

Leishmanicidal Activity of Oleuropein: *Leishmania donovani* Promastigote Cell Death through a Possibly ROS-Independent Mechanism

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Abstract

The development of potent and inexpensive antiparasitic agents for common use is imperative due to the absence of an effective and safe treatment for visceral leishmaniasis, a devastating parasitic disease. *Oleuropein*, a secoiridoid, exerts an antileishmanial effect on promastigotes of *Leishmania infantum*, *L. major* and *L. donovani*, as well as on amastigotes of *L. donovani* in *in vitro* and *in vivo* experimental models. In this study, our aim was to characterize how oleuropein drives parasites into cell death. Our hypothesis is that oleuropein promotes an apoptosis-like cell death which restrains the inflammatory processes that facilitate parasitic dissemination.

Logarithmic-phase *L. donovani* promastigotes were treated with oleuropein for 24, 48 and 72 h, and parasitic cell cycle, parasitic membrane asymmetry and intracellular generalized oxidative stress were assessed via flow cytometry. Morphological alterations were analyzed with confocal microscopy.

Cell-cycle analysis revealed that oleuropein fragmented parasite DNA within the first 24 h of incubation. *In vitro* treatment of promastigotes with oleuropein resulted in several morphological alterations and significant annexin V binding. Interestingly, oleuropein treatment did not increase the levels of intracellular reactive oxygen species until 48 h of incubation.

Our findings suggest that oleuropein promotes apoptosis-like cell death in *L. donovani* promastigotes that is not being mediated by an induced endoparasitic oxidative stress.

Keywords: Oleuropein, *Leishmania donovani*, Apoptosis, Leishmaniasis

Abbreviations:

Ca²⁺: Calcium Ion; CM-H₂DCFDA: 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; FITC: Fluorescein Isothiocyanate; gMFI: Geometric Mean Fluorescence Intensity; PI: Propidium Iodide; ROS: Reactive Oxygen Species.

Introduction

Nature is the major source of novel chemical compounds with potential antiparasitic activity [1]. In particular, the olive tree, *Olea europaea* L. (Oleaceae), is of great interest because it contains chemical compounds with various biological activities. Oleuropein, the main phenolic compound in *O. europaea*, is a secoiridoid glucoside (Figure 1) [2]. It is able to scavenge free radicals in order to protect cells from oxidation, leading to anti-aging and cardioprotective effects; it has also shown antitumor, antiviral, and antibacterial activities [2,3]. Previously, we demonstrated that oleuropein exerts *in vitro* antileishmanial activity on promastigotes and amastigotes of the genus *Leishmania* [4]. Moreover, we have showed that intraperitoneal

administration of oleuropein diminishes the parasite burden in the visceral tissues *in vivo*, an effect that was maintained even 6 weeks after treatment [4]. The antiparasitic effect of oleuropein merits further investigation because its mechanism(s) of its action remains unknown. It is important to determine whether oleuropein causes leishmanicidal or leishmanistatic effects and to determine the type of cell death that is initiated in *Leishmania* parasites.

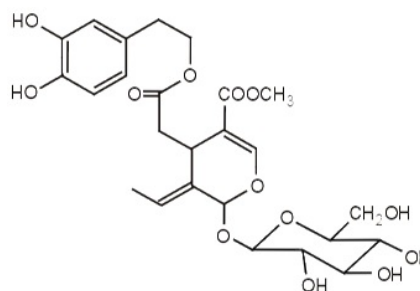


Figure 1: Molecular formula of oleuropein.

The Nomenclature Committee on Cell Death put forward a list of phenotypical manifestations that define cell death [5], enabling us to use the term “apoptosis” when the cell death induced in parasites is circumscribed by these hallmarks. Oleuropein induces apoptosis in human skin carcinoma A-431 cells, a process that involves the activation of caspase-3 [20], and it inhibits cancer-cell proliferation by arresting the cell cycle phase in G0/G1 phase [6,7]. Moreover, oleuropein drives human colon adenocarcinoma HT-29 cells into a p53-dependent apoptosis [8]. Although *Leishmania* parasites do not contain any canonical regulators of apoptosis, such as the caspases and p53 [9], it is interesting to examine the mechanism by which oleuropein inhibits parasite growth. Parasite apoptosis is orchestrated mainly by a “biochemical triangle” that includes calcium ion (Ca^{+2}) levels, mitochondrial membrane potential and the production of intracellular reactive oxygen species (ROS). ROS production could be the stimulus that promotes alterations to Ca^{+2} homeostasis or the loss of physiological mitochondrial membrane potential, but it could also be the outcome of these biochemical activities [10].

Parasites of the genus *Leishmania* cause leishmaniasis, an inflammatory disease with a wide spectrum of clinical manifestations. Leishmaniasis is characterized by the WHO as a neglected disease; nearly 2 million new cases are diagnosed every year, leishmaniasis is endemic in 98 countries on five continents and the disease mostly affects “the poorest of the poor” [11]. Antileishmanial drugs such as pentavalent antimonials, paromomycin sulfate, miltefosine, and liposomal amphotericin B are associated with numerous drawbacks that extend from restricted activity and development of drug resistance to systemic toxicity and an intolerable cost for impoverished sufferers [12]. Due to these problems, in recent years there has been more interest in the study of ethnomedicines as a source of new chemotherapeutic compounds capable of eliminating parasites in the infected host with minimal side effects. Several natural products have been tested for antiparasitic activity and for the promotion of direct apoptosis-like death in *Leishmania* parasites [13].

During the initial establishment of parasitic infection, a subpopulation of *Leishmania* protozoans becomes altruistically apoptotic [14], enabling the rest of the population to infect host cells without activating defense mechanisms via inflammatory molecules. In contrast, after the onset of *Leishmania* infection, a non-inflammatory environment is not strategically correct for *Leishmania* parasites because tissue inflammation favors their spread. For that reason, a potent chemotherapeutic compound should promote apoptosis-like death of *Leishmania* in order to allow the host to utilize dynamic cellular immune responses to exterminate invasive parasites [15,16]. Our hypothesis is that oleuropein promotes an apoptotic-like cell death since it is already demonstrated in our previous research that oleuropein is able to promote a selective anti-inflammatory and anti-oxidant gene regulation in the environment of *L. donovani*-infected BALB/c mice that is responsible for the induction of the desired Th1 immune response [17]. The aim of the present study was to shed light on biochemical and morphological alterations in *Leishmania donovani* Laveran and Laveran et Mesnil, 1903, Ross (Trypanosomatidae) promastigotes treated with oleuropein.

Materials and Methods

Parasites

The *L. donovani* reference strain (zymodeme MON-2, strain MHOM/IN/1996/THAK35) was used in this study. Promastigotes were

cultured in complete medium consisting of RPMI-1640 (low phenol red content; Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM NaHCO_3 , 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Paisley, UK) at 26°C. Cultures were evaluated daily for parasite growth and promastigotes were collected at the middle of the logarithmic phase or at the beginning of the stationary phase of growth.

Oleuropein

Oleuropein was obtained from olive leaves (*O. europaea* var. microcarpa alba or koroneiki) as described elsewhere [4]. Purity was determined using high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy (Figure 2). Quantitative determination of oleuropein purity was performed with a Thermo Finnigan HPLC system (Thermo Electron Corporation, San Jose, California, San Jose, CA, USA) coupled with a Spectra System UV6000LP PDA detector (Thermo Electron Corporation). A two-solvent gradient method was used: solvent A, H_2O + 1% acetic acid (v/v); solvent B, methanol. The flow rate was 1 ml/min and the following elution program was applied: 0-2 min, linear gradient to 5% solvent B; 2-10 min, linear gradient to 25% solvent B; 10-20 min, linear gradient to 40% solvent B; 20-30 min, linear gradient to 50% solvent B; 30-34 min, 50% solvent B, isocratic; 34-45 min, linear gradient to 90% solvent B; 45-50 min, 90% solvent B, isocratic; 50-60 min, linear gradient to 100% solvent B; 60-65 min, 100% solvent B, isocratic. A standard solution of oleuropein was prepared in 50:50 H_2O /methanol. Separation was performed at 25°C and the injection volume was 20 μl . Detection was performed at 248 nm with a Supelco Analytical Discovery HS C18 column (Sigma-Aldrich, Taufkirchen, Germany) (25 cm, 4.6 mm, inside diameter 5.0 μm). *O. europaea* var. koroniki is one of the most popular cultivates in Greece; and its leaves are a rich source of oleuropein, which and its percentage can constitute nearly 10% of dry weight. Xynos et al. Oleuropein was diluted in distilled water before use, filtered with a 0.45 μm filter (Millex[®]; Merck, Billerica, MA, USA), and stored at 4°C. Logarithmic-phase *L. donovani* promastigotes were exposed to two concentrations of oleuropein: 128.4 μM (69.4 $\mu\text{g}/\text{ml}$), which is the half-maximal inhibitory concentration, and 256.8 μM (138.8 $\mu\text{g}/\text{ml}$), which is twice the half-maximal inhibitory concentration [4]. Higher concentrations were deemed impractical and without scientific importance because similar concentrations previously displayed a cytotoxic effect (CC50) against J774A.1 macrophages [4].

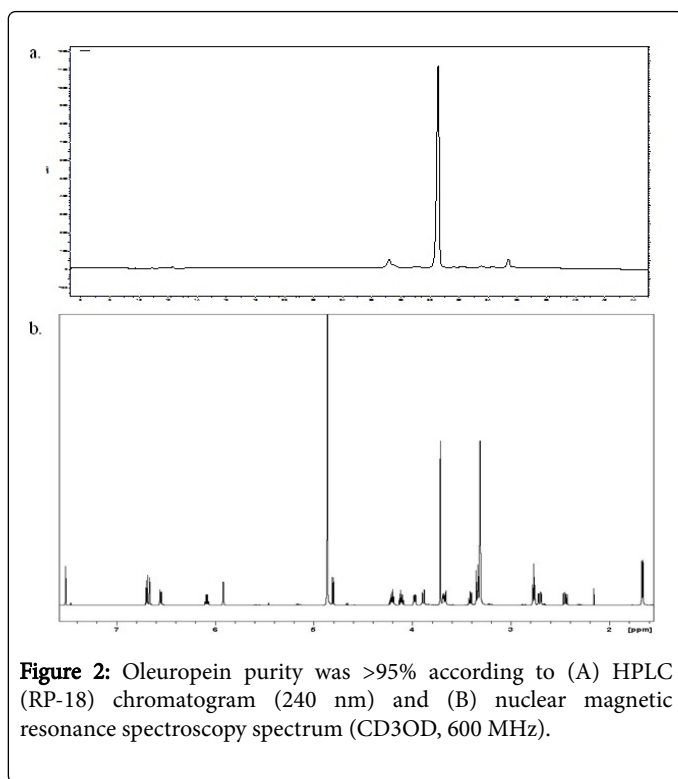


Figure 2: Oleuropein purity was >95% according to (A) HPLC (RP-18) chromatogram (240 nm) and (B) nuclear magnetic resonance spectroscopy spectrum (CD3OD, 600 MHz).

Promastigote cell-cycle analysis

We used flow cytometry to evaluate the effects of oleuropein on the *L. donovani* promastigote cell cycle. Oleuropein (128.4 μM and 256.8 μM) was added to cultures of 2×10^6 parasites/ml of *L. donovani* promastigotes in logarithmic growth and the cultures were incubated at 26°C. The negative control consisted of similar parasite cultures without oleuropein. After 24 h, 48 h, and 72 h of incubation, 1 ml was removed from each culture and the parasites were counted. Parasites (10^6) from each group were incubated with 100% ethanol (v/v) for 2 min and washed with phosphate-buffered saline supplemented with 100 $\mu\text{g}/\text{ml}$ RNase A (Promega, Madison, WI, USA) and 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI). Cells were incubated for another 1 min at room temperature and placed at 4°C until use. Flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA) was used to evaluate 10,000 promastigotes per experimental group and the results were plotted with Cell Quest Pro Version 5.2.1. (Becton Dickinson) and Flowjo V.10.0.8 (TreeStar Inc., Ashland, OR, USA).

Staining with annexin V-fluorescein isothiocyanate (FITC)

In order to evaluate the effect of oleuropein on *L. donovani* membrane integrity, we used annexin V-FITC and PI from the Apoptosis Detection Kit I (Becton-Dickinson) in conjunction with flow cytometry. Briefly, 128.4 μM and 256.8 μM oleuropein were added to fresh parasite cultures (starting inoculum of 2×10^6 *L. donovani* promastigotes/ml in mid-logarithmic growth), which were incubated at 26°C in 25-cm² culture flasks. The negative control lacked oleuropein. After 24 h, 48 h, and 72 h of incubation, 1 ml was removed from each culture and parasites were counted. As a positive for inducing necrosis, we added Triton X-100 (0.1%, v/v) to the culture medium for 5 min to perforate the cell membranes of the promastigotes. Subsequently, 10^6 promastigotes from each group (negative control, positive control,

128.4 μM oleuropein, and 256.8 μM oleuropein) were washed with ice-cold phosphate-buffered saline and then with annexin binding buffer. One hundred microliters (10^5 promastigotes) from each group were placed in separate eppendorf tubes and were incubated for 15 min at 26°C with 10 μl annexin V-FITC and 10 μl PI in accordance with the manufacturer's instructions. Samples were placed at 4°C for flow cytometry (10,000 promastigotes per experimental group). Cells that were not stained with both annexin V-FITC and PI were considered healthy. Cells that were stained with annexin V-FITC but not with PI were considered to be in early apoptosis. Cells that were stained with both annexin V-FITC and PI were alive or already dead and considered to be in late apoptosis or in necrosis respectively.

Confocal microscopy

Oleuropein (256.8 μM) was added to a culture with 2×10^6 parasites/ml (*L. donovani* promastigotes in logarithmic growth) at 26°C for 24 h. The negative control consisted of a similar culture without oleuropein. After incubation, the parasites were washed in phosphate-buffered saline and resuspended in paraformaldehyde 2% (v/v) for 15 min at room temperature. Parasites were washed again with phosphate-buffered saline (50 $\mu\text{l}/\text{sample}$) to remove paraformaldehyde and placed on coverslips coated with 1 mg/ml poly-L-lysine (Sigma-Aldrich). After an 18-h incubation at room temperature to permit the promastigotes to attach to the coverslips, the cells were incubated for 1 min in 0.1% Triton X-100 (v/v) to perforate cellular membranes. Parasites were incubated with 50 $\mu\text{g}/\text{ml}$ RNase A for 1 h at room temperature, and then with 100 $\mu\text{g}/\text{ml}$ PI for another hour under the same conditions. The coverslips were mounted with 10 μL Mowiol 4-88 (25% v/v glycerol, 100 mM Tris-HCl, pH 8.5; Calbiochem[®]-Merck), on microscope slides, sealed with nail polish and stored at 4°C. After 18 h at 4°C, slides were subjected to confocal fluorescence microscopy (TCS-SP; Leica Microsystems, Wetzlar, Germany). At least 15 cells from three independent experiments were observed from each experimental group.

ROS production in parasites

We also used flow cytometry to assess the effect of oleuropein on intracellular ROS production in logarithmic phase *L. donovani* promastigotes. Oleuropein (128.4 μM and 256.8 μM) was added to cultures of 2×10^6 parasites/ml, and the cultures were incubated at 26°C. The negative control lacked oleuropein. After 2 h, 4 h, 8 h, 12 h, 18 h, 24 h, 48 h, and 72 h incubation, 1 ml was removed from each culture and the parasites were counted. Then, 10^6 parasites from each group were incubated with 5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA; Thermo Fisher Scientific, Waltham, MA, USA) for 45 min at 26°C in the dark. Positive-control cells were parasites from the negative-control culture that were incubated with 10 μM hydrogen peroxide for another 30 min under the same conditions. Parasites from each experimental group were washed with phosphate-buffered saline and placed at 4°C until use. Flow cytometry was used to evaluate 10,000 promastigotes per experimental group.

Statistical analysis

Three independent experiments were carried out in duplicate for each analysis. The nonparametric Mann-Whitney test (SPSS; Statistical Package for Social Sciences[®], Version 14.0) was used to identify significant differences between *in vitro* experiments. P values < 0.05 were considered significant.

Results

Oleuropein causes DNA fragmentation in *L. donovani* promastigotes

First, we investigated whether oleuropein influences cell-cycle progression and DNA integrity in *L. donovani* promastigotes (Figure 3). After only 24 h of incubation, both oleuropein concentrations caused a significant increase ($P=0.05$) in the number of parasites with incomplete DNA load (in the subG0 phase). At this time point, parasites in subG0 treated with 128.4 μM and 256.8 μM oleuropein constituted $26.4 \pm 3.5\%$ and $35.7 \pm 3.7\%$ of the population, respectively, while only $8.4 \pm 5\%$ of negative-control parasites were in subG0 (Figure 3). At 48 h and 72 h, the percentage of parasites in subG0 in each experimental group was lower than at 24 h, but significantly more parasites exposed to oleuropein were in subG0 than were negative-control parasites at these time points (Figure 3). This decrease was expected because the rest of the population continues to divide, thus decreasing the percentage of the population in subG0. Consistent with this observation, at 24 h, the percentage of the parasite population in G1 was significantly lower ($P=0.05$) after exposure to 128.4 μM ($55.8 \pm 5.2\%$) and 256.8 μM oleuropein ($47.6 \pm 0.4\%$) than after no exposure ($67.1 \pm 10.2\%$; Figure 3). At 48 h and 72 h, the percentages of G1-phase parasites exposed to the two oleuropein concentrations were significantly higher than the percentage of negative-control parasites in G1 (Figure 3). The few significant between-group differences in S and G2-M phases did not suggest that either oleuropein concentration affected cell-cycle progression in *L. donovani* promastigotes.

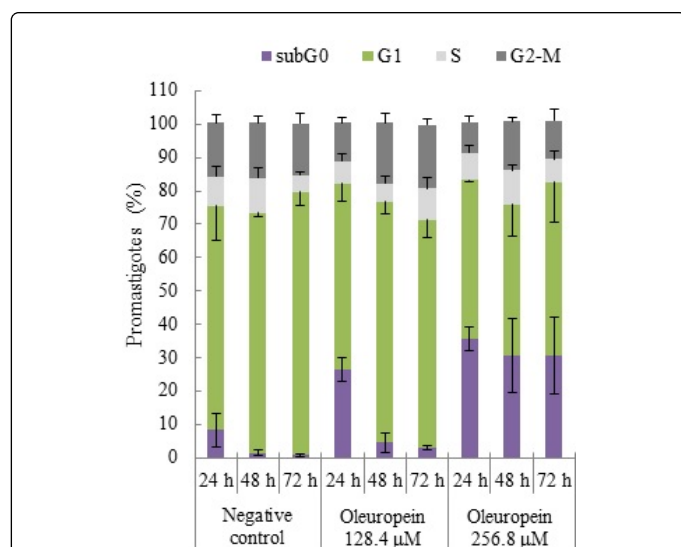


Figure 3: Effect of oleuropein on the cell cycle of *L. donovani* promastigotes. * $P<0.05$ versus the negative control. ^ $P<0.05$ versus the other oleuropein concentration. Data represent the average \pm standard deviation of three independent experiments.

The effect of oleuropein on parasite membrane asymmetry

The DNA fragmentation detected in our cell-cycle analysis (Figures 3 and 4) suggests that oleuropein exerts leishmanicidal rather than leishmaniostatic effect. To confirm the presence of apoptosis-like cell death, we sought to identify membrane asymmetry and membrane impermeability via annexin V staining (Figure 5). At 24 h, only 2 \pm

0.5% of negative-control parasites appeared to be annexin V+/PI- (early apoptosis). Parasites exposed to the low dose of oleuropein exhibited a similar rate of annexin V+/PI- staining ($4.7 \pm 4\%$), while exposure to the high concentration was associated with a significant increase ($9.3 \pm 5.5\%$, $P=0.05$; Figure 5). At 24 h, only $1.9 \pm 0.5\%$ of negative-control cells were annexin V+/PI+ (in late apoptosis or necrotic; Figure 5). Incubation with the low dose of oleuropein did not alter the percentage of annexin V+/PI+ parasites, but incubation with 256.8 μM oleuropein induced a significant 3-fold increase ($6.1 \pm 2.4\%$, $P=0.05$) versus the other two experimental groups (Figure 5).

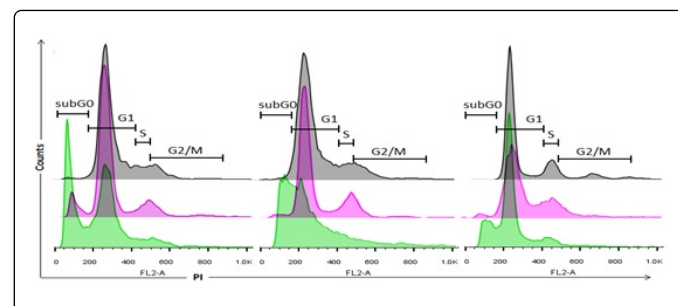


Figure 4: Semi-merged histograms of flow-cytometry data from oleuropein-treated *L. donovani* (left to right, 24 h, 48 h, and 72 h of oleuropein exposure). Black, negative control (no exposure); purple, 128.4 μM oleuropein; green, 256.8 μM . Data represent one of three independent experiments.

At 48 h, the rates of negative-control parasites in early ($1.6 \pm 1.1\%$) and late apoptosis or necrosis ($1.3 \pm 0.9\%$) remained low (Figure 5); these percentages were higher in the presence of 128.4 μM oleuropein ($4.5 \pm 1.8\%$ and $3.6 \pm 1.2\%$, respectively, $P=0.05$; Figure 5). However, 256.8 μM oleuropein significantly increased the percentage of annexin V+ cells ($4.7 \pm 0.9\%$ and $18.7 \pm 5.8\%$, respectively, $P=0.05$; Figure 5).

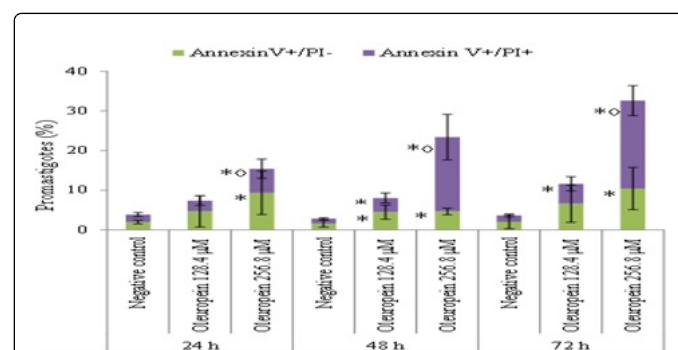


Figure 5: Effect of oleuropein on membrane asymmetry in *L. donovani* promastigotes, as assayed via double staining with annexin V and PI. * $P<0.05$ versus the negative control. ^ $P<0.05$ versus the other oleuropein dose. Data represent the average \pm standard deviation of three independent experiments.

At 72 h, few parasites in the negative-control group were in early ($2.1 \pm 1.7\%$) or late apoptosis/necrosis ($1.6 \pm 0.4\%$; Figure 5). There was a three-fold increase in annexin V+ staining after exposure to 128.4 μM oleuropein, but only the percentage of annexin V+/PI+ cells ($5 \pm 1.8\%$) was significantly ($P=0.05$) higher than the respective percentage of parasites without exposure to oleuropein (Figure 5). In contrast, in parasites exposed to 256.8 μM oleuropein, the percentages

of annexin V+/PI- cells ($10.5 \pm 5.3\%$) and annexin V+/PI+ cells ($22.1 \pm 3.8\%$) significantly increased ($P= 0.05$). The positive control, which consisted of promastigotes exposed to Triton X-100, contained $2.4 \pm 1.3\%$ annexin V+/PI- cells and $44.4 \pm 3.5\%$ annexin V-/PI+ (necrotic) cells (Figure 6).

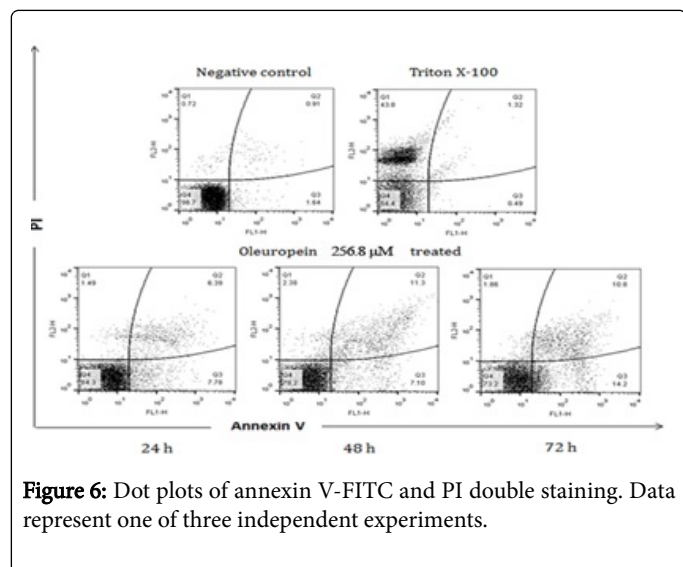


Figure 6: Dot plots of annexin V-FITC and PI double staining. Data represent one of three independent experiments.

Morphological alterations in oleuropein-treated *L. donovani* promastigotes

The biochemical markers examined above highlight the ability of $256.8 \mu\text{M}$ oleuropein to promote apoptosis in *Leishmania* parasites after only 24 h of incubation. Using confocal microscopy and PI staining, we examined the morphological changes in the cells and nuclei of *L. donovani* promastigotes after 24 h of exposure to oleuropein (Figure 7).

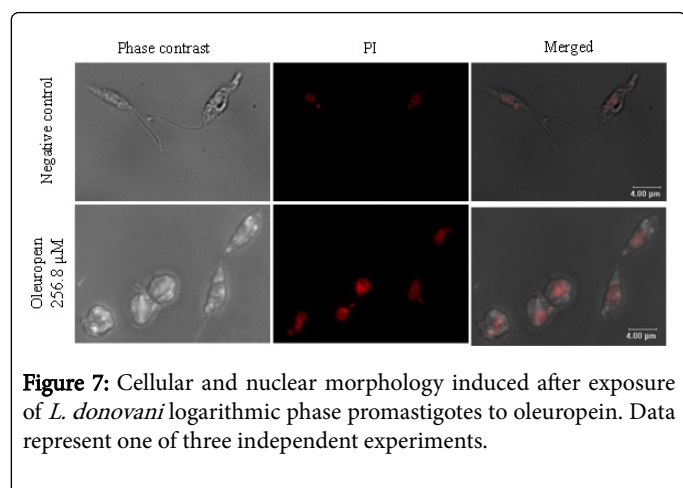


Figure 7: Cellular and nuclear morphology induced after exposure of *L. donovani* logarithmic phase promastigotes to oleuropein. Data represent one of three independent experiments.

In the absence of oleuropein, *L. donovani* promastigotes exhibited canonical morphological manifestations, including a typical elongated cell shape with a long flagellum and a spherical and well-stained nucleus and kinetoplast (Figure 7). In contrast, parasites treated with $256.8 \mu\text{M}$ oleuropein for 24 h were round, had decreased cellular volume, and had lost a substantial portion of their flagella; in some cases, the flagellum was completely missing (Figure 7). After exposure to oleuropein, the fluorescence of apoptotic nuclei was more intense

than the fluorescence of the nuclei of non-treated parasites (Figure 7). This intensity, which was also higher in the kinetoplast of oleuropein-exposed parasites, indicated chromatin condensation. These phenotypes demonstrate that oleuropein promotes apoptosis-like death in these parasites.

Endoparasitic ROS Production in Oleuropein-Treated *L. donovani* Promastigotes

Intracellular production of ROS may constitute the cause or the effect of apoptosis in normal eukaryotic parasites. The time course of ROS production in terms of other biochemical parameters is critical in determining whether the apoptotic mechanism involves ROS generation (Figure 8). We selected CM- H_2DCFDA as an indicator of general oxidative stress [18], and our flow-cytometry experiments evaluated all possible cell populations (live cells, apoptosis-like cells, and dead cells). ROS may not be detectable in dead protozoans [19].

At each timepoint, the ROS levels in the positive-control cultures significantly differed ($P= 0.05$) from those of the other three experimental groups, with values ranging from 9.5 ± 0.8 geometric mean fluorescence intensity (gMFI) to 10.5 ± 0.3 gMFI (Figure 8). Apart from the positive-control we did not observe any increase in the level of endoparasitic ROS up to 48 h among the rest experimental groups (Figure 8); at 48 h, only the high dose of oleuropein prompted a significant ($P=0.05$) change in ROS levels (2.6 ± 0.9 gMFI) versus the negative control (0.6 ± 0.2 gMFI; Figure 8). At 72 h, both oleuropein concentrations caused significant ($P= 0.05$) increases in ROS levels (1.6 ± 0.6 gMFI and 7.3 ± 0.1 gMFI, respectively) versus the negative control (Figure 8).

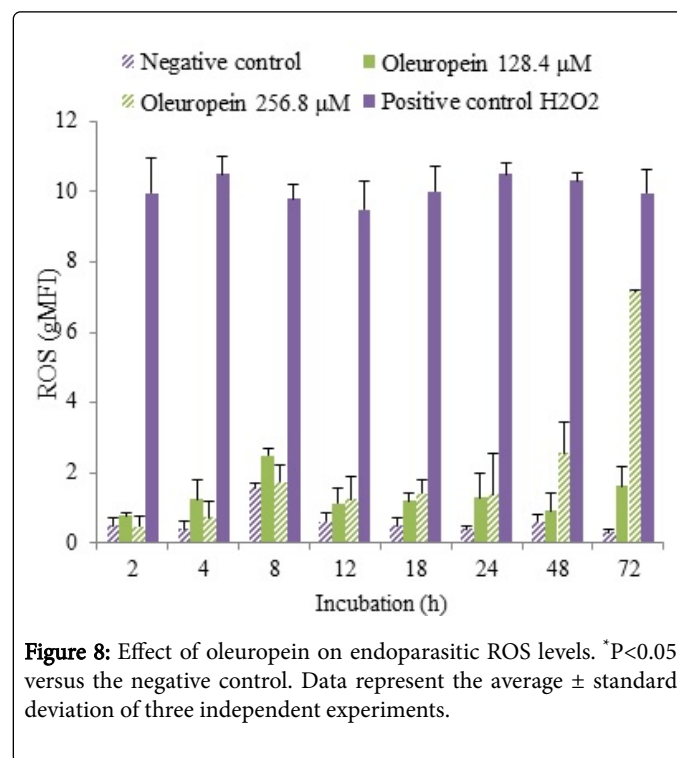


Figure 8: Effect of oleuropein on endoparasitic ROS levels. * $P<0.05$ versus the negative control. Data represent the average \pm standard deviation of three independent experiments.

Discussion

The ability of oleuropein to promote apoptosis in various mammalian cell lines through caspases, p53, and Bcl-2 [8,20]

prompted our interest in the mechanism by which this biophenol drives *L. donovani* parasites into apoptosis. Parasites of the *Leishmania* genus lack these regulators of apoptosis, suggesting that protozoan apoptosis should be driven by different mechanisms [9,10]. The parasite transition to apoptosis is expected to be associated with a non-inflammatory environment during parasite clearance *in vivo*, favoring the required immune response by type 1 T helper cells [14,16,21]. In a previous study, oleuropein reduced the proliferation of *L. donovani* promastigotes, as revealed with the AlamarBlue[®] assay [4]. This colorimetric method detects decreases in the metabolism of cultured protozoans without providing mechanistic details. These decreases may be due to the leishmanicidal or leishmaniostatic action of the potential pharmaceutical agent.

Notably, we observed inconsistent results when we attempted to assay DNA fragmentation via gel electrophoresis and DNA staining with ethidium bromide (data not shown). We therefore chose to assess antileishmanial activity by evaluating the cell cycle using flow cytometry. Previously, this method was used to detect the leishmanicidal activity of pure constituents of *Asparagus racemosus* (Asparagaceae) (racemoside A) and *Artemisia annua* L. (Asteraceae), (artemisinin) [22,23]. These natural extracts induced genome fragmentation, which resulted in the production of parasites with incomplete DNA load. On the other hand, taxol, which is derived from *Taxus baccata* L. (Taxaceae), halts *L. donovani* mitosis at G2-M phase, thus exerting leishmaniostatic activity [24]. Oleuropein appears to exhibit leishmanicidal activity since it is able to increase the percentage of *L. donovani* promastigotes with incomplete DNA load after 24 h of incubation (Figures 3 and 4). Other flavonoids, such as luteolin and quercetin, display the same activity against *Leishmania* promastigotes, but the effect becomes pronounced after more than 24 h of exposure [22,23,25]. After 72 h of incubation, oleuropein, luteolin, and quercetin all lead to cell-cycle arrest in 30% of the promastigote culture (Figure 3) [25].

The extent of *L. donovani* DNA fragmentation detected here is not sufficient to indicate that these cells undergo apoptosis (Figure 3). Additional phenotype and biochemical information is needed to characterize apoptosis in promastigotes, a process that is followed, to some extent, by eukaryotic parasites [9]. The basic phenotype of apoptosis in these parasites is membrane asymmetry; annexin V-binding phospholipids such as phosphatidylinositol, ethanolamine, phosphatidylglycerol, and phosphatidic acid are redistributed in the plasma membrane [26,27]. Racemoside A, *Aloe vera* (L.) Burm. f. (Xanthorrhoeaceae) extract, and the methanolic extract of *Piper betle* L. (Piperaceae) exhibit different patterns of action over time [22,28]. Racemoside A induces plasma-membrane asymmetry after 30 min of incubation, and after 6 h, the majority of parasites are annexin V-/PI+, reflecting necrosis. *Aloe vera* extract promotes the highest percentages of annexin V+ cells at 24 h, whereas luteolin and quercetin cause the highest percentages of annexin V+ cells after 48 h of incubation, due to delayed genome fragmentation [25]. In the present study, oleuropein exposure led to phenotypes that were similar to those induced by *Aloe vera* extract, since the percentage of annexin V+ parasites was significantly higher after only 24 h of incubation (Figure 5). However, at 48 h and 72 h, it seemed that cells moved from an early apoptotic state (annexin V+/PI-) to a late-apoptotic or necrotic state (Annexin V+/PI+), after which the population underwent a new parasitic entry into the early apoptotic stage (Figure 6).

Here, DNA fragmentation and membrane asymmetry, the classic hallmarks of apoptosis, were confirmed via confocal microscopy. The

cellular and nuclear morphologies of *L. donovani* were altered after a 24-h incubation with a high dose of oleuropein (Figure 7). Exposed parasites were rounded, had lost part of their flagella, had diminished cellular volume, and harbored condensed chromatin. Previously, racemoside A, taxol, luteolin, and quercetin caused similar cellular damage, abrogating cellular cohesion and disrupting the architecture of the parasite's nucleus [22,24,25]. In contrast, exposure to *Aloe vera* extract previously led to chromatin condensation only (Dutta et al., 2007a), and 10-deacetylbaicatin III, a taxol derivative, did not alter parasite morphology [19,24].

The disturbance of cellular physiology in the parasite is orchestrated by a "biochemical triangle" that includes endoparasitic ROS levels, Ca⁺² homeostasis, and mitochondrial membrane potential [10]. Numerous stimuli have the ability to raise endoparasitic Ca⁺² levels, a phenomenon that promotes the disruption of mitochondrial membrane potential. This biochemical cascade is followed by ROS production due to oxidative phosphorylation [29]. Other stimuli, such as camptothecin, curcumin, thenoyltrifluoro-acetone, and hydrogen peroxide, increase endoparasitic ROS levels and lead to lipid peroxidation [30]. This process affects membrane asymmetry and Ca⁺² channels and is followed by a loss of mitochondrial membrane potential. On the other hand, antimony potassium tartrate promotes ROS production, which first disrupts mitochondrial membrane potential and then increases Ca⁺² levels [31]. *Aloe vera* extract employs a different mechanism of action; the disruption of mitochondrial membrane potential is not followed by increases in ROS and/or Ca⁺² [19]. The mechanism of oleuropein seem to be similar to that of *Aloe vera*, since endoparasitic ROS levels were significantly higher after 48 h of exposure (Figure 8). After 48 h, membrane asymmetry was advanced (Figure 5), a high number of parasites were in subG0 phase (Figure 3), and the parasites displayed morphological alterations (Figure 7). Our data suggest that ROS production derives from extensive damage to the parasite and does not activate mechanisms that lead these protozoans into apoptosis.

Conclusions

The data presented here constitute the first evidence that oleuropein promotes the apoptosis of *L. donovani* promastigotes, a process that may not interfere with ROS production. This outcome may contribute to the effective suppression of amastigote proliferation in the infected host, which in a previous *in vivo* visceral model resulted in a lower parasite burden in the spleen and liver [4]. Controlled parasite apoptosis likely does not trigger inflammatory responses within the parasite's niche, enabling the host's immune response to undergo type 1 T helper cell polarization and to activate the production of host microbicidal molecules such as ROS and reactive nitrate intermediates.

Conflict of interest

The authors declare no financial or commercial conflicts of interest.

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