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Effect of Olive Leaf Components on the Expansion of Human Hematopoietic Stem and Progenitor Cells

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Abstract
Hematopoietic stem cells (HSCs) transplantation and/or the infusion of hematopoietic cells are currently the best alternative for the treatment of a wide variety of hematological disorders. Novel modulators of HSCs fate continue therefore to be identified in an attempt to optimize the *in vitro* culture of these cells. In this study, main olive leaf components, oleuropein, apigenin 7-glucoside, and luteolin 7-glucoside, and their combination, were investigated for their effect on the viability, self-renewal and differentiation of CD34+ hematopoietic cells. High concentrations of up to 50 µM of these olive leaf phytochemicals, as well as their combination, did not decrease CD34+ cell viability suggesting that these compounds are not cytotoxic on HSCs. Flow cytometric analysis revealed a decrease in the expression of CD34 on the cell surface of HSCs after 9 days of treatment with these compounds indicating a decrease in the proportion of the stem population. Results of the colony-forming unit assay showed an increase in the number of different colonies following treatment with olive leaf phytochemicals. These findings suggest that olive leaf phytochemicals, used alone or in combination, enhance the differentiation of hematopoietic stem cells rather than stimulating their self-renewal providing the first evidence of the potential differentiation effect of main olive leaf compounds on HSCs.

Keywords: Hematopoietic stem cells, olive leaf components, cell viability, self-renewal, differentiation.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are the cells that give rise to all blood cells. As with all stem cells, HSCs have the ability for self-renewal and have sufficient plasticity to give rise to progenies that has the potential to differentiate into at least nine different mature blood cell types which function in some biological activities such as control of homeostasis balance, immune function, and response to microorganisms and inflammation [1]. HSCs are found in the bone marrow of adults where they represent less than 0.01% of all cells [2]. They are also found in umbilical cord blood and, in small numbers, in peripheral blood. In their un-differentiated state, HSCs express some surface markers, one of which is CD34, a glycophosphoprotein considered as a hallmark of hematopoietic stem/progenitor cells (HSPCs), which are accordingly referred as CD34-positive cells (CD34+ cells) [3].

Hematopoietic stem cells (HSCs) are probably the most extensively characterized somatic stem cells and are the only stem cells that have been clinically used to treat diseases such as leukemia, germ cell tumors, and congenital immune deficiencies [4]. As HSC transplantation and/or the infusion of blood cellular components has been proven to be a life-saving modality for a broad range of hematological disorders, huge efforts have been dedicated to optimize protocols for expanding hematopoietic progenitors (CD34+ cells) or generating blood cellular components from these cells. Great successes have been achieved and several cytokines, growth factors and developmental regulators have been identified for their effect on HSCs fate decision [2]. However, no established methods are yet available, and thus thousands of molecules, including plant-derived compounds, are being screened for their potential role in *in-vitro* stem cell expansion or differentiation [5, 6, 7].

Olive leaf extract contains many bioactive compounds which contribute to its health promoting effects. Olive leaf extract has antioxidant, anti-diabetic, anti-hypertension anti-cancer and neuroprotective effects [8, 9, 10, 11].The primary constituents that contribute to the health benefits of olive leaves are oleuropein and its derivatives, hydroxytyrosol, as well as several other flavonoids apigenin 7-glucoside and luteolin 7-glucoside [12, 13]. In our previous study, olive leaf extract from a Tunisian variety, Chemlali, showed anti-leukemia effect on the chronic myelogenous leukemia K562 cells by inducing their differentiation towards the monocyte/macrophage lineages [14]. HPLC analysis of the extract revealed three main compounds, oleuropein, apigenin 7-glucoside and luteolin 7-glucoside [14]. In this study, we investigated the effect of these compounds on the cell viability of hematopoietic stem/progenitor cells and we got insight into their effects on the self-renewal and differentiation of HSPCs.

MATERIALS AND METHODS

Sample preparation and cell culture

Oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) were purchased from Sigma Aldrich. Stock solutions of 100 mM of each compound were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C until use. Cryopreserved CD34+ hematopoietic progenitor cells were purchased from Life Technologies™(GIBCO, Life Technologies™) and cultured in StemPro®-34 serum-free medium supplemented with StemPro®-34 Nutrient Supplement. The culture was supplemented with stem cell factor (SCF), interleukin-3 (IL-3) and granulocytes macrophages colony-stimulating factor (GM-CSF) at final concentrations of 100 ng/mL, 50 ng/mL and 25 ng/mL, respectively (GIBCO, Life Technologies™).

Cell proliferation assay

Cell proliferation was investigated using MTT (3-(4, 5-Dimethylthiazol – 2 – yl) – 2 , 5 - diphenyltetrazolium bromide) assay. CD34+ cells were seeded in 96-well plates at 2×10^4 cells/mL and incubated for 24 h before treatment. Main olive leaf compounds, Olp, Api7G and Lut7G, diluted in growth medium, were then added at final concentrations of 5 µM, 10 µM, 25 µM, 50 µM and 75 µM. In order to see the effect of the combination (Comb) of these three compounds, HSPCs were treated with a mixture of Olp, Api7G and Lut7G at 55 µM, 5 µM and 5 µM, respectively. The concentrations of the compounds in the mixture were calculated based on the compounds' actual concentration in the extract and the extract's effective concentration as reported in our previous study [14]. Control cells (Control) were treated with 0.05% DMSO (vehicle). MTT was added after the cells were incubated with the compounds for 3 days and the resulting formazan was completely dissolved by adding 100 µL of 10% sodium dodecyl sulfate (SDS). The absorbance was determined at 570 nm in a multidetection microplate reader (Power-scan HT). Absorbance caused by the ability of the sample to reduce MTT or by its color, was corrected using plates prepared in the same conditions in the absence of cells.

Cell viability assay

The viability of HSPCs cells was assessed using flow cytometry on the 3rd day of treatment with each compound Olp, Api7G and Lut7G, as well as their combination (Comb). Briefly, CD34+ cells were cultured at 2×10^4 cells/mL in 24-well plates. After 24 h incubation, CD34+cells were treated with 10 µM, 25 µM and 50 µM of each compound (Olp, Api7G and Lut7G) as well as with their combination (Comb). DMSO-treated CD34+ cells served as control cells

(Control). After incubation for 3 days, treated cells were harvested, suspended in Guava ViaCount reagent and allowed to be stained for at least 5 min in darkness. The cell number and viability were measured by flow cytometry (Guava Technologies). The cells were examined under a phase contrast microscope (Leica Microsystem) in order to detect any morphological changes following treatment with olive leaf phytochemicals. The viability of cells treated with 50 µM of each compound, as well as with their combination, for prolonged period (9 days) was assessed following the same protocol.

CD34 marker expression

To investigate if the primitive forms of hematopoietic cells were conserved after treatment with olive leaf phytochemicals, we analyzed the treated cells for the expression of CD34. Briefly, hematopoietic stem/progenitor cells were incubated with olive leaf compounds, used alone at 50 µM or in combination (Comb), for 9 days. Then, the expanded control and treated cells were collected, the number adjusted to 1×10^5 cells and washed with 1x PBS. After the addition of anti-CD34 (Abcam), the cells were incubated for 40 min on ice, washed twice with 1x PBS and the expression level of CD34 was measured by flow cytometry using the Guava Express software of the Guava PCA.

Colony forming unit assay

Cells incubated for 9 days (2×10^3 cells) treated with olive leaf phytochemicals, used alone at 50 µM or combined, were suspended in methylcellulose serum-free medium supplemented with SCF, IL-6, IL-3, GM-CSF, G-CSF and EPO (R&D Systems), and plated in duplicate in 35-mm culture dishes. Each plate was scored using an inverted microscope for colony-forming unit of granulocytes-macrophages (CFU-GM), burst-forming unit erythroids (BFU-E) and mixed colonies of granulocyte-erythroid-macrophage-megakaryocytes (CFU-GEMM) after 10 and 14 days of incubation at 37°C in humidified incubator containing 5% CO_2. Colonies made up of more than 30 cells were scored as "colony-forming units".

Statistical analysis

All data are presented as the mean ± SD of three independent experiments. Statistical analyses of the differences compared to the control were performed using a paired two-tailed Student's test. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Effect of olive leaf components on CD34+ cell proliferation

In order to investigate if treatment with olive leaf phytochemicals affects the cell growth of HSPCs, CD34+ cells were treated for three days with different concentrations of Olp, Api7G and Lut7G, or their combination (Comb), and the cell proliferation was assessed using MTT assay. Results showed that treatment with Olp did not affect CD34+ cell proliferation even when treated at up to 75 µMOlp (Figure 1A). On the other hand, Api7G (Figure 1B) showed a slight anti-proliferative effect on CD34+ cells when Api7G concentration is 50 µ Mor higher (89.63% ± 5.57 at 50 µM and 84.36% ± 6.27 at 75 µM compared to the untreated or control cells), while the anti-proliferative effect of Lut7G was observed only when the concentration was 75 µM, significantly decreasing the cell proliferation by up to 74.37% ± 6.41(Figure 1C). Treatment with the combination of the compounds (Comb)did not have any effect on the proliferation of treated CD34+ (Figure 1D).

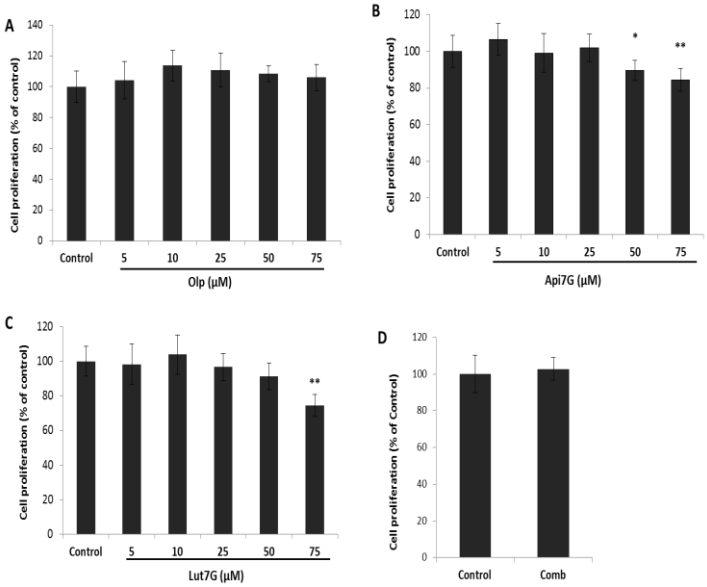

Figure 1. Cell proliferation of hematopoietic stem/progenitor cells cultured with or without olive leaf phytochemicals. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) or luteolin 7-glucoside (Lut7G) used alone (5, 10, 25, 50 and 75 µM) or combined (Comb) for 3 days. Cell proliferation was assessed by MTT assay. Control represents cells treated with the vehicle (0.05% DMSO). Results are presented as the mean ± SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

Effect of olive leaf components on CD34+ cell viability

The MTT assay is based on the mitochondrial enzymes' activity and therefore does not give a clear idea whether the decrease in cell proliferation is a result of CD34+ cell death or the inhibition of their growth rate. To resolve this, we conducted more specific cytotoxicity assay using flow cytometric analysis. This assay distinguishes between viable and non-viable cells based on the differential permeability of DNA-binding dye propidium iodide in the ViaCount reagent.CD34+ cells were treated with different concentrations of each compound from 10 µM up to 50 µM, as well as with the combination of the compounds. Results indicated that treatment with olive leaf main phytochemicals used alone or in combination at the concentrations used, did not decrease the CD34+ cell viability (Figure 2A). These

findings suggest that olive leaf components, used alone at concentrations up to 50 µM or in combination, did not exhibit any cytotoxicity on HSPCs. Consistent with these results, microscopic observation did not reveal any significant morphological changes in the treated and control cells. CD34+ cells treated with 50 µM of each compound or Comb had the same morphology as control cells and no signs of cytotoxicity have been detected (Figure 3).Prolonged treatment up to 9 days with 50 µM of each compound or with Comb did not decrease the cell viability either (Figure 2B).

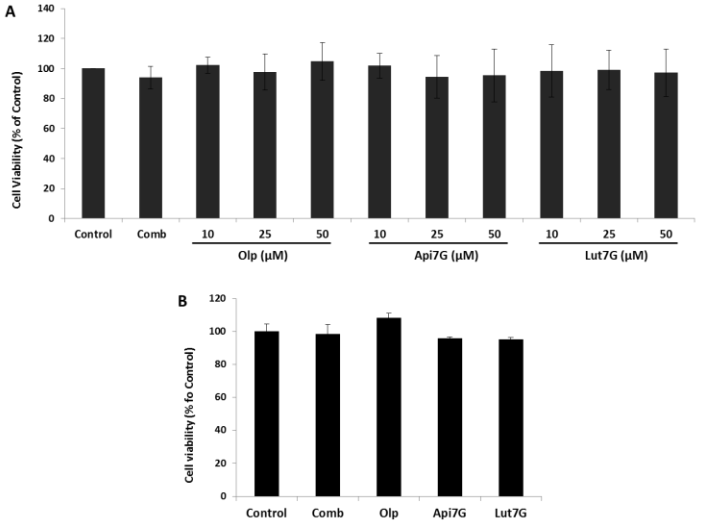

Figure 2. Cell viability of hematopoietic stem/progenitor cells cultured with olive leaf phytochemicals for 3 days **(A)** and 9 days **(B)**.Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 10, 25 and 50 µM) or combined (Comb), for 3 days**(A)**. Cell viability was also assessed for longer period (for 9 days) after treatment with high concentration (50 µM)of Olp, Api7G or Lut7G, as well as with Comb **(B)**. Cell viability was measured by flow cytometry. Control represents cells treated with 0.05% DMSO. Results are presented as the mean ± SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

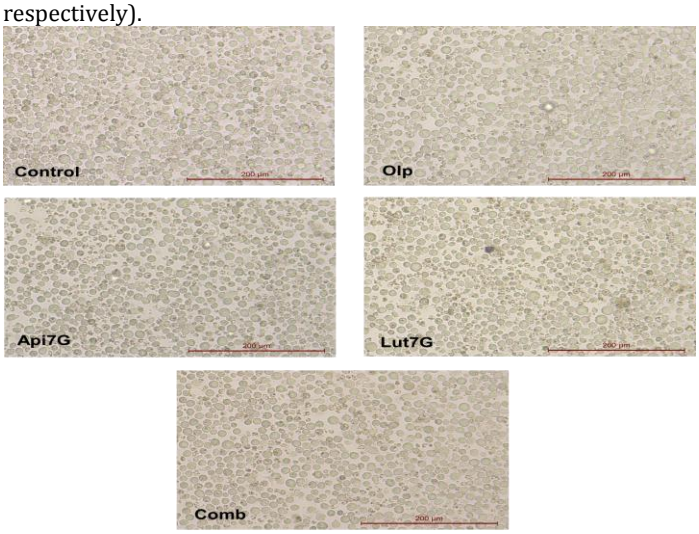

Figure 3.CD34+ cells cultured with olive leaf phytochemicals for 3 days. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (50 µM) or combined (Comb). Cells were observed under a phase contrast microscope at 100X magnification. Scale bars represent 200 µm.

CD34 expression after treatment with olive leaf compounds

The glycophosphoprotein CD34 is the hallmark of HSPCs, and its expression level indicates the presence of the primitive forms of hematopoietic cells. Results showed that treatment with olive leaf compounds for 9 days inhibited the expression of CD34 on the cell surface of treated cells, especially for cells treated with Comb, Olp and Lut7G, indicating a decrease in the proportion of the stem population while treatment with Api7G did not have the same effect (Figure 4). The non-decrease in the cell viability of hematopoietic cells after treatment with olive leaf components for 9 days (Figure 3B) suggests that the decrease in CD34 expression is due to the effect of olive leaf compounds and not to cytotoxicity after having been in culture for 9 days.

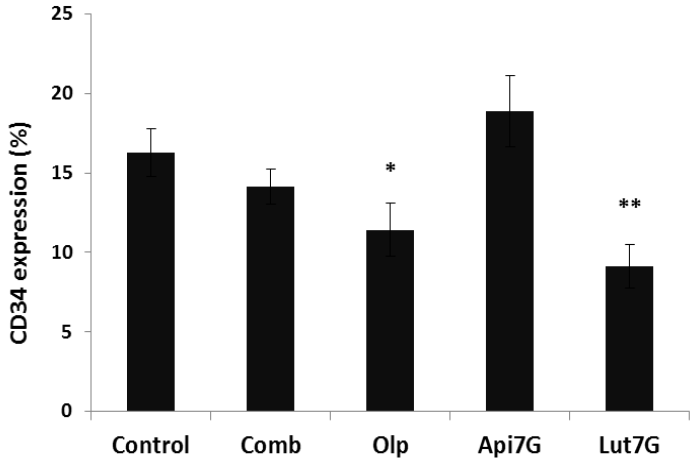

Figure 4. Expression of CD34 treated with or without olive leaf compounds. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 µM) or in combination (Comb) for 9 days. CD34 expression was evaluated using flow cytometry. Control represents cells treated with 0.05% DMSO. Results are presented as the mean ± SD of three independent experiments. * and ** significantly different from the control ($p < 0.05$ and $p < 0.01$ respectively).

CFC potential of CD34+ cells incubated with olive leaf compounds

The colony-forming cell (CFC) assay reveals the presence of early progenitors in the culture which form different colonies in the presence of a combination of growth factors. The CD34+ cells cultured with or without olive leaf phytochemicals were seeded in semi-solid methylcellulose medium as described above. An increase in the number of CFU-GM was detected in cells treated with Olp and Lut7G. The number of mixed colonies, indicative of the presence of more primitive and multi-potent progenitors, was increased in CD34+ cells cultured with olive leaf phytochemicals. While they were absent in control cells, BFU-E colonies were detected in cells treated with Olp, Api7G and Lut7G at 50 µM (Figure 5).

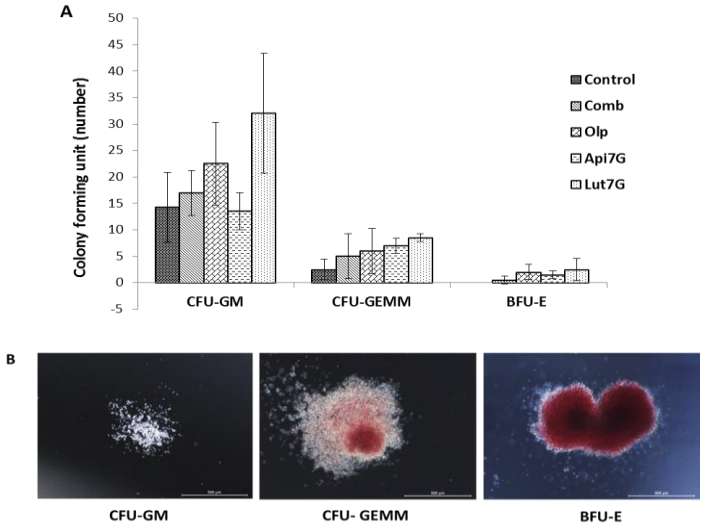

Figure 5. Colony-forming unit potential of CD34+ cells cultured with olive leaf components. (A) Number of colony-forming unit of CD34+ cells cultured with or without olive leaf components. Cells were treated with oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) used alone (at 50 µM) or combined (Comb) for 9 days before they were cultured in methylcellulose medium for 14 days. (B) Example of the obtained colonies visualized under a phase contrast microscope at 40X magnification. Scale bars represent 500 µm.

DISCUSSION

Several lines of evidence suggest that certain nutrients, vitamins and flavonoids could have important roles in maintaining the self-renewal of stem cells and stimulating their differentiation in order to provide the continuous replacement of mature cells in blood, brain and other tissues. This study was undertaken in order to investigate the effect of olive leaf compounds, well known for their health benefits, on the cell proliferation and viability of HSPCs and to explore their potential for stimulating the self-renewal and/or differentiation of HSPCs. In olive leaves, the secoiridoidoleuropein is the most prominent compound making up to 19% (w/w) of olive leaves, followed by flavonoids which represent up to 1.8% (w/w) [15]. Oleuropein has several pharmacological properties, including antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer, antimicrobial, and antiviral activities [15]. Oleuropein was shown to be a highly bioavailable compound which is rapidly absorbed after oral administration[16]. Main flavonoids in olive leaves are the apigenin 7-glucoside and luteolin 7-glucoside. These flavonoids are absorbed in the intestine after hydrolysis to apigenin and luteolin which have been recognized as bioactive compounds due to their anti-inflammatory, antioxidant and anti-cancer properties [17, 18, 19].

Treatment with olive leaf phytochemicals of up to 50 µM did not affect the proliferation of HSPCs, with the exception of Api7G which slightly decreased the cell proliferation when used at 50µM. To investigate the effect of olive leaf compounds on the viability of HSPCs, flow cytometry, a more specific assay compared to the MTT assay, was performed. Results showed that olive leaf phytochemicals or Comb were not cytotoxic on HSPCs at all the tested concentrations. And even though 50 uM of Api7G decreased the cell proliferation, the cell viability of Api7G-treated cells at the same concentration had the same viability as the control cells. These findings suggest that the slight decrease in cell proliferation was actually a reduction in the growth rate of CD34+ cells rather than cell death. These data indicate that the main compounds of olive leaf and their combination were not cytotoxic on HSPCs. Previous studies focusing on the anti-leukemia effects of Api7G showed a drastic decrease in cell proliferation of leukemia cells after treatment with Api7G or apigenin at 50 µM for 3 days, approximating 90% decrease in the human promyeolocytic leukemia HL-60 cells and 60% decrease in the human chronic myeloid leukemia K562 cells [20, 21]. A reduction in cell viability was also observed in K562 cells treated with 50 µM apigenin for 3 days [21]. Ruela-de-Sousa *et al*. [22] reported that apigenin both stops cell proliferation and drives HL-60 cells into cell death by inhibiting the PI3K/PKB pathway and inducing caspase-dependent apoptosis, while no significant effect has been observed in normal human peripheral blood lymphocytes. Takahashi *et al*.,[23] reported that the growth inhibition of luteolin on HL-60 leukemia cells was seen at about 50 µM and above. Incubation of HL-60 cells with luteolin caused DNA fragmentation, the hallmark of apoptosis, which appeared at 20 µM of luteolin treatment, and continue dose-dependently from 20 to 60 µM treatment[24].Oleuropein has been shown to inhibit the growth of TF-1a erythroleukemia cells and oleuropein-rich extract of olive leaf showed an important anti-proliferative effect of the chronic myeloid leukemia K562 cells associated with an induction of apoptosis and cell differentiation [25, 14].The results of this study demonstrate that olive leaf components are not cytotoxic on hematopoietic stem cells even at high concentration of up to 50 µM treatment and for prolonged treatment periods of up to 9 days, while the same treatment-concentration has been reported to cause a significant decrease in the cell viability of leukemia cells, suggesting that olive leaf components cause cell death in leukemia cell lines but not on normal hematopoietic stem cells.

HSCs are characterized by two main features: self-renewal and multipotency giving rise to all types of blood cells. The expression of CD34 on hematopoietic cells decreases over time as the cells differentiate, while other lineage-specific proteins appear gradually on the surface of the committed cells as a mark of their differentiated state. Thus, the decrease in CD34 expression on the 9th day, particularly in cells treated with Olp and Lut7G, which implies a decrease in the

stem population, could also indicate an increase in the differentiated population. This fact is in correlation with the increase in the number of BFU-E and CFU-GM in methylcellulose cultures suggesting that Lut7G and Olp enhance the differentiation of hematopoietic stem cells. Even though it did not decrease the expression of CD34, Api7G treatment increased the number of CFU-GEMM and BFU-E suggesting that Api7G enhance the differentiation of hematopoietic stem cells without inhibiting their self-renewal. Together, these findings suggest that olive leaf phytochemicals have the potential to enhance the differentiation of hematopoietic stem/progenitor cells.

Despite the decrease in the stem cell population, indicating an inhibition of self-renewal, results of the colony-forming cell assay, which measure changes in the numbers of different subsets of progenitor cells, did not imply a decrease in the total colony number resulted from cultures with olive leaf compounds compared to the control cells. Moreover, the number of the CFU-GEMM colonies derived from multi-potent progenitors was increased in cultures supplemented with olive leaf phytochemicals. These results suggest that progenitors from treated cells are likely to be more competent to form differentiated cell colonies than those of the control cells. This suggests that olive leaf phytochemicals had a protective effect on hematopoietic progenitors allowing them to survive and give rise to different colonies. This is not surprising considering that the protective effect of these compounds has been previously described but in other cell lines and was attributed to their antioxidant activity [26, 27, 28]. Shytle et al. (2007) reported that natural compounds reduced the oxidative stress-induced apoptosis of primary murine cells *in vitro* and *in vivo* which explains the protective effects of these compounds on stem cells [29].

CONCLUSION

The present study is the first report on the effect of olive leaf phytochemicals on hematopoietic stem cells. The main components of olive leaf, used alone or combined, were not cytotoxic on CD34+ cells. Furthermore, we showed that olive leaf phytochemicals modulate the fate of hematopoietic stem cells by enhancing their differentiation potential rather than their self-renewal. This study introduces olive leaf components as novel modulators of the fate of hematopoietic stem cells and open possibilities for more investigations on their differentiation-inducing effects on stem cells.

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