.45 ± 0.61
Miltefosine	5.15 ± 0.50	9.63 ± 0.50	–
Benznidazole	–	–	25.591 ± 2.728

IC_{90}: Effective Concentration that inhibits 90 % of the growth of the tested parasite.
IC_{90}: Means ± Standard deviation.

3. Results and discussion

3.1. Antikinetoplastid activity

Research on antikinetoplastid drugs has been intensified in recent years exploiting natural products from medicinal plants [26,27]. The leishmanicidal and trypanosomal activities of plant extracts have been attributed to several compounds belonging to different chemical groups such as monoterpenes, triterpenes, alkaloids, lignans and flavonoids [28–30]. In special, sesquiterpenoids and flavonoids isolated from various plants have widely exhibited a prominent leishmanicidal [31] and trypanocidal activity [32]. In the present work, ethanolic extracts of sixteen plants used in traditional medicine for treatment of infectious diseases such as skin disorders, in the central north of Morocco, were evaluated. Roots of *Alkanna tintoria*, *Berberis hispanica*, *Ephedra altissima* and *Rubia tinctorium*; aerial parts of *Crataegus oxyacantha* and *Urtica dioica*; leaves of *Ammi majus, Globularia alypum*, *Inula viscosa*, *Lavandula dentata*, *Nerium oleander*, *Origanum majorana*, and *Rhamnus alaternus*; seeds of *Eruca sativa* and *Juniperus oxycedrus* and peel of *Punica granatum*. The extracts were firstly screened at 400, 200, 100; 50 and 25 μg/mL against promastigote stage of *L. donovani* and *L. amazonensis*, and epimastigote form of *T. cruzi.* The crude ethanolic extract of *I. viscosa* showed the most interesting activity against the tested strains, with IC_{50}s between 10.87 and 18.10 μg/mL (Table 1) which was a promising fact to continue with the bioassay-guided fractionation in order to isolate and identify the main active molecules involved in the anti-parasitic effects. Therefore, the ethanolic extract was submitted to liquid chromatography on silica gel affording six fractions, F1-F6 (Fig. 1). Indeed, samples that led to a percentage of growth inhibition (%GI) > 50 % were assayed in different concentrations to determine the half maximal inhibitory concentration (IC_{50}) and were classified as highly active (IC_{50} < 25 μg/mL), active (25 < IC_{50} < 50 μg/mL), moderately active (50 < IC_{50} < 100 μg/mL) and not active when IC_{50} > 100 μg/mL. In addition, miltefosine and benznidazole were evaluated for comparative purposes as reference drugs against leishmaniasis and Chagas disease respectively. Miltefosine showed IC_{50}s of 2.64 μg/mL and 1.35 μg/mL against *L. amazonensis* and *L. donovani*, respectively, whereas benznidazole showed an IC_{50} of 1.81 μg/mL against *T. cruzi*.

The IC_{50} values varied upon the fractions and the parasitic strains showing different magnitudes of the inhibitory potential. The most active fractions, F2 (IC_{50} values ranging from 8.67 to 35.66 μg/mL), F3 (IC_{50} values ranging from 3.80 to 6.61 μg/mL) and F4 (IC_{50} values ranging from 3.63 to 27.12 μg/mL), exhibited potent activity against the three studied parasites, similar to the reference drugs (Table 1 and Fig. 1), highlighting these three fractions as the most promising ones. Subsequently, these fractions were further chromatographed on a sephadex LH-20 column yielding five (F2/1-F2/5), six (F3/1-F3/6) and ten sub-fractions (F4/1-F4/10), respectively, which were assayed against the three parasites. From Fraction 2, F2/4 exhibited a potent antikinetoplastid effect on *L. amazonensis* (IC_{50} 15.95 μg/mL) and *T. cruzi* (IC_{50} 13.74 μg/mL). From fraction F3, sub-fractions F3/1 and F3/2 were the most actives against the three parasites, showing IC_{50}s between 6.44 and 12.72 μg/mL for *L. amazonensis* and *L. donovani*, respectively, and an IC_{50} of 9.04 μg/mL on *T. cruzi* for F3/1.While from fraction F4, sub-fractions F4/3, F4/4, F4/5 and F4/6 were the most actives against the three parasites, showing IC_{50}s between 6.60 and 19.60 μg/mL for *L. amazonensis* and *L. donovani*, respectively and IC_{50}s between 8.59 and 11.18 μg/mL for *T. cruzi.* Therefore, sub-fraction F2/4 was submitted to purification steps, affording the sesquiterpenoids **1** and **3**. Additionally, sub-fraction F3/1 showed to be the most potent one, yielding the compound **3** and sub-fraction F3/2 afforded

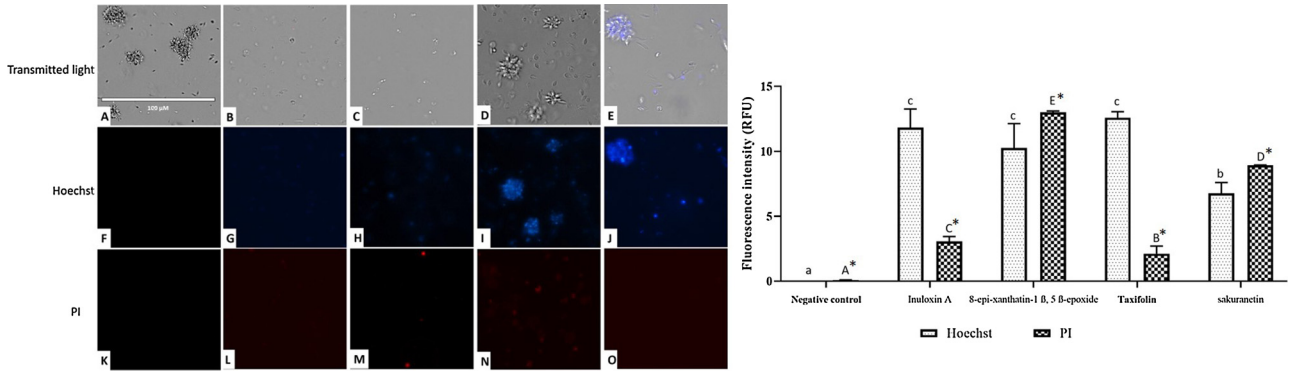

Fig. 3. *Leishmania amazonensis* promastigotes incubated with IC_{90} of the four active compounds for 24 h: Inuloxin A (**B**, **G**, **L**), sakuranetin (**C**, **H**, **M**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**, **N**), taxifolin (**E**, J, **O**). Images (40X) are representative of the cell population observed in the performed experiments using an EVOS FL Cell Imaging System AMF4300, Life Technologies, USA. Hoechst stain is different when comparing negative control (**A**, **F**, **K**) with treated cells where the nuclei are bright blue. Red fluorescence corresponds to the propidium iodide stain. Hoechst stain (**F**-J), Propidium iodide stain (**K**-**O**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

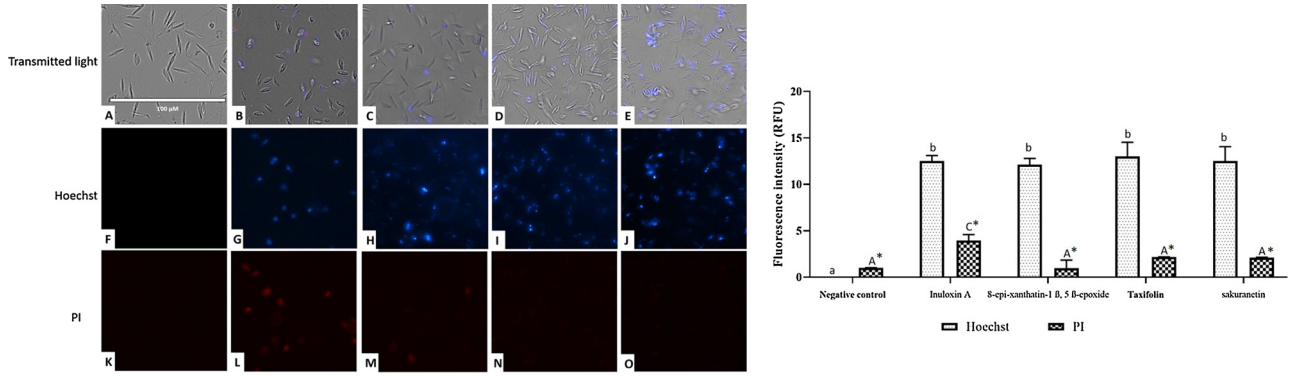

Fig. 4. Images (40X) obtained from an EVOS FL Cell Imaging System showing fluorescence when promastigotes of *Leishmania donovani* incubated with IC_{90} of the four tested compounds for 24 h: Inuloxin A (**B**, **G**, **L**), sakuranetin (**C**, **H**, **M**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**, **N**), taxifolin (**E**, J, **O**) and stained with Hoechst-propidium iodide. Hoechst corresponds to the chromatin condensation (blue) in treated cells. Red fluorescence corresponds to the propidium iodide stain. A control without treatment has been used (**A**, **F**, **K**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

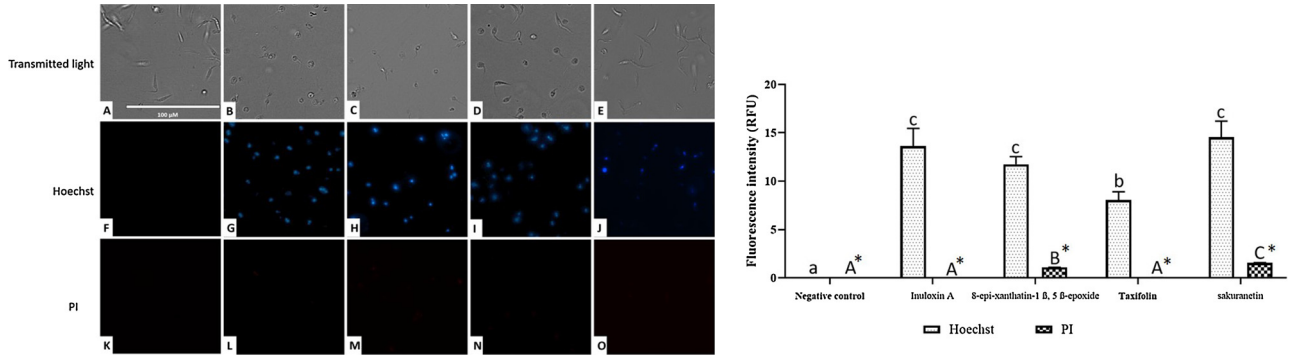

Fig. 5. Images (40X) obtained from EVOS FL Cell Imaging System showing the effect of Inuloxin A (**B**, **G**, **L**), sakuranetin (**C**, **H**, **M**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**, **N**), taxifolin (**E**, J, **O**) at the IC_{90} against epimastigotes of *Trypanosoma cruzi* after 24 h of incubation in comparison to the control (**A**, **F**, **K**), then using Hoechst-propidium iodide double stains. Hoechst corresponds to the chromatin condensation (blue) in treated cells. Red fluorescence corresponds to the propidium iodide stain. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

compounds **4**, **5** and **7**. Sub-fraction F4/10 yielded compounds **2**, **6** and **8** (Figs. 1,2). Hence, in some cases, the IC_{50} was higher than the crude extract which could be explained by a possible synergism between *I. viscosa* components since the complex mixtures of plant components may provide different outcomes: an additive, a synergistic or an antagonistic effect. The chemical structures of the known isolated

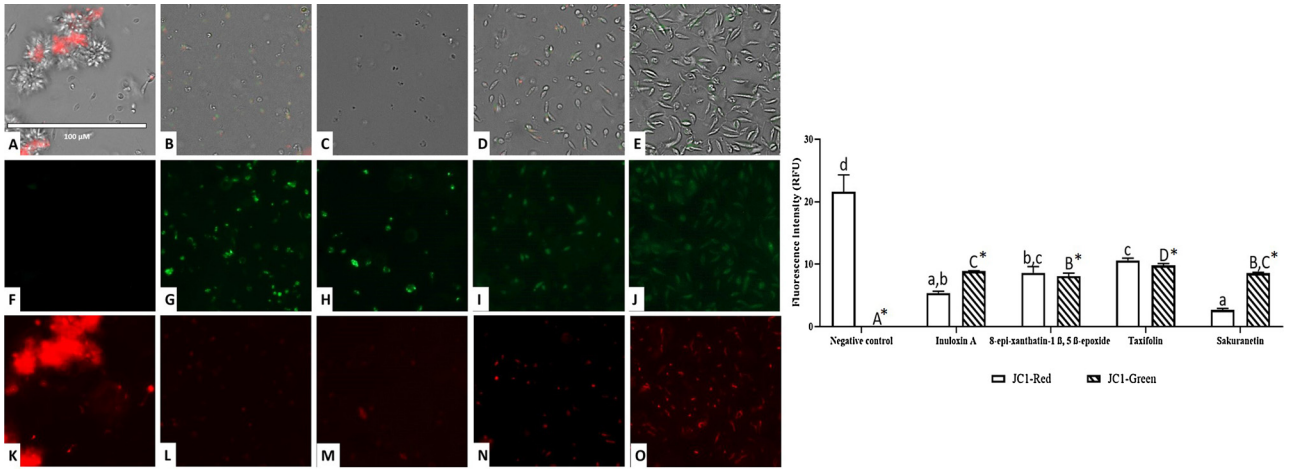

Fig. 6. The effect of inuloxin A (**B**, **G**, **L**), sakuranetin (**C**, **H**, **M**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**, **N**), taxifolin (**E**, J, **O**) on the mitochondrial potential of *Leishmania amazonensis* promastigotes compared to the control (**A**, **F**, **K**). JC-1 dye accumulates in the mitochondria of healthy cells as aggregates (red fluorescence) in cells treated with the IC_{90} of compounds for 24 h, due to collapse of mitochondrial potential, the JC-1 dye remained in the cytoplasm in its monomeric form, green fluorescence. Images (40X) are representative of the parasites observed in the performed experiments using an EVOS FL Cell Imaging System. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

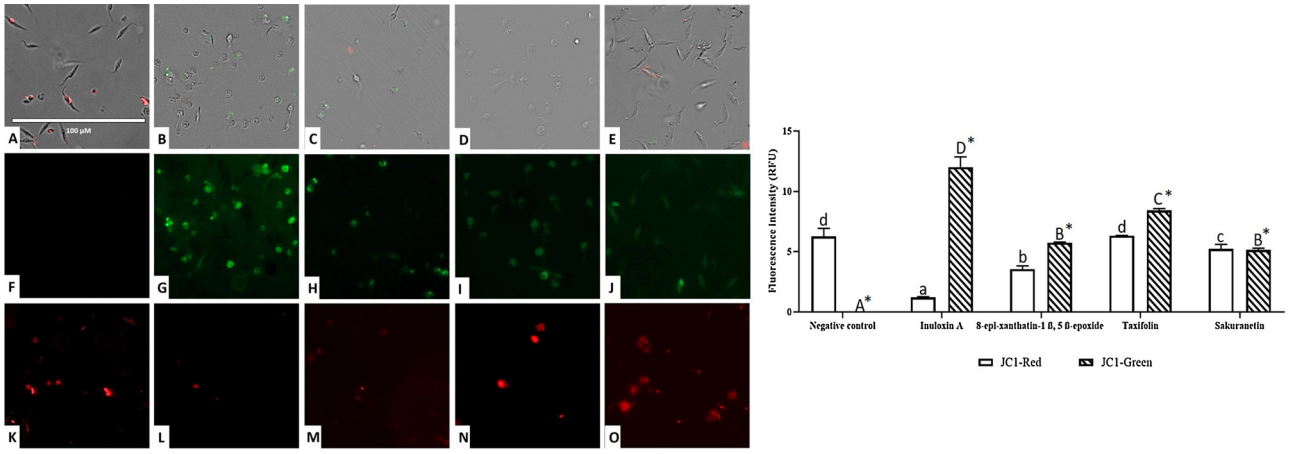

Fig. 7. Images (40X) obtained from EVOS FL Cell Imaging System showing the effect of IC_{90} of inuloxin A (**B**, **G**, **L**), sakuranetin (**C**, **H**, **M**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**, **N**), taxifolin (**E**, J, **O**) on the mitochondrial potential of *Trypanosoma cruzi* epimastigotes compared to the control (**A**, **F**, **K**) using JC1 kits after 24 h of incubation. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$).

compounds (**1**-**8**) were identified as isocostic acid (**1**) [33], ilicic acid (**2**) [34], 8-*epi*-xanthatin-1β,5β-epoxide (**3**) [35], inuloxin A (**4**) [36], sakuranetin (**5**) [37], taxifolin (**6**) [38], 3-*O*-acetyl-7-*O*-methylaromadendrin (**7**) [38], and quercetin (**8**) [39] using spectrometric and spectroscopic data, including 1D and 2D NMR experiments, in addition to comparison with data reported in the literature. Compounds **1**-**8** were evaluated against the three parasites. In addition, the cytotoxicity on murine macrophages was also assessed. Miltefosine showed a CC_{50} of 72.18 µM, whereas benznidazole showed a CC_{50} of 399.91 µM (Table 2). The results, over the promastigote stage of *Leishmania*, showed that compounds **3** and **4** displayed potent activity in µM range and were slightly less potent than the reference drug. Moreover, compounds **3** and **4** showed similar potency to benznidazole against epimastigotes of *T. cruzi*. Regarding the selectivity index (SI) towards macrophages (Table 2), the compounds **3** and **4** showed a moderated cytotoxic profile on the three parasites (SI values ranging from 1.66 to 3.67). Based on the *in vitro* results on promastigote forms, compounds **3**-**6** were selected to be evaluated on intracellular amastigotes of *L. amazonensis*. The results revealed that compounds (**4**-**6**) exhibited higher potency than miltefosine (IC_{50} 3.11 µM), showing IC_{50} values from 0.64 to 2.13 µM. In fact, sesquiterpene lactone **4** was 4.8-fold more potent than the reference drug. Besides, the three compounds showed higher selectivity index than miltefosine (Table 3), highlighting compound **6** with a SI of 181.81 versus 23.16 for miltefosine.

To the best of our knowledge, only one investigation described the leishmanicidal effect of *I. viscosa* where series of inuloxin derivatives were evaluated against promastigotes of *L. donovani* [40]. Similar to our finding, compound **4** displayed important antiparasitic activity (6.89 µM). Based on literature, there are no investigations that reported the activity of this compound against neither amastigote form nor other *Leishmania* spp. or *T. cruzi*. In addition, the characterization of the sesquiterpene lactone **3** in *I. viscosa* is described for the first time and when isolated from other plant species, it was reported as the most active against *T. cruzi* and *L. donovani* [41,42]. Furthermore, the results obtained in the current study confirm the noteworthy leishmanicidal and trypanosomal potency of compounds **5** and **6** commonly found in *I.*

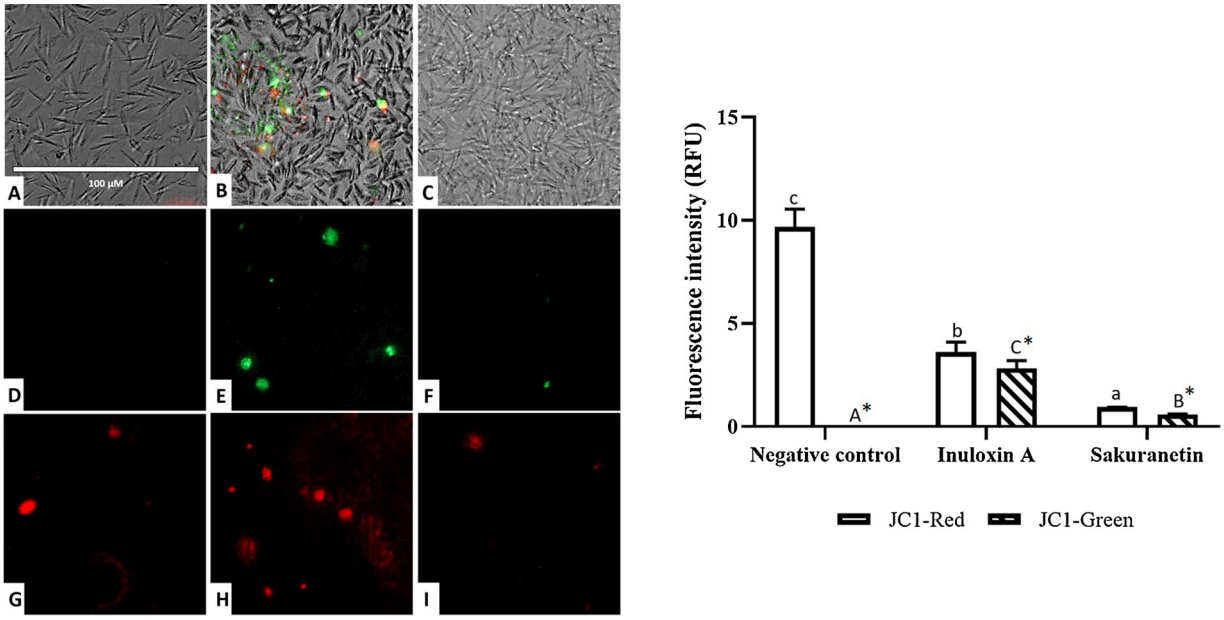

Fig. 8. *Leishmania donovani* (40X) stained with JC-1 kits after 24 h incubation of promastigotes with IC_{90} of inuloxin A (**B**, **E**, **H**) and sakuranetin (**C**, **F**, **I**). Images (40X) are representative of the effect on the mitochondrial potential of the parasites and observed using an EVOS FL Cell Imaging System. A control without treatment has been used (**A**, **D**, **G**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e or A*-E* reflect that means within compounds with different letters are significantly different ($p < 0.05$).

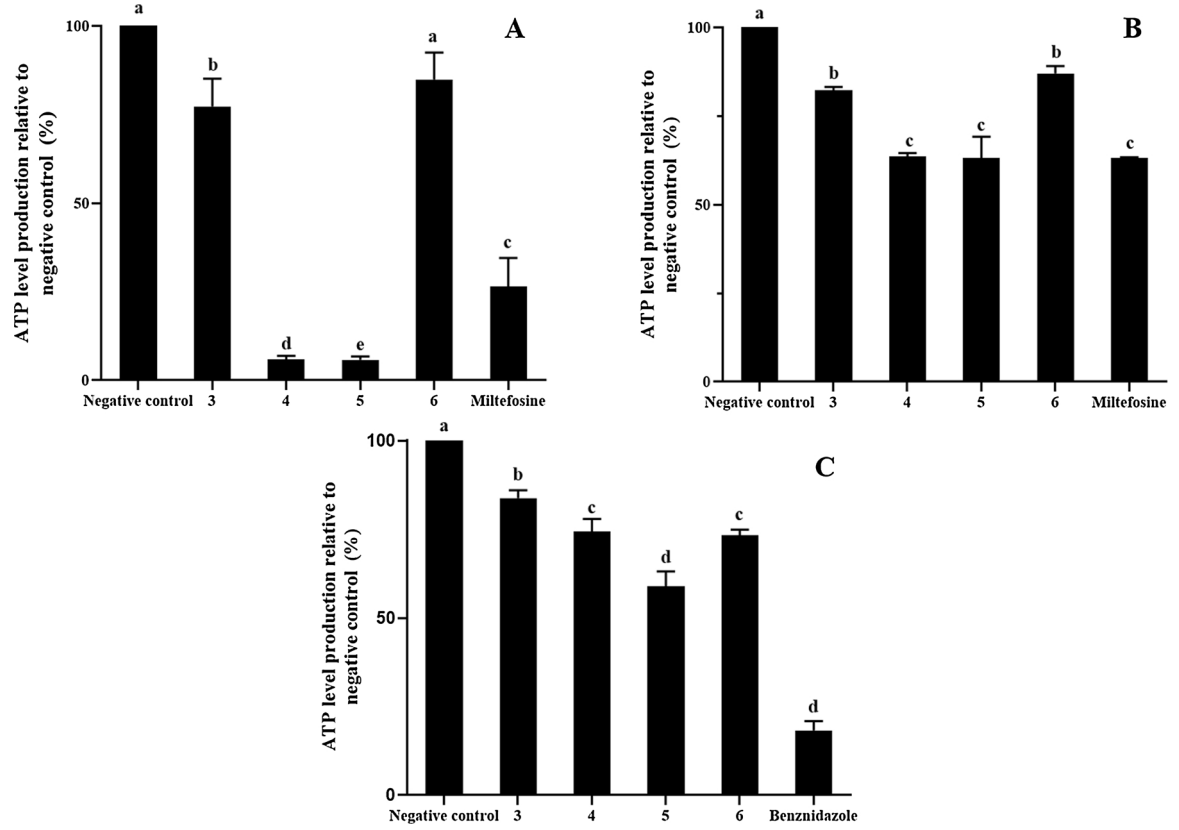

Fig. 9. The effect of 8-*epi*-xanthatin-1β,5β-epoxide (**3**), inuloxin A (**4**), sakuranetin (**5**) and taxifolin (**6**) on the ATP production of *Leishmania amazonensis* (**A**), *Leishmania donovani* (**B**) and *Trypanosoma cruzi* (**C**), using CellTiter-Glo Luminescent Cell Viability Assay. Results are representing in percentage relative to the negative control. Cells were treated by the IC_{90} concentration for 24 h. Values are given as mean ± SD (N = 3). a-e: means within compounds with different letters are significantly different ($p < 0.05$).

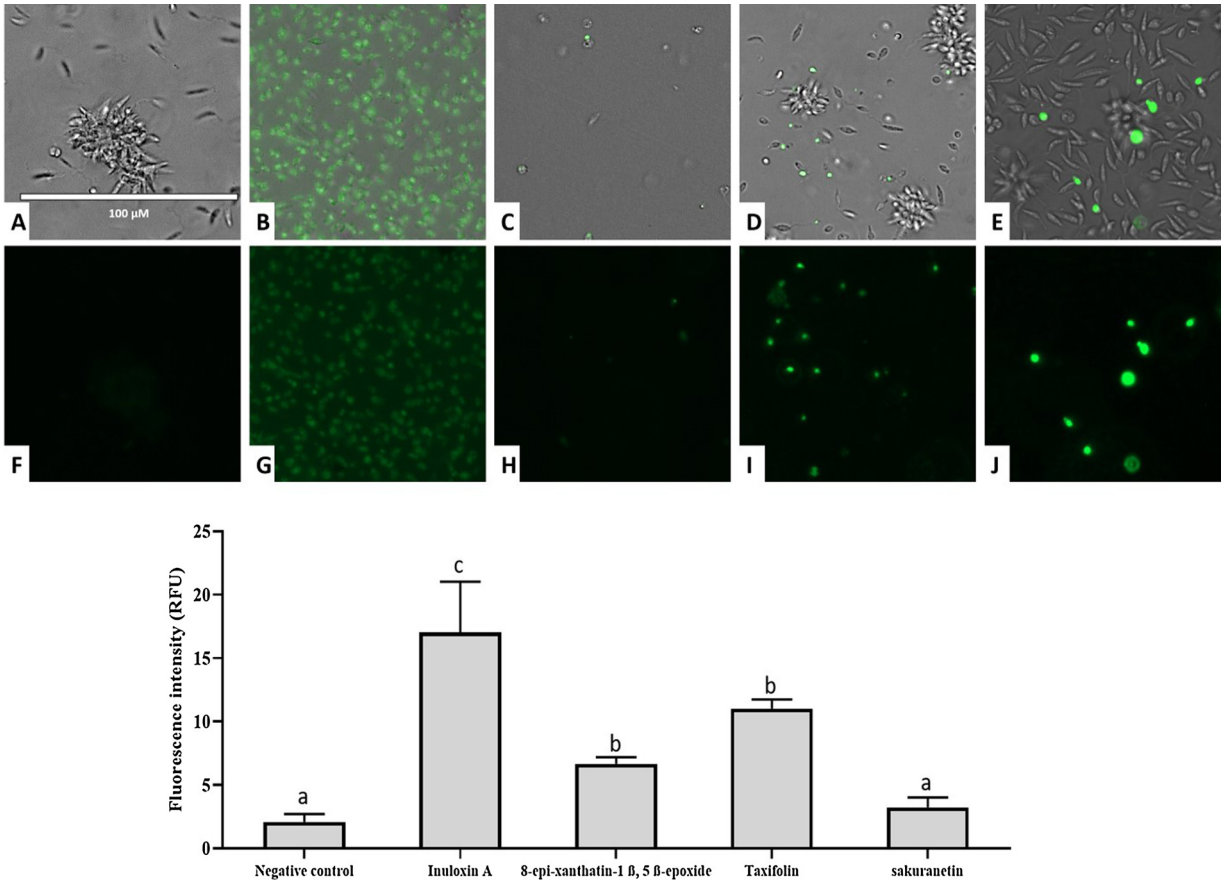

Fig. 10. Images (40X) presenting the effects of IC_{90} concentration of inuloxin A (B, G), sakuranetin (C, H), 8-*epi*-xanthatin-1β,5β-epoxide (D, I) and taxifolin (E, J) on the plasma membrane permeability of *Leishmania amazonensis* promastigotes after 24 h incubation. Cells were labeled with Sytox® Green. Images were obtained using an EVOS FL Cell Imaging System and untreated strains were used as negative control (A, F). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

viscosa [43,44].

The influence of the substitution pattern in the sesquiterpene and flavonoid skeleton on the antikinetoplastid activity was studied, revealing the following trends on the preliminary structure-activity relationship of these natural compounds. In one hand, the influence of the substitution pattern in the sesquiterpene scaffold on the antikinetoplastid activity seems to be linked to the lactone ring (**3** and **4** versus **1** and **2**) which is a critical functional group for the activity. Previous data demonstrated the essential function of furanone ring in the biological activities [40,45]. On the other hand, the SAR analysis of the flavonoids showed that the overall oxidation level are important trends of this series, compound **6** containing five oxygenated positions has increased potency compared to their parent compounds **5** and **8**. Moreover, the acetylation and methylation revealed detrimental effect on the activity, when comparing effect of compound **6** to that of compound **7**. These results highlighted that the best functional group is the hydroxyl, which can act as an H-bond donor suggesting that the hydrophilicity of the molecule contributes to its biological activity. Previous investigations confirmed that the hydrophobic character of molecules decreases the cell permeability of the target reflecting a weak activity [46]. In the same vein, it was suggested that the association of hydroxyl group at C-4′ associated to one methoxyl group at C-7 in compound **5** is necessary for the antiparasitic potency [47]. The presence of such groups could constitute interesting sites of interaction and/or reaction related to the antiparasitic activity. Furthermore, characteristics such as acetylation were involved in a better transport and distribution in the biological system which enhanced the leishmanicidal effect [48].

3.2. Programmed cell death pathway

Results obtained from the in vitro antiparasitic activities and the selectivity index of compounds over amastigote stage (Tables 2 and 3), were encouraging to analyze their possible mechanism of action. (Table 4). Indeed, cell death of different developmental stages of kinetoplastids such as *Trypanosoma* and *Leishmania* genus were coupled to the occurrence of apoptotic events [20]. Among the most relevant apoptotic phenotypes of *Leishmania* spp. *and Trypanosoma* spp., there are DNA fragmentation, cell shrinkage, plasma membrane modifications and mitochondrial depolarization [21,49]. Hence, in the present work, different experiments were investigated in order to obtain insights into the apoptotic potential of the four active compounds against *L. donovani*, *L. amazonensis* and *T. cruzi*.

We first analyzed the DNA condensation that provides evidence of death through apoptosis. The properties of the performed assay indicate that a normal state of chromatin allows light blue when cells stained with Hoechst 33,342, while the early stained apoptotic cells show bright blue nuclei corresponding to chromatin condensation. While, PI was used to stain the dead cells showing dense bright red nuclei. The staining pattern resulted from this kit leads to distinguish normal, apoptotic and dead parasites. As displayed in Figs. 3, 4, 5 , the treatment of *L. amazonensis* with compounds **3–6** induced a highly noticeable condensation of chromatin, compared with the control ($p < 0.05$). Moreover, the red fluorescence indicated that PI penetrated the cell

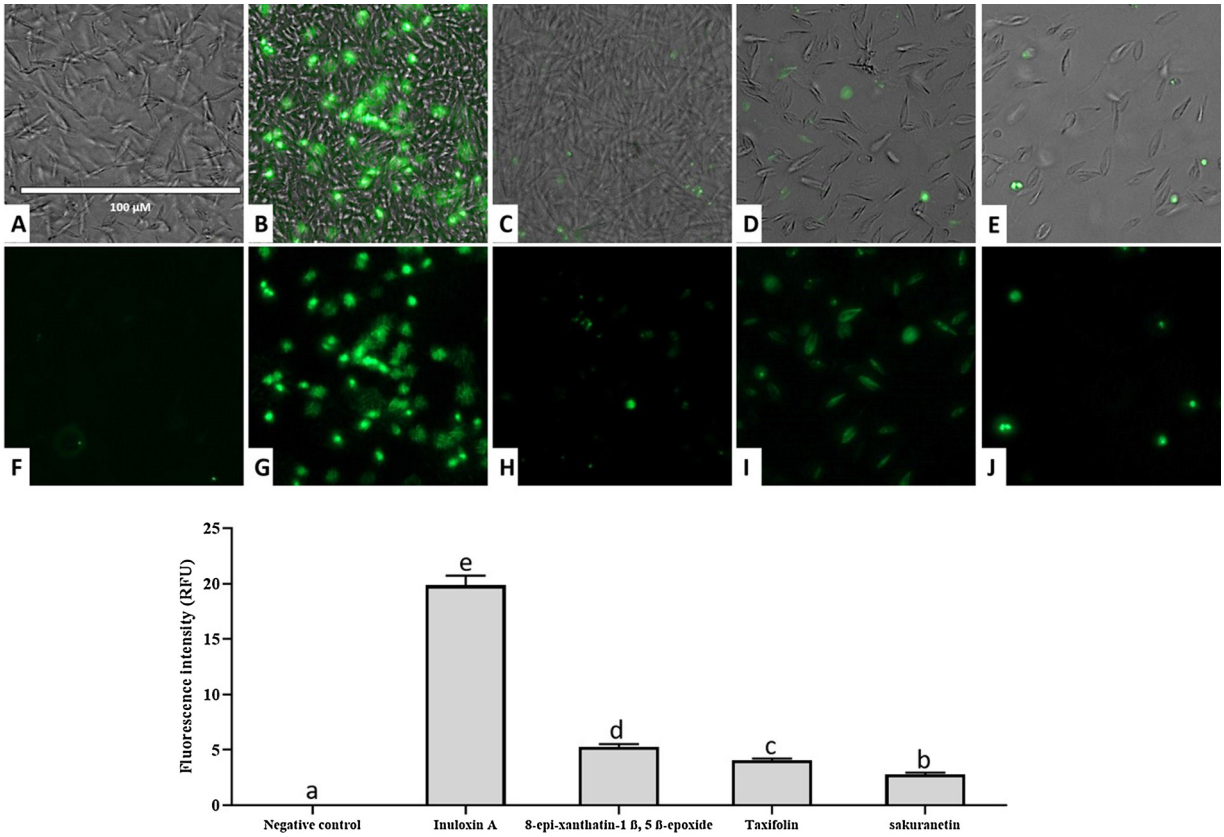

Fig. 11. Representative images (40X) of *Leishmania donovani* promastigotes stained with Sytox® Green kits. Parasites were incubated for 24 h with IC_{90} of inuloxin A (**B**, **G**), sakuranetin (**C**, **H**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**) and taxifolin (**E**, J). Negative control (untreated strains) was used (**A, F**). The effect on plasma membrane permeability was observed using an EVOS FL Cell Imaging System. The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

membrane of dead cells of *L. amazonensis* (Fig. S1). However, *L. donovani* when treated with compounds **3**,**5**,**6** and *T. cruzi* with compounds **4** and **6,** cells were able to exclude PI but not Hoechst indicating an apoptotic event (Fig. S2 and S3).Previous investigations confirmed the fragmentation of DNA of *Leishmania* spp. and *Trypanosoma* spp. treated with sesquiterpene lactones [50,51], which was not elucidated previously in literature for compounds **3–6**. In kinetoplastids, the apoptotic process involved the intrinsic pathway via mitochondria [52,53],a key event for the parasites survival, the effect of the tested molecules on the mitochondrial membrane potential (ΔΨm) was determined using JC-1 fluorescence measure. JC-1 is a probe that can aggregate within mitochondria and record red fluorescence at higher ΔΨm. However, in lower ΔΨm, this probe cannot accumulate within the mitochondria showing a green fluorescence since it stays in the cytosol area as monomers. As displayed in Figs. 6,7, the treatment of *L. amazonensis* and *T. cruzi* with the four compounds reduced the red fluorescence and enhanced the green one, when compared with the untreated parasites ($p < 0.05$) indicating a remarkable reduction in ΔΨm (Fig. S4 and S5). However, compounds **3** and **6** were unable to interrupt the mitochondrial membrane, while compounds **4** and **5** indicated a significant depolarization ($p < 0.05$) (Fig. 8, Fig. S6). To verify whether ATP production was affected by the loss of ΔΨm, the ATP level generated in 24 h was quantified and compared to positive and negative controls. It was observed that the treatment of *L. amazonensis* by compounds **4** and **5** caused the most significant decrease in the total ATP level ($p < 0.05$) (Fig. 9A). The effect of these both molecules was moderate in *L. donovani* and *T. cruzi* (Fig. 9B). However, the inhibition of ATP production by the three parasites was weaker when treated with compounds **3** and **6** (Fig. 9).

In order to investigate other events that complete the pathway of the PCD induced by the four compounds, we analyzed the plasma membrane integrity (Figs. 10, 11, 12). This experiment was monitored by the increase in SYTOX® Green fluorescence which is a high-affinity nucleic acid stain able to penetrate cells with compromised plasma membranes [54]. We found that the treatment of both *Leishmania* spp. And *T. cruzi* with the four compounds increased the green fluorescence confirming that the plasma membrane permeability was significantly affected ($p < 0.05$) (Fig. S7, S8 and S9). Permeability of the plasma membrane was identified as important target for drug discovery studies in *Leishmania* and *Trypanosoma* genus [55–57].

The generation of free radicals in parasites is deleterious, since the regulation of oxidative stress is crucial for the survival of parasites. Hence, the analysis of this factor showed that treatment of *L. amazonensis* and *T. cruzi* but not *L. donovani* with compounds **3**-**6** noticeably enhanced the red fluorescence reflecting a high generation of ROS (Figs. 13,14, Fig. S10 and S11). Similar to our findings, previous studies reported the generation of ROS in *T. cruzi* epimastigotes and *L. amazonensis* promastigotes induced by compounds isolated from natural products [58,59]. However, it was reported that phytochemicals induced ROS production in *L. donovani* promastigotes [60] which is in disagreement with our data. This dissimilarity could be explained by the different active moiety in compounds, phytochemical families, experimental conditions and concentrations. In addition, protein and lipid oxidation induced by ROS were widely involved in mitochondrial dysfunction by enhancing proton permeability, then decreasing ATP level [49]. It was noted that mitochondrial dysfunction and ROS

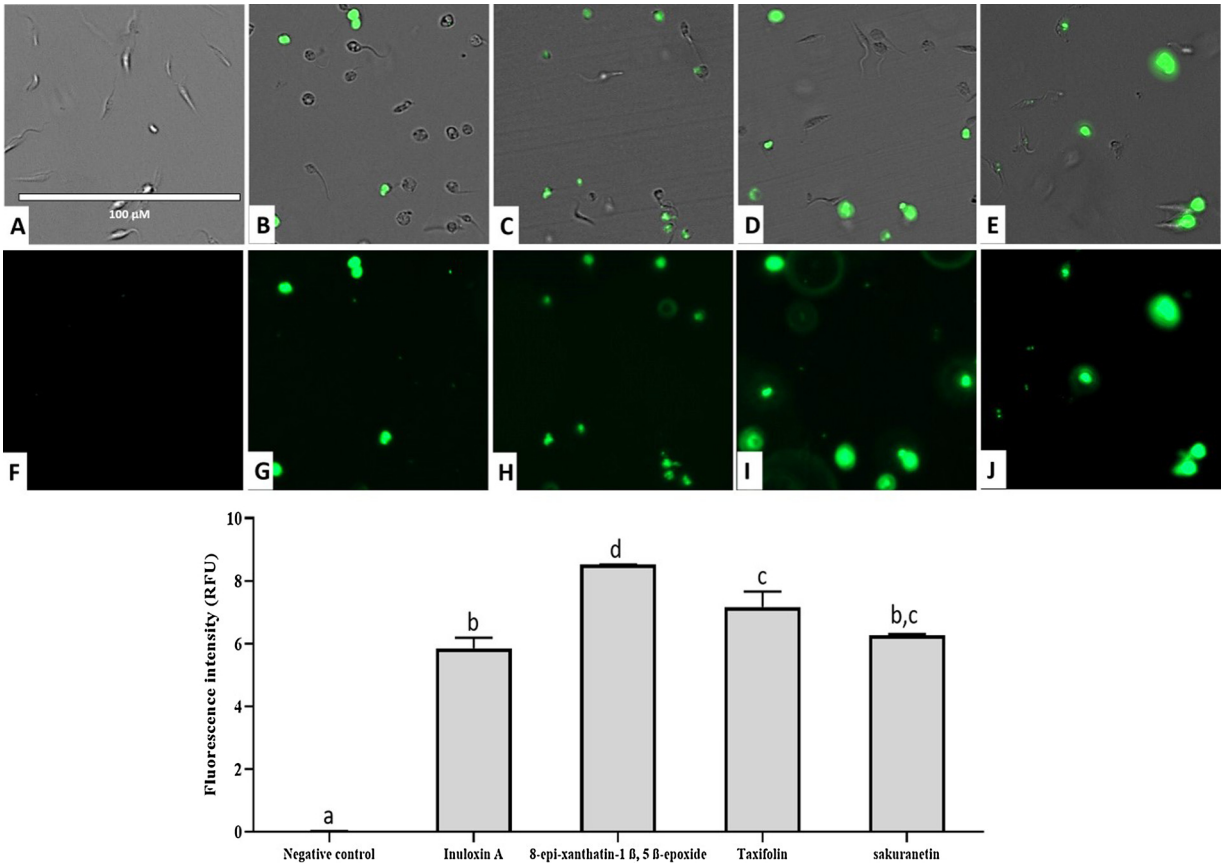

Fig. 12. Images (40X) obtained from an EVOS FL Cell Imaging System showing fluorescence when epimastigotes of *Trypanosoma cruzi* were incubated for 24 h with IC_{90} of inuloxin A (**B**, **G**), sakuranetin (**C**, **H**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**) and taxifolin (**E**, J) and stained with Sytox® Green kits. Effects of compounds on plasma membrane permeability were compared to the negative control (**A, F**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

production are related and crucial for the inhibitory potential of *T. cruzi* [61] and *L. amazonensis* [62]. This is in line with our results, the tested compounds failed to generate ROS, to decrease ATP level and to rich mitochondria of *L. donovani,* especially in the case of compounds **3** and **6**.

3.3. PCD and structure-activity relationship

Based on the observed results in the current work, the four isolated compounds showed significant activities through the induction of apoptosis against the evaluated parasites. The physicochemical and molecular proprieties of these compounds could affect the drug affinity towards the target, particularly for kinetoplastid protozoa [63]. For instance, sesquiterpenoids displayed a significant leishmanicidal effect and previous investigations linked their mode of action to the chemical structures. Barrera et al. demonstrated the inhibitory potential of the functional group α-methylene-γ-lactone in this family [64]. Furthermore,sesquiterpenes lactones react with sulfhydryl groups through the Michael-type addition way leading to an inhibition of the protective enzymes against oxidative damage [65,66], which could explain the generation of ROS by *L. amazonensis* and *T. cruzi* when treated with sesquiterpenoids **3** and **4.** In addition, the nonpolar character of lactones could probably correlate their mode of action which facilitates the passage of compounds into the membrane as clearly observed using the SYTOX® Green assay. Moreover, the presence of reactive α,β-unsaturated carbonyl group in sesquiterpene lactones enhancedthe antiprotozoal potency but also the cytotoxicity [67] which is in accordance with our data in the case of compound **3**.

Regarding the group of flavonoids presented by compounds **5** and **6**, we suggest that the apoptotic pathway is related to the lipophilicity character of these molecules, which facilitates the absorption and distribution of the molecule into the parasites. The lipophilicity was mathematically demonstrated to increase the biological activities [68]. According to literature data, lipophilicity is an important factor involved in the antiparasitic activity, the most lipophilic compound was the more active against amastigotes and promastigotes of *L. amazonensis* due to the presence of four prenyl groups [69]. On the other hand, and similar to our results, compound **6** displayed interesting antiparasitic activities. However, its mode of action was principally based on the inhibition of trypanothione reductase in *L. infantum* [70] and arginase in *L. amazonensis* [16]. These findings could explain the maintained ATP levels mentioned above indicating that the mitochondria was intact.

The obtained data also showed that *L. donovani* was more resistant than *L. amazonensis* and *T. cruzi*. We did not observe any accumulation of ROS into the parasite which was also related to mitochondria function and ATP production. As highlighted in bibliography, *L. donovani* has developed different mechanism of defense in order to neutralize the host response namely the A2 proteins that protect the parasite against oxidative stress. Mccall et al. conducted a comparative study between normal and low level of A2 expression, highlighting that A2 was involved in *L. donovani* pathogenicity and resistance [71].

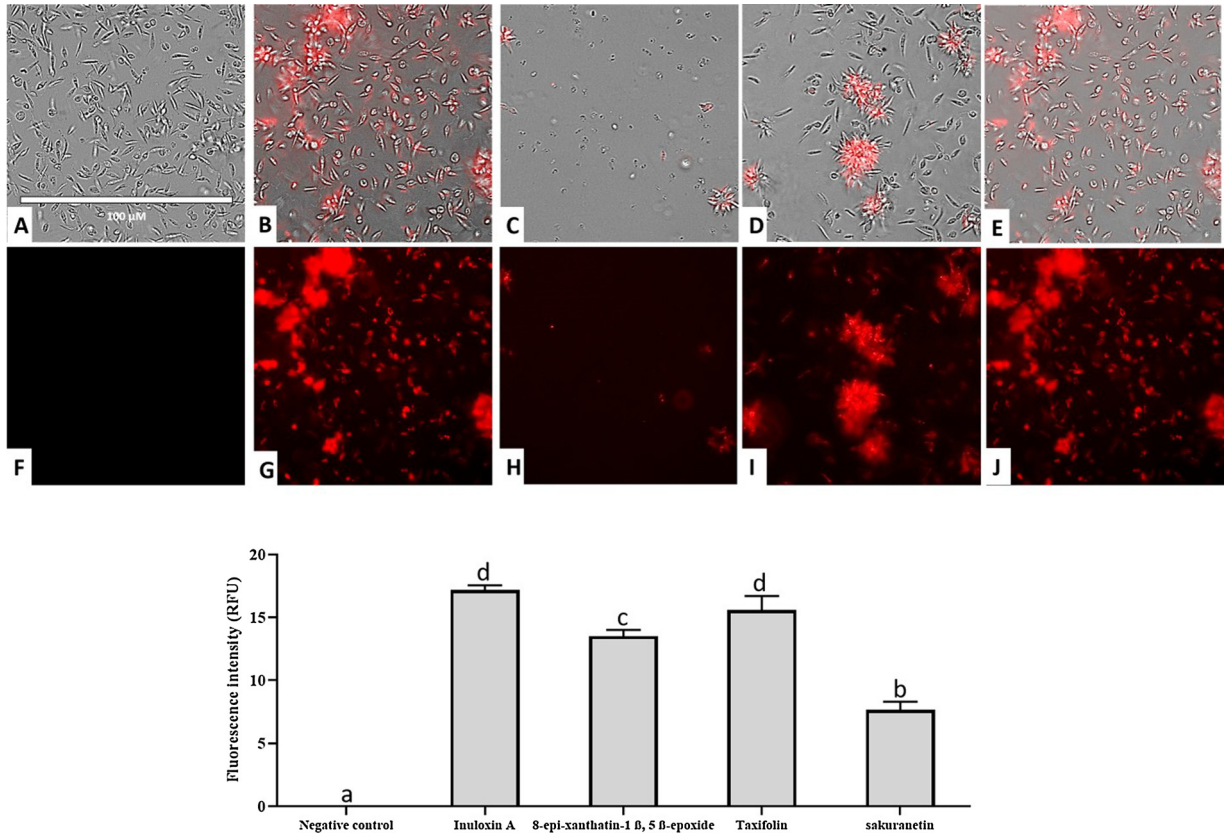

Fig. 13. Increased level of ROS production in promastigotes of *Leishmania amazonensis* after treatment with IC_{90} of inuloxin A (**B**, **G**), sakuranetin (**C**, **H**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**) and taxifolin (**E**, J) for 24 h. After CellROX Deep Red staining, images (40X) were obtained using an EVOS FL Cell Imaging system. Results were analyzed in comparison to the negative control (**A, F**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e reflect that means within compounds with different letters are significantly different ($p < 0.05$). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

4. Conclusion

Neglected tropical diseases such as Chagas disease and leishmaniasis cause negative impact on social and economic aspects, especially in developing countries. Overall, in the current study we proved the promising leishmanicidal and trypanosomal activities of sesquiterpenoids and flavonoids isolated from *Inula viscosa*. In general, the characterized compounds induce apoptosis via chromatin condensation, plasma membrane permeability, alteration of the mitochondrial membrane potential and oxidative damage. Moreover, the structure-activity relationship analysis of the anti-protozoal activity suggests that lactonization or oxidation enhances the biological profile. However, further studies are needed to validate other mechanism of action such as enzymes production and proteins expression.

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CRediT authorship contribution statement

Ikrame Zeouk: Data curation, Investigation, Methodology, Writing - original draft. **Ines Sifaoui:** Data curation, Investigation, Writing - original draft. **Atteneri López-Arencibia:** Data curation, Writing - original draft. **María Reyes-Batlle:** Investigation. **Carlos J. Bethencourt-Estrella:** Investigation. **Isabel L. Bazzocchi:** Funding acquisition, Supervision, Writing - review & editing. **Khadija Bekhti:** Methodology, Supervision. **Jacob Lorenzo-Morales:** Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **Ignacio A. Jiménez:** Funding acquisition, Supervision, Writing - review & editing. **José E. Piñero:** Funding acquisition, Supervision, Writing - review & editing.

Declarations of Competing Interest

The authors declare no conflict of interest.

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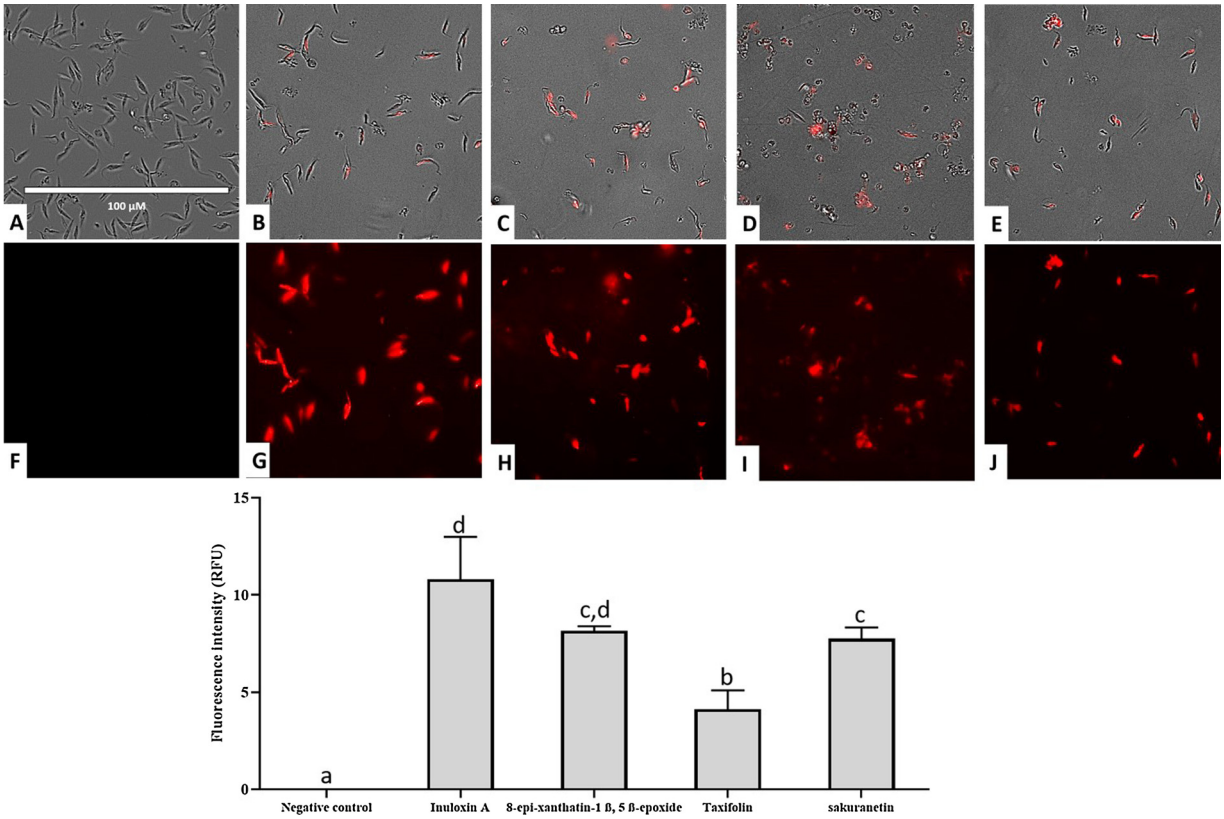

Fig. 14. Representative images (40X) of CellROX Deep Red staining of *Trypanosoma cruzi* epimastigotes after treatment with IC_{90} of inuloxin A (**B**, **G**), sakuranetin (**C**, **H**), 8-*epi*-xanthatin-1β,5β-epoxide (**D**, **I**) and taxifolin (**E**, J) for 24 h. Images were obtained using an EVOS FL Cell Imaging system and compared to the negative control (**A, F**). The bar graph includes the calculated values of RFU (Fluorescence intensity). Differences between the values were assessed using one-way analysis of variance (ANOVA). Data are presented as means ± SD (N = 3) and letters a-e reflect that means within compounds with different letters are significantly different ($p < 0.05$).

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.2020.110518.

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