# Antiproliferative, Antioxidant and Anti-inflammatory Effects of Hydroxytyrosol on Human Hepatoma HepG2 and Hep3B Cell Lines

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**Abstract.** *Background. Olive oil intake has been shown to induce beneficial effect on health. This study aimed to investigate the effects of olive oil polyphenol hydroxytyrosol (HT) on cell proliferation and its antioxidant and anti-inflammatory capacity in human hepatoma Hep3B and HepG2 cell lines. Materials and Methods. Cell growth after HT treatment was measured by 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Lipogenic enzyme activity was evaluated by radiochemical assay. Cell total antioxidant activity and cell interleukin-6 (IL-6) levels were measured by enyme linked immunosorbent assay (ELISA) methods. Results. HT caused an evident antiproliferative effect mediated by inhibition of lipogenic enzymes. Moreover, HT induced activation of the cell antioxidant system and reduced cellular IL-6 levels. Conclusion. Our findings provide insights into the mechanisms of action of HT in the context of inhibition of cancer cell proliferation and prevention of oxidative stress in human hepatoma cells. Our results also show a down-regulation of lipogenic enzymes involved in cell proliferation.*

The traditional Mediterranean diet is characterized by high consumption of foods of plant origin, relatively low consumption of red meat and high consumption of olive oil and its products.

Different studies have shown beneficial effects of olive oil on human health. In fact, it has been demonstrated that people who consume virgin olive oil present a lower incidence of breast, endometrial and gastrointestinal cancer (1-3). These beneficial effects were initially believed to be due to the oleic acid content of olive oil; however, there has been a shift in research interest, with a growing focus on other minor components of olive oil, such as the polyphenols (4).

The major phenols identified in olive oils include hydroxytyrosol (HT) and tyrosol, secoiridoids and lignans. HT is a relatively well-characterized olive oil polyphenol (5) and has been shown to be a potent antioxidant, with ability to scavenge oxygen and nitrogen free radicals, to inhibit platelet aggregation, and endothelial cell activation, and to protect against DNA damage (6, 7). Moreover, HT has been shown to have anticancer properties, confirmed *in vitro,* in different cell line studies (8-10). It was reported that HT may exert a pro-apoptotic effect, modulating expression of genes involved in proliferation of promyelocytes (11).

Our previous study (12) showed that the main olive oil polyphenols, HT and oleuropein had antiproliferative effects and induced apoptosis of human colorectal cancer cells by suppressing fatty acid synthase (FAS) activity.

Several alterations of lipid metabolism are often found in tumors, where neoplastic lipogenesis is essential for cancer cell survival (13). Cancer cells esterify fatty acids predominantly to phospholipids, essential components of cell membranes. The main pathway through which proliferating cells gain lipids for membrane synthesis is the endogenous mevalonate pathway. Increased synthesis of mevalonate and mevalonate-derived isoprenoids supports increased cell proliferation through the activation of growth-regulatory proteins and oncoproteins and by promoting DNA synthesis (13, 14).

Expression of FAS and other lipogenic enzymes, such as farnesyl diphosphate synthase (FPPS) appear to play an important role in the pathogenesis and growth of cancer (15, 16). Several studies have demonstrated that drug inhibitors of lipogenic enzymes may provide a feasible tumor-targeting strategy across a wider spectrum of tumors (17).

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*Key Words:* Cell proliferation, oxidative stress, inflammation, olive oil, cancer, lipid metabolism.

However, despite the fact that HT has been linked to cancer prevention, there is little evidence to clarify its effect on liver carcinoma. Olive oil polyphenols are quickly absorbed by the human intestine, but the biotransformation of absorbed HT should take place mostly in the liver.

The present study attempted to provide new insights into the *in vitro* effects of HT on cell proliferation and its antioxidant and anti-inflammatory capacity in human hepatoma Hep3B and HepG2 cell lines as a model system of the human liver.

## Materials and Methods

*Cell culture conditions*. HepG2 and Hep3B cell lines derived from human liver tissue with a well-differentiated hepatocellular carcinoma. HepG2 cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA and Hep3B cells were kindly gifted by professor Brian Irvine Carr. Both cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, in monolayer culture, and incubated at 37˚C in a humidified atmosphere containing 5% $CO_2$ in air. At confluence, the grown cells were harvested by means of trypsinization and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

*HT treatment*. HepG2 and Hep3B cells were seeded at a density of $3\times10^5$ cells/5 ml of DMEM containing 10% FBS in 60-mm tissue culture dishes (Corning Costar Co. Milan, Italy). The cells were incubated for 24 h to allow attachment and then, the medium was replaced by fresh culture medium, containing HT dissolved in 2% methanol, at 30, 80, 100 and 200 μM. Under these experimental conditions, the cells were allowed to grow for 48 and 72 h and then processed for subsequent analyses. Each experiment included an untreated control and a control with the equivalent concentration of methanol (2%). Triplicate cultures were set up for each HT concentration and for controls, and each experiment was repeated three times. Cell viability, determined using the trypan blue exclusion test, always exceeded 90%.

*Assessment of cell proliferation*. After HT treatment, the proliferative response of HepG2 and Hep3B cells was estimated at each time point by the colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. In brief, MTT stock (5 mg/ml in medium) was added to each dish at a volume of one tenth the original culture volume and cells were incubated for 2 h at 37˚C in humidified $CO_2$. At the end of the incubation period, the medium was removed, and blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol). MTT conversion to formazan by metabolically viable cells was monitored by a spectrophotometer, at an optical density of 570 nm.

*FAS activity assay*. FAS enzymatic activity was evaluated in HepG2 and Hep3B treated with HT at different concentrations (30, 80, 100 and 200 μM ) for 48 h. Parallel experiments were conducted in untreated control cells. The cells were washed twice in phosphate buffered saline (PBS) and then trypsinized and centrifuged at 200 g. The cell pellets were resuspended in 20 mM Tris-HCl (pH=7.5), 1 mM dithiotrheitol (DTT), 1 mM $MgCl_2$, and 1 mM EDTA, and centrifuged at 10,000 ×g for 10 min at 4˚C. Sample protein content was determined using Lowry's method (18). An aliquot of supernatant (50 μl) was preincubated with 100 mM potassium phosphate buffer (pH=7) for 15 min at 37˚C. Subsequently, 20 μl of reaction mix [2.5 mM NADPH, 1.25 mM acetyl-CoA, 1.25 mM malonyl-CoA and 0.02 mM 2-$^{14}$C-malonyl-CoA (52 mCi/mmol, Amersham Biosciences, Little Chalfont UK)] was added and samples were incubated for 10 min at 37˚C. Reactions were stopped by the addition of 500 μl of 1 N HCl/methanol (6:4 v:v); fatty acids were extracted with 1 ml of petroleum ether and incorporation of 2-$^{14}$C-malonyl-CoA was analyzed by scintillation counting. FAS activity was expressed as picomoles of incorporated 2-14C-malonyl-CoA per minute per milligram of total protein (pmol/min/mg protein). Parallel samples were assayed to evaluate total and non-specific radioactivity. In all experiments, enzyme assays were carried out in duplicate. The coefficients of intra- and interassay variation were 3% and 4%, respectively.

*FPPS assay*. FPPS activity was evaluated in HepG2 and Hep3B treated with HT at different concentrations (30, 80, 100 and 200 μM) for 48 h. Briefly, the cells were washed twice with 1 ml ice-cold PBS and scraped in 0.2 ml ice-cold lysis buffer (40 mM imidazole and 50 mM dithiotrheitol (DTT)). The cell lysate was centrifuged at 10,000 ×g for 5 min and the supernatant was used for FPPS assay. FPPS was assayed in 150 μl containing 25 mM HEPES, (pH=7), 2 mM $MgCl_2$, 1 mM DTT, 5 mM KF, 1% noctyl-β-glycopyranoside, 3.3 μM [4-$^{14}$C]-isopentenyl pyrophosphate (IPP) (18 Ci/mmol), 3 μM unlabeled IPP and 20 μM geranyl diphosphate. Reactions were started by adding 40 μl of lysate containing 100 μg of total protein and the mixture was then incubated for 45 min at 37˚C. Reactions were stopped by the addition of 150 μl 2.5 N HCl in 80% ethanol containing 100 μg/ml farnesol as a carrier. The samples were hydrolyzed for 30 min at 37˚C to convert the FPP to farnesol and neutralized by the addition of 150 μl of 10% NaOH. The reaction product (farnesol) was extracted into 1 ml of n-hexane and an aliquot (200 μl) of the organic phase was used for radioactivity counting. One unit of enzyme activity is defined as the amount of enzyme required to synthesize one pmol of FPP per min. Parallel samples were assayed to evaluate the total and the non-specific radioactivity. In all experiments, enzyme assays were carried out in duplicate. The coefficients of intra- and inter-assay variation were 3% and 4%, respectively.

*Total antioxidant activity assay*. After HT treatment for 48 h, total antioxidant activity assay was measured using a commercially available Antioxidant Assay Kit (Cayman Chemical Company Ann Arbor, USA), according to the manufacturer's recommendations. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin. The suppression of the absorbance, measured at 750 nm, is proportional to the concentration of the antioxidants, which is expressed relative to trolox, a water-soluble tocopherol analog, and is quantified as molar trolox equivalents.

Briefly, the cells were washed twice with 1 ml ice-cold PBS and centrifuged at 10,000 ×g for 5 min. The cell pellet was homogenized in 5 mM potassium phosphate (pH=7.4), containing 0.9% sodium chloride and 0.1% glucose and centrifuged at 10,000 ×g for 15 min at 4˚C. The supernatant obtained was used for total antioxidant activity assay. Trolox standard and cell samples were pipetted into the wells of a plate in duplicate. Metmyoglobin and chromogen

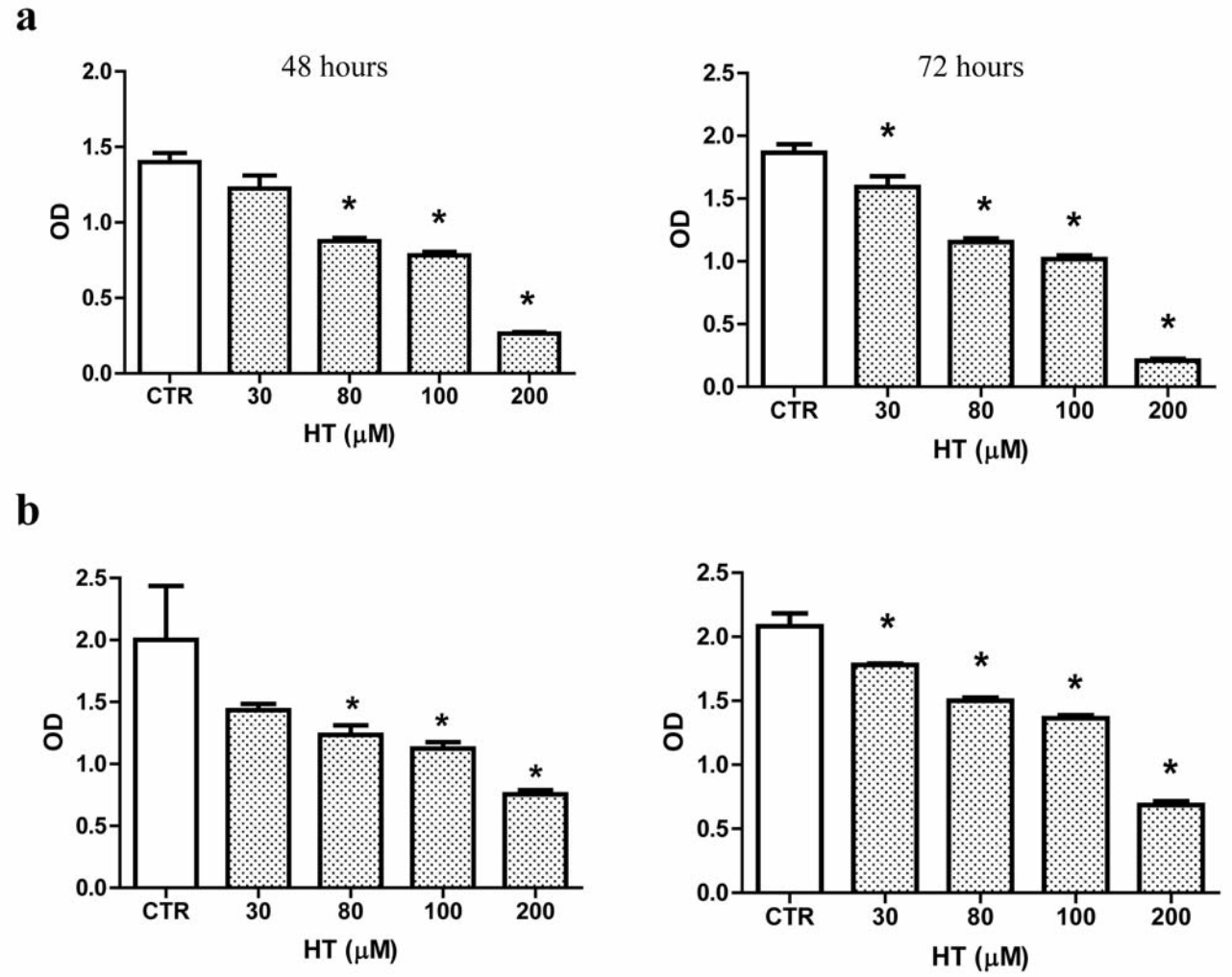


Figure 1. *Effect of increasing hydroxytyrosol (HT) concentration on the conversion of 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in HepG2 (a) and Hep3B (b) cells at 48 and 72 h. All data are the mean±SE of three consecutive experiments. Significance was determined by ANOVA with Dunnett's post hoc test. *p<0.05 versus control.*

were added and the reactions were started by adding of hydrogen peroxide working solution to all the wells being used. The plate was covered and then incubated for 5 min at room temperature on a shaker. The amount of trolox produced was monitored by reading the absorbance at 750 nm using a plate reader. The assay was performed in duplicate. The intra and inter-assay coefficients of variation were 3.4% and 3%, respectively.

*Interleukin-6 (IL-6) determination*. Detection and quantitative measurement of human IL-6 in cell culture supernatants were performed by the AviBion Human IL-6 ELISA kit (Orgenium Laboratories, Vantaa, Finland) following the manufacturer's recommendations. The assay uses an antibody specific for human IL-6 coated on a 96-well plate. Briefly, after HT treatment for 48 h, the cells were washed twice with 1 ml ice-cold PBS and centrifuged at 10,000 ×*g* for 5 min. The cell pellet was resuspended in 0.4 mM of phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100, vortexed and centrifuged at 10,000 ×g for 15 min at 4˚C. The supernatant obtained was used for IL-6 determination. Standards, sample diluent (blank), cell samples and biotinylated anti-human IL-6 antibody were pipetted into the wells and the plate was incubated for 90 min at room temperature. IL-6 present in the samples were captured by the antibodies immobilized on the wells and by the biotinylated specific detection antibodies. The plate was then washed five times with wash buffer. Streptavidin-horseradish peroxidise conjugate was pipetted into each well and the plate was incubated for 30 min at room temperature and the wash was repeated. Tetramethyl-benzidine substrate was pipetted into each well and the color development was stopped by adding stop solution. The absorbance value was read at 450 nm using a spectrophotometer and human IL-6 concentrations were determined by interpolation from the standard curve. The expressed data were the mean of two determinations of the sample. Sensitivity of the test was <7 pg/ml, and the intra and inter-assay coefficients of variation were ≤9.4% and ≤8.6%, respectively.

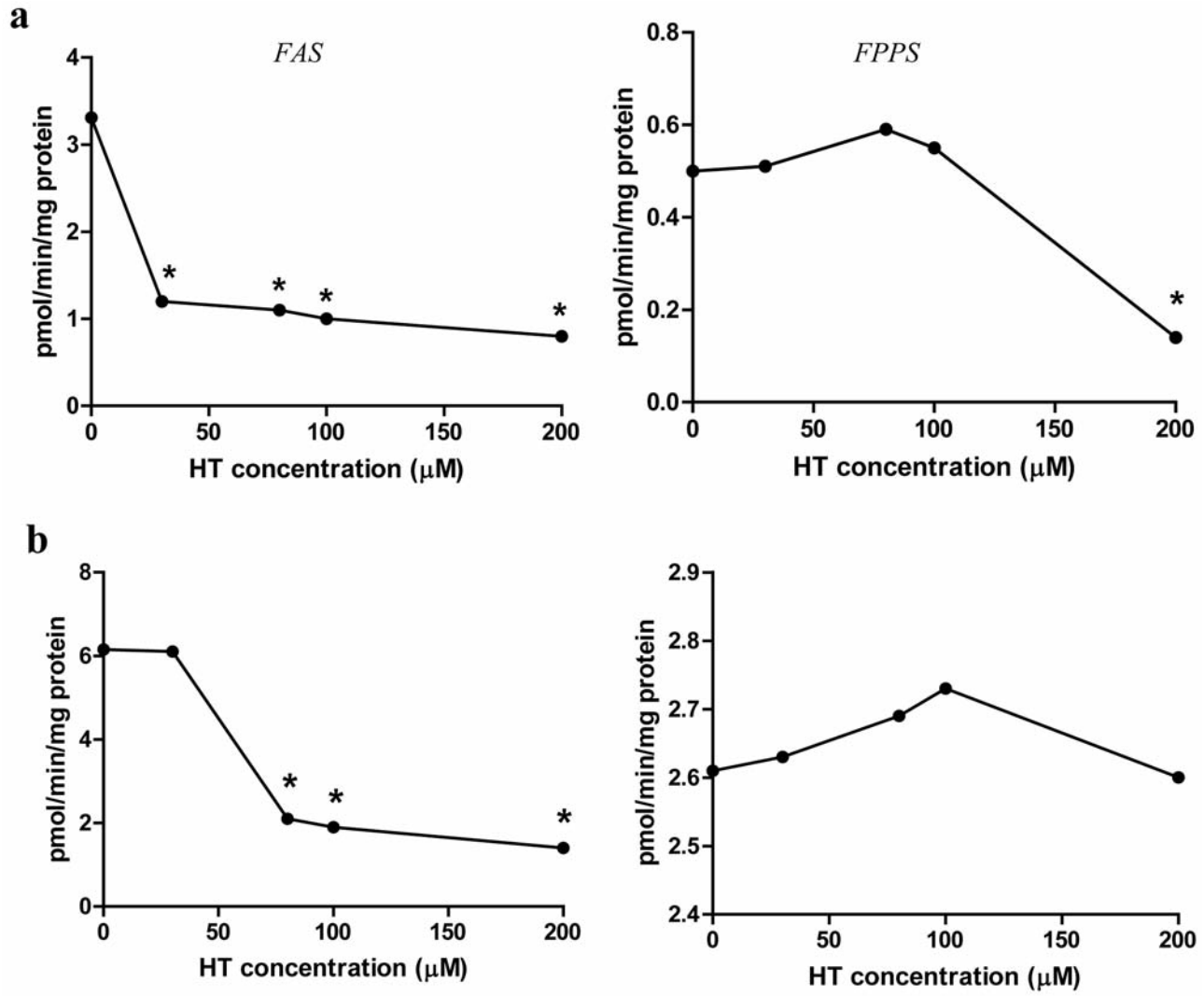


Figure 2. *Effects of hydroxytyrosol (HT) on fatty acid synthase (FAS) and farnesyl diphosphate synthase (FPPS) activity in HepG2 (a) and Hep3B (b) cells. Enzyme activity levels are expressed as pmol/min/mg of total protein. Significance was determined by ANOVA with Dunnett's post hoc test. *p<0.05 versus control.*

*Statistical analysis*. The significance of the differences between the control group *versus* each experimental group (concentration) was evaluated with one-way analysis of variance (ANOVA) and Dunnett's post hoc test. Differences were considered significant at a 5% probability level.

## Results

Exposure of HepG2 and Hep3B cells to increasing concentrations of HT (from 30 to 200 μM) caused an evident antiproliferative effect, in a dose- and time-dependent manner. For both cell lines, after 48 h of treatment the antiproliferative effect was statistically significant from concentrations of 80 μM ($p<0.05$, one-way ANOVA test and Dunnett's post hoc test) (Figure 1a and b). Treatment with HT for 72 h showed a statistically significant reduction, in cell proliferation, even at 30 μM for HepG2 and Hep3B cells (Figure 1a and b).

The inhibition of proliferation detected after HT treatment was mediated by an inhibition of FAS activity in both cell lines. FAS inhibition was statistically significant at 80 μM HT for Hep3B cells (Figure 2b) and at 30 μM for HepG2 cells (Figure 2a). Moreover, the antiproliferative effect exerted by HT in HepG2 cells seems to involve FPPS activity only at higher concentrations of HT (200 μM) (Figure 2a). In Hep3B cells, HT inhibited cell proliferation with a mechanism that did not appear to involve FPPS activity (Figure 2b).

In HepG2 cells, treatment with different concentrations of HT for 48 h induced the cell antioxidant system in a dose-

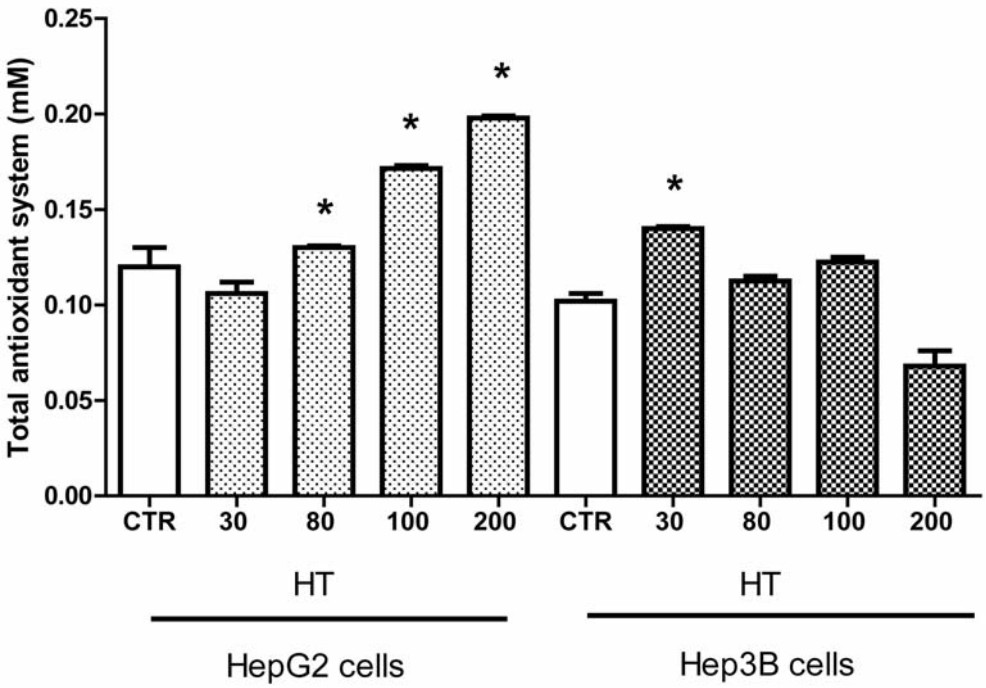


Figure 3. *Effects of hydroxytyrosol (HT) on the total antioxidant system in HepG2 and Hep3B cells. All data represent the mean±SE of three consecutive experiments. Significance was determined by ANOVA with Dunnett's post test. *p<0.05 versus control.*

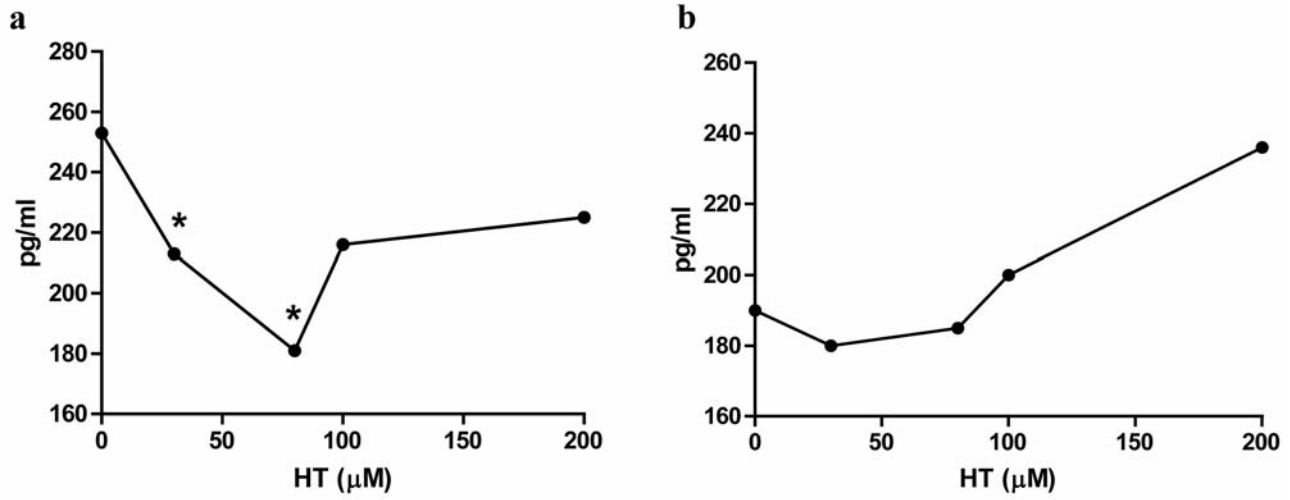


Figure 4. *Levels of interleukin-6 (IL-6) after treatment with increasing concentrations of hydroxytyrosol (HT) in HepG2 (a) and Hep3B cells (b) cells. Data represent the mean value of three consecutive experiments. Significance was determined by ANOVA with Dunnett's post hoc test. *p<0.05 versus control.*

dependent manner, starting from 80 μM. In Hep3B cells, HT treatment only significantly increases the activity of the cell antioxidant system at 30 μM (Figure 3).

In these hepatoma cells, HT induced a reduction of cellular IL-6 concentration. The anti-inflammatory activity of HT was more evident in HepG2 cells, where the effect was maintained up to 80 μM of HT. After cell treatment with high doses of HT, IL-6 levels increased in both HepG2 and Hep3B cells, probably due to cellular toxic effect (Figure 4 a and b).

## Discussion

It is generally accepted that HT is an extremely potent antioxidant, which induces cell death and apoptosis (9, 11).

In accordance to this acceptance, our findings show that HT caused an evident antiproliferative effect in a dose- and time-dependent manner in two human hepatoma cell lines and regulated the activity of enzymes associated with cell proliferation, and the antioxidant system, and a cytochine involved in inflammation.

This study points out that the intake of molecules with phenolic structure such as HT contained in extra virgin olive oil may contribute to a chemopreventive action through rapid modification of mitogenic signals. The antiproliferative effect of HT detected in HepG2 and Hep3B cells was mediated by down regulation of FAS and FPPS activity. The overexpression and increased activity of lipogenic enzymes have been recognized as some of the most frequent molecular alterations in cancer cells (19, 20). Enhanced endogenous lipogenesis, involving FAS, may represent a previously unrecognized oncogenic stimulus that drives progression of human epithelial cells towards malignancy (21). Importantly, high levels of FAS positively correlate with aggressive behavior, and a poorer prognosis in various types of cancer (15).

Several lines of evidence show increased FPPS activity in tumor tissues, suggesting its possible contribution to the neoplastic process (22, 23) and since an FPP and FAS-related lipogenic phenotype emerges early in carcinogenesis, it might represent a possible target for antineoplastic interventions. Previously, we demonstrated that activity of FAS and FPPS, as well as the expression of their mRNAs is up-regulated in colorectal cancer tissue (16, 24).

In light of this evidence, the inhibition of FAS and FPPS represents an effective means of reducing the cell proliferation rate and inducing apoptosis ofcancer cells. In our experiments, the inhibition of cell proliferation detected in HepG2 and Hep3B cell lines after HT treatment reinforces the role of lipogenic enzymes in the regulation of hepatic cellular metabolic processes, offering a mechanism for olive oil-related cancer preventive effects.

Olive oil is believed to exert its biological benefits mainly *via* the antioxidant activity of polyphenols. Here, we detected an increase in total cellular antioxidant activity after HT treatment. In HepG2 cells, the effect of HT on the cell antioxidant system was dose-dependent, whereas in Hep3B cells, the effect was independent of HT concentration. Moreover, in Hep3B cells, the HT dose of 200 μM led to a reduction of total antioxidant activity. Certain studies (10, 25) have demonstrated that phenolic compounds in olive oil can act not only as antioxidant but also as pro-oxidants, thus generating ROS. It is interesting to note that oleuropein at high doses was found to have cytotoxic activity in a prostate cancer cell line (3), involving oxidative stress.

It has been reported that the prolonged activation of inflammatory cells generates ROS which can damage tissues and contribute to carcinogenesis. Our results show that IL-6 levels increase with high doses of HT both in HepG2 and Hep3B cells. Persistent inflammation, which can be mediated by IL-6, facilitates the production of inflammatory oxidants, inducing oxidative stress.

In conclusion, the results reported in this study provide insights into the mechanisms of action of the HT in the context of inhibition of cancer cell proliferation and prevention of oxidative stress and inflammation in human hepatoma cells. These results significantly highlight a down-regulation of enzymes involved in cell proliferation. This indicates that as well as being an antiproliferative agent, HT promotes the cells own defences against oxidative stress.

## Acknowledgements

The Authors would like to thank Professor Brian I. Carr (Laboratory of Cellular and Molecular Biology, National Institute for Digestive Diseases, Castellana Grotte, Bari, Italy) for kindly providing the Hep3B cell line.

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*Received September 12, 2012*
*Revised October 18, 2012*
*Accepted October 19, 2012*