# Inhibition of p38/CREB activity and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects

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**Abstract**

We investigated the anti-proliferative effects of an olive oil polyphenolic extract on human colon adenocarcinoma cells. Analysis indicated that the extract contained hydroxytyrosol, tyrosol and the various secoiridoid derivatives, including oleuropein. This extract exerted a strong inhibitory effect on cell proliferation, which was linked to the induction of a G2/M phase cell cycle block. Following treatment with the extract (50 µg/ml) the number of cells in the G2/M phase increased to 51.82 ± 2.69% relative to control cells (15.1 ± 2.5 %). This G2/M block was mediated by the ability of olive oil polyphenols (50 µg/ml) to exert rapid inhibition of p38 (38.7 ± 4.7%) and CREB (28.6 ± 5.5%) phosphorylation which led to a downstream reduction in COX-2 expression (56.9 ± 9.3 %). Our data suggest that olive oil polyphenols may exert chemopreventative effects in the large intestine by interacting with signalling pathways responsible for colorectal cancer development.

Keywords: Olive oil; polyphenol; colon cancer; large intestine; MAPK kinase; COX-2

## Introduction

The regular consumption of olive oil has been suggested to be one of the factors associated with a lower incidence of coronary heart disease and certain cancers, in particular colon cancer, in Mediterranean countries compared to those in Northern Europe [1]. For example, olive oil consumption has been shown to reduce the incidence of aberrant crypt foci in azoxymethane-treated rats [2] and dimethyl-benz(a)antracene-induced mammary carcinogenesis [3]. Furthermore, olive oil intake has been shown to induce significant levels of apoptosis in large intestinal cancer cells [4]. These anti-cancer properties [5] are thought to be mediated by phenolic compounds present in olive oil, such as hydroxytyrosol [6], lignans [7] and secoiridoids [8]. For example, a polyphenolic fraction extracted from olive oil has been shown to inhibit human promyelocytic leukemia cell (HL60) proliferation by inducing apoptosis and differentiation [9]. Although the exact mechanism by which olive oil exerts its anticancer action is unknown, the down-regulation of COX-2 and Bcl-2 expression, which plays a critical role in colorectal carcinogenesis, has been suggested [4].

The biological properties of olive oil polyphenols *in vivo* will depend on the extent of their absorption and metabolism in the gastrointestinal tract. We have recently shown that although hydroxytyrosol and tyrosol are absorbed and metabolised in the small intestine, oleuropein, a major olive oil polyphenol, is not transferred across the jejunum or ileum [10]. Instead secoiridoids, such as oleuropein, reach the large intestine where they are degraded by the microflora to yield hydroxytyrosol and may express biological activity towards large intestinal cells [10]. The cellular mechanisms by which olive oil polyphenols may express anticancer activities in the large intestine are poorly understood, although they may act by modulating the activity of COX-2, as has been reported for other polyphenols [11]. COX-2 is over-expressed in colorectal cancer, and this over-expression has a strong association with colorectal neoplasia, by promoting cell survival, cell growth, migration, invasion and angiogenesis [12; 13]. In addition, the MAPK signalling pathway, which is known to regulate COX-2 activity [14], has long been viewed as an attractive pathway for anticancer therapies, due to its central role in regulating the growth and survival of cells from a broad spectrum of human cancers [15]. The aim of this study was to investigate the potential anti-proliferative effects of an olive oil polyphenolic extract on human colonic cancer cells. We show that their ability to inhibit cancer cell proliferation is associated with their potential to induce G2/M cell cycle block via the inhibition of p38 and CREB activity and downstream COX-2 expression.

**Material and methods**

*Materials:* The polyphenolic extract was kindly extracted by Professor Visioli and obtained by processing extra virgin olive oil by methanol/water extraction as previously described [16]. All the primary antibodies used, anti phospho-p38 MAP Kinase ($Thr^{180}/Tyr^{182}$) Ab, anti total-p38 MAP Kinase Ab, anti phospho-CREB ($Ser^{133}$) Ab, anti total-CREB Ab and anti COX-2 Ab, were from Cell Signalling (New England Biolabs, Hertfordshire UK). Pre-stained molecular-mass markers were purchased from Biorad (Hemel Hempstead, UK). Anti-Bromodeoxyuridine Ab was from Becton Dickinson (Cowley, Oxford UK). Secondary horseradish peroxidase-conjugated goat anti-rabbit Ab, secondary rabbit anti-mouse FITC Ab, Ponceau Red solution, Sulforhodamine B based assay kit, 5-Bromo-2′-deoxyuridine (BrdU), RNAse and propidium iodide were from Sigma (Poole, Dorset UK). ECL reagents were from Chemicon (Millipore, Watford UK). Hyperfilm-ECL and nitrocellulose membrane were from Amersham Biosciences (Chalfont St.Giles UK). All other reagents used were obtained from Sigma.

*HPLC analysis:* HPLC analysis was carried out with an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD) linked to the HP ChemStation Software system as previously described [10]. Calibration curves of the commercially available compounds were constructed using authentic standards (0.1 to 100μM) and in each case were found to be linear with correlation coefficients of >0.995.

*Cell culture and proliferation:* Caco-2 cells (ECACC Salisbury, Wiltshire UK) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% heat-inactivated bovine serum, 2mM L-glutamine, 1% nonessential amino acids, 100U/ml penicillin, and 100μg/ml streptomycin. The ability of the olive oil polyphenol extract to inhibit cellular proliferation was tested using the sulforhodamine B (SRB) assay. Cells were seeded in 24-well plates ($2.5 \times 10^4$ per well) and low confluence cells (8h after seeding) were exposed to the olive oil polyphenolic extract (10-100μg/ml) or vehicle (methanol 2%). Cells were harvested following 24h, 48h, 72h and 96h in culture and fixed by the addition of 125μl ice-cold TCA (10% final concentration; 4°C; 1h). After fixing, media was removed, cells were washed and total biomass was determined using SRB (250μl of 0.4% SRB; 0.5h). Unincorporated dye was discarded by washing with 1% acetic acid, whilst cell incorporated dye was solubilised using Tris Base (10mM). Dye incorporation, reflecting cell biomass, was measured at 492nm, using a GENios microplate reader (TECAN, Reading UK).

*Cell cycle analysis:* Caco-2 cells were seeded on 6 well plates ($1 \times 10^5$ cells/well), grown until 80% confluence and treated with the polyphenol extract (10 and 50μg/ml; 24h). The cells were analyzed to ascertain the cell cycle phase distribution with the two-colour flow cytometric method described by Poolman and Brooks [17]. Samples were analysed by flow cytometry using a FACS Calibur benchtop flow cytometer (Becton-Dickinson, Oxford UK) equipped with a 15mW blue argon laser source (excitation wavelength: 488nm). The percentages of the cells in G0/G1, S and G2/M phases were assessed using the FL3-A channel (675nm), and the number of cells was sorted (count) at 200 for the G0/G1 population, at 400 for the G2/M and the S phase in between using CellFIT Cell-Cycle Analysis Version 2.0.2 software.

*Western Immunoblotting:* Caco-2 cells ($1 \times 10^5$ cells/well) were grown on 6-well plates (80% confluent) and were exposed to either the polyphenol extract (10-100μg/ml) or to vehicle (2% methanol) for 15 min for p38 and CREB phosphorylation or for 24h for COX-2 expression. The Bradford assay was performed to assess protein content and Western immunoblotting was performed as described previously [18]. Samples containing 20μg of protein were run on 9% SDS-acrylamide gel and proteins were transferred to nitrocellulose membranes by semi-dry electroblotting ($1.5mA/cm^2$) . Blots were incubated with anti phospho-p38 MAP Kinase ($Thr^{180}/Tyr^{182}$) Ab (1:1000 dilution), anti total-p38 MAP Kinase Ab (1:1000 dilution), anti phospho-CREB ($Ser^{133}$) Ab (1:1000 dilution), anti total-CREB Ab (1:1000 dilution) and anti COX-2 Ab (1:1000 dilution) in TTBS containing 1%(w/v) skimmed milk powder. Blots were exposed to hyperfilm for 2 minutes in an autoradiographic cassette and developed, before protein bands were quantified using Quantity One software. For p38 and CREB analysis, the relative band intensity of the phosphorylated form was divided by the relative band intensity of the total form to obtain the ratio phospho/total.

*Statistical analysis:* Data are expressed as means±S.D. Statistical analysis was performed using Graph Pad Instat software by one-way ANOVA followed by Student's t-test with a confidence level of 95% to identify significantly different means. Significance level was set at $p < 0.05$.

**Results**

*Polyphenolic content of the olive oil extract:* Chromatographic separation of the olive oil polyphenolic extract by reverse phase HPLC-DAD (Figure 1) revealed the presence of the phenolic alcohols, hydroxytyrosol (6.3%, Peak 1) and tyrosol (3.0%, Peak 2), and the secoiridoid oleuropein (16.3%, Peak 3), which were

identified and quantified by retention time and spectra comparison with authentic phenolic standards. The extract also contained other secoiridoid derivatives, eluting at 40-60min RT (combined peaks identified by 4).

*Assessment of cytotoxicity and cell cycle:* Significant inhibition of Caco-2 cell growth was observed following exposure to the polyphenolic extract, as indicated by reductions in cell biomass determined by the SRB assay (Figure 2). Growth inhibition was statistically significant for all concentrations tested (10-100µg/ml) at 48h, 72h and 96h and for 50-100 µg/ml at 24h. Untreated sub-confluent cells revealed a consistent cell cycle phase distribution of cells in G2/M (15.1 ± 2.5 %), G0/G1 (82.8 ± 4.2 %) and S (1.9 ± 1.7 %) phases (Figure 3). Treatment with the polyphenol extract (10 µg/ml) had no significant effect on cell cycle distribution whilst 50µg/ml significantly blocked the cell cycle at the G2/M phase (51.82 ± 2.69), with a concomitant reduction in the number of cells on G0/G1 phase (40.86 ± 3.02), and did not significantly alter S phase (7.31 ± 5.03) (Figure 3).

*COX-2 expression, p38 and CREB:* COX-2 expression was dose dependently reduced following exposure of cells to the polyphenol extract (10-100µg/ml) for 24h (Figure 4A). The reduction in COX-2 expression was significant at concentrations of 50-100 µg/ml ($p<0.001$), with COX-2 levels reduced by 56.9 ± 9.3 % relative to basal levels in response to the 50 µg/ml exposure. The phosphorylation state of p38, which was probed using a phospho-specific antibody that recognizes the dually phosphorylated motif $Thr^{180}$-Pro-$Tyr^{182}$ within activated p38, was also observed to decrease in a dose-dependent manner in response to the extract (10-100 µg/ml; 15min) (Figure 4B). Inhibition of p38 activation was significant at concentrations of 50 µg/ml (38.7 ± 4.7 %) and higher. Parallel immunoblots with an antibody that detects total levels (non phosphorylated and phosphorylated p38) indicated that there were no significant changes in total p38. The phosphorylation state of CREB, which was determined by probing membranes with a phospho-specific antibody which recognises the phosphorylated motif $Ser^{133}$, was also observed to be reduced in a dose-dependent manner in response to the polyphenol extract (10-100 µg/ml; 15min) (Figure 4C). The ratio of phospho/total CREB, reflecting its activation, was significant at concentrations of 50 µg/ml (28.6 ± 5.5 %) and above.

**Discussion**

Olive oil consumption has been proposed to contribute to the lower incidence of large intestinal cancer in Mediterranean countries [1]. It is known to contain a variety of phenolic compounds (phenolic alcohols, lignans, secoiridoids), which are thought to be responsible for its reported anti-carcinogenic effects [5]. The

unbalanced control of cellular proliferation is a primary characteristic of cancer cells. Therefore, compounds which are able to inhibit cancer cell proliferation may be useful as chemopreventive and/or chemotherapeutic agents. In the present study, we demonstrate that a mixture of phenolic compounds, representative of those that reach the large intestine, significantly inhibit the proliferation of colonic cancer cells. This reduction in cancer cell growth was mediated by the induction of a G2/M phase cell cycle block, which is in agreement with previous studies showing that hydroxytyrosol, a polyphenol rich in our extract, induces inhibition of the proliferation of human colon carcinoma cells [6; 19]. Other polyphenols, such as flavan-3-ols and their nitrosated derivatives have also been found to express potent anti-proliferative effects in human colon adenocarcinoma cells [20]. These findings are significant as whilst simple olive oil polyphenols are only partially absorbed in the small intestine, resulting in plasma concentrations of hydroxytyrosol, tyrosol and related metabolites of 1–2 μM [21; 22], the large intestinal epithelium is exposed to higher concentrations of a variety of polyphenols, such as hydroxytyrosol and various secoiridoid derivatives [23].

The cellular mechanisms by which polyphenols exert anti-proliferative effects are poorly understood. Our results indicate that olive oil polyphenols induce a cell cycle block in the G2/M phase in colonic adenocarcinoma cells and that this inhibition of proliferation may be mediated by an inhibition of COX-2. This is in agreement with previous studies which show that polyphenols present in red wine and black tea also inhibit colonic cancer cell growth via a mechanism involving COX-2 [11]. COX-2 expression is known to be regulated in a cell cycle-dependent manner and mRNA levels of COX-2 are lower when cells accumulate in the G2/M phase [24]. Furthermore, whilst COX-1 is constitutively expressed in the majority of tissues, COX-2 expression is induced by many different stimuli including mitogens and tumour promoters in a discrete number of cell types [25]. COX-2 is aberrantly over-expressed in many human cancers, most notably of colonic origin, and has been demonstrated to play a role in tumour progression and metastasis [12]. Multiple studies have revealed a role for COX-2 inhibitors in decreasing the risk of colon cancer development and in suppressing tumour formation and growth. Thus, COX-2 is considered an important therapeutic target for cancer prevention [26] and its inhibition has been demonstrated for a number of other dietary components, such as carotenoids [27], celecoxib, [28], selenomethionine [29] and curcumin [30]. In agreement with our data, previous studies have indicated that tyrosol and oleocanthal (the dialdehydic form of (-)-deacetoxy-ligstroside aglycone), may also inhibit COX-2 activity [31; 32].

The regulation of COX-2 gene expression in a variety of tissues is controlled by signalling through a number of pathways [14], including the mitogen-activated protein kinase (MAPK) pathway [14]. MAPK kinase enzymes, including p38 MAPK, p42/44 MAPK, and c-Jun N-terminal kinase (JNK), have been implicated in the regulation of COX-2 gene expression in a variety of tissues, including the large intestine [33] and are involved in both transcriptional and post-transcriptional regulation of COX-2 [33]. The control of COX-2 transcription is regulated by p38 and/or other signalling pathways through the activation of transcription factors, such as cyclic AMP response element binding protein (CREB), NF-κB, C/EBP, NFAT or AP-1 [14]. We demonstrate that the olive oil polyphenols are capable of down-regulating COX-2 expression in colonic cancer cells by a mechanism involving the early inhibition of p38 and downstream inhibition of the transcription factor CREB. These observations agree with other Caco-2 cells studies which demonstrate that inhibition of p38 significantly reduces COX-2 expression [34]. The induction of COX-2 via the p38/CREB pathway has been also demonstrated in human non-transformed colonocytes exposed to *Clostridium difficile* toxin A, which induces reactive oxygen species production and COX-2 induction. We suggest that the anti-proliferative effects of olive oil polyphenols, representative of those reaching the large intestine, may be mediated, in part, by the inhibition of p38 and CREB phosphorylation/activation leading to a reduction in COX-2 expression. Such a mechanism is likely to contribute to the anticancer activity of olive oil in the large intestine.

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**Figure 1**

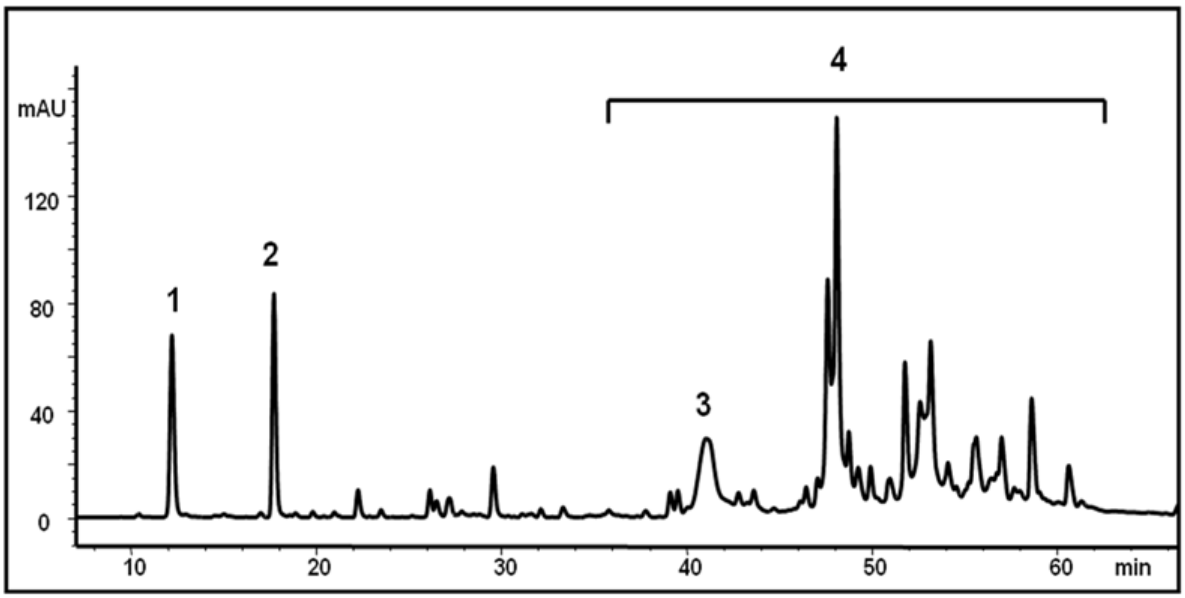

**Figure 2**

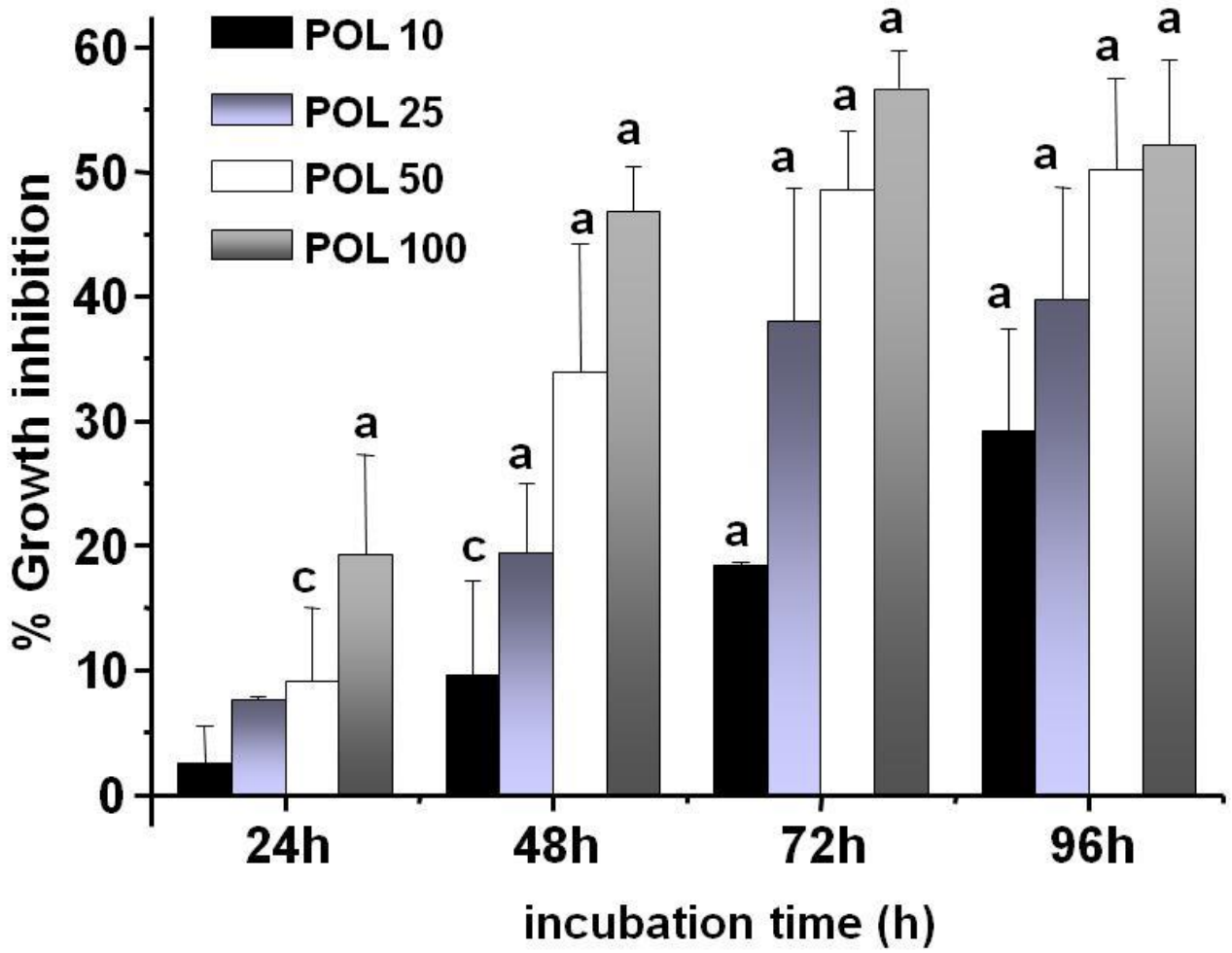
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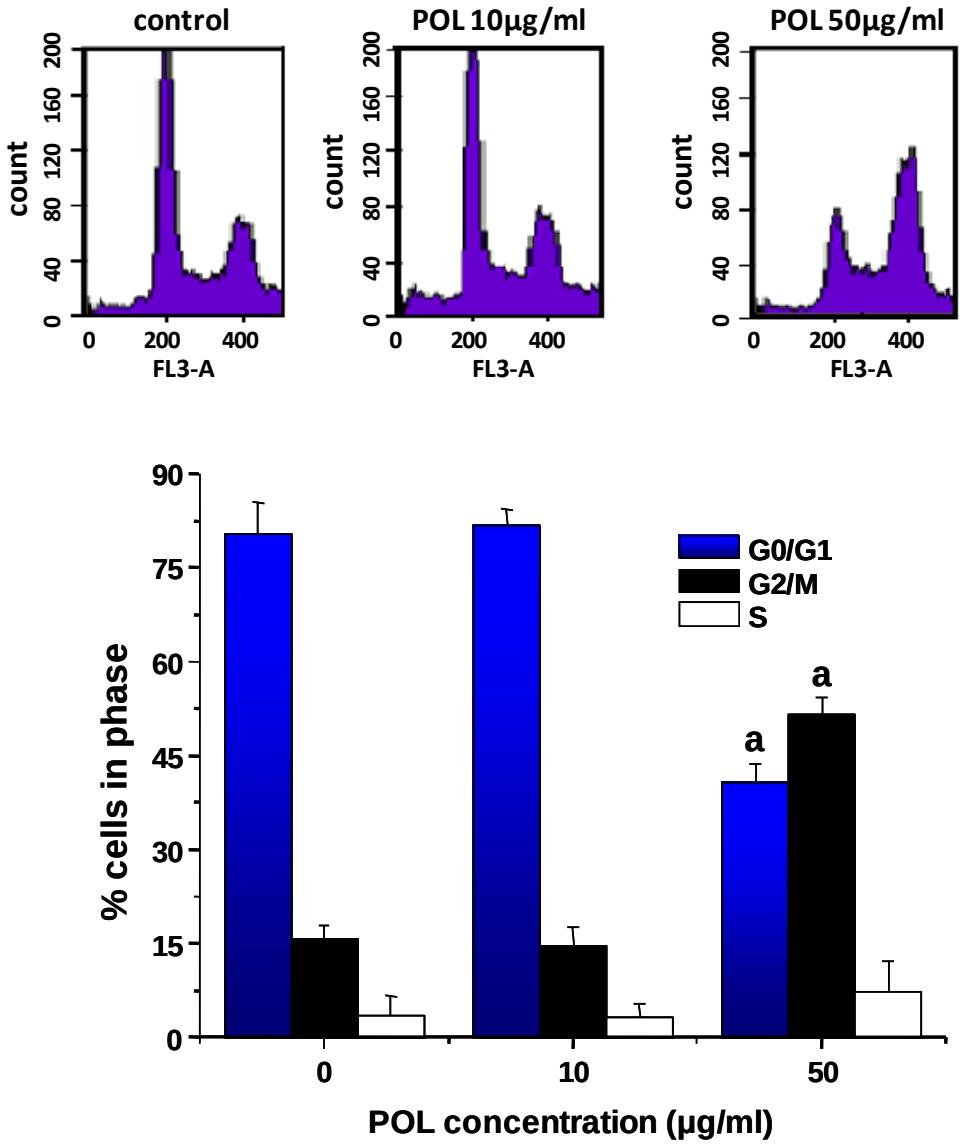
Figure 3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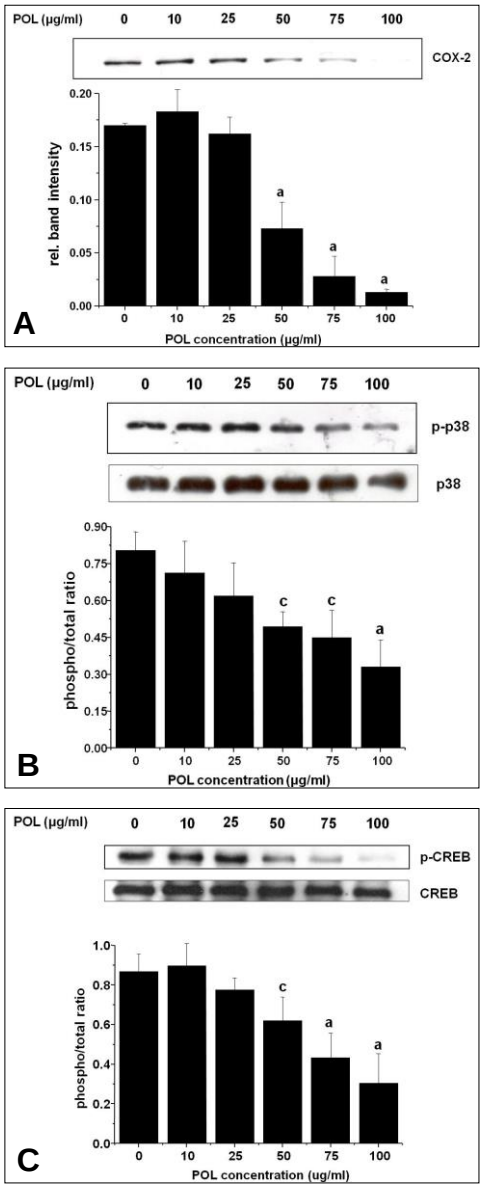
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**Figure Legends**

**Figure 1**: Typical HPLC-DAD chromatogram (280 nm) obtained from the separation of the olive oil polyphenolic extract. 1: hydroxytyrosol (6.3%); 2: tyrosol (3%); 3: oleuropein aglycone (16.3%). Peaks at RT 40-60min relate to secoiridoid derivatives (4).

**Figure 2**: Growth inhibition induced by olive oil polyphenols treatment. Caco-2 cells were exposed to the polyphenol extract (10-100µg/ml) for 24h, 48h, 72h and 96h before Sulforhodamine B assays were conducted. Data are means of 3 separate experiments, each performed in quadruplicate (n=12), and presented as mean ± SD. a = $p < 0.001$; c = $p < 0.05$ represent a significant increase in growth inhibition relative to vehicle treated cells.

**Figure 3**: Effects of the olive oil polyphenolic fraction on cell cycle phase distribution. Sub-confluent Caco-2 cells were exposed to vehicle (MeOH) or the polyphenol extract (10 and 50µg/ml) for 24h. Cells were trypsinized, pelleted, and collected prior to fixing/digestion with RNase. Cellular DNA was stained with PI and the distribution of cells in G0/G1, S and G2/M phase was analyzed by flow cytometry. Results are obtained from three independent experiments and presented as means ± SD. a = represents a significant decrease in cells in G0/G1 relative to vehicle treated cells, $p<0.001$; b = represents a significant increase in cells in G2/M relative to vehicle treated cells, $p<0.001$

**Figure 4**: Immunoblotting analysis on Caco-2 cells exposed to polyphenolic extract. Crude lysates (20 µg) prepared from Caco-2 cells exposed to vehicle (MeOH) or the polyphenol extract (10-100 µg/ml) for 24h (COX-2) or 15min (p38 and CREB) were immunoblotted with antibodies that specifically recognize COX-2 (panel A), phosphorylated and total p38 (panel B), and phosphorylated and total CREB (panel C). Data obtained from experiments were analyzed using Bio-Rad Quantity One 1-D Analysis software. Results are obtained from four independent experiments and presented as means ± SD. a = $p<0.001$; c= $p<0.05$ represent significant decrease relative to vehicle treated cells.