Virgin Olive Oil Phenols Inhibit Proliferation of Human Promyelocytic Leukemia Cells (HL60) by Inducing Apoptosis and Differentiation

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ABSTRACT Although epidemiologic evidence and animal studies suggest that olive oil may prevent the onset of cancer, the components responsible for such an effect and their mechanisms of action remain largely unknown. In the present study, we investigated the effect of a virgin olive oil phenol extract (PE) on proliferation, the cell cycle distribution profile, apoptosis, and differentiation of the human promyelocytic cell line HL60. PE inhibited HL60 cell proliferation in a time- and concentration-dependent manner, as demonstrated by the viable cell count and 3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide (MTT) metabolism. Cell growth was completely blocked at a PE concentration of 13.5 mg/L; apoptosis was also induced as detected by fluorescence microscopy and flow cytometry. Determination of the cell cycle distribution by flow cytometry revealed an accumulation of cells in the G0/G1 phase. Two compounds isolated from PE, the dialdehydic forms of elenoic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and to tyrosol (pHPEA-EDA), were shown to possess properties similar to those of PE; they account for a part of the powerful effects exerted by the complex mixture of compounds present in PE. The concentrations of the different compounds in PE were determined by HPLC, and the purity of 3,4-DHPEA-EDA and pHPEA-EDA was ascertained by NMR. Treatment with PE induced a differentiation in HL60 cells, which subsequently acquired the ability to produce superoxide ions and reduce nitroblue tetrazolium to formazan. These results support the hypothesis that polyphenols play a critical role in the anticancer activity of olive oil. J. Nutr. 136: 614–619, 2006.

KEY WORDS: • olive oil • phenols • apoptosis • differentiation • chemoprevention

Evidence supports the hypothesis that the health-promoting properties of the Mediterranean diet and its ability to decrease the incidence of some degenerative diseases, including coronary heart diseases and cancer, may be attributed, at least in part, to virgin olive oil (1). In particular, recently published epidemiologic studies demonstrated a clear association between the consumption of olive oil and a reduced risk of cancer in different sites such as breast (2–4), prostate (5), lung (6), larynx (7), ovary (8) and colon (9). In addition, studies conducted in animals agree with these observations and show that olive oil can prevent the development of azoxymethane-induced aberrant crypt foci and colon carcinomas (10), and reduce the incidence of dimethylbenz(a)anthracene-induced mammary tumor in rats (11). Furthermore, it was demonstrated that olive oil can suppress spontaneous liver tumorigenesis in mice (12) and reduce skin carcinogenesis when applied topically both before and after exposure of mice to UV light (13).

Although the above-reported studies clearly indicate a cancer-protective activity of virgin olive oil, the components responsible for such an effect and their mechanisms of action remain largely unknown. Although the beneficial health effects of olive oil have been attributed mainly to the high content of oleic acid, greater attention has recently focused on minor components such as phenolic compounds, which have a strong antioxidant activity (14,15), as well as several other biological properties (16). The phenolic composition of olive oil is rather complex and includes different classes of hydrophilic phenols including phenolic acids, phenolic alcohols, flavonoids, secoiridoids, and lignans (17). Among the phenolic alcohols, hydroxytyrosol (3,4-dihydroxyphenyl-ethanol; 3,4-DHPEA) and tyrosol (p-hydroxyphenyl-ethanol; pHPEA) have been the subject of numerous investigations. Secoirdoid derivatives are the most abundant phenols in olive oil. This class of compounds includes mainly the dialdehydic form of elenoic acid linked either to hydroxytyrosol (3,4-DHPEA-EDA) or to tyrosol (pHPEA-EDA) and the isomer of oleuropein aglycon (3,4-DHPEA-EA).

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**Abbreviations used:** ATRA all-trans retinoic acid; Cdk cyclin-dependent kinase; 3,4-DHPEA, 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol; 3,4-DHPEA-EDA, dialdehydic form of elenic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycon; DMSO, dimethyl sulfoxide; MTT, [3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide]; NBT, nitroblue tetrazolium; PE, phenol extract; pHPEA, p-hydroxyphenyl-ethanol or tyrosol; pHPEA-EDA, dialdehydic form of elenic acid linked to tyrosol; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate.


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Relatively high concentrations of lignans, such as (+)-1-acetoxypinoresinol, (+)-1-pinoresinol, and (+)-1-hydroxypinoresinol were also reported (18).

Recently, the cancer-protective activity of 3,4-DHPEA was suggested after it was shown that this compound can completely inhibit the proliferation of promyelocytic HL60 leukemia cells and induce apoptosis in a concentration range of 50–100 μmol/L (19,20). Treatment with 3,4-DHPEA also arrested HL60 cells in the G2/M phase of the cell cycle (19). In contrast to the effects of 3,4-DHPEA, pHPEA, which lacks the ortho-hydroxyl group on phenol ring, did not inhibit proliferation or induce apoptosis. Although 3,4-DHPEA and pHPEA are present in virgin olive oil mainly in a linked form (3,4-DHPEA-EDA, pHPEA-EDA, and 3,4-DHPEA-EA), no studies were performed to investigate the anticancer properties of these compounds either as components of a complex mixture isolated from olive oil or as purified single species. The quantity of 3,4-DHPEA-EDA and pHPEA-EDA depends on the different olive cultivars, but their concentration is 10–100 times higher than the respective phenolic alcohols 3,4-DHPEA and pHPEA (17).

The aim of this research was to investigate the antiproliferative and apoptosis-inducing activity on HL60 cells of a complex phenolic extract obtained from virgin olive oil. In addition, the effect of the single purified compounds, i.e., 3,4-DHPEA-EDA and pHPEA-EDA, was determined to quantify their contribution to the response obtained with the whole phenolic extract. The ability of the phenolic extract to block the cell cycle in the G2/M phase and induce differentiation in the promyelocytic leukemia HL60 cells, is also reported.

MATERIALS AND METHODS

Extraction and separation of phenolic compounds from virgin olive oil. The mechanical oil extraction process was performed using an industrial plant as follows: olives (Moraído) were crushed using a hammer crusher; the malaxation was carried out for 40 min at 25°C, and the oil was extracted by centrifugation (9600 × g; 20 min) using a decanter (Rapanelli Model 400 ECO/G) at a low level of water addition. A phenol methanolic extract was obtained from the virgin olive oil, which contained 650 mg/kg of total phenols, as reported by Montedoro et al. (21); the separation of phenols was performed by semipreparative HPLC as previously reported (22). The purity of the 3,4-DHPEA-EDA and pHPEA-EDA preparations was evaluated by HPLC and confirmed by NMR (22). The PE and purified 3,4-DHPEA-EDA were dissolved in ultra-pure (Milli-Q) water at 100 mg/mL. The molar concentrations of the different compounds, the growth rates were quantified by the MTT assay as previously described (23).

Measurement of apoptosis by flow cytometry. Next, the various treatment-time aliquots of the HL60 cell suspension, treated with the different compounds as described above, were transferred into centrifuge tubes, washed once with PBS (400 × g; 7 min), and then processed for the apoptosis assay. Apoptosis was evaluated by PI staining and flow cytometry as previously reported (19).

Quantification of apoptosis by fluorescence microscopy. Aliquots (100 μL) of the HL 60 cell suspensions were taken at each treatment-time and the percentages of viable, apoptotic, and necrotic cells were assessed using a fluorescent microscopy assay (24). The cell suspensions were centrifuged (400 × g; 7 min) and the pellets were resuspended in complete RPMI-medium containing the DNA binding dyes Hoechst 33342 (HO 342, 20 mg/L in PBS) and PI (10 mg/L in PBS). After 10 min of incubation at room temperature, the cells were examined as previously reported (19).

DNA cell cycle analysis. After treatment of HL60 cells with different concentrations of the compounds in RPMI-medium for 24 h, the cells were recovered by centrifugation (400 × g; 7 min) and washed twice with cold PBS. The pellet was resuspended in 50 μL cold PBS + 450 μL cold methanol and incubated for 1 h at 4°C. The cells were then centrifuged (400 × g; 7 min) and the pellet was washed twice with cold PBS, resuspended in 500 μL PBS and 5 μL RNase (20 mg/L, final concentration) and incubated for 30 min at 25°C. After incubation, the cells were chilled on ice for 10 min, stained with PI (50 mg/L, final concentration) for 1 h, and analyzed by flow cytometry using a FACScan flow cytometer at a wavelength of 488 nm. The percentages of cells in the G2/M, S and G1/M phases were calculated using CellFIT Cell-Cycle Analysis Version 2.0,2. Software.

Evaluation of differentiation. The differentiation of HL60 cells was evaluated by the nitroblue tetrazolium (NBT) reduction assay as previously described (25). Briefly, after treatment with different concentrations of PE for 7 d, aliquots (4 mL) of the cell suspensions were centrifuged (400 × g; 7 min); the pellet was suspended in 1 mL of complete medium and stimulated with phorbol 12-myristate 13-acetate (PMA; 0.2 μmol/L, final concentration) in the presence of NBT (1.2 mmol/L) for 1 h at 37°C and 5% CO2. The cells were then washed twice with 5 mL cold bovine serum solution (137 mmol/L NaCl, 5 mmol/L KCl, 0.8 mmol/L MgSO4, 10 mmol/L HEPES, pH 7.4) to remove unreacted NBT, and the insoluble formazan deposits in the resulting pellet were solubilized in 1 mL of a mixture containing 90% (v/v) dimethyl sulfoxide (DMSO), 0.1% (wt/v) SDS, and 0.01 mol/L NaOH by vigorous mixing on a vortex. The samples were centrifuged (1500 × g; 5 min) to remove the cellular debris, and then the absorbance of supernatants was measured at 715 nm (A715nm); data are expressed as change in A715nm/106 cells.

Statistical analyses. All tests were run in triplicate for each experimental condition and each experiment was repeated at least 3 times; the results are reported as means ± SD. Significant differences among treatments were tested using a 1-way ANOVA. When a significant (P < 0.05) treatment effect was detected, means were compared using Tukey’s post hoc comparisons.

RESULTS

The HPLC analysis of the crude extract of hydrophilic phenols (PE) from the virgin olive oil showed that, according to the literature data, PE contains low amounts of both 3,4 DHPEA and pHPEA compared with the secoiridoid derivatives. The quantitative analysis of compounds, expressed as mg/kg extract, present in the PE (Table 1) showed that 3,4 DHPEA-EDA was the most abundant, followed by 3,4 DHPEA-EA and pHPEA-EDA. The molar concentrations of the different compounds are also reported (Table 1); these are quantified when the PE is dissolved in the cell culture medium at a concentration of 13.5 mg/L to obtain a total concentration of 3,4 DHPEA + 3,4 DHPEA-containing compounds (3,4 DHPEA-EDA and 3,4 DHPEA-EA) corresponding to 12.5 μmol/L.

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In a previous study, we showed that 3,4 DHPEA, isolated from virgin olive oil, inhibited HL60 cell growth in a dose-dependent manner, resulting in a complete block of proliferation at 100 \( \mu \text{mol/L} \) (19). On the basis of these results, preliminary experiments were conducted in which HL60 cells were incubated with the PE dissolved in the culture medium at a concentration of 10\( \mu \)g/L (total concentration of 3,4 DHPEA-containing compounds corresponding to 100 \( \mu \text{mol/L} \)) to check the proliferation on subsequent days. After 24–48 h of treatment, 100% of the cells were necrotic as demonstrated by both the trypan blue exclusion method and the MTT assay (data not shown). Further experiments were then performed by incubating the cells with lower concentrations of PE (0–25 \( \mu \text{mol/L} \)).

The HL60 proliferation results (Fig. 1A) show a clear dose-dependent effect with complete cell growth inhibition at a PE concentration of 12.5 \( \mu \text{mol/L} \) of 3,4 DHPEA-containing compounds. At 25 \( \mu \text{mol/L} \), a slow decline in cell viability was evident; this reached the zero value after 96 h of incubation. The MTT assay used to evaluate further the inhibitory activity of PE on cell growth after 72 h of treatment showed a dose-dependent inhibition of MTT metabolism (Fig. 1B). The effect was significant at 6.25 \( \mu \text{mol/L} \) (26.3 ± 2.4% of inhibition, \( P < 0.001 \)) and 12.5 \( \mu \text{mol/L} \) (80.1 ± 0.9% of inhibition, \( P < 0.0001 \)). At 25 \( \mu \text{mol/L} \), the MTT reduction was completely abolished, confirming the cytotoxic activity of PE at this concentration.

In the same concentration range, PE induced apoptosis in HL60 cells after 24 h of treatment as demonstrated by flow cytometry and fluorescence microscopy (Fig. 2A). Both methods gave comparable results, thereby demonstrating that there was a weak effect on apoptosis at the lowest concentrations, which became more evident at 12.5 and 25 \( \mu \text{mol/L} \). Under these experimental conditions PE also caused a notable alteration in the cell cycle distribution (Fig. 2B); the percentage of cells in the G\( _0 \)/G\( _1 \) phase increased, whereas those in the S phase decreased. Although the effect on the distribution of the cells in the G\( _0/G_1 \) and S phases was moderate, it was significant at the highest PE concentrations. At 12.5 \( \mu \text{mol/L} \), PE induced a 13.4% increase (\( P < 0.05 \)) in the proportion of cells in the G\( _0/G_1 \) phase, and this effect was associated with an 11.4% decline (\( P < 0.05 \)) of cells in the S phase. Surprisingly, the G\( _2/M \) phase was not affected by the treatment (Fig. 2B).

Because PE was more potent than hydroxytyrosol in both inhibiting proliferation and inducing apoptosis, the effect of PE cannot be justified by the presence of hydroxytyrosol. We therefore investigated the effects exerted by 3,4-DHPEA-EDA and \( \phi \)HPEA-EDA, the 2 main components of PE, on proliferation and apoptosis of HL60 cells. These compounds were purified from PE extract and the results for purity, demonstrated by HPLC and confirmed by NMR, were 97 and 92%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/kg extract</th>
<th>( \mu \text{mol/L} ) culture medium</th>
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<tr>
<td>3,4 DHPEA</td>
<td>1</td>
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<td>3,4 DHPEA-EDA</td>
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<td>3,4 DHPEA-EA</td>
<td>110</td>
<td>3.94</td>
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<tr>
<td>Total 3,4 DHPEA-containing compounds</td>
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<td>( \phi )HPEA</td>
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<td>0.069</td>
</tr>
<tr>
<td>( \phi )HPEA-EDA</td>
<td>20</td>
<td>0.89</td>
</tr>
</tbody>
</table>

**TABLE 1**

**Different compounds present in the phenol extract (PE) obtained from virgin olive oil**

FIGURE 1  Effect of increasing concentrations of PE (expressed as 3,4-DHPEA + 3,4-DHPEA-EDA and 3,4-DHPEA-EA) on proliferation of HL60 cells measured over time by the trypan blue method (panel A) and after 72 h of incubation by the reduction of MTT (panel B). Values are means ± SD, \( n = 4 \); means (at each time, panel A) without a common letter differ, \( P < 0.05 \).

FIGURE 2  Effect of increasing concentrations of PE (expressed as 3,4-DHPEA + 3,4-DHPEA-EDA and 3,4-DHPEA-EA) on apoptosis (panel A) and cell cycle distribution (panel B) of HL60 cells. Values are means ± SD, \( n = 4 \); means within a method (panel A) and within each phase of the cell cycle (panel B) without a common letter differ, \( P < 0.05 \).
respectively. The effect of 3,4-DHPEA-EDA and pHPEA-EDA on proliferation of HL60 was determined after incubating the cells for 72 h with increasing concentrations of the 2 compounds, by both the cell count with trypan blue (Fig. 3A) and by the reduction of MTT (Fig. 3B). Both methods showed that the 2 compounds induced a clear dose-response inhibition of cell growth. pHPEA-EDA was more potent than 3,4-DHPEA-EDA in suppressing cell growth; in fact, the concentrations at which the compounds caused 50% inhibition of cell proliferation were 7–8 and 30–35 μmol/L, respectively. When the cells were treated with 3,4-DHPEA-EDA (8.465 μmol/L) and pHPEA-EDA (0.89 μmol/L), concentrations present in 12.5 μmol/L PE (Table 1), the proliferation was inhibited by 14 ± 3% (P < 0.01) and 5 ± 4% (P = 0.056), respectively. Combining the 2 compounds at these concentrations, the cell growth inhibition was 20 ± 3% (P < 0.01), demonstrating a clear additive effect.

3,4-DHPEA-EDA and pHPEA-EDA induced apoptosis in HL60 cells 24 h after treatment (Table 2). Similar to the effect on proliferation, pHPEA-EDA was more potent than 3,4-DHPEA-EDA, inducing apoptosis in 90% of the cells at a concentration of 60 μmol/L (Table 2), whereas a 256 μmol/L concentration of 3,4-DHPEA-EDA was required to have the same effect (Table 2). At the highest concentrations, both compounds were cytotoxic as shown by the increased percentage of necrotic cells (Table 2).

To examine the effect of PE on differentiation, HL60 cells were treated with increasing concentrations of PE, harvested after 24, 48, and 72 h, and then subjected to the NBT reduction assay (Fig. 4). The ability of cells to reduce NBT, a functional marker of granulocyte/monocyte differentiation of HL60, increased significantly after 48 and 72 h of treatment with PE at a concentration of 9 and 12.5 μmol/L. Treatment of cells with DMSO, a well-known differentiation agent of HL60 cells, produced a reduction of NBT similar to that of PE after 48 h of incubation, whereas at 72 h, the effect was much more evident (Fig. 4).

**DISCUSSION**

Because unbalanced control of proliferation, apoptosis, and differentiation are the main characteristics of cancer cells, compounds that induce apoptosis and differentiation are good candidates as cancer chemopreventive and/or chemotherapeutic agents.

In the present study, we demonstrated clearly that a complex mixture of phenolic compounds (PE), isolated from virgin olive oil, inhibited proliferation and induced apoptosis in the human promyelocytic cell line HL60. In addition, PE treatment altered the cell cycle by increasing cells in the G0/G1 phase while decreasing those in the S phase. The G0/G1 ratio, a sensitive marker indicating the arrest in G1, increased from 2 in the control cells to 5 in the PE-treated cells (12.5 μmol/L) suggesting a block of the cell-cycle at the G0/S transition. Similar effects were shown previously to be induced by purified 3,4-DHPEA (19). However, a complete inhibition of cell growth was observed at a PE concentration of 12.5 μmol/L, whereas the same effect was observed with 3,4-DHPEA at 100 μmol/L. At a PE concentration of 12.5 μmol/L, the 3,4-DHPEA concentration was only 0.1 μmol/L (Table 1); therefore, we concluded that the PE effect was not due to 3,4-DHPEA.

Instead, the inhibition of proliferation and induction of apoptosis may be caused by other compounds present in the olive oil extract. Indeed, the HPLC chromatogram shows that the chemical composition of PE is particularly complex and contains several components other than 3,4-DHPEA. Supporting the results reported in other studies (17), we found that PE contains relatively high concentrations of 3,4-DHPEA-EDA, 3,4-DHPEA-EA, and pHPEA-EDA compared with 3,4-DHPEA and pHPEA. We were able to obtain sufficient amounts of highly purified 3,4-DHPEA-EDA and pHPEA-EDA from this mixture to test their effect on cell proliferation and apoptosis. Both compounds inhibited proliferation and induced apoptosis in tumor cells. A complete inhibition of proliferation was obtained with 3,4-DHPEA-EDA and pHPEA-EDA at concentrations of 130 and 60 μmol/L, respectively, suggesting that the PE effect is only partially attributable to these compounds. This conclusion is further supported by the experiment in which both compounds were tested at the concentrations present in the PE and demonstrating the absence of a synergistic action. Whether 3,4-DHPEA-EDA and pHPEA-EDA act synergistically with other compounds of PE remains to be determined. Other bioactive components that are present in the PE could be responsible for the above described anticancer effects. Lignans, which are present in substantial amounts in the phenolic fraction of olive oil (18) and found in our extract, are also worthy of consideration. Lignans were shown to inhibit skin, breast, colon, and lung cancer cell growth (26,27) and induce apoptosis in colon tumor cells (28).

The observation that pHPEA-EDA is more powerful than 3,4-DHPEA-EDA for both inhibition of proliferation and induction of apoptosis is intriguing because tyrosol does not affect these parameters at concentrations as high as 250 μmol/L (19). The possible contamination of the purified compounds by other bioactive molecules can be excluded because the purity and structural characteristics of both compounds were tested by NMR. On the other hand, it has to be considered that when PE and 3,4-DHPEA are combined with the dialdehydic
form of elenoic acid, the physicochemical and structural properties of the resulting compounds may be strongly modified.

Another important result of the present investigation is that PE can induce differentiation in HL60 cells. Although several previous studies showed that phenols present in wine and tea can induce differentiation in promyelocytic cell lines (29,30), as well as in other systems (31), to the best of our knowledge, our results are the first to show such an effect exerted by phenols from olive oil. The differentiation assay, based on NBT reduction, measures the PMA-induced superoxide ion production as a functional marker for granulocyte/monocyte differentiation of HL60 cells (32). This assay cannot distinguish whether the cells acquire a granulocyte or monocyte phenotype. However, microscopic observation showed that cells did not form aggregates and did not adhere to the flask. These results suggest that PE treatment induced HL60 cells to differentiate along the granulocytic lineage. A similar effect was induced by DMSO (33), which was used as positive control in our experiments, and by different agents such as all-trans retinoic acid (ATRA) (34), which is currently used for differentiation therapy of acute promyelocytic leukemia (35). It is interesting to note that, similar to the PE effect, ATRA inhibits cell proliferation and arrests the cells in the G0/G1 phase (36). The mechanisms by which PE inhibits proliferation and induces HL60 differentiation are not known, but recent evidence supports the existence of a link between the regulation of the cell cycle machinery and cell differentiation (37). The cell cycle is regulated by the sequential activation of various cyclin-dependent kinases (Cdk) and the selective induction of different Cdk inhibitors (38,39). Thus, it is possible that PE treatment interferes directly with these processes by inhibiting Cdk or inducing Cdk inhibitors. In this context, 2 properties of olive oil phenols may be important; the first could be related to their potent antioxidant activity (40) and the second to the metal-chelating ability recently ascribed to these compounds (41). There is evidence that cancer cells produce large amount of reactive oxygen species (42), which may act as essential intracellular second messengers for the mitotic signal transduction of growth factors (43). Therefore, olive oil phenols, acting as free-radical scavengers, may inhibit these cellular process and cancer cell proliferation. On the other hand, it should be noted that EDTA, a well-known metal chelator, induces differentiation and suppresses the proliferation of HL60 cells (44), thus suggesting that the olive oil phenols could act in a similar way. Further studies are currently in progress to clarify these points.

Although no epidemiologic studies were conducted to determine the possible effect of olive oil on the incidence of leukemia, there is evidence that olive oil exerts a protective activity against carcinogenesis in other sites. In the present study, a clear anticancer activity of some phenolic compounds isolated from olive oil was demonstrated. Such activity was evident at phenol concentrations relatively higher than those observed in vivo after a normal daily intake of olive oil (45); however, it is possible that the regular low life-time intake of olive oil results in an overall protective effect. This phenomenon was demonstrated by recent clinical trials showing that short-term consumption of olive oil in humans (50 mL/d) can change several oxidative stress markers (46,47), although the concentrations of phenols are lower than that required to show biological activity in vitro. In addition, our results giving basic knowledge about interactions of phytochemicals with biological systems may be useful for the design of “functional foods.”

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### TABLE 2

<table>
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<th>Compound</th>
<th>Concentration</th>
<th>Apoptosis</th>
<th>Necrosis</th>
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<td></td>
<td>µmol/L</td>
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<td>Late</td>
</tr>
<tr>
<td>Control</td>
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<td>1.8 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>pHPEA-EDA</td>
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<sup>1</sup> Values are means ± SD, n = 6. Means in a column without a common letter differ, P < 0.05.
LITERATURE CITED


