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In Vitro Antitumor Activity of Olive Oil Tyrosol and Hydroxytyrosol and their Methyl Carbonate Derivatives

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ABSTRACT

A Mediterranean diet rich in olive oil has been associated with health benefits in humans. The phenolic compounds found in virgin olive oil have demonstrated antitumor activity and antioxidant properties. The aim of this study was to confirm the *in vitro* antiproliferative effect of tyrosol (TYR) and hydroxytyrosol (HTYR) on human cancer cell line and to investigate and compare the antitumor effect of methyl carbonate derivatives of these natural compounds as tyrosol methyl carbonate (TYRMC) and hydroxytyrosol methyl carbonate (HTYRMC) synthesized in our laboratory by an eco-friendly procedure. Four human tumor cell lines of melanoma (M14), pulmonary (H125), colon (WiDr) and promyelocytic leukaemia (HL60) were growth in the presence of 3.125, 6.25 12.5, 25, 50 and 100 mcg/ml of TYR, HTYR, TYRMC and HTYRMC for 24, 48 and 72 h. The antiproliferative activity was assayed by counting the cells in trypan blue, the apoptosis induction and cell cycle profiles were evaluated by flow cytometry analysis. A significant growth inhibition was observed in each tumour cell line exposed to these molecules in the experimental conditions described above. In addition this study demonstrated that HTYRMC results more effective in cell growth inhibition and apoptosis induction reaching 98.2% at the concentration of 100 mcg/ml. However, further studies are necessary to better understand the mechanisms of these molecules on tumour cell growth.

Keywords: antiproliferative activity, olive oil, phenolic compounds
Abbreviations: **HTYR**, hydroxytyrosol; **HTYRMC**, hydroxytyrosol methyl carbonate; **TYR**, tyrosol; **TYRMC**, tyrosol methyl carbonate

INTRODUCTION

Natural products have long been considered as excellent sources for drug discovery given their structure diversity and wide variety of biological activities. Over the last five decades, a large number of papers have reported that natural compounds isolated from plants, including polyphenols, exhibit healthy effects on humans (Manach 2005). In the Mediterranean area, the daily consumption of wine and virgin olive oil is related to the protection against several kinds of cancer (Kontou 2011). The main responsible of these benefits are phenolic compounds such as tyrosol (TYR) and hydroxytyrosol (HTYR) (**Fig. 1A**) present in these food and beverage (Cornwell 2008). In virgin olive oil, they derive from the hydrolysis of secoiridoids during olive oil storage. Their concentration is related to the olive tree variety, climatic and agronomic conditions, degree of maturation at harvest, and the manufacturing process (Allouche 2007).

Currently, many studies reported the *in vitro* and *in vivo* biological activities of phenolic compounds naturally present in virgin olive oil. Among them, anti-inflammatory, cardioprotective antioxidant and chemopreventive effects in various types of cancers were claimed (Owen 2000; Visioli 2002).

It has been well established that HTYR is a potent antioxidant capable to scavenge oxygen and nitrogen free radicals, to inhibit Low Density Lipoprotein (LDL) oxidation, platelet aggregation and endothelial cell activation and to protect against DNA damage. Moreover, HTYR is able to reduce the synthesis of prostaglandin E2 blocking the transcription of COX-2 and 5-lipooxygenase, thereby reducing the chronic influence associated with diseases such as cancer (Loru 2009; Rodríguez-Ramiro 2011; Bayram 2012).

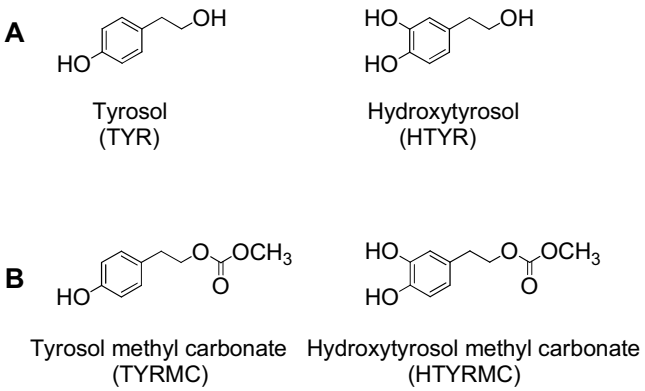

Fig. 1 Natural and synthetic phenolic compounds. (**A**) Phenolic compounds present in virgin olive oil: tyrosol (TYR) and hydroxytyrosol (HTYR). (**B**) Methyl carbonate tyrosol (TYRMC) and methyl carbonate hydroxytyrosol (HTYRMC) derivatized on the alcoholic chain.

More recently, the anti-proliferative and pro-apoptotic properties of HTYR on breast (MDA and MCF-7), prostate (LNCap and PC3) colon (HT-29, SW480 and HCT116) gastric (AGS) and melanoma (M14) cancer cell lines were described (D'Angelo 2005; Corona 2009; Obied 2009; Bernini 2011; Bouallagui 2011). Conversely, the *in vitro* antitumor effects of TYR are rarely described (Ahn 2008; Deiana 2010; Warleta 2011).

Studies in human, animal and cellular systems suggest that phenols from virgin olive oil are capable of inhibiting several stages in carcinogenesis, including metastasis (Hashim 2008). Unfortunately, the bioavailability of TYR and HTYR in humans is poor, and they are found in biological fluids mainly as conjugated metabolites (Khymenets

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2010). Recently, we have synthesized more lipophilic derivatives such as tyrosol methyl carbonate (TYRMC) and hydroxytyrosol methyl carbonate (HTYRMC) by an efficient and ecofriendly procedure (Bernini 2007, 2008, 2009) as illustrated in **Fig. 1B**. These compounds could be a response to the food, cosmetic and pharmaceutical industries increasing demand for new lipophilic antioxidants.

In this study we investigated the *in vitro* antiproliferative effects of TYRMC and HTYRMC compared to those of the corresponding natural compounds TYR and HTYR. The pro-apoptotic properties and cell cycle modulation were also investigated.

MATERIALS AND METHODS

Chemicals and instruments

Reagents and solvents of high analytical grade were purchased from Sigma Aldrich (Milan, Italy). 2-Iodoxybenzoic acid (IBX) was prepared in laboratory as described in the literature (Frigerio 1999). Silica gel 60 F254 plates and silica gel 60 were furnished from Merck (Milan, Italy).

HPLC analyses were performed on a Varian Prostar 325 apparatus equipped with a Varian Pursuit 5 μm C_{18} column (150 × 4.6 mm) and a dual wavelength UV-Vis detector selected on λ= 280 nm. Elutions were carried out at a 1 ml/min flow rate using a H_2O/CH_3CN mixture (90:10, v/v) for the first minute and a gradient to 40:60 within the following 30 min. GC-MS analysis were performed on a Shimadzu VG 70/250S apparatus equipped with a CP-SIL 8 CB-MS column (25 m, 0.25 mm and 0.25 mm film thickness). The analyses were performed using an isothermal temperature profile of 100°C for 2 min, followed by a 10°C/min temperature gradient until 280°C for 15 min. The injector temperature was 280°C. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ (99.8% in deuterium) and in CD_3OD (99.8% in deuterium) using a Bruker 400 MHz spectrometer.

Cell culture

Solid human tumour cell lines H125 (non–small cell lung carcinoma obtained from American Type Culture Collection, Rockville, MD, ATCC), WIDR (colon carcinoma, obtained from ATCC) and M14 (melanoma donated by S. D'Atri IDI IRCCS, Rome, Italy) were maintained in RPMI 1640 Euroclone (H125 and M14) or DMEM Euroclone (WIDR) containing 10% fetal calf serum (Invitrogen, Irvine CA, USA) 1% L-glutamine (Invitrogen) 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma chemical co. St. Louis, MO) hereafter referred as Complete Medium (CM).

Human promyelocitic leukemic cell line (HL-60 obtained from ATCC) employed for the present study was cultured in CM containing RPMI 1640 Medium Euroclone.

All cell lines were maintained in 5% CO_2 in air humidified at 37°C.

Cells were routinely sub-cultured and cells in exponential growth phase were used for all experiments. Cells that were grown as monolayers were detached from cultures by trypsinization. Cells were washed twice with PBS and treated with 0.25% trypsin-EDTA (Invitrogen) for 10 min at 37ºC in 5% CO_2 humidified atmosphere. The trypsinization effect was neutralized with CM. The cells were collected after centrifugation at 1000 rpm for 5 min and washed with PBS for viability assays.

Drug treatment and cell proliferation assay

Aliquots of TYR, HTYR, TYRMC and HTYRMC were stored at –20°C in ethanol (EtOH). The compounds were thawed just before use and diluted in RPMI 1640 to desiderated concentrations. For the inhibition of tumour cell proliferation experiments, the tumour cells HI25, WIDR, H125 and HL60 were placed in a 24-well (Falcon Becton Dickinson) tissue culture plate (1×10^5cells/well) and incubated 3 h at 37°C in 5% CO_2 in CM to permit the adherence of cell growing in monolayer. Than the polyphenols were added to cultures at the concentration of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml. Controls were performed by adjusting the respective EtOH concentration to that of the treated samples. Cells with only RPMI medium were included as controls. All cells were treated with the compounds at 37°C in 5% CO_2 for 24, 48 and 72 h.

Cell proliferation, measured as the cellular growth of treated cells *vs.* untreated controls, was performed after 24, 48 and 72 h of treatment. After trypsinization, the cells were harvested and counted in the presence of Trypan Blue in a Burker chamber. Each experiment was repeated three independent times.

Cell sensitivity to TYR, HTYR, TYRMC and HTYRMC treatment was expressed in terms of IC_{50} (i.e. the compound concentration producing 50% of inhibition cell growth, calculated on the regression line in which the number cells values were plotted against the logarithm of drug concentration).

Cell cycle assay and apoptosis

Tumor cells were seeded in 24-well culture plates at 1×10^5 cells/well and treated with different doses of TYR, HTYR, TYRMC and HTYRMC for 24, 48 and 72 h.

After incubation, 1×10^6cells/ml were washed in cold PBS, fixed with cold 70% ethanol and stored at -20°C for 24 h. At least 1×10^4 events per sample were acquired and analyzed on an FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA USA) after propidium iodide labeling (PI, Sigma) and 100 KU/ml of RNase A (Sigma) exposure. The percent coefficient of variation (CV) relative to G_1 peak distribution was around 3%. The percentage of cells in G0/G1, S and G2/M phases were calculated using ModFitLT software (Becton-Dickinson). Each experiment was repeated three independent times.

Flow cytometry was also used to detect apoptosis in non-treated or TYR, HTYR, TYRMC and HTYRMC treated cells for 24, 48 and 72 h. Cells were then washed in PBS and fixed with 50% acetone/methanol (1/4) in 50% PBS at 4°C for 18 h. After two washes in PBS, the cells were suspended in PBS (1×10^6 cells/ml) containing 50 μg/ml of propidium iodide (PI, Sigma) and 100 KU/ml of RNase A (Sigma) and incubated in the dark at room temperature for 30 min. The DNA content per cell was evaluated by PI fluorescence measured on a linear scale using the FACScan flow cytometer (Becton-Dickinson, San Jose, CA USA) with an argon ion laser emitting at 488 nm. Data collection of 10,000 cells/sample was gated utilizing forward light scatter and side light scatter dot plot to exclude cell debris and aggregates. Apoptotic cells were identified as the sub-G1 hypochromic peak in DNA staining profiles. All data were stored and analysed by using the Cell Quest Software.

Statistical analysis

All test were run in triplicate for each experimental condition and each experiment was repeated at least 3 times; the results are reported as means ± SD. Data were analyzed by a one-way ANOVA with a *post hoc* Tukey's test.

RESULTS

In vitro antiproliferative activity on the human cancer cell lines

Human M14 melanoma cell line and human promyelocitic leukemic cell line HL60 were tested for sensitivity to TYR, HTYR, TYRMC and HTYRMC.

For this purpose, M14 and HL60 cells were incubated with different doses of these compounds at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml, for 24, 48 and 72 h. Cell viability was assessed by trypan blue dye exclusion assay. The results show that all polyphenols induced a time and dose dependent inhibition of M14 cell growth. However, the rate of growth inhibitory effect changed among the different compounds when compared at the same doses (data not shown). After 72 h of treatment the cytotoxic effects significantly increased with all drugs used. The percentages of cell growth inhibition, as illustrated in **Table 1** show that, respect to other compounds HTYRMC induced a marked significantly inhibitory activity at all doses examined reaching 98.2% at a concentration of 100 mcg/ml. Conversely, excluding the dose of 100 mcg/ml, no statis-

Table 1 Percent growth inhibition of M14 melanoma cell line treated with TYR, HTYR, TYRMC and HTYRMC for 72 h of culture.

Treatment	TYR	TYRMC	HTYR	HHTYR MC
12.5 mcg/ml	31.2	38.1	22.4	41.8
25 mcg/ml	40.3	41	31.4	65.9
50 mcg/ml	41.9	47.5	41.2	87.2
100 mcg /ml	55.1	77	81.2	98.2

Effect of TYR, HTYR, TYRMC and HTYRMC on tumor cell growth of M14 melanoma cell line. Cells were cultured in CM or CM containing the indicated concentrations of TYR, HTYR, TYRMC and HTYRMC for 72 h. Control group were treated with EtOH alone. Cell proliferation was determined by the tryptan blue dye exclusion assay. Measurements were done in triplicate. Data are expressed in terms of percent growth inhibition of M14 treated cell line respect to the control. Values were calculated by the mean of three separate experiments

Table 2 Percent growth inhibition of HL60 cell line treated with TYR, HTYR, TYRMC and HTYRMC.

	TYR	TYRMC	HTYR	HTYRMC
24 h				
25 mcg/ml	3.3	15.4	2.7	7.9
50 mcg/ml	9.7	31.9	10.9	19.6
100 mcg/ml	47.5	46.1	34.2	59.6
48 h	**TYR**	**TYRMC**	**HTYR**	**HTYRMC**
25 mcg/ml	3.8	**7.5**	**9.8**	13.9
50 mcg/ml	22.7	20.7	17.1	42.5
100 mcg/ml	41.6	48.4	66.8	79.5
72 h	**TYR**	**TYRMC**	**HTYR**	**HTYR MC**
25 mcg/ml	3.4	12.3	12.7	18.6
50 mcg/ml	13.9	39.8	14. 9	35.8
100 mcg/ml	48.9	55.2	78.3	89.1

Effect of TYR, HTYR, TYRMC and HTYRMC on tumor cell growth of HL60 cell line. Cells were cultured in CM or CM containing the indicated concentrations of TYR, HTYR, TYRMC and HTYRMC for 24, 48 and 72 h. Control group were treated with EtOH alone. Cell proliferation was determined by the trypan blue dye exclusion assay. Measurements were done in triplicate. Data are expressed in terms of percent growth inhibition of HL60 treated cell respect to the control. Values were calculated by the mean of three separate experiments

Table 3 IC_{50} values of TYR, TYRMC HTYR, HTYRMC.

	M14	H125	WIDR	HL60
			HTYR	
24 h	69.6	42	76.7	223.9
48 h	41.7	48.5	42.2	78.9
			HTYRMC	
24 h	55.9*	39.8	46.3**	87.7**
48 h	9.9**	3.4**	39.3	55.2**

Tumor cell lines were treated with HTYR or HTYRMC at concentrations of 3.125, 6.25 12.5, 25, 50 and 100 mcg/ml for 24 and 48 hours. Data are expressed as IC_{50} values, the compound concentration producing 50% of cell growth inhibition calculated on the regression line in which the number cells values were plotted against the logarithm of drug concentration. Data are representative of at least three experiments. Significant differences among IC_{50} value of HTYR and HTYRMC treatments for each tumor cell: * $P < 0.05$ and ** $P < 0.01$

tical differences were observed between TYR and TYRMC treatment.

Similar inhibitory effects were obtained with all polyphenols against HL60 cell line as illustrated in **Table 2**. The results show an "inhibition trend" similar to those observed in M14 cell line. Moreover, they exhibit significant differences in growth inhibition between HTYR and HTYRMC at all doses and times examined. In the case of TYR and TYRMC, the significant differences were observed only using 25 and 50 mcg/ml.

In order to evaluate the individual chemosensitivity to HTYR and HTYRMC, all tumor cell lines were treated with these compounds at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml for 24 and 48 h. The corresponding IC_{50} values are illustrated in **Table 3**. The data show that: a) after 24 h HTYRMC was significantly more effective in inhibiting cell growth of WIDR and HL60 lines; conversely, not significant differences were observed in H125 and M14 cell line; b) after 48 h HTYRMC in M14, H125 and HL60 is significantly more effective in inhibiting tumour growth; c) notably the M14 and H125 cell line show a strong chemosensitivity to HTYRMC.

Cell cycle and apoptosis induction

In order to further investigate the mechanism underlying the anti-proliferative activity of HTYR and HTYRMC the cell cycle distribution has been analysed by flow cytometry. The cell cycle progression was assessed following treatment with 100 mcg/ml of both compounds for 24 h.

As depicted in **Fig. 2**, both HTYR and HTYRMC induced a significant increase in the proportion of cells in the G1 cell cycle phase. The results of a representative experiment illustrated in the figure have been confirmed by further similar experiments. Consequently, HTYR and HTYRMC induced an arrest of tumour lines in the G1 phase with a concomitant reduction in the number of cells in S/G2 phases. More interestingly, the differences between the control and treated cells were statistically significant within all lines treated with HTYR (100 mcg/ml). Furthermore, differences between HTYR and HTYRMC treatments were significant ($P < 0.01$) in M14 and WIDr cell lines that showed a marked inhibition in cell cycle progression. None statistical differences were observed between HTYR and HTYRMC in blocking in G1/S phase the HL60 and H125 cell line.

The effect of HTYR and HTYRMC on induction of apoptosis was examined in all four cancer cell lines M14, WIDR, H125 and HL60. The percentage of apoptosis was determined using a staining assay with propidium iodide (PI). Flow cytometric analysis revealed that treatment with HTYR at the concentration of 50 and 100 mcg/ml for 72 h induce a very limited apoptosis (2-15%) in all examined tumor lines (data not shown).The treatment with HTYRMC at the same concentration of HTYR, 50 and 100 mcg/ml for 72 h induce apoptosis levels significantly higher respect to those obtained with HTYR. **Fig. 3** illustrates the percent increase of apoptosis obtained by the treatment with HTYRMC respect to HTYR used at the same concentration (25 mcg/ml or 50 mcg/ml) for 72 h. These findings, expressed as mean of percent increase, indicate a significant increase of apoptosis induction in all tumor lines examined. This increase is not significant with the treatment of 25 mcg/ml whereas it becomes noticeable and totally significant with 50 mcg/ml of HTYRMC. In particular this effect is considerable on H125 and WIDR cell line in witch the apoptosis induction by HTYR at the same concentration is very low (< 4% and < 6%, respectively).

DISCUSSION

Epidemiological and prospective studies have illustrated the beneficial effects of bioactive compounds that naturally occur in many vegetable foods of the Mediterranean diet (Patil 2009). In particular, some of these studies have shown an inverse correlation between olive oil consumption and incidence of various types of cancers e.g. breast (Trichopoulou 1995), prostate (Hodge 2004), oral cavity (Francheschi 1999), and colon (Braga 1999). Recently, it was hypothesized that the cancer preventive capacity of olive oil could be mediated by the presence of potent antioxidant phenolic compounds that were shown to possess several biological activities (Raederstorff 2009).

Among the different phenols known to be present in olive oil, TYR and HTYR have received particular attention because of their association with the inhibition of both initiation and promotion in carcinogenesis (Cornwell 2008; Hashim 2009). However, they have been reported to have a poor bioavailability in humans since they are found in biological fluids mainly as conjugated metabolites (Khymenets 2010).

In the present study we investigated the antitumor activity of two additional derivatives of TYR and HTYR such as TYRMC and HTYRMC synthesized in our laboratory. These compounds are more lipophilic than TYR and HTYR showing the alcoholic group derivatized by a carboxymethylated moiety. To evaluate and compare the antitumor activity of these four polyphenols, a human melanoma cell

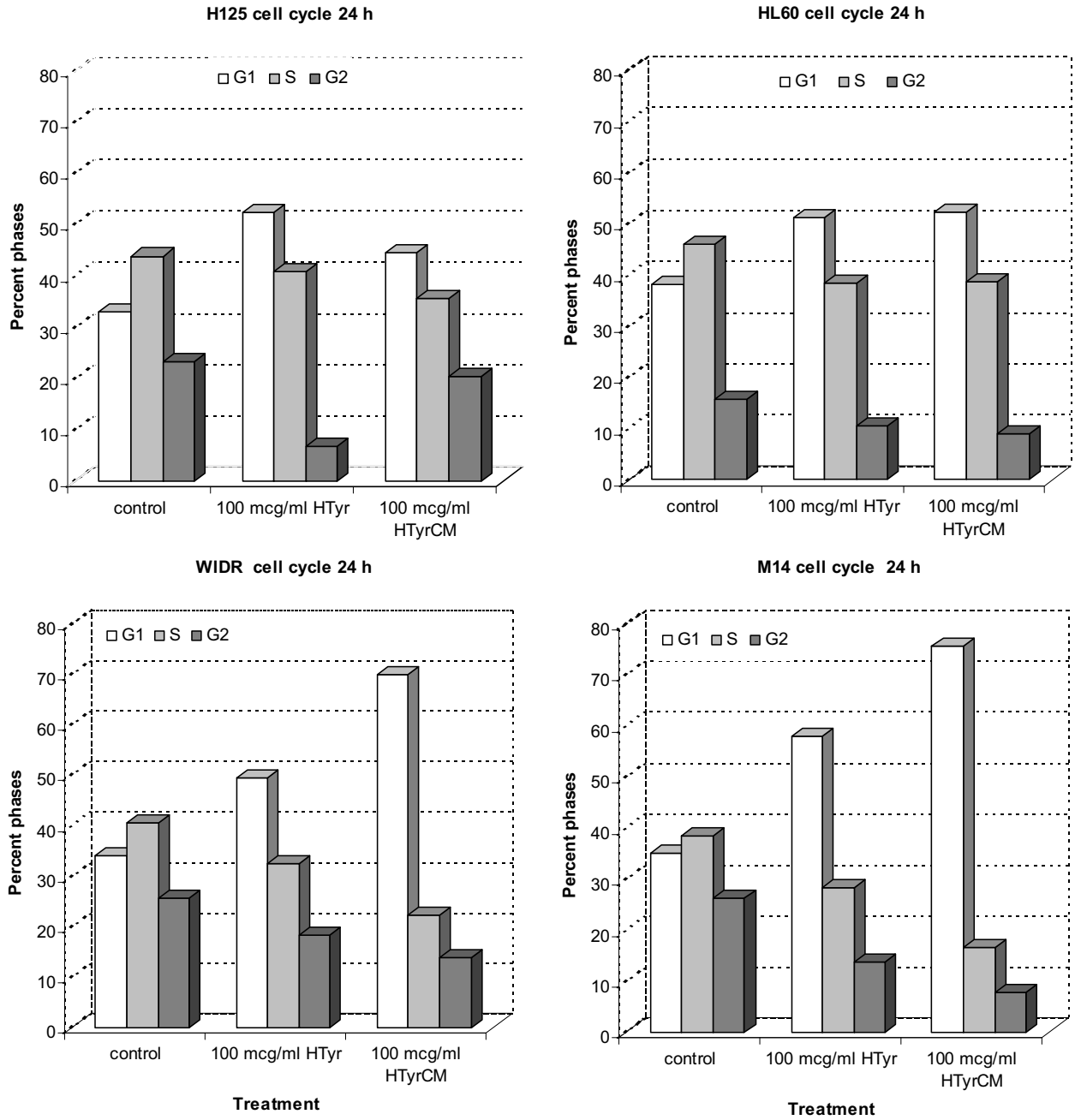

Fig. 2 Effect of treatment with 100 mcg/ml of HTYR or HTYRMC on cell cycle phase distribution of M14, WiDR, H125 and HL60 cell lines. The cells were cultured in CM containing the indicated concentration of HTYR or HTYRMC for 24 h, harvested, stained with PI and processed for flow cytometric analysis of DNA content. Control groups were treated with EtOH alone. Data are representative from at least three independent experiments. The results illustrated in the figure have been confirmed by further similar experiments. The increase of the percentage of cells in G1 cell cycle phase reached a statistical ($P < 0.05$) as shown by student's *t*-test.

line (M14) and a human promyelocitic leukemic cell line (HL-60) were treated for 24, 48 and 72 h with increasing amounts of TYR, HTYR, TYRMC and HTYRMC. The results showed that the growth inhibition increased with concentration and time of exposure to every one polyphenols tested as illustrated in **Table 1** and **Table 2**. After 72 h of exposure the cell growth inhibition reaches more than 60-90%, in particular the HTYRMC exposure showed the most antiproliferative effect. Comparison of the inhibitory effect of HTYRMC respect to HTYR on four tumor cell line (**Table 3**) showed that after 48 h of treatment the IC_{50} of HTYRMC in M14, H125 and HL60 are significantly ($P < 0.01$) lower respect to the IC_{50} of HTYR. Moreover it is interesting to observe that the IC_{50} value is strictly dependent on the cell type used.

Additionally, we observed significant morphological changes in all tumor cells treated with HTYRMC. Cell shrinkage and formation of apoptotic bodies were clearly observed suggesting the cell growth arrest could be related to apoptosis induction (data not shown).

Previous studies demonstrated that HTYR causes growth arrest and apoptosis in various cell line among which M14 (D'Angelo 2005) and HL60 cell lines (Fabiani 2009). Our results (**Fig. 3**) confirm the proapoptotic effect of HTYR and show a significative percent increase of the number cells in apoptosis evaluated in tumor lines treated with HTYRMC for 72 h. Particularly, the percent of increase of number cells in apoptoss depends on the tumor line examined.

Recently published work suggests that the anti-proliferative and pro-apoptotic properties of HTYR on HL60 cells may be mediated by the accumulation of hydrogen peroxide (H_2O_2) in the culture medium (Fabiani 2009). Fabiani (2011) suggest that the culture medium components interfere with H_2O_2-produced by HTYR that are the basis of the proapoptotic mechanism. In our experiments, the cell lines were grown and treated with polyphenols in different culture medium, this could explain the different level of

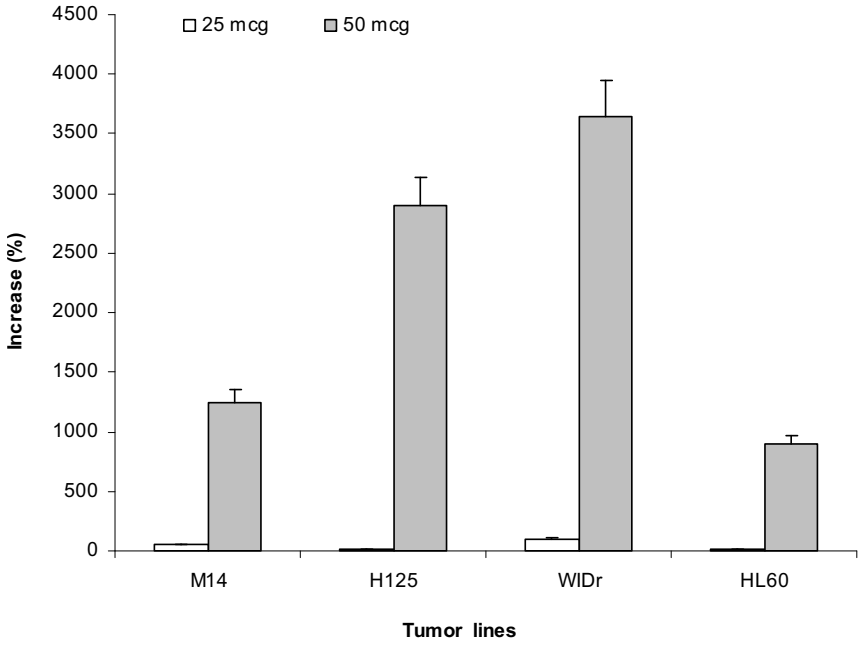

Fig. 3 Apoptosis induction in M14, H125, WiDR and HL60 tumor cell lines treated with 25 or 50 mcg/ml of HTYRMC or HTYR for 72 h. The results are expressed as percent increase (mean of three experiments) of apoptosis induced by 50 mcg of HTYRMC (grey) or 25 mcg of HTYRMC (white) respect to HTYR used at the same concentration for 72 h. The percent increase was calculated as: $= \frac{\text{HTYRMC value- HTYR value}}{\text{HTYR value}} X\ 100$

apoptosis that we founded.

It is reasonable to hypothesize that the differences in the level of apoptosis induction in different cell lines may depend on different mechanisms involved. HTYR interferes with several signalling pathways that control proliferation and apoptosis, among these the FAS inhibition is related to cell line used (Notarnicola 2011).

The inhibition of cell cycle progression by HTYR has been previously described (Rafehi H 2012). The results obtained in our experiment showed that in all line used the treatment with HTYR exhibited statistically significant block of G_1 to S phase transition manifested by the increase of cell number in G_0/G_1 phase. Our results show that only in WIDR and M14 cell line HTYRMC induced a significantly stronger block of the cell cycle at the G_0/G_1 phase respect to those obtained with HTYR.

These results are in agreement with previous results that show that HTYR inhibits the proliferation of HL60 human promyelocytic leukemia cells and alters the cell cycle progression, inducing an accumulation of cells in the G_0/G_1 phase (Fabiani 2002). In contrast, previous studied have been found that HTYR induces growth arrest and apoptosis in human colon carcinoma HT-29 cells and the growth arrest was associated with the accumulation of cells in S and G_2/M phases of the cell cycle (Guichard 2006). The molecular mechanisms by which HTYR interferes with the cell cycle and induces apoptosis on tumour cells are not known; however, it is well known that HTYR is able to modify the expression of important proteins involved in the regulation of these processes. The cell cycle is regulated by the sequential expression of cyclins, which activate various cyclin-dependent kinases (CDK) and by the selective induction of different CDKi (Sherr 1999). It is known that among the different proteins involved in the regulation of the cell cycle, HTYR reduces the level of CDK 6 and increases the activity of cyclin D3. Moreover, HTYR increase the expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ at both protein and mRNA levels (Fabiani 2008).

In addition, the upregulation of numerous antioxidant proteins and enzymes, and the alteration in the expression of transcription factors induced by HTYR may also contribute to its anti-cancer effects described in numerous studies (Rafehi 2012).

In this study we have confirmed that most important phenolic compounds of olive oil show anticancer activity by antiproliferative and proapoptotic activity and that this is detectable in four different cell lines. Our findings may also support the hypothesis that novel compounds might exert a more antitumour activity than the natural compounds by inducing a greater number of cells in cell cycle arrest and apoptosis. This is particularly evident in the case of HTYRMC suggesting a marked and antitumor effect of this synthetic compound.

CONCLUSIONS

This study showed that the phenolic compounds TYR, HTYR, TYRMC and HTYRMC exert a significant antiproliferative effect in different tumor cell lines *in vitro*. Particularly the synthesized methyl carbonate derivatives had the most antitumor activity. However, the subcellular events contributing to HTYR and HTYRMC antiproliferative activity on tumor cells remain completely unknown therefore, further studies are necessary to explore molecular mechanisms of tumor suppressive effect of these derived compounds as well as other olive oil products.

REFERENCES

Ahn EY, Jiang Y, Zhang Y, Son EM, You S, Kang SW, Park JS, Jung JH, Lee BJ, Kim DK (2008) Cytotoxicity of *p*-tyrosol and its derivatives may correlate with the inhibition of DNA replication initiation. *Oncology Reports* **19**, 527-534

Allouche Y, Jiménez A, Gaforio JJ, Uceda M, Beltrán G (2007) How heating affects extra virgin olive oil quality indexes and chemical composition. *Journal of Agricultural and Food Chemistry* **55**, 9646-9654

Bayram B, Ozcelik B, Grimm S, Roeder T, Schrader C, Ernst IM, Wagner AE, Grune T, Frank J, Rimbach G (2012) A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression. *Rejuvenation Research* **15**, 71-81

Bernini R, Crisante F, Merendino N, Molinari R, Soldatelli MC, Velotti F (2011) Synthesis of a novel ester of hydroxytyrosol and α-lipoic acid exhibiting an antiproliferative effect on human colon cancer HT-29 cells. *European Journal of Medicinal Chemistry* **46**, 439-446

Bernini R, Mincione E, Barontini M, Crisante F (2008) Convenient synthesis of hydroxytyrosol and its lipophilic derivatives from tyrosol or homovanillyl alcohol. *Journal of Agricultural and Food Chemistry* **56**, 8897-8904

Bernini R, Mincione E, Crisante F, Barontini M, Fabrizi G (2009) A novel use of the recyclable polymer-supported IBX: An efficient chemoselective and regioselective oxidation of phenolic compounds. The case of hydroxyltyrosol derivatives. *Tetrahedron Letters* **50**, 1307-1310

Bernini R, Mincione E, Crisante F, Fabrizi G, Gentili P (2007) Chemoselec-

tive and efficient carboxymethylation of the alcoholic chain of phenols by dimethyl carbonate (DMC). *Tetrahedron Letters* **63**, 7000-7003

Bouallagui Z, Han J, Isoda H, Sayadi S (2011) Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food and Chemical Toxicology* **49**, 179-184

Braga C, La Vecchia C, Franceschi S, Negri E, Parpinel M, Recarli A, Giocosa A, Trichopoulos D (1998) Olive oil, other seasoning fats, and the risk of colorectal carcinoma. *Cancer* **82**, 448-453

Cornwell DG, Ma J (2008) Nutritional benefit of olive oil: the biological effects of hydroxytyrosol and its arylating quinone adducts. *Journal of Agricultural and Food Chemistry* **56**, 8774-8786

Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, Spencer JP (2009) Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. *Molecular Nutrition and Food Research* **53**, 897-903

D'Angelo S, Ingrosso D, Migliardi V, Sorrentino A, Donnarumma G, Baroni A, Masella L, Tufano MA, Zappia M, Galletti P (2005) Hydroxytyrosol, a natural antioxidant from olive oilprevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. *Free Radical Biology and Medicine* **38**, 908-919

Deiana M, Corona G, Incani A, Loru D, Rosa A, Atzeri A, Paola Melis M, Assunta Dessì M (2010) Protective effect of simple phenols from extravirgin olive oil against lipid peroxidation in intestinal Caco-2 cells. *Food and Chemical Toxicology* **48**, 3008-3016

Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G (2002) Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *European Journal of Cancer Prevention* **11**, 351-358

Fabiani R, Fuccelli R, Pieravanti F, De Bartolomeo A, Morozzi G (2009) Production of hydrogen peroxide is responsible for the induction of apoptosis by hydroxytyrosol on HL60 cells. *Molecular Nutrition and Food Research* **53**, 887-896

Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Morozzi G (2008) Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and with induction of differentiation in HL60 cells. *Journal of Nutrition* **138**, 42-48

Fabiani R, Sepporta MV, Rosignoli P, De Bartolomeo A, Crescimanno M, Morozzi G (2011) Anti-proliferative and pro-apoptotic activities of hydroxyltyrosol on different tumour cells: The role of extracellular production of hydrogen peroxide. *European Journal of Nutrition* **51**, 455-464

Franceschi S, Favero A, Conti E, Talamini R, Volpe R, Negri E, Barzan L, La Vecchia C (1999) Food groups, oils and butter, and cancer of the oral cavity and pharynx. *British Journal of Cancer* **80**, 614-620

Frigerio M, Santagostino M, Sputore S (1999) A user-friendly entry to 2-iodoxybenzoic acid (IBX). *Journal of Organic Chemistry* **64**, 4537-4538

Guichard C, Pedruzzi E, Fay M, Marie JC, Braut-Boucher F, Daniel F, Grodet A, Gougerot-Pocidalo MA, Chastre E, Kotelevets L, Lizard G, Vandewalle A, Driss F, Ogier-Denis E (2006) Dihydroxyphenylethanol induces apoptosis by activating serine/threonine protein phosphatase PP2A and promotes the endoplasmic reticulum stress response in human colon carcinoma cells. *Carcinogenesis* **27**, 1812-1827

Hashim YZ, Rowland IR, McGlynn H, Servili M, Selvaggini R, Taticchi A, Esposto S, Montedoro G, Kaisalo L, Wähälä K, Gill CI (2008) Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells *in vitro*. *International Journal of Cancer* **122**, 495-500

Hodge AM, English DR, McCredie MR, Severi G, Boyle P, Hopper JL, Giles GG (2004) Foods, nutrients and prostate cancer. *Cancer: Causes and Control* **15**, 11-20

Khymenets O, Fitó M, Touriño S, Muñoz-Aguayo D, Pujadas M, Torres JL, Joglar J, Farré M, Covas MI, de la Torre R (2010) Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. *Drug Metabolism and Disposition* **38**, 1417-1421

Kontou N, Psaltopoulou T, Panagiotakos D, Dimopoulos MA, Linos A (2011) The Mediterranean diet in cancer prevention: A review. *Journal Medical of Food* **14**, 1065-1078

Loru D, Incani A, Deiana M, Corona G, Atzeri A, Melis MP, Rosa A, Dessì MA (2009) Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicology Industrial Health* **25**, 301-310

Manach C, Williamson G, Morand C, Scalbert A, Rémésy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition* **81**, 230S-242S

Notarnicola M, Pisanti S, Tutino V, Bocale D, Rotelli MT, Gentile A, Memeo V, Bifulco M, Perri E, Caruso MG (2011) Effects of olive oil polyphenols on fatty acid synthase gene expression and activity in human colorectal cancer cells. *Genes and Nutrition* **6**, 63-69

Obied HK, Prenzler PD, Konczak I, Rehman AU, Robards K (2009) Chemistry and bioactivity of olive biophenols in some antioxidant and antiproliferative *in vitro* bioassays. *Chemical Research in Toxicology* **22**, 227-234

Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B, Bartsch H (2000) Olive-oil consumption and health: The possible role of antioxidants. *The Lancet Oncology* **1**, 7-12

Patil BS, Jayaprakasha GK, Chidambara Murthy KN, Vikram A (2009) Bioactive compounds: Historical perspectives, opportunities and challenges. *Journal of Agricultural and Food Chemistry* **57**, 8142-8160

Raederstorff D (2009) Antioxidant activity of olive polyphenols in humans: A review. *International Journal for Vitamin and Nutrition Research* **79**, 152-165

Rafehi H, Smith AJ, Balcerczyk A, Ziemann M, Ooi J, Loveridge SJ, Baker EK, El-Osta A, Karagiannis TC (2012) Investigation into the biological properties of the olive polyphenol, hydroxytyrosol: Mechanistic insights by genome-wide mRNA-Seq analysis. *Genes and Nutrition* **7**, 343-355

Rodríguez-Ramiro I, Martín MÁ, Ramos S, Bravo L, Goya L (2011) Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress. *Toxicology* **288**, 43-48

Sherr CJ, Roberts JM (1999) CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes and Development* **13**, 1501-1512

Trichopoulou A, Katsouyanni K, Stuver S, Tzala L, Gnardellis C, Rimm E, Trichopoulos D (1995) Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. *Journal of the National Cancer Institute* **87**, 110-116

Visioli F, Poli A, Gall C (2002) Antioxidant and other biological activities of phenols from olives and olive oil. *Medicinal Research Reviews* **22**, 65-75

Warleta F, Quesada CS, Campos M, Allouche Y, Beltrán G, Gaforio JJ (2011) Hydroxytyrosol protects against oxidative DNA damage in human breast cells. *Nutrients* **3**, 839-857