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Cytotoxic activity of olive leaf extract against leukemia cell lines U937 and THP1

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ABSTRACT

This study investigates the possible mechanism of induction of apoptosis in human leukemia U937 and THP-1 cells through the use of olive leaf extract (*Olea europaea L.*) Olive leaf extract, as the treatment in U937 and THP-1 cells, results in growth inhibition and induction of cell death measured by MTT assay and apoptosis confirmed by fluorescence microscopy, flow cytometry analysis and the activity of caspases-3 and -6. The data indicate that olive leaf extract causes a decrease in cell viability in both cancer cell lines tested in a concentration-dependent way, with percentage rates of 24.21 ± 9.18 for U937 and 31.47 ± 4.99 for THP-1 at a concentration of 800 µg/mL in 48 hours. The percentage increase in apoptosis, which was 100% for U937 and 56,11 ± 7 for THP-1 7 at a concentration of 800 µg/mL in a time period of 48 hours, has been associated with changes in mitochondrial membrane potential, exceeding 85% for U937 and 40% for THP-1 at the same concentration and time period, suggesting the involvement of the intrinsic pathway of cell death by apoptosis. We also evaluated the activation of caspases-3 and -6 and confirmed the pro-apoptotic activity of the extract. The results suggest that the extract of olive leaves affects the cellular viability in the tested cell lines U937 and THP-1, inducing cell death by apoptosis.

Keywords: *Leukemia cell lines U937 and THP-1, apoptosis, mitochondrial potential, caspases-3 and -6.*

1. INTRODUCTION

Chemoprevention, a relatively new and promising strategy in cancer prevention, is defined as the use of compounds that are nutritionally natural and/or synthetic substances that block, inhibit or retard carcinogenesis [1]. Chemopreventive agents can induce apoptosis in malignant cells, *in vitro* and *in vivo,* which is considered an anticancer mechanism [2]. The olive tree (*Olea europaea L.*) has been known for its medicinal properties for numerous years. The leaves contain many potentially active compounds that have antioxidant activity [3]. Olive leaf extract has been reported for having antihypertensive and anti-carcinogenic activity, also showing anti-diabetic and anti-atherosclerosis properties, among others [4] and was recently introduced to the Pharmacopoeia Ph.Eur. 5 [5]. The main physiological substances of the olive leaf extract are hydroxytyrosol, tyrosol, caffeic acid, p-coumaricacid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, glycosylatedluteolin-7, glycosylatedapigenin-7, and glycosylateddiosmetin-7 [5,6]. Several studies have reported that compounds isolated from the olive leaf extract, such as hydroxytyrosol and its derivatives, have antioxidant activity, preventing conditions such as oxidative stress and cancer [7, 8].

As it is a type of programmed cell death, apoptosis is a normal physiological process which involves elimination of undesirable cells such as cancer cells [9, 10]. There are two classical apoptosis pathways: the extrinsic pathway which requires transmembrane receptors of cell death and the intrinsic pathway, which starts with the route via mitochondrial stimuli. Both pathways activate caspases, such as caspase-3, inducing the cell death and the inactivation of these pathways is associated with tumor activity [11, 12].

Numerous studies have demonstrated that oleuropein and hydroxytyrosol, the most abundant phenolic compounds in olive leaves, are anticarcinogenic agents by presenting antioxidant activity and inducing cell death by apoptosis [13, 14, 15].

The present study investigated whether olive leaf extract has anticarcinogenic activity against leukemia cell lines U937 and THP-1 and the possible induction mechanism of cell death by apoptosis.

2. EXPERIMENTAL SECTION

2.1. Material and Preparation of Plant Extract.

Vincristine, 3-(4,5-dimethyl-thiazol-2) 2,5-diphenyl-2 H-tetrazom bromide (MT), acridine orange and ethidium bromide were purchased from Sigma Chemicals (St. Louis, MO , USA).

Individual adult leaves were collected with the help of pruning shears in a fragment of the Atlantic Forest, which were found in the Garden of Olives Farm, located in the Forest Zone, located in the southeastern state of Minas Gerais, Brazil. Control samples were deposited in the Rioclarense herbarium (HRCB), at Sao Paulo State University, under the registration number VIC: 36,631. Olive leaves (*Olea europaea L.*) were subjected to drying in forced ventilation under a constant temperature of 40°C to stabilize the weight for the dry product to the aforementioned materials.

The dried material was crushed with a mortar and pestle; in this procedure 1.0 kg of olive leaves were used. The material was subjected to an extraction by macerating it in absolute ethyl alcohol (PA) diluted in distilled water 1:1 for seven days and at room temperature for 30 days. The extract was concentrated in a rotary evaporator under controlled temperature between 40 and 50ºC and subjected to freeze drying, resulting in a dry material. Finally, the yield of the extract was found to have contained 200 grams (20%) and they were stored in an opaque plastic container, in the presence of a drying agent.

2.2. Cell lines and cell culture.

The U937 cells (leukemia lymphoid origin), and THP-1 cells (monocytic leukemia) were obtained from the Behavioral Biology Laboratory at the Center for Biotechnology and Biosciences, at the North Fluminense State University, at the Goytacazes Campus, RJ. Cells were cultured in D-MEM F12 (Gibco, BRL) according to the methodology described by [16].

2.3. MTT and LDH assays.

The U937 and THP-1 cell lines were plated into a volume of 100 µL/well $1x10^6$cels/mL) in 96-well plates, treated with the olive leaf extract at final concentrations of 50, 100, 200, 400 and 800 µg/mL for testing cell viability using MTT according to the methodology described by [16]. Part of the culture supernatant was used for the determination of lactate dehydrogenase (LDH). The determination of the LDH enzyme is proportional to the number of dead cells by necrosis *in vitro.* For quantification of LDH, KIT Doles was used.

2.4. Assessment of apoptosis by fluorescence microscopy.

The U937 and THP-1 cells were incubated for 24, 36 and 48 hours with the olive leaf extract, then were stained with a solution of 10 µg/ml of acridine orange (Sigma) and 10 µg/ml of ethidium bromide (Sigma) according to the methodology described by [16].

2.5. Analysis of mitochondrial membrane potential by flow cytometry.

The mitochondrial membrane potential was examined using the lipophilic cationic fluorescent dye,JC-1 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide). The tumor cell lines were treated with the olive leaf extract (800 µg/mL) and the cells were incubated for 48 hours in an incubator (37°C under 5% CO_2) according to the methodology described by [16].

2.6. Analysis of caspases-3 and -6 activity.

In order to prove that the olive leaf extract induced apoptosis, a Colorimeter Sampler Kit (Invitrogen) containing substrates for caspases-3 and -6 was used. The cultures containing $1x10^6$ cels/ml were incubated with a concentration of 400 µg/mL for the olive leaf extract for a time period of between 36 and 48 hours. Subsequently, the cells were centrifuged for 5 minutes at 1500 rpm and resuspended in 50 µL of a lysis buffer. Samples were centrifuged for 1 min (10,000xg) and then added 50 µL of substrate (4 µM). The samples were incubated at 37 ° C for 2 hours in the dark and was read in a spectrophotometer (ThermoLabsystemsMultiskan, Model 352) using a wavelength of 405 nm. As negative controls, cells were incubated with DMEM F12 (Gibco, BRL) supplemented with 20 µg/mL of gentamycin (Gibco, BRL) and 10% fetal bovine serum (Gibco BRL). To extract the cells they were incubated with DMSO (SIGMA®) at 1%.

2.7. Statistical Analysis.

The results were expressed as a mean ± standard deviation, and independently analyzed. The assays were analyzed by ANOVA - One way -analysis followed by Tukey, using GraphPad Version 5.0. Significant differences were considered as P <0.05.

3. RESULTS SECTION

3.1. Determining the viability of U937 and THP-1 cells treated with olive leaf extract (MTT).

To investigate the effect of olive leaf extract on cell viability of the leukemia cell lines U937 and THP-1, cells were exposed to various concentrations of the extract for a period of 48 hours with the cell viability being measured by MTT assay. Seen in Figure 1, the extract reduced the viability of cell line U937 in a concentration-dependent manner to values of 43.00 ± 12.61%, 40.38% ± 6.13 and 24.21 ± 9.18% at concentrations of 200, 400 and 800 µg/mL in 48 hours, respectively. On the other hand, with the THP-1 cell line, the olive extract reduced the percentage of cell viability rates to 51.9 ± 9.85% and 31.47 ± 4.99% at concentrations of 400 and 800 µg/mL, respectively, being less active than the U937 lines. For the purpose of a better comparison of the activity of the olive leaf extract against both of the tested cell lines the effective concentration (EC_{50}) was calculated based on the viability test, MTT. EC_{50} was determined from the dose-response curves and demonstrated that the olive leaf extract showed greater cytotoxicity with the U937 lines (254.7 ± 1.09 µg/ml) when compared to the treatment with the THP-1 cells (475.3 ± 1.08 µg/mL).

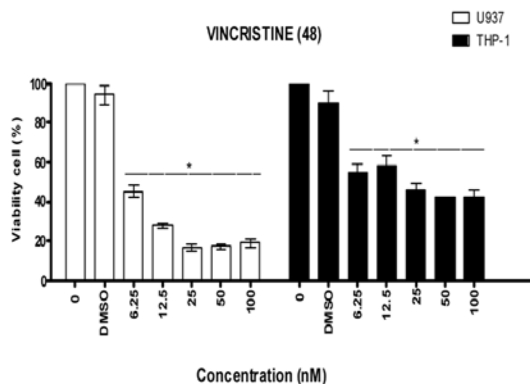

Figure 1. The cytotoxic effect of the olive leaf extract against human cells THP-1 and U937 after 48 hours in incubation by the colorimetric assay using MTT (n-3). The zero stands for the negative control test (cell culture medium). The DMSO concentration was 1%. * P <0:05; a statistically significant value in relation to the negative control (zero) through the Tukey test.

These values represent the dosage of the olive leaf extract that were able to kill 50% of the tested cells. The EC_{50} values indicate that the U973 line is more sensitive to the olive leaf extract than theTHP-1 line (Table 01).

Tested drugs	U937	THP-1
Olive leaf extract (µg/mL)	254,7 ± 1,09	475,3 ± 1,08
Vincristine (nM)	6.46 ± 1,13	26,15 ± 1,24

As a positive control (Figure 2) the cytotoxic effect of the compound vincristine was evaluated in leukemia cell lines U937 and THP-1 at nanomolar concentrations, and this organic compound was used in the therapy of the two tested cell lines. In table01 it can be observed that vincristine showed lower values of EC_{50} (6.46 ± 1.13nM and 26.15 ± 1.24nM) than EC_{50} from the olive leaf extract for U937 and THP-1 cell lines (254.7 ± 1.09 mg/mL and 475.3 ± 1.08 µg/mL), and therefore it is more efficient in reducing cell viability, however, its side effects cease aggressive treatment (Figure 2).
Several studies have shown that most anticancer drugs promoted death in cancer cells through the mechanism of apoptosis. Thus, compounds that promote apoptosis as a means of induction of cell death are strong candidates for drugs with anti-tumoral activity [17, 18].

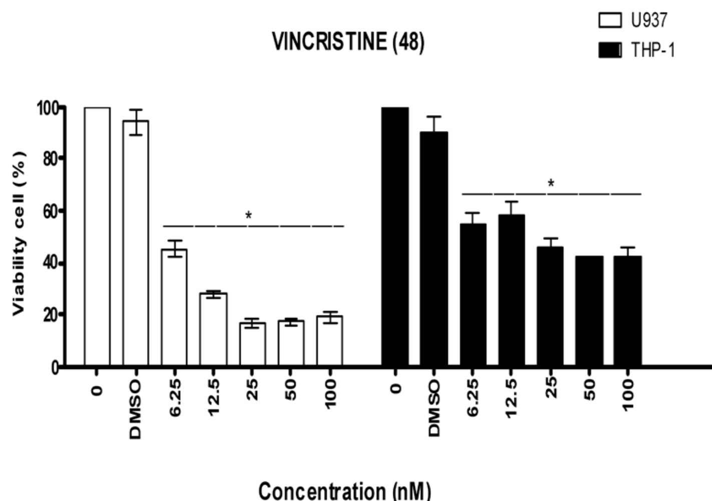

Figure 2. The cytotoxic effects of the compound Vincristine (positive control) against human cells U937 and THP-1, after 48 hours of incubation. The evaluation was performed through the micro colorimetric assay using MTT. The zero stands for the negative control test (cell culture medium). The DMSO concentration was 0.2%. *P <0:05, statistically significant values compared to the negative control (zero) through the Tukey test.

3.2. Assessment of cell viability through lactic dehydrogenase release.

The LDH enzyme released by tumor cells (U937, THP-1) was measured with a spectrophotometer after 48 hours of treatment with the olive leaf extract in different concentrations of 50, 100, 200 and 800 µg/mL. In Figure 3 it can be seen that the lines treated with the extract showed an increased release of the LDH enzyme at concentrations of 800 µg/mL for U937 cells and at 400 and 800 µg/mL for THP-1, indicating that they were able to induce the release of LDH in the cell lines that were tested in a concentration-dependent manner and promoted disruption of the plasma membrane in higher concentrations, confirming the reduction of cell viability through the MTT method.

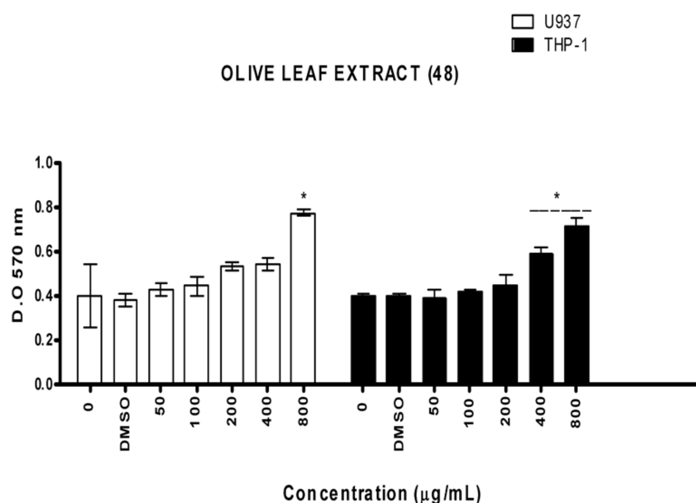

Figure 3. Assessment of the release of LDH (lactate dehydrogenase). The U937 and THP-1 cells were treated with different concentrations of olive leaf extract for 48 hours and evaluated for the amount of LDH released. The zero stands for the negative control test (cells and culture medium). The DMSO concentration was 1%. *P <0:05, statistically significant values compared to the negative control (zero) through the Tukey test.

Despite the presence of the LDH released into the medium confirming the results of cell viability by the MTT assay, its presence suggests necrotic cell death. However, this result may be due to an artifact, since cells undergoing apoptosis *in vitro* may later enter into necrosis (Figure 3). The release of LDH indicates that cell death was caused by necrosis, however, the analysis by fluorescence microscopy and caspases -3 and -6 (Figure 4 and 5) indicates that the cells died by apoptosis and that necrosis was secondary to apoptosis.

3.3. Evaluation of the cell death mechanism induced by the olive leaf extract (fluorescence microscopy)

In order to confirm death by apoptosis promoted by the olive leaf extract in the U937 and THP-1lines, quantitative and qualitative analyses were performed taking into consideration the recommendations of the Nomenclature Committee on Cell Death (NCCD) [10]. Figure 4 shows the percentage of apoptosis induced by the olive leaf extract in the two human leukemia cell lines tested (U937 and THP-1). The olive leaf extract was able to induce apoptosis in cell line U937 at about 21± 4% and 51.57 ± 5.06% at a concentration of 400 µg/mL in a time period of 36 and 48 hours and 71.5 ± 13% and 100% at the concentration of 800 µg/mL at 36 and 48 hours, respectively. For the THP-1 strain the extract was able to induce apoptosis in 14.55 ± 3.76% at a concentration of 400 µg/mL at 48 hours and 16.75 ± 5.1 and 56.11 ± 7.11% % at a concentration of 800 µg/mL for 36 and 48 hours, respectively. Analyzing the two graphs of figure 4, it can be seen that the THP-1 line is less sensitive to the extract tested at the same concentrations and time periods. The major components of olive oil have been identified as effective agents in the intervention stages of initiation, promotion and progression of carcinogenesis. Studies have shown that oleuropein inhibits the growth of differentiated glioblastoma cells, erythroleukemia cells,

renal cell carcinoma, malignant melanoma, colorectal adenocarcinoma and sarcoma [19]. Studies showed that olive leaves induce apoptosis in K562 leukemia cells in relation to the monocyte line age being one of the possible therapeutic effects of the olive leaf in cancer cells [20]. Oleuropein, the main component of the olive leaf, induced cell death by apoptosis in human HeLa cervical carcinoma cells [21]. These mechanisms of action corroborate the results of reduction of cell viability promoted by the olive leaf extract tested.

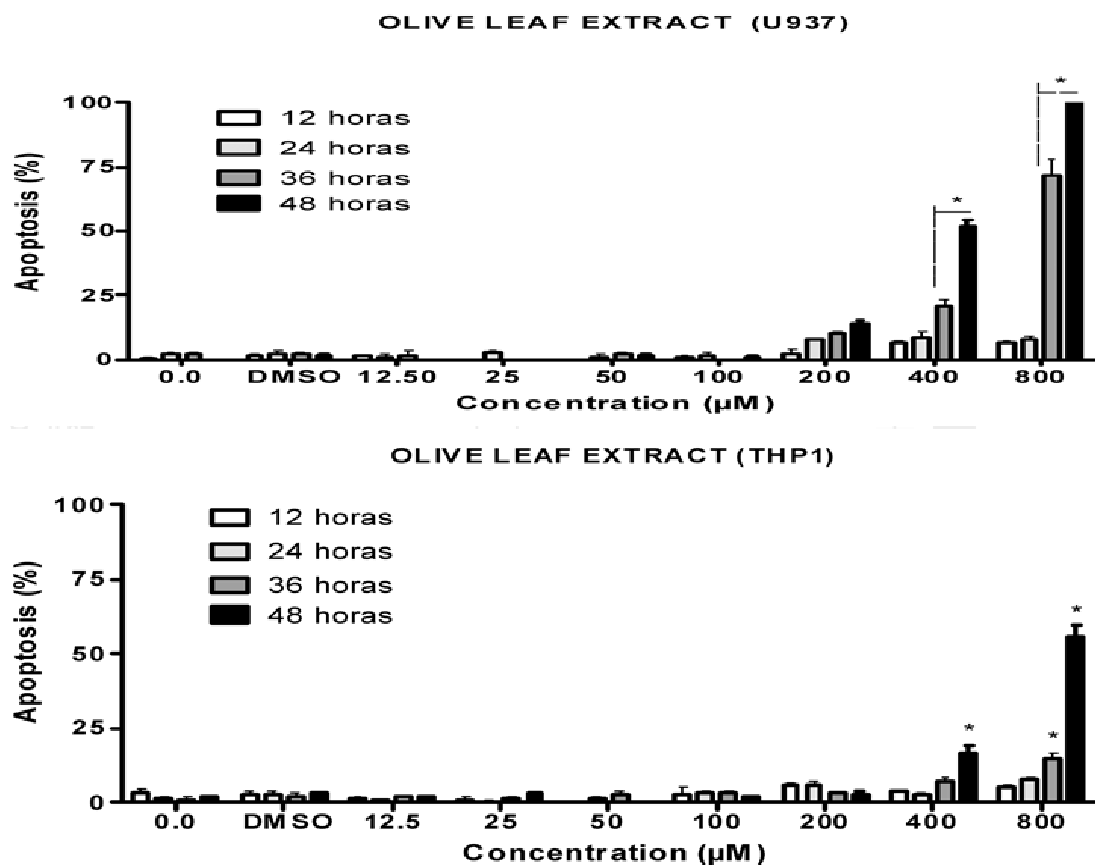

Figure 4. Percentage of apoptosis induced by the olive leaf extract in the U937 and THP-1neoplasticcell lines determined by fluorescence microscopy in three different times of 12, 24, 36 hours. The zero stands for the negative control test (cells and culture medium). The DMSO concentration was 0.2%. * P <0:05, statistically significant values compared to the negative control (zero) through the Tukey test.

3.4. Alteration of mitochondrial membrane potential.

It can be seen from the results of the assessment of mitochondrial membrane potential that by using the lipophilic cationic fluorescent marker JC-1, the cell population control group for the strain is evaluated (98%) in the quadrant "C" which corresponds with the graphic region Dot-plot where there are cells with viable mitochondria (live cells) (Figures 6, 7). For cells of the cell lines subjected to treatment with the olive leaf extract and the compound vincristine (positive controls) a shift in the cell population was observed in quadrant "C" to quadrant "D" which are cells with dissipation of mitochondrial membrane potential (FL1).

The olive leaf extract was able to reduce the mitochondrial membrane potential for more than 85% in the U937 line at concentrations of 400 and 800 μg/mL in 48 hours and over 40% for the THP-1 cell line at a concentration of 800 μg/mL in 48 hours (Figures 5, 6). These data confirm that the U937 cell line is more sensitive to the extractthanTHP-1 cells. The olive leaf extract contains appreciable amounts of polyphenolic compounds, with oleuropein being the dominant compound [22, 23].

The compound oleuropein reduced the viability of MCF-7 cells by inhibiting the rate of proliferation and induction of apoptosis. Furthermore, oleuropein blocked the cell cycle in the transition phaseG1/S.

3.5. Evaluation of the activity of caspases -3 and -6.

According to figure 7, it can be seen that the olive leaf extract was able to activate caspases -3 and -6 for cell line U937. The values for caspases -3 and -6 were higher (0.46 ± 0.08 and 0.36 ± 0.03) compared to the control (0.10 ± 0.02). The results confirm an increase inactivity of these enzymes to the olive leaf extract, after 48 hours of treatment for cell line U937, which proves its ability to promote cell death by apoptosis. These results corroborate the results obtained by flow cytometry and fluorescence microscopy, showing the cytotoxic effect of the olive leaf extract through the induction of cell death by apoptosis in a concentration-dependent manner. Antiproliferative work of raw olive leaf extracts and phytochemicals (oleuropein) against cancer cell lines at micromolar concentrations inhibited the proliferation of human breast cancer and human bladder carcinoma [24]. The antitumor effect of the phenolic compounds of the olive extract has been studied because of its ability to inhibit proliferation and promote apoptosis in various cell lines by several mechanisms. Studies show that phenolic compounds of olive oil inhibit cell proliferation and block the cell cycle in leukemia cells as in HL-60 [25, 26]. The results obtained from this work show that the olive

leaf extract is promising in the search for new anti-neoplastic agents, but further tests should be performed to elucidate the pathways of induction of apoptosis promoted by olive leaf extract in these neoplastic cell lines.

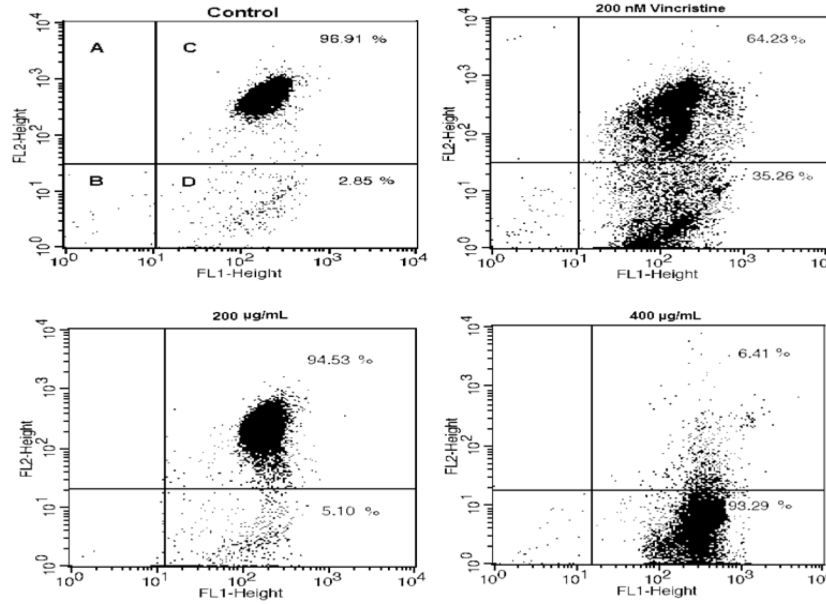

Figure 5. Dot-plots of the analysis of mitochondrial membrane potential, flow cytometry, U937 cells subjected to 48 hours of incubation with the olive leaf extract (E). Quadrant C, containing cells having mitochondria with normal membrane potential, quadrant D, containing cells having mitochondria with a loss of membrane potential through the Tukey test.

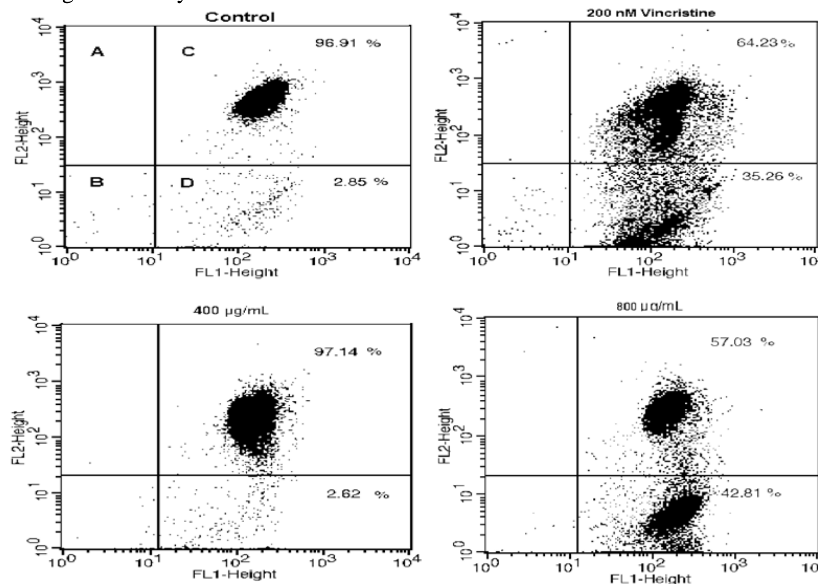

Figure 6. Dot-plots of the analysis of mitochondrial membrane potential by flow cytometryforTHP-1 cells subjected to48-hourincubationwith the olive leaf extract (E). Quadrant “C” cells having mitochondria with normal membrane potential, quadrant “D” cells having mitochondria with a loss of membrane potential through the Tukey test.

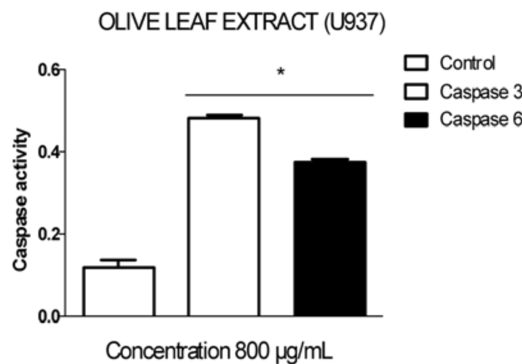

Figure 7. Activity of caspases -3 and -6 with the olive leaf extract in human cell lines U937 after 48 hours of incubation. The evaluation was performed using the *ColorimeterSampler Kit* (invitrogen) containing substrates for caspase-6 and caspase-3. The zero stands for the negative control test (cells and culture medium). The DMSO concentration was 0.2%. *P <0:05, statistically significant values compared to the negative control (zero) through the Tukey test.

4. CONCLUSIONS

The olive leaf extract shows cytotoxic activity against human leukemia cell lines U937 and THP-1, decreasing cell viability by inducing apoptotic cell death through alteration of mitochondrial membrane potential and activation of caspases -3 and -6. The results of fluorescence microscopy and flow cytometry showed that the olive leaf extract alters the mitochondrial membrane potential by inducing cell death by apoptosis, being the most active U937 line to the olive leaf extract. Lastly, the results suggested that the olive leaf extract is promising in the search for drugs with anticancer activity by inducing cell death by apoptosis.

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