Antiproliferative Activity of Olive Oil Phenolics against Human Melanoma Cells

A.L.M. Batista de Carvalho, F. Caselli, V. Rodrigues, F. Paiva-Martins and M.P.M. Marques

Abstract: Background: Virgin olive oil, the main source of lipids in a mediterranean diet, is broadly recognised to possess health-beneficial features, namely a protective role against cancer. It comprises several phenolic compounds, the major ones being tyrosol (p-hydroxyphenylethanol), hydroxytyrosol (3,4-dihydroxyphenylethanol), lignans and secoiridoids, namely oleuropein (OP) and the oleuropein aglycones 3,4-(dihydroxyphenyl) ethanol elenolic acid ester and 3,4-(dihydroxyphenyl)ethanol elenolic acid di-aldehyde.

Objective: The goal of this work is the in vitro evaluation of the anti-proliferative activity against human amelanotic melanoma (C32 cell line) for hydroxytyrosol and the most important secoiridoids of olive oil. The effect of hydroxytyrosol on non-neoplastic cells (BJ fibroblast) was also investigated.

Methods: Inhibition of cell proliferation was assessed by the Sulforhodamine colorimetric method, in both neoplastic and non-tumorigenic cells.

Results: 3,4-(Dihydroxyphenyl)ethanol elenolic acid di-aldehyde and 3,4-(dihydroxyphenyl)ethanol elenolic acid ester secoiridoid aglycones were found to display growth-inhibiting activity (at ca. 100 µM), as opposed to oleuropein that elicited a strong protective effect at all concentrations (100 to 1000 µM). 3,4-Dihydroxyphenylethanol evidenced a dual effect (strongly dose-dependent) – cytoprotective for lower dosages and cytotoxic at high concentrations.

Conclusions: Attending to the recognised structural dependence of the biological activity of phenolic derivatives, the previously gathered conformational data on the olive oil constituents presently investigated assisted the interpretation of their biological properties. This type of studies, coupling structural characterisation to biological assessment, allows the establishment of reliable structure activity relationships for polyphenolic compounds, ruling their cytoprotective vs cytotoxic activity and therefore their potential use as natural-based pharmaceutical agents.

Keywords: Virgin olive oil, hydroxytyrosol, secoiridoid polyphenols, anticancer, melanoma, fibroblasts.

INTRODUCTION

Virgin olive oil (VOO), the main source of lipids in a mediterranean diet, is broadly recognised to possess health-beneficial features, namely a protective role against cancer [1-4]. It comprises several phenolic compounds, the major ones being hydroxytyrosol (3,4-dihydroxyphenylethanol, 3,4-DHPEA), tyrosol (p-hydroxyphenylethanol, p-HPEA), lignans (such as (1)-pinoresinol and (1)-1-acetoxy-pinoresinol) and secoiridoids, namely oleuropein (OP) and the oleuropein aglycones 3,4-(dihydroxyphenyl) ethanol elenolic acid ester (3,4-DHPEA-EA) and 3,4-(dihydroxyphenyl)ethanol elenolic acid di-aldehyde (3,4-DHPEA-EDA) (Fig. 1). Epidemiological evidence, in vitro and animal studies indicates a protective role of olive oil phenolics (OVPs) against cancer [5-10], as well as an inhibitory effect regarding different stages of carcinogenesis, including angiogenesis and metastasis. Actually, a clear association between the consumption of olive oil (namely in a Mediterranean diet) and a reduced risk of cancer has been reported, namely regarding breast [11, 12], prostate [13], lung [14], larynx [15], ovary [16], colon [17] and bladder cancer [9]. Also, a study by Owen and coworkers suggested that a fraction of the ingested OVPs may reach the colon and confer chemopreventive effects against colorectal cancer [1]. In addition, olive oil phenolic extracts have recently been shown to exert
an anti-proliferative effect towards human promyelocytic leukemia cells (HL60) [18-20], as well an inhibitory action towards cell attachment and invasion in HT115 human colon carcinoma [21].

Vissers and colleagues [23] suggested that an important step in the metabolism of oleuropein and oleuropein aglycones is their hydrolysis into either hydroxytyrosol or tyrosol. As a consequence, it has been observed that 3,4-DHPEA rises early after virgin olive oil ingestion, reaching a peak after ca. 1 h in plasma and 0-2 h in urine [24-26], undergoing an extensive intestinal/hepatic metabolism [24, 27-30]. Hydroxytyrosol was reported to exert a marked antioxidant activity and prevent metal ion catalysed production of reactive oxygen species in bulk oils [31-34], and was also found to avoid LDL oxidation both in vitro and ex vivo [35-37], and to reduce colon cancer growth [10]. In addition, there are some studies evidencing a significant antioxidant effect from this compound towards cultured cancer cells, from epithelial colorectal [38] and melanoma [39] to prostate adenocarcinoma [40].

The biological activity of this type of olive oil phenolic compounds towards cancer is mainly due to their high antioxidant capacity [25, 41], through free radical scavenging, metal chelation [42] and modulation of cell signalling pathways and gene expression [43]. However, there are other mechanisms likely to be associated to this antitumour activity, namely at the invasion and metastasis level, possibly involving prevention of attachment of cancer cells to extracellular matrices [21]. The secoiridoid aglycones 3,4-DHPEA-EA and 3,4-DHPEA-EDA, which are present in olive oil at higher concentrations than hydroxytyrosol, are thought to be the main responsible for the high antioxidant capacity of this food product.

However, despite the widely accepted chemopreventive and antineoplastic properties of olive oil phenolics, the specific components responsible for them and the mechanisms underlying their action remain largely unknown. Actually, very few studies have been reported to this date on the molecular basis of this health-beneficial activity of OVPs. It is generally accepted that the antioxidant properties of these phenolic constituents are conferred by their ortho-diphenolic (catecholic) moieties [44, 45] (Fig. 1). These structural and conformational characteristics, which determine biological activity, namely chemoprevention against oxidative-induced pathologies, have been previously studied by the authors through Raman spectroscopy coupled to DFT calculations [22].

Although melanoma represents only 4-5% of all skin cancers, it contributes to 85% of skin cancer deaths, with rising rates mainly due to sunlight overexposure (UV radiation). Amelanotic melanoma is the most dangerous form of skin cancer, with a very poor prognosis and short survival periods. Moreover, metastasis from malignant melanoma occurs mainly in lung, which drastically increases the mortality attributed to this kind of neoplasia.

The present work reports the in vitro evaluation of the anti-proliferative activity against human amelanotic melanoma (C32 cell line) for 3,4-DHPEA and the most important secoiridoids present in olive oil – OP, 3,4-DHPEA-EA and 3,4-DHPEA-EA. Inhibition of cell proliferation was assessed by the Sulforhodamine (SRB) colorimetric method, that measures cell density. The effect of 3,4-DHPEA on non-neoplastic cells (BJ fibroblast cell line) was also investigated.

MATERIALS AND METHODS

Chemicals

Antibiotics (penicillin-streptomycin 100x solution), dimethyl sulfoxide (DMSO), Dulbecco’s Modified Eagle’s Medium – high glucose (DMEM-HG, 4500 mg/L glucose), ethylenediaminetetraacetic acid (EDTA, disodium salt, dihydrate), phosphate buffered saline (PBS), Sulforhodamine B (SRB, monosodium salt, 0.5% (w/v) solution), TRIS (tris(hydroxymethyl)aminoethane), Trypan blue (0.04% (w/v) solution), trypsin-EDTA (1x), and inorganic salts and acids (of analytical grade) were purchased from Sigma-Aldrich Chemical S.A. (Sintra, Portugal). Fetal bovine serum (FBS) was obtained from Gibco-Life Technologies (Porto, Portugal).

Phenolic Compounds

Hydroxytyrosol (3,4-DHPEA) was synthesised from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich, S. A. Sintra, Portugal) according to Baraldi et al. [46]. Oleuropein and 3,4-DHPEA-EDA were extracted from olive leaves, according to the procedures of Gariboldi [47] and Paiva-Martins [48], respectively. The aglycone 3,4-DHPEA-EA was obtained from oleuropein by an enzymatic reaction...
SRB (0.5% in 1% acetic acid) was added to each well, and staining assay to obtain the cellular protein content

methanol (1% (v/v) in DMSO). Following a 72 h incubation period, the growth media was aspirated, the cells were washed, fixed and stored at 20°C. Briefly, fixation was carried out overnight with ice-cold methanol (1% (v/v) in acetic acid), after which methanol was decanted and the plate was air-dried.

Cell proliferation was then evaluated through the SRB staining assay to obtain the cellular protein content [50-52]. SRB (0.5% in 1% acetic acid) was added to each well, and the cells were incubated at 37°C for 1 hour. Plates were rinsed with 1% acetic acid, air-dried, and the bound dye was eluted with 1 ml of 10 mM TRIS buffer (pH 10). Absorbance was measured at 540 nm, the amount of dye released being proportional to the number of cells in each well and thus a reliable indicator of cell proliferation.

Statistical Analysis

All experiments were performed in triplicate. The results were expressed as mean values±standard deviation, (the corresponding error bars being displayed in the graphical plots). Statistical analyses were performed using ANOVA, followed by Dunnett post hoc test. Statistical comparison between the data was based on the Pearson correlation coefficient, values less than 0.05 being considered as significant.

RESULTS AND DISCUSSION

The main phenolic components of virgin olive oil – 3,4-DHPEA, OP, 3,4-DHPEA-EDA and 3,4-DHPEA-EA – were screened as to their growth-inhibitory effect towards the C32 human melanoma cell line, and the results interpreted in the light of a previously performed conformational analysis of these compounds by Raman spectroscopy coupled to quantum mechanical calculations (which yielded their most stable structures at physiological conditions) [22]. Actually, it is well recognised that the structural preferences of this type of systems are determinant of their biological function, such as their cytotoxic or cytoprotective activities. In fact, only minor modifications in their structural characteristics (such as the number and position of the ring hydroxyl substituents) are known to drastically affect their bioactivity, similarly to what was verified for other dietary phenols (e.g. hydroxycinnamic and gallic acid derivatives [53-58]).

3,4-DHPEA-EA and 3,4-DHPEA-EDA displayed a clear dose-response inhibition of C32 cell growth already for the lower concentration tested (100 μM) (Fig. 3(A) and (B)). In contrast, oleuropein elicited a strong cytoprotective effect.
studies by Fabiani et al., as well as with reported previous data gathered by the authors for colon and breast cancer cells (although the exact proportion depends on the olive cultivar, including the red ones).

Fig. (4). Dose-dependent cytoprotective effect of OP towards the C32 human melanoma cell line, for an incubation time of 72 h. The cell density was assessed by the SRB method (as described in Methods). The results are the mean±standard deviation obtained from experiments performed in triplicate, and are expressed as a percentage of the control±standard deviation. The one-way ANOVA statistical analysis was used, and the Dunnett’s post-test was carried out to verify the significance of the obtained results (*p<0.05 versus control at 72 h).

Fig. (5). Dose-dependent antiproliferative effect of 3,4-DHPEA towards the C32 human melanoma cell line, for an incubation time of 72 h. The cell density was assessed by the SRB method (as described in Methods). The results are the mean±standard deviation obtained from experiments performed in triplicate, and are expressed as a percentage of the control±standard deviation. The one-way ANOVA statistical analysis was used, and the Dunnett’s post-test was carried out to verify the significance of the obtained results (***p<0.001 versus control at 72 h).

Regarding the latter, a complete inhibition of growth was reported for a 3,4-DHPEA-EDA concentration equal to 130 µM, while a viability decrease to ca. 35% at 100 µM was presently observed for the C32 cells, evidencing a marked selectivity of the compound as regards the type of cancer cell.

The much higher anti-proliferative activity of the conjugated species 3,4-DHPEA-EDA is suggested to be related to their distinct structural characteristics as compared to hydroxytyrosol and oleuropein, which establish their lypophilicity versus hydrophilicity and consequently determine their transport processes into the cell and subsequent bioavailability. In fact, linkage of hydroxytyrosol to the di-aldehydic form of elenoic acid involves a drastic change in the physicochemical and structural properties of the resulting conjugates, as was previously shown by the authors through spectroscopic and theoretical methods [22]. The balance between lipophylic and hydrophylic in these systems is of paramount importance for their widespread biodistribution and potential effect within the cellular medium, hydroxytyrosol and oleuropein (comprising a glucose moiety) being significantly more hydrophilic than the corresponding aglycones, which may prevent their prompt cellular uptake. The presence of aldehydic groups in both 3,4-DHPEA-EDA and 3,4-DHPEA-EDA, known to be involved in an efficient interaction with proteins (often through lysine residues), may then justify their observed cytotoxicity towards melanoma cells, the compound containing two such moieties displaying a much higher effect at lower dosages (100 µM, Fig. 3). Furthermore, the strong lipophilic character of these oil phenolics allows them to interact promptly with the cell membrane, leading to disruption and cell growth impairment, as previously evidenced in red blood cell membranes [62].

The results currently obtained for 3,4-DHPEA-EDA regarding the melanoma cell line are in accordance with previous data gathered by the authors for colon adenocarcinoma (unpublished data), as well as with reported studies by Fabiani et al. on HL60 leukemia cells [18, 19].
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studies, coupling structural characterisation to biological activity of phenolic derivatives, namely at the molecular level. This dual effect presently measured for hydroxytyrosol towards the BJ human fibroblast cell line, for an incubation time of 72 h. The cell density was assessed by the SRB method (as described in Methods). The results are the mean±standard deviation obtained from experiments performed in triplicate, and are expressed as a percentage of the control±standard deviation. The one-way ANOVA statistical analysis was used, and the Dunnett’s post-test was carried out to verify the significance of the obtained results (*p<0.05 versus control at 72 h).

CONCLUSION

The main phenolic constituents of virgin olive oil – 3,4-DHPEA, OP, 3,4-DHPEA-EDA and 3,4-DHPEA-EA – were assessed as to their in vitro effect on human amelanotic melanoma cells. The aglycones 3,4-DHPEA-EDA and 3,4-DHPEA-EA were found to display a significant growth-inhibitory activity even at the lowest dosage tested (100 μM), as opposed to oleuropein that elicited a strong protective effect at all concentrations (100 – 1000 μM). The smaller oily phenolic 3,4-DHPEA, in turn, led to either a cytoprotective or a cytotoxic effect, in a concentration-dependent way, which was coupled to a modest cytotoxicity against non-neoplastic (fibroblast) cells.

This dual effect presently measured for hydroxytyrosol towards amelanotic melanoma cells is in agreement with the known twofold behaviour of phenolic systems, that may act either as antioxidants or pro-oxidants in a concentration-dependent manner [63]. Hence, their overall biological activity is the result of a tight balance between these two abilities, leading to either a cytotoxic or an antiproliferative activity [53]. Moreover, it is expected that in virgin olive oil 3,4-DHPEA, 3,4-DHPEA-EA and 3,4-DHPEA-EDA act synergistically with other phenolic components (e.g. lignans), although this has not yet been unequivocally demonstrated and needs further clarification through studies at the molecular level.

According to the recognised structural dependence of the biological activity of phenolic derivatives, namely as chemopreventive agents oxidative-induced carcinogenesis, the previously gathered conformational data on the olive oil constituents presently investigated [22] assisted the interpretation of their biological properties. This type of studies, coupling structural characterisation to biological assessment, allows the establishment of reliable structure activity relationships (SAR’s) for natural based polyphenols, ruling their potential cytoprotective role against deleterious oxidative processes vs cytotoxicity towards neoplastic cells [64].

Since unbalanced control of cell growth and differentiation are the main characteristic of neoplasia, natural compounds such as the ones presently studied, consumed in a daily basis (e.g. in a Mediterranean diet) and capable of inhibiting cell proliferation, are promising candidates as chemoprotective and/or chemotherapeutic agents. In particular, the knowledge gathered on the interference of this kind of phytochemicals on cell growth may be the basis for the development of topical skin formulations (e.g. for protection against deleterious sunlight UV radiation), as well as for the rational design of functional foods, aiming at cancer prevention (e.g. specifically skin cancer, mainly in populations under increased risk).

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from the Portuguese Foundation for Science and Technology – UID/MULTI/00070/2013.

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Fig. (6). Dose-dependent antiproliferative effect of 3,4-DHPEA towards the BJ human fibroblast cell line, for an incubation time of 72 h. The cell density was assessed by the SRB method (as described in Methods). The results are the mean±standard deviation obtained from experiments performed in triplicate, and are expressed as a percentage of the control±standard deviation. The one-way ANOVA statistical analysis was used, and the Dunnett’s post-test was carried out to verify the significance of the obtained results (*p<0.05 versus control at 72 h).
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