

## Involvement of Hydrogen Peroxide Formation on Apoptosis Induction by Olive Oil Phenolic Compounds

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**Abstract:** In the present investigation the ability of different phenolic compounds, either present or not in olive oil, to induce both apoptosis on tumour cells and  $H_2O_2$  accumulation in cell culture medium was assessed. Among the phenols studied we found that tyrosol (*p*-HPEA), homovanillic alcohol and protocatechuic, *o*-coumaric, vanillic, homovanillic, ferulic and syringic acids did not induce either apoptosis on HL60 cells or  $H_2O_2$  accumulation, while hydroxytyrosol (3,4-DHPEA), 3,4-dihydroxyphenylacetic acid (3,4-DHPA), 3,4-dihydroxy-hydrocinnamic acid (3,4-DHHC) and gallic acid induced both apoptosis and accumulation of  $H_2O_2$  in the culture medium which were significantly reduced by catalase. In contrast, the dialdehydic form of elenoic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and to tyrosol (*p*-DHPEA-EDA) induced high level of apoptosis not reduced by catalase. Finally, oleuropein exerted a weak pro-apoptotic effect not mediated by  $H_2O_2$  release. From these results it is evident that: (i) the catechol moiety of phenols is necessary but not sufficient to induce apoptosis and  $H_2O_2$  accumulation; (ii) the 3,4-DHPEA metabolism may partially reduce its pro-apoptotic potential; (iii) the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA is not mediated by  $H_2O_2$  releasing activity.

**Keywords:** olive oil; phenols; apoptosis; hydrogen peroxide

### INTRODUCTION

Olive oil contains different phenolic compounds which have been shown to possess preventive activities toward chronic-degenerative diseases such as cardiovascular diseases and cancer (KRIS-ETHERTON *et al.* 2002). Hydroxytyrosol (3,4-DHPEA) and tyrosol (*p*-HPEA) are the most representative olive oil phenols present both as free compounds and linked to the dialdehydic form of elenoic acid (3,4-DHPEA-EDA and *p*-HPEA-EDA) (SERVILI & MONTEDORO 2002). Previous studies carried out in our laboratory have showed that these compounds exert a pro-apoptotic activity toward cancer cell lines (FABIANI *et al.* 2002, 2006). In particular, we have found that 3,4-DHPEA at high concentrations (100  $\mu$ M) induces apoptosis on HL60 cells through an oxidative stress caused by the extracellular production of hydrogen peroxide ( $H_2O_2$ ) (FABIANI *et*

*al.* 2008). The apoptotic effect of 3,4-DHPEA was effectively inhibited by N-acetyl-cysteine, dietary antioxidants (ascorbate and  $\alpha$ -tocopherol) and the enzyme catalase (FABIANI *et al.* 2008).

In this study, preliminary results on the pro-apoptotic activities of different phenolic compounds structurally similar to 3,4-DHPEA are reported, together with the extracellular  $H_2O_2$  accumulation and the effect of catalase on the apoptosis induction in HL60 cells.

### MATERIALS AND METHODS

**Materials.** 3,4-DHPEA was obtained from Cayman Chemicals Ltd; protocatechuic, vanillic, ferulic, 3,4-dihydroxy-hydrocinnamic, *o*-coumaric and syringic acids were obtained from Fluka Co. (Buchs, Switzerland); gallic acid was from Carlo

Erba (Milan) while oleuropein was purchased by Extrasynthese (Genay, France). 3,4 DHPEA-EDA and *p*-HPEA-EDA were purified by semi-preparative HPLC from a methanolic extract obtained from a virgin olive oil containing 650 mg/kg of total phenols as previously reported (FABIANI *et al.* 2006). Tyrosol (*p*-HPEA) and all other reagents were purchased from Sigma-Aldrich (Irvine, UK) unless differently specified

**Cell treatment and apoptosis analysis.** Human promyelocytic leukemia cells (HL60) were cultured in complete RPMI 1640 medium supplemented with 10% FCS, 2.0mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were seeded at a density of  $0.25 \times 10^6/\text{ml}$  and incubated for 24 h at 37°C and 5% CO<sub>2</sub> with the different phenolic compounds (100μM) in the absence and in the presence of catalase (CAT: 100 U/ml). The percentage of apoptotic cells was determined by a fluorescent microscopy assay as previously described (FABIANI *et al.* 2006).

**Measurement of H<sub>2</sub>O<sub>2</sub> concentration in the culture medium.** The concentration of H<sub>2</sub>O<sub>2</sub> in the culture medium was measured by the ferrous ion oxidation-xylenol orange method as follows: medium (20 μl) was mixed with a reaction solution (200 μl) containing 250μM ammonium iron (II) sulphate, 25mM H<sub>2</sub>SO<sub>4</sub>, 100mM sorbitol and 125μM xylenol orange and incubated at room temperature for 30 min. The absorbance was then read at 595 nm and the concentration of H<sub>2</sub>O<sub>2</sub> was derived from a standard curve.

## RESULTS AND DISCUSSION

Among the different phenols tested, at 100μM concentration, tyrosol (*p*-HPEA), homovanillic alcohol and protocatechuic, *o*-coumaric, vanillic, homovanillic, ferulic and syringic acids did not induce either apoptosis on HL60 cells or H<sub>2</sub>O<sub>2</sub> accumulation in the culture medium (results not shown). Since homovanillic acid and homovanillic alcohol are important methoxy metabolites of 3,4-DHPEA, derived from the reaction catalysed by the catechol-*O*-methyltransferase (D'ANGELO *et al.* 2005), the present data suggest that the 3,4-DHPEA biotransformation may in part decrease its activity. The results obtained with the other phenolic compounds regarding both the pro-apoptotic activity and the ability to release H<sub>2</sub>O<sub>2</sub> in the culture medium are reported in Figure 1A and 1B, respectively. It is clear that hydroxytyrosol (3,4-DHPEA), 3,4-dihydroxyphenylacetic acid (3,4-DHPA), 3,4-dihydroxy-hydrocinnamic acid (3,4-DHHC) and gallic acid induced both apoptosis and accumulation of H<sub>2</sub>O<sub>2</sub> in the culture medium. Such activities were efficiently reduced by catalase so suggesting that the pro-apoptotic effect is mediated by H<sub>2</sub>O<sub>2</sub>. In contrast, 3,4-DHPEA-EDA and *p*-HPEA-EDA induced high level of apoptosis which was not reduced by catalase (Figure 1A). This result is in accordance with the low accumulation of H<sub>2</sub>O<sub>2</sub> in the culture medium induced by 3,4-DHPEA-EDA and *p*-HPEA-EDA (Figure 1B). It should be noted that, in olive oil, these two compounds are about

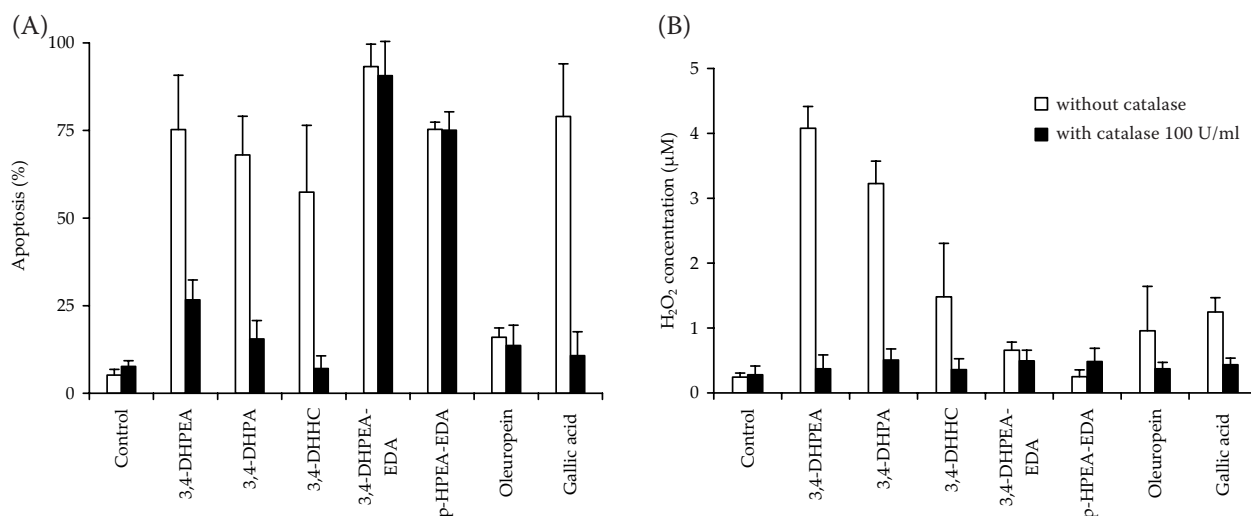


Figure 1. Effect of different phenolic compounds on apoptosis of HL60 cells (A) and H<sub>2</sub>O<sub>2</sub> accumulation in the cell culture medium (B)

100 and 10 times more abundant than 3,4-DHPEA and *p*-HPEA, respectively (FABIANI *et al.* 2006), therefore they could significantly influence the cancer preventive activity of olive oil. Finally, oleuropein exerted a weak pro-apoptotic effect which was not reduced by catalase and therefore it is not mediated by  $H_2O_2$  release. The low cytotoxicity of oleuropein has been previously observed in human salivary gland (BABICH & VISIOLI 2003)) and breast cancer cells (MENENDEZ *et al.* 2007). All together these results suggest that; (i) the catechol moiety of phenols is necessary but not sufficient to induce apoptosis and  $H_2O_2$  accumulation; (ii) the 3,4-DHPEA metabolism may partially reduce its pro-apoptotic potential; (iii) the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA is not mediated by the  $H_2O_2$  releasing activity. Further studies are currently in progress to elucidate the mechanisms involved in the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA.

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