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Cytotoxic activity of olive leaf extract against human melanoma (SK-MEL-5) and morin melanoma cell lines (B16F10)

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ABSTRACT

This study investigates possible induction of the apoptosis mechanism in SK-MEL-5 and B16F10 leukemia cells by the extract of olive leaves (*Olea europaea L.*). The treatment with the olive leaf extract in SK-MEL-5 and B16F10 cells results in growth inhibition and induction of apoptosis measured and confirmed by the following methods: MTT assay, fluorescence microscopy, flow cytometry analysis and the activity of caspases-3 and -6. The data indicate that the olive leaf extract promotes a decrease in cell viability in a concentration-dependent manner for both cancer cell lines tested, with percentage rates of 10.71 ± 4.79 for SK-MEL-5 and 18.45 ± 7.05 for B16F10 at a concentration of 800μg/mL in 48 hours. The percentage increase in apoptosis, which was 77.73 ± 4.72% for SK-MEL-5 and 79.34 ± 1.56 for B16F10 at a concentration of 800μg/mL in 48 hours was associated to an alteration of the mitochondrial membrane potential being higher than 82.08% for SK-MEL-5 and 66.25% for the same concentration and time period for B16F10, suggesting the involvement of the intrinsic pathway of cell death by apoptosis. Together, these results suggest that the olive extract affects cell viability of the SK-MEL-5 and B16F10 cell lines tested by inducing cell death through apoptosis.

Keywords: *apoptosis, SK-MEL-5 and B16F10 cell lines, olive leaf extract, antitumor activity.*

1. INTRODUCTION

Malignant melanoma is the most aggressive form of skin cancer and has shown an increase in frequency, especially among the young Caucasian population, and therefore, has become a public health problem [1, 2, 3, 4]. Although being the less frequent cancer of the skin, its lethality is the highest because of its great possibility of metastasis, which is a resistance to the existing therapeutic approaches [5].

There are few chemotherapeutic agents available for the treatment of metastatic melanoma, and those which have been used produced unsatisfactory results, in addition, the treatment is invariably accompanied by side effects including nausea, hair loss and increased susceptibility to infections [6].

The main physiological substances of the olive leaf are hydroxytyrosol, tyrosol, caffeic acid, p- coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, luteolin-7-glycosylate, glycosylated apigenin-7, 7-glycosylated diosmetin [7, 8]. Oleuropein and its derivatives such as hydroxytyrosol and tyrosol are the major components of the olive leaf (*Olea Europaea*) and are present in the fruit and oil. Oleuropein and its derivatives are responsible for its pharmacological effects and contain anticlastogenic, anti-inflammatory and antioxidant activity and inhibit the tumor growth of various cancers [9, 10].

There are two classical pathways for apoptosis: the extrinsic pathway which requires transmembrane receptors, and the intrinsic cell death pathway, which starts the route via mitochondrial stimuli. Both pathways activate caspases, such as caspase-3, effecting cell death and inactivation of these pathways are associated with tumor activity [11, 142, 13, 14]. Countless studies have shown that oleuropein and hiroxitirosol, the most abundant phenolic compounds in olive leaves, are anticarcinogenic agents for presenting antioxidant activity and induce apoptotic cell death [15, 16].

The need for new approaches to the treatment of melanoma includes the gathering of drugs whose benefits are confronted with toxicity, in the search for a favorable therapeutic index. Therefore, the present study investigated whether olive extract has anticarcinogenic activity against SK-MEL-5 and B16F10 leukemia cell lines and the possible induction mechanism of cell death by apoptosis. These compounds may be used in the future as drugs in cancer prevention and therapy.

2. EXPERIMENTAL SECTION

2.1. Material and obtaining the olive leaf extract.

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

Individual adult leaves were collected with pruning shears in the Atlantic forest in a fragment found at the Olive Garden Farm, located in the Forest Zone, in the southeastern state of Minas Gerais. Control samples were deposited in the Rioclarense

herbarium (HRCB), at São Paulo State University, under the registration number VIC: 36,631.

Olive leaves from the *Olea europaea L*. species were dried in a forced ventilation greenhouse oven at a constant temperature of 40°C until the weight was stabilized in order to obtain the dry materials for the above.

The dried material was crushed with a mortar and pestle; 1.0 kg of olive leaves were used in this procedure. The small grain size material was placed in an amber bottle and subjected to an extraction by maceration in absolute ethyl alcohol (PA) diluted in 1:1 distilled water for seven days at room temperature. The period for the exhaustive extraction was 30 days. Every seven days, the extraction liquid was filtered and a new liquid extractor was added. The extract was concentrated on a rotary evaporator under controlled temperature between 40 and 50°C. The leaf extract drying process was finalized under direct ventilation, controlled temperature between 40 and 50^0C and then subjected to a freeze-dryer giving rise to a dried material. At the end, the performance of the extract had an outcome of 200g and they were stored in an opaque plastic container with the presence of a drying agent.

2.2. Cell lines and cell culture.

The SK-MEL-5 (human melanoma) and B16F10 (morin melanoma) cells were obtained from the Center of Biosciences and Technology, at the State University of Norte Fluminense (Campos dos Goytacazes, RJ). The cells were cultured in D-MEM F12 (Gibco, BRL) supplemented with 20 µg/mL gentamicin (Gibco, BRL) and 10% fetal bovine serum (Gibco BRL). The cultures were replicated every 2 days and kept in an incubator (Forma Scientific Inc., Model 3159) at 37°C with 5% of CO_2 and controlled humidity.

2.3. MTT assay and LDH material and preparation of the plant extract.

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

Individual adult leaves were collected with the aid of pruning shears in Atlantic forest in a fragment found at the Olive Garden Farm, located in the Zona da Mata Mineira, in the southeastern state of Minas Gerais. Control samples were deposited in the herbarium Rioclarense (HRCB), at the São Paulo State University, under the registration number of VIC: 36,631. Olive leaves (*Olea europaea L.)* were dried in a forced ventilation oven at a constant temperature of 40°C until the weight was stabilized in order to obtain the dry materials for the aforementioned. The dried material was crushed with a mortar and pestle; 1.0 kg of Olive leaves was used in this procedure. The material was subjected to an extraction by maceration in absolute ethyl alcohol (PA) diluted in distilled water 1:1 for seven days at room temperature for 30 days. The extract was concentrated on a rotary evaporator at a temperature controlled between 40 and 50°C and subjected to a freeze-dryer giving rise to a dried material. Finally, the extract was found that the yield was 200 g (20%) and they were stored in an opaque plastic container, in the presence of drying agent.

2.4. Cell lines and cell culture.

The SK-MEL-5 (human melanoma) and B16F10 (murine melanoma) cells were obtained from the Laboratory of Recognizing Biology and The Biosciences and Biotechnology Center of the State University of Norte Fluminense, Campos dos Goytacazes, RJ. The cells were cultured in D-MEM F12 (Gibco, BRL) according to the methodology described by ref. no [17].

2.5. MTT and LDH assays.

The SK-MEL-5 and B16F10 cell lines were plated in 100 µL/well ($1x10^6$ cells/mL) in 96-well plates, treated with the olive leaf extract at final concentrations of 50, 100, 200 and 400 and 800 µg/mL for the cell viability test with MTT according to the methodology described by ceva/cineva [16]. A 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 of *in vitro* necrosis. For quantification of LDH, KIT doles were used.

2.6. Evaluation of apoptosis by fluorescence microscopy.

The SK-MEL-5 and B16F10 cells were incubated for 24, 36 and 48 hours with the olive leaf extract, then stained with a solution of 10 µg/mL acridine orange (Sigma) and 10 µg/mL bromide ethidium (Sigma), according to the methodology described by ref. no [17].

2.7. Analysis of the potential of mitochondrial membrane by flow cytometry.

The mitochondrial membrane potential was assessed using the fluorescent lipophilic cation JC-1 marker (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol carbocyanine iodide. The tumor cell lines were treated with the olive extract (800 µg/mL) and cells were incubated for 48 hours in an incubator (37°C and 5% of CO_2) according to the methodology described by ref. no [17].

2.8. Analysis of caspase-3 and -6.

To prove that the olive leaf extract induces apoptosis, a *Colorimeter Sampler Kit* (Invitrogen) was used containing substrates for caspases-3 and -6. The cultures containing $1x10^6$ cells/mL were incubated with a concentration of 400 µM 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 the lysis buffer. Samples were centrifuged for 1 min (10,000xg) and then 50µL of substrate (4 mM) was added. The samples were incubated at 37°C for 2 hours in the dark and were read using a spectrophotometer (Thermo Lab systems-Multiskan, model 352) using a wavelength of 405 nm.

As negative controls, the cells were incubated with DMEM F12 (Gibco, BRL), supplemented with 20µg/mL gentamycin (Gibco, BRL) and 10% fetal bovine serum (Gibco BRL). For the extraction, the cells were incubated with DMSO (SIGMA®) to 1%.

2.9. Statistical analysis.

The results were expressed as mean ± standard deviation and analyzed independently. The assays were analyzed by ANOVA - One way - followed by Tukey using GraphPad software version 5.0. The differences were considered significant at $P < 0.05$.

3. RESULTS SECTION

3.1. Reduction of cell growth in the SK-MEL-5 and B16F10 cell lines (MTT).

To investigate the effect of the olive leaf extract on cell viability of SK-MEL-5 and B16F10 leukemia cell lines, the cells were exposed to various concentrations of the extract for a period of 48 hours and the cell viability was measured by MTT assay. It can be seen in Figure 1 that the extract reduced cell viability of the SK-MEL-5 strain in a concentration dependent manner to values of 37.07 ± 17.09%, 21.84 ± 1.49% and 10.71 ± 4.79 at concentrations of 200, 400 and 800 µg/mL in 48 hours, respectively. In the B16F10 line the olive leaf extract reduced cell viability percentage rates to 63.34 ± 8.89% and 18.45 ± 7.05% at concentrations of 400 and 800µg/mL, respectively, and was less active than in the SK-MEL-5 strains.

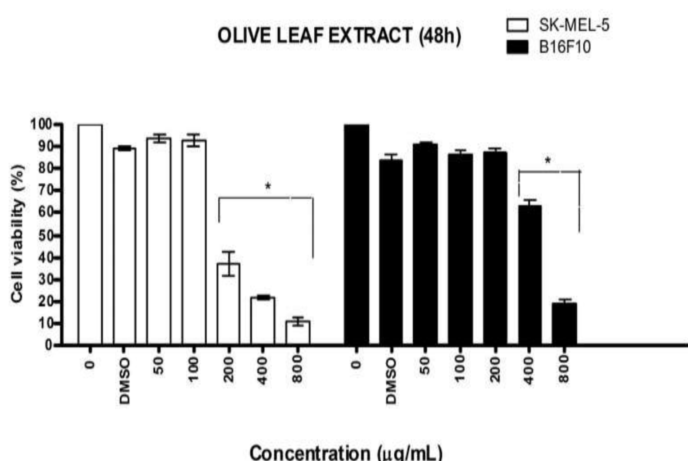

Figure 1. Cytotoxic effect of olive leaf extract against human melanoma cell lines (SK-MEL-5) and morin (B16F10) after 48 hours of incubation by the colorimetric assay using MTT (n-3). Zero stands for the negative control test (cell culture medium). The DMSO concentration was 1%. * P <0.05; statistically significant value compared to the negative control (zero).

For the purpose of a better comparison of the olive leaf extract activity against both cell lines tested the effective concentration (EC_{50}), was calculated, based on the MTT viability test. EC_{50} was determined from the dose response curves and demonstrated that the olive leaf extract showed greater cytotoxicity from the SK-MEL-5 strain (191.7 ± 1,08µg/mL) compared to treatment with B16F10 cells (479, 0 ± 1,06 µg/mL).

Table 1. EC_{50} (µg) of the olive leaf extract on cell viability based on the cellular cytotoxicity assay by MTT.

Drogs tested	SK-MEL-5	B16F10
Olive leaf extract (µg/mL)	191,7± 1,08	479,0 ± 1,06
Cisplatin (µM)	18,57 ± 1,08	>100

ND – Not determined

These values represent the olive leaf extract dose able to kill 50% of the tested cells. EC50 values indicate that the SK-MEL-5 strain is more sensitive to the olive leaf extract than the B16F10 line (Table 1).

As a positive control (Figure 2) the cytotoxic effect of the compound cisplatin was evaluated on SK-MEL-5 and B16F10 leukemia cell lines at nanomolar concentrations, and this organic compound was used in the treatment of both cell lines tested. It can be seen in Table 01 that cisplatin showed lower EC_{50} (18.57 ± 1.08 nM to > 100 nM) than the EC_{50} of the olive leaf extract for lines SK-MEL-5 and B16F10 (191.7 ± 1 08 µg/mL and 479.0 ± 1.06 µg/mL) and therefore is more efficient at reducing cell viability (Figure 2).

Several studies have shown that most antitumor drugs promoted death of cancer cells through the mechanism of apoptosis, a type of programmed cell death. Thus, compounds that promote apoptosis as a pathway of induction of cell death are strong candidates for drugs with antitumor activity [18,19].

Recent studies show that the olive leaf extract inhibits cell proliferation and apoptosis, reduces cell viability and blocks the cell cycle at the G1 phase in MCF-7 cells [20]. Another study found that Hydroxytyrosol, a major component of the olive leaf extract, reduced the level of free radicals and caused damage to the DNA molecule human chronic myelogenous leukemia K562 cells [21].

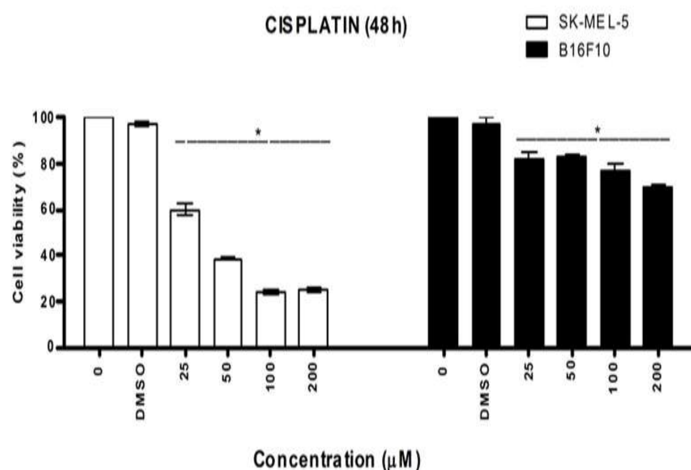

Figure 2. Cytotoxic effect of the compound cisplatin (positive control) against human SK-MEL-5 cells and B16F10 morin after 48 hours of incubation. Zero stands for the negative control test (cell culture medium). The DMSO concentration was 0.2%. * P < 0.05, statistically significant values compared to the negative control (zero).

3.2. Evaluation of cell viability by the release of dehydrogenase lactate.

The LDH enzyme was released by neoplastic cells SK-MEL-5 and B16F10. Figure 3 shows that both strains treated with the olive leaf extract showed an increase of LDH enzyme released in the concentrations of 200, 400 and 800 µg/mL for SK-MEL-5 and 800 for B16F1, indicating that they were able to induce the release of LDH in the cell lines tested in a concentration-dependent manner, which can be deduced by the fact that there was disruption of the plasma membrane in higher concentrations, thus confirming the reduction of cell viability by the MTT method.

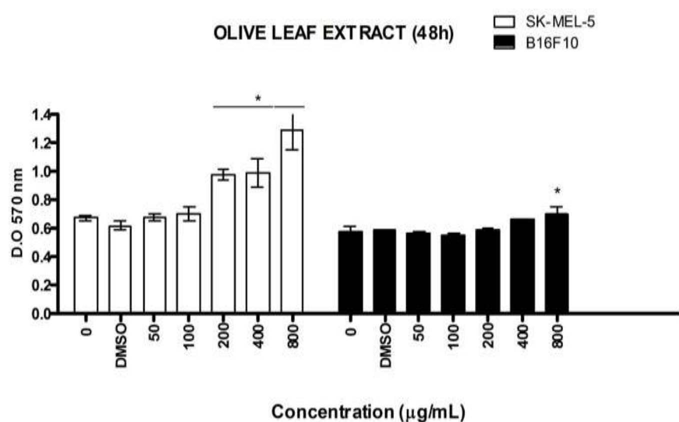

Figure 3. Evaluation of the release of LDH (lactate dehydrogenase). The SK-MEL-5 and B16F10 cells were treated with different concentrations of the olive leaf extract for 48 hours and evaluated for the amount of LDH released. The zero stands for the negative control test (the cell culture medium). The DMSO concentration was 1%. * P< 0.05, statistically significant values compared to the negative control (zero).

Despite the presence of LDH released into the medium confirming the results of cell viability by the MTT assay, the negative result does not invalidate the positive result of this test, since the membranes from the dead cells, by apoptosis, are not ruptured.

3.3. Evaluation of the mechanism of cell death induced by the tested compounds (fluorescence microscopy).

The figure 4 shows the percentage of apoptosis induced by the olive leaf extract in two of the strains of human and morin melanoma cells (SK-MEL-5 and B16F10), respectively. It is observed that the olive leaf extract was able to induce apoptosis in the SK-MEL-5 cell line at 48.95 ± 6.0 and 66.89 ± 1.14% at a concentration of 400 µg/mL for a time period of 36 and 48 hours and 77.73 ± 4.72 and 79.34 ± 1.56% at a concentration of 800 µg/mL at 36 and 48 hours, respectively. For the B16F10 line the extraction was able to induce apoptosis in 14.95 ± 22,42 and 2.05% at the concentration of 400 µg/mL at 48 hours and 38.15 ± 12.3% and 70.49 ± 5.91% at a concentration of 800 µg/mL for 36 and 48 hours, respectively. Analyzing the two graphs of Figure 4, it is noted that the B16F10 strain is less sensitive to the extracts tested at the same concentrations and time periods.

Most of the olive leaf oil components have been identified as effective agents in the intervention phases of initiation, promotion and progression of carcinogenesis. Studies have shown that oleuropein inhibits the growth of differentiated cells of glioblastoma, erythroleukemia cells, renal cell carcinoma, malignant melanoma, colorectal adenocarcinoma and sarcoma [22].

Studies have shown that olive leaves induce apoptosis in K562 leukemia cells in relation to monocyte lineage being one of the possible therapeutic effects of olive leaves in cancer cells [23]. Oleuropein, the main component of olive leaves, induced cell death by apoptosis in human HeLa cervical carcinoma cells [24]. These action mechanisms corroborate the cell viability reduction results promoted by the olive leaf extract.

The olive leaf extract displays in its composition various flavonoids such as luteolin [25]. The best known antioxidant activity of flavonoids is their ability to deactivate reactive molecules of singlet oxygen. They may also protect cell membranes from lipid peroxidation ensuring, in this way, membrane fluidity and integrity, reducing the formation of peroxides and immunosuppressive preventing changes in intracellular signaling and cell function.

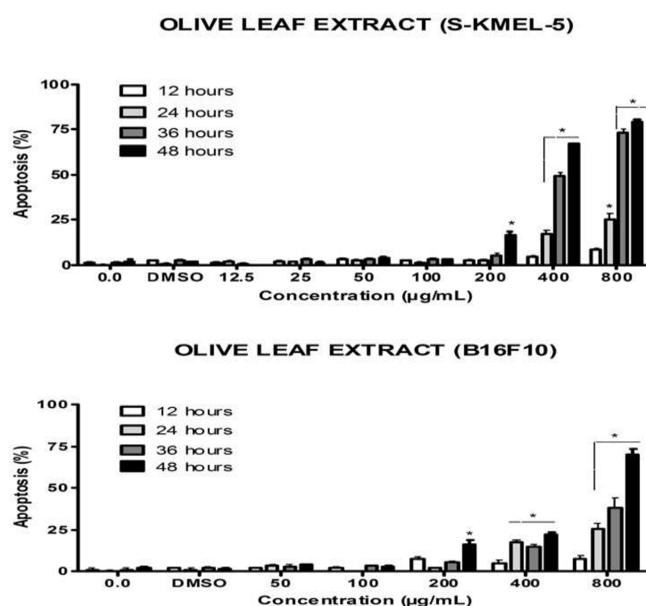

Figure 4. Percentage of apoptosis induced by the olive leaf extract in tumor lines SK-MEL-5 and B16F10 determined by fluorescence microscopy in three different times 12, 24, 36. The zero stands for the negative control test (cell culture medium). The DMSO concentration was 0.2%. * P <0.05, statistically significant values compared to the negative control (zero).

Flavonoids also act in cell apoptosis and angiogenesis. The action in angiogenesis and cell apoptosis is interesting and may explain the *in vitro* antitumor action of these substances [26]. Several works on the flavonoid luteolin have proven effective in reducing the viability of lung (LNM35), colon (HT29), liver (HepG2) and breast (MCF7 / 6) cancer cell lines [27].

Studies have shown that compounds present in the olive leaf extract, such as oleuropein and hydroxytyrosol, are capable of activating the mechanism of programmed cell death by apoptosis in several types of cancer [28].

3.4. Alteration of the mitochondrial membrane potential (flow cytometry).

It can be observed from the results of the evaluation of the mitochondrial membrane potential, using a fluorescent lipophilic cation JC-1 marker, that the population of cells in the control group for the assessed lines (SK-MEL and B16F10-5) are at 84%, 12% and 86.81% in the "C" quadrant, respectively, which corresponds to a *Dot-plot* graph region where there are cells with viable mitochondria (live cells) (Figures 6 and 7).

For cells of the strains subjected to treatment with the olive leaf extract and the compound Vincristine (positive control), a shift of the cell population from quadrant "C" to quadrant "B" was observed, where cell dissipation from the mitochondrial potential membrane was found (FL1). The Olive leaf extract was able to reduce the mitochondrial membrane potential in more than 50.80 and 82.08 % in the SK-MEL-5 strain at concentrations of 400 and 800 µg/mL in 48 hours, and greater than 50.98 and 66.25% for cells from strain B16F10 at a concentration of 400, and 800 µg/mL at 48 hours (Figure 5 and 6).

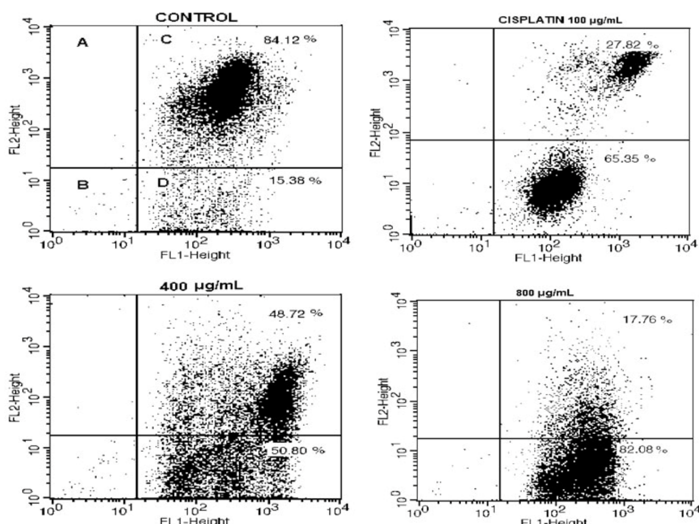

Figure 5. *Dot-plots* of the analysis of mitochondrial membrane potential, flow cytometry, for SK-MEL-5 cells subjected to 48-hour incubation with the olive leaf extract (E). Quadrant C cells having mitochondria with normal membrane potential, quadrant D, cells having mitochondria with loss of membrane potential by the Tukey test.

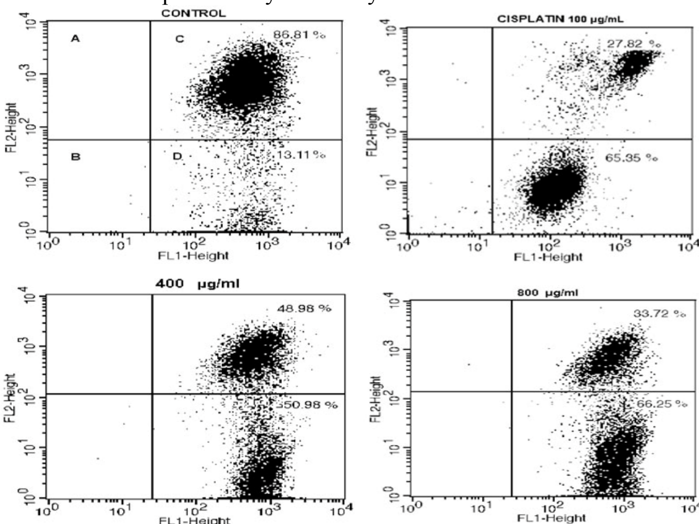

Figure 6. *Dot-plots* of the analysis of mitochondrial membrane potential, flow cytometry, B16F10 cells subjected to 48 hours of incubation with the olive leaf extract (E). Quadrant B cells having mitochondria with normal membrane potential, quadrant C cells having mitochondria with loss of membrane potential using the Tukey test.

These data confirm that the SK-MEL-5 cell line is more sensitive to the extract than B16F10 cells. The olive leaf extract contains an appreciable amount of polyphenolic compounds, with oleuropein as the dominant compound [29]. The compound oleuropein reduced the viability of MCF-7 cells by inhibiting the proliferation rate and apoptosis induction. In addition, oleuropein blocked the cell cycle in the G1/S transition [30].

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 in the SK-MEL-5 cell line. The activity values of caspases-3 and -6 were higher (0.34 ± 0.04 and 0.27 ± 0.01) compared to the control (0.11 ± 0.03). The results confirmed an increase in the activity of these enzymes to the olive leaf extract, after 48 hours of treatment for SK-MEL-5 cell line, which demonstrates its ability to promote cell death by apoptosis.

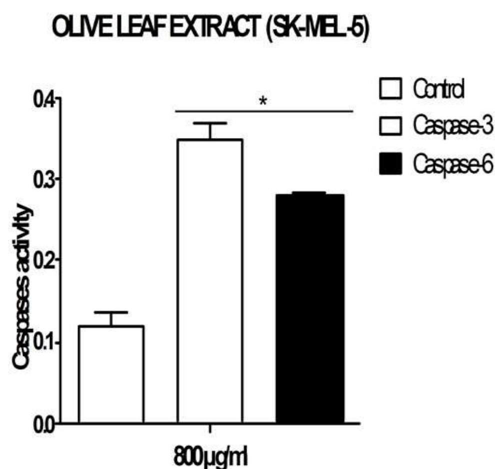

Figure 7. Activity of caspases-3 and -6 through the olive leaf extract against human cell line SK-MEL-5, after 48 hours of incubation. Zero stands for the negative control test (cell culture medium). The DMSO concentration was 0.2%. * P <0.05, statistically significant values compared to the negative control (zero).

These results corroborate the results obtained by the fluorescence microscopy and flow cytometry, showing the cytotoxic effect of the olive leaf extract by inducing cell death through apoptosis in a concentration-dependent manner.

Antiproliferative studies of raw extracts of olive leaves and phytochemicals (oleuropein) against cancer cell lines at micromolar concentrations inhibited the proliferation of human breast cancer and human bladder carcinoma [31].

The antitumor effect of the phenolic compounds of the olive leaf extract has been studied due to its ability to inhibit proliferation and promote apoptosis in various cell lines by several mechanisms. Studies have shown that phenolic compounds of olive oil inhibit cell proliferation and block the cell cycle in leukemia cells HL-60 type [32, 33].

The results of this work show that the olive leaf extract is promising in the search for new anticancer agents, however more tests should be conducted to elucidate the induction of apoptotic pathways promoted by olive leaf extract in these neoplastic lines.

4. CONCLUSIONS

This study demonstrated that the extract from olive leaves showed antitumor activity against SK-MEL-5 and B16F10 human melanoma cell lines, decreasing cell viability in a concentration-dependent manner. The results of the fluorescence microscopy, flow cytometry and caspases-3 and -6 showed that the olive leaf extract altered mitochondrial membrane potential of inducing cell death by apoptosis, and thus, the S-KMEL-5 human strain was more active because of the olive leaf extract. Finally, the results suggested that olive leaf extract is promising in the search for drugs with anticancer activity by inducing cell death through apoptosis.

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