

## Anticancer effect of an olive tree extract through his cytotoxic, antioxidant and antiangiogenic activities

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### ABSTRACT

The aim of the current study was to evaluate the anticancerous potential of a phenolic olive tree extract through his cytotoxic, antioxidant and antiangiogenic activities. The *in vitro* cytotoxic activity of a crude olive tree extract and his major constitutive components has been evaluated against human KB, HL60 cancer cell lines using MTS and flow cytometry. ROS production was estimated with the DCFH- DA assay. Antiangiogenic activity was evaluated *in vitro* on endothelial cells tube formation and the pro-angiogenic factor expression was quantified using qRT-PCR. *In vitro* cellular assays have demonstrated the cytotoxic effect of the crude olive tree extract. This extract

reduces significantly ( $p < 0.05$ ) ROS produced in cells exposed to oxidative stress. Beside this, olive tree extract has demonstrated a strong anti-angiogenic activity, which was correlated with a significant decrease ( $p < 0.05$ ) in VEGF, angiopoietin and HIF1 $\alpha$  expression. Basically, the evaluation of anti-proliferative, antioxidant and anti-angiogenic activities could be the first step to formulate an efficient pharmaceutical product with preventive and/or curative properties against cancer.

**Key words:** Olive tree extract; Anticancerous; Cytotoxic; Antioxidant; Antiangiogenic.

## INTRODUCTION

Olive is a generic name for about 35 species of evergreen shrubs and trees of *Olea* genus and *Oleaceae* family, native to tropical and warm temperate regions. The name is particularly used to describe *Olea europaea* L. space cultivated for its edible fruits (olives). The use of olive fruits and leaves for alimentary, therapeutic and cosmetic purposes was a well-known practice in the Mediterranean basin for more than 7 000 years (Evagelia *et al.*, 2004). Although polyphenols are present in olive leaves, olive fruits has been known for several years as a major source of phenolic compounds with high biological activities (Japón-Luján *et al.*, 2007; Mario-Casas *et al.*, 2003; Visioli *et al.*, 2000). Actually, combined of olive fruits and leaves (10% of the total weight of the olives produced generated during olive harvest and olive tree size) to biophenols extracting enhance both qualitative and quantitative extraction yield.

The phenolic fraction of olive tree (fruits, leaves and very young leaves) is a complex mixture of chemical compounds with different structures and molecular weight obtained using mainly liquid-liquid extraction (methanol-water).

Olive tree polyphenols belong generally to the following classes: (a) tyrosol, hydroxytyrosol and their secoiridoide derivatives; (b) 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid and 4-hydroxycinnamic acid derivatives; (c) lignans and (d) flavonoids. These various compounds have antioxidant (Nardia *et al.*, 2014; Benavente-García *et al.*, 2000), antimicrobial (Micol *et al.*, 2005) and antiproliferative (Taamalli *et al.*, 2012) properties. Today, data collected from *in vitro*, *in vivo* and clinical studies reveal the benefits of these bioavailable compounds for human health. These antioxidant compounds are useful agents for preventing cellular aging and age-related diseases by improving mitochondrial function (Raederstorff *et al.*, 2010). The benefits of olive phenolic compounds on the cardiovascular system have been well documented (Covas, 2007, 2006; Esturch *et al.*, 2006). Beside, these compounds also modulate the inflammatory response associated to several diseases (Killeen *et al.*, 2014; Scoditti *et al.*, 2012; Richard *et al.*, 2011).

Published data have reported that apoptosis and/or necrosis are implicated in the cytotoxicity of olive tree polyphenols. Thus, it has been demonstrated that oleuropein and hydroxytyrosol induce apoptosis in HT-29 human colon adenocarcinoma, HL-60 promyelocytic leukemia) and MCF-7 breast cancer cell lines (Fabiani *et al.*, 2002, 2006, Han *et al.*, 2009). More recently, LeGendre *et al.*, (2015) stated that oleocanthal rapidly and selectively induces apoptotic or necrotic processes *via* lysosomal membrane permeabilization. Beside this, through an *in vivo* study, Hamdi and Castellon (2005) showed that oleuropein inhibits tumor growth in tumor-bearing mice. Additionally, many epidemiological studies suggest the possible correlation between the consumption of olive products and the incidence of breast cancer (Giacosa *et al.*, 2013).

In the present study, we investigated the cytotoxic effect of an olive tree extract against several cancer cell lines through commonly used cellular assays. Additionally, the current study includes the determination of antiangiogenic and antioxidant effects of olive phenolic compounds contained in their stable and natural matrix.

## MATERIALS AND METHODS

### Extract preparation

Olive leaves and fruits were mixed to obtain the aqueous olive tree extract (OTE) by solid-liquid extraction 100% natural, 100% mechanical using water (no use of alcohol, no use of ethanol, no use of chemicals). Actually, OTE is marketed in the world (France, Switzerland, Belgium, ...) under the brandname OLIVIE FORCE / OLIVIE RICHE (see more in [www.olivie.ma](http://www.olivie.ma)). The extract consists in a dark brown solution whose major constituents have been determined (table 1). It comes from organic olive trees that are planted in the middle of a rocky desert where trees suffer and trigger a self-defence mechanism where they produce abnormally high quantities of anti-oxydants to survive.

Table 1. Total phenols and major constituents of olive tree extract. Mean  $\pm$  standard deviation (corresponds to the initial first version of OLIVIE FORCE / OLIVIE RICHE).

Parameter	Average value (g per100g)
Density	1.3
Dry matter	98.0 $\pm$ 7.8
Mineral matter	12.9 $\pm$ 0.7
Fat	< 1
Total polyphenols	7.90 $\pm$ 1.9
Hydroxytyrosol	2.09 $\pm$ 0.14
ORAC ( $\mu$ mol Te kg)*	3848100 $\pm$ 38481

\*Antioxidant activity determined by ORAC assay (Oxygen Radical Absorbance Capacity).

## Cell culture

The HSkMEC (human skin immortalized endothelial cells) was kindly provided by C Kieda (UPR4301 CNRS, Orleans, France) (Kieda 2002), the KB cell line (human nasopharyngeal epidermis carcinoma) was obtained from NCI and HL60 (promyelocytic leukemia) and HT-29 cells (human colon adenocarcinoma) were purchased from ATCC. KB cells in D-MEM, HL60 and HT29 in RPMI and HSkMEC in OPTI-MEM were cultured in complete medium containing 10% (except HSkMEC 2%) fetal bovine serum, penicillin, streptomycin and fungizone in a humidified incubator under 5% CO<sub>2</sub> atmosphere at 37°C. Phenolic compounds (used as reference molecules in this study): oleuropein, hydroxytyrosol, tyrosol, caffeic acid, apigenin, and luteolin were purchased from Sigma Alderich (Paris, France).

## MTS assay

HSkMEC, KB and HL60 cell lines were used to determine the cytotoxicity of OTE and reference molecules (phenolic compounds used as standards). Cells were plated in 96-well tissue culture microplates (10<sup>3</sup> to 2.10<sup>3</sup> cells/well in 200 µl of culture medium).

After 24 hours of incubation at 37°C under 5% CO<sub>2</sub> atmosphere, cells were treated with increasing concentrations of OTE (diluted in H<sub>2</sub>O) and phenolic compounds dissolved in DMSO (1, 10, and 100 µM). Control cells were treated with either water or DMSO (1% of final volume). These operations were carried out using an automated workstation (Biomek 3000 Laboratory Automation Workstation, Beckman- Coulter). After 72h of exposure to olive tree extract or chemicals, MTS reagent (CellTiter 96® AQueous One, Promega) was added and incubated for 3 h at 37 °C. Experiments were performed in triplicate: the absorbance was monitored

at 490 nm and results were expressed as the inhibition of cell proliferation calculated as the ratio  $[(1-(OD_{490} \text{ treated}/OD_{490} \text{ control})) \times 100]$ .

### **FACS analysis**

Apoptotic and necrotic cells were analyzed by Fluorescence-Activated Cell Sorting (FACS). KB cells ( $10^4$  cells in 100  $\mu\text{L}$  medium/well in 96-well microplate) were exposed for 24 and 48 hours at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  to olive tree extract at different concentrations. 50nM doxorubicin was used as positive control. Microplates were centrifuged to collect floating cells. Adherent cells were trypsinized, washed with PBS, mixed with floating cells and spun down. Cells were resuspended into 100 $\mu\text{L}$  medium prior the addition of 50 $\mu\text{L}$  of freshly prepared reaction mixture consisting in the fluorescent agent 7-aminoactinomycin D (7-AAD) (6.5  $\mu\text{L}$  of 1 mg/ml ethanol solution) and recombinant human protein annexin-V-PE (6.5  $\mu\text{L}$ , Bender). After 20 min of incubation in the dark at room temperature, cells were analyzed with a Guava EasyCyte flow cytometer (Millipore). Cells were classified according to their fluorescence and the results were expressed as percentage of cells in each group.

### **LDH release assay**

Necrosis was estimated through the release of LDH in the culture medium. 20.000 KB cells were incubated for 24 and 48 h in the presence of OTE in 96-well microplates containing 100  $\mu\text{L}$  medium. After centrifugation at 300g for 1 min, 25  $\mu\text{L}$  of culture medium was added with 25  $\mu\text{L}$  Cytotox-ONE reagent (Promega) and kept in the dark at room temperature for 20 min. Fluorescence was recorded (exc 560 nm, em 590 nm): results are expressed as the residual activity in the presence of OTE compared to activity in the presence of vehicle alone.

## Caspase activity assay

Caspase activity was determined in KB cells after 24 and 48h of treatment by olive tree extract. Cells ( $20 \cdot 10^3$  cells in 180  $\mu\text{L}$  of complete medium per well) were plated in a 96-well microplate under 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$ . Lysis buffer (20  $\mu\text{L}$  of a 10x stock solution consisting of 250 mM HEPES buffer, pH 7.5, 5 mM EDTA, 0.5% NP40, 0.1% SDS and 50 mM dithiothreitol) was added before adding caspase-3 substrate (DEVD-AMC at final concentration of 50  $\mu\text{M}$ ). The plates were incubated at  $37^\circ\text{C}$  and fluorescence was recorded ( $\lambda_{\text{ex}}$  360 nm,  $\lambda_{\text{em}}$  465 nm) after 0, 30, 60, 120, and 180 min. The reaction rate was calculated from the slope of the linear regression (fluorescence vs time) and expressed as the ratio of activation compared to the control (cells treated with DMSO). Doxorubicin (1 $\mu\text{M}$ ) was used as positive control.

## ROS production

HT29 cells were grown in 200  $\mu\text{L}$  RPMI complete medium ( $20 \cdot 10^3$  cells/well in black 96-well microplates). After 24 hours, cells were incubated with increasing concentrations of olive tree extract (1 to 100% diluted in  $\text{H}_2\text{O}$ ) and 100  $\mu\text{M}$  of reference phenolic compounds (hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin, caffeic acid dissolved in DMSO). Control cells received DMSO (1% of final volume). The culture medium was removed and cells were washed with PBS buffer before addition of 100  $\mu\text{L}$  of RPMI containing 50 mM of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were subsequently incubated for 30 minutes at  $37^\circ\text{C}$  before washing with PBS. The fluorescence was monitored after the addition of 100  $\mu\text{L}$  of PBS ( $\lambda_{\text{ex}}$  485 nm,  $\lambda_{\text{em}}$  530 nm). The positive control was untreated HT29 cells incubated in PBS containing 1 mM of  $\text{H}_2\text{O}_2$ .

### ***In vitro* endothelial cell tube formation**

In this study, the *in vitro* test of tube formation on Matrigel was used to assess the anti-angiogenic activity of olive tree extract. The collagen matrix (Matrigel) was diluted in ice-cold Opti-MEM without fetal calf serum and 30µl of this mix were transferred into a 96-well microplate. The plate was incubated at 37°C under 5% CO<sub>2</sub> atmosphere for 60 min to form the gel. HSkMEC cells (50.103 cells/well) were seeded in 200µl medium containing increasing concentrations of olive tree extract and phenolic compounds (100µM). Endothelial cells cultured on Matrigel formed a three-dimensional micro-network of tubes within 16 h of culture. Cell organization was documented photographically with an inverted microscope at 10 magnification.

### **Quantification of pro-angiogenic factors by qRT-PCR**

The quantitative RT-PCR was used to measure expression levels of genes coding for the pro-angiogenic factors, vascular endothelial growth factor (VEGF: A, B and C isoforms), hypoxia-inducible factor (HIF-1α), angiopoietin (ANGPT 1 and 2 isoforms) and VEGF receptor (VEGFR-2) as previously reported (Kieda *et al.*, 2006, Kieda *et al.*, 2013). The cells were lysed and total RNA was extracted from each well of the 96-well microplate using RNA extraction kit according to manufacturer's instructions (Promega, Madison, WI). Two micrograms of RNA were used to prepare cDNA with oligo (dT) and primers of Myeloblastoma Avian Virus (AMV) and reverse transcriptase (Promega). Quantitative real-time PCR was performed using the Roche Light Cycler system and kit FastStart DNA Master SYBR Green-I. Primers were supplied by Qiagen. The data were analyzed with LightCycler 480 Software and values were normalized to the relative amounts of the cDNA of GAPDH gene.



## Statistical analysis

All cellular tests were performed in triplicate. Statistical analyzes were performed using GraphPad Prism version 5.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by post-hoc Dunnet test for normal distributed data or by the Kruskal-Wallis if the sample distribution does not follow the normal law. Differences between groups were considered as statistically significant at  $p < 0.05$ .

## RESULT

### OTE cytotoxicity

A cytotoxic extract is defined as a substance (or mixture of several phytochemical compounds) that is toxic for the cell, acting at several levels from simple metabolic disorders to apoptosis and/or necrosis induction. Actually, studying of plant extract's cytotoxicity is essential to determine appropriate dose prior to its biological uses. Nevertheless, the cytotoxic character is often sought in pharmaceutical field during the research for new drugs with various biological properties. The results presented in this section are those obtained with the commercial MTS test, e.g. the formazan formation followed at 490 nm. The measured absorbance values are directly proportional to the number of living cells and the results were expressed as the inhibition of cell proliferation and viability (Figure 1). Due to its intrinsic color which can quench or conceal measurements, serial dilutions of OTE were applied to cells: the highest concentration 100 indicates that the pure OTE was used at a final concentration of 1  $\mu$ l OTE into 100  $\mu$ l of medium, whereas the lowest concentration 1 indicates that the pure OTE was diluted 1 to 100 and used at a final concentration of 1  $\mu$ l diluted OTE into 100  $\mu$ l of medium.

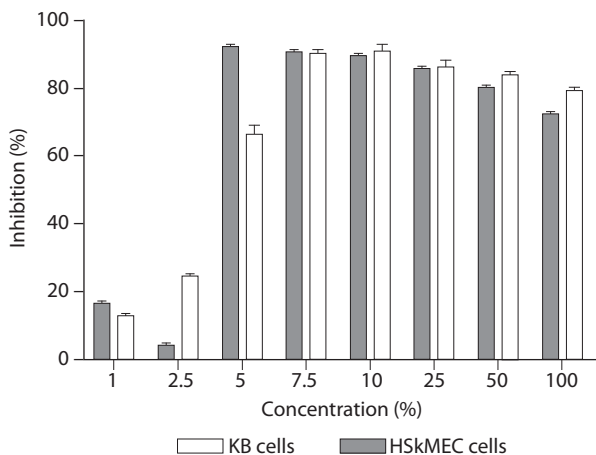


Figure 1. Inhibition of cell viability (%), illustrating the cytotoxicity of olive tree extract in KB cell line.

After 72 hours of treatment, the olive tree extract showed a strong cytotoxic effect on both KB and HSkMEC cell lines. Thus, the olive tree extract was toxic up to 5% dilution with a percentage cell viability inhibition over than 80%. The EC<sub>50</sub>, concentration of the olive tree extract corresponding to 50% of cell viability inhibition, was subsequently evaluated. KB cells were more sensitive to the olive tree extract (EC<sub>50</sub> = 39%) than endothelial cells (EC<sub>50</sub> = 43%), although the observed difference was not significant. Such toxic effect was also observed against other human cancer cells (HL60, MCF-7, HCT116) and non-cancerous cell lines (MRC5 and EPC data not shown). This cytotoxicity activity could be attributed to the phenolic fraction which is present in olive tree extracts (fruits and leaves). Actually, the profile of the olive tree extract revealed a complex mixture of phenolic compounds. Among of them, the components identified as oleuropein, hydroxytyrosol (3',4'-DHPEA) and tyrosol

(*p*-DHPEA) were the most represented and flavonoids such as rutin, quercetin, luteolin and apigenin were present in lower concentrations (Laaboudi *et al.*, 2015). For this reason, oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin, and caffeic acid were used as pure individual molecules for comparison in the current study (Table 2).

Table 2. Inhibition of cell proliferation induced by pure molecules contained in OTE on KB cells. Results are the mean  $\pm$  SD of experiments performed in triplicate and expressed as a percentage of inhibition compared to cells treated with DMSO only.

Compound	Concentration		
	100 $\mu$ M	10 $\mu$ M	1 $\mu$ M
Caffeic acid	0 $\pm$ 10	3 $\pm$ 5	6 $\pm$ 6
Apigenin	83 $\pm$ 1	30 $\pm$ 5	5 $\pm$ 1
Luteolin	81 $\pm$ 1	22 $\pm$ 6	4 $\pm$ 2
Oleuropein	0 $\pm$ 9	0 $\pm$ 1	0 $\pm$ 10
Tyrosol	0 $\pm$ 6	5 $\pm$ 3	0 $\pm$ 8
Hydroxytyrosol	11 $\pm$ 11	1 $\pm$ 10	0 $\pm$ 6

Results of table 2 show, surprisingly, that only flavonoids (apigenin and luteolin) were potent inhibitors of KB cell proliferation, while other molecules (tyrosol, hydroxytyrosol, oleuropein and caffeic acid) had no or negligible effect. However flavonoids have no more antiproliferative activity at 1 $\mu$ M. Similar results were obtained with other studied cell lines, including HSkMEK (data not shown). This does not exclude the effect on cell proliferation of non-identified compounds (at this stage) present in OTE, including secoiridoids derivatives of hydroxytyrosol and tyrosol especially oleacein (3',4'-DHPEA-EDA) and oleocanthal (*p*-DHPEA-EDA).

## FACS analysis of cell death

Apoptosis and necrosis are two distinct and redundant forms of cell death occurring in response to chemical aggression. These two cellular processes featured different and complementary modes of action: apoptosis (or programmed cell death) is a tightly regulated cell death program that plays a pivotal role in a variety of biological processes, generally executed in animal cells *via* the activation of cellular proteases leading to the cleavage of chromatin into nucleosomal fragments [43], while necrosis involves the destruction of the plasma membrane leading to the release of cytosolic enzymes and cofactors into the external medium. Flow cytometry was used at first to investigate the cell death process: it enabled to discriminate between intact “healthy” cells, apoptotic cells and necrotic cells in an advanced state of degradation.

In line with cytotoxicity experiments, KB cells were treated by diluted OTE (50-1%) for 24 and 48h and analyzed by FACS. According to the fluorescence monitored in FACS, cells can be classified as intact, in early apoptosis, in late apoptosis or necrotic (dead cells). The percentage of cells into each group is shown in Table 3. As it can be inferred from results of table 3, significant changes were observed in cell population distribution after 24 h of exposure to OTE. Intact cell population progressively decreased when the OTE concentration increased, associated with a transient raise in early apoptotic cells at low OTE concentration and a massive outburst of necrotic cells at higher OTE concentrations. After 48h of exposure, the necrotic cell population predominated even with low OTE concentrations to the detriment of intact and early apoptotic cell populations. Therefore it can be concluded that the effects of olive tree extract are dose- and time-dependent.

**Table 3. Quantification by FACS of cell death induced in KB cell lines by olive tree extract.**

Concentration (%)	Cell population (%)							
	Intact		Early apoptotic		Late apoptotic		Necrosis	
	24h	48h	24h	48h	24h	48h	24h	48h
DMSO	90	90	3.1	2.1	0.5	1.2	6.5	7.2
Doxo 100 mM	53	32	36	45	0.2	1.0	11	21
OTE 50	3.4	12	4.1	1.8	0.1	0.3	92	86
OTE 25	3.6	3.9	6.6	2.2	0.8	0.3	89	94
OTE 10	20	2.3	34	8.0	0.4	0.4	46	89
OTE 7.5	37	3.4	57	12	0.2	0.4	6.0	84
OTE 5	54	19	42	33	0.2	0.4	3.4	47
OTE 2.5	86	84	7.6	8.1	0.7	2.0	5.4	57
OTE 1	83	82	11	9.5	0.3	0.9	4.7	7.2

Caspases (cysteine-aspartic acid proteases) are regarded as the most common apoptotic effector activated during apoptosis in many cell types. It was reported that caspase-3 is the terminal effector in the apoptotic cascade and is the major contributor to cellular DNA fragmentation.[33] To go further in the elucidation of the cell death pathway induced by OTE, the catalytic activity of caspase-3 was explored in KB cells treated for 24 and 48 h with OTE at the same concentrations as in FACS analysis. Caspase-3 activity can be determined by the cleavage of the pro fluorescent DEVD peptide which is catalyzed by both caspase-3 and 7, albeit more efficiently by the former. As expected, 1 $\mu$ M doxorubicin elicited a potent activation of caspase-3 activity after a 48 h treatment (figure 3A). Although FACS analysis showed the apoptotic effect of olive tree extract at low concentrations (7.5 and 5%), results of figure 3A show that caspase-3 activity was moderately increased after a 48h exposure of KB cells to OTE. This suggest that apoptotic activity of olive tree extract was marginal compared to its necrotic effect and

led us to evaluate the release of cytosolic release of lactic dehydrogenase used as a hallmark for necrosis.

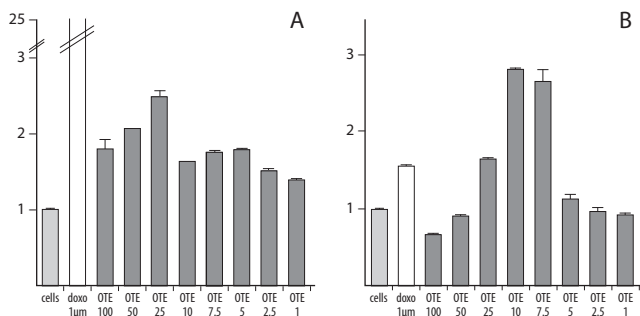


Figure 2. Activation of caspase-3 (A) and release of LDH (B) from KB cells after 48h of treatment with olive tree extract, vehicle only (cells) and doxorubicin (1µM).

Results are expressed as the relative activity in duplicate experiments ± SD, compared to activity in untreated KB cells.

As shown in figure 2B, the release of LDH was enhanced after 48h of exposure to increasing OTE concentrations, before to decline at high concentrations, indicative of a complete cell destruction. This confirms that the olive tree extract induces a transient and moderate activation of apoptosis preceding a massive necrotic cell death.

### ROS production

The production of reactive oxygen species can be estimated with the DCFH-DA assay using the fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, the non-fluorescent DCFH-DA diffuses spontaneously into cells and is deacetylated by cellular esterase to 2',7'-Dichlorodihydrofluorescein (DCFH), which is rapidly oxidized by ROS to the highly fluorescent 2',7'-dichlorodihydrofluoresceine (DCF). The emitted fluorescence intensity (Irel) is proportional to the levels of ROS produced into cell cytosol.

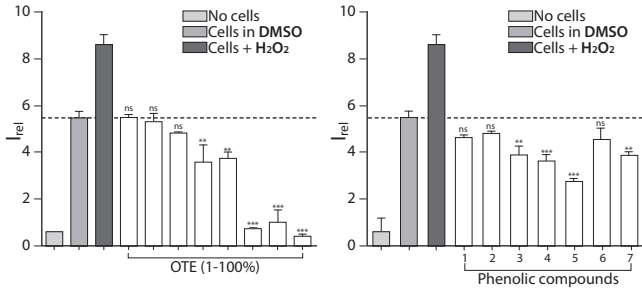


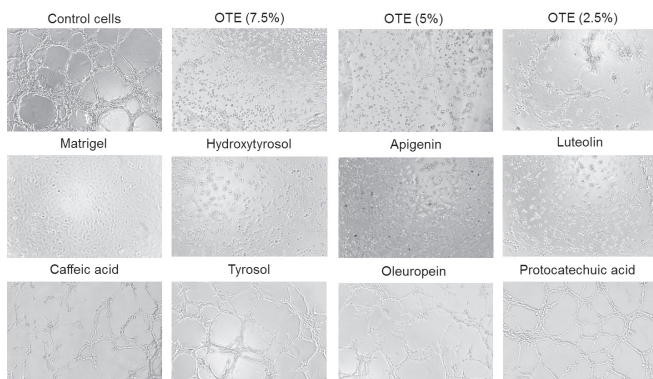
Figure 3. Intracellular ROS production after incubation of HT29 cells for 24h with (A) increasing concentrations of olive tree extract and (B) pure phenol compounds (100  $\mu$ M). Results are the mean  $\pm$  SD of 3 individual assays. \*\*\* ( $p < 0.001$ ), \*\* ( $p < 0.01$ ) \* ( $p < 0.05$ ); ns, not significant from fluorescence produced in cells treated with vehicle only. (1), oleuropein; (2), tyrosol; (3), hydroxytyrosol; (4), apigenin; (5), luteolin; (6), protocatechuic acid; (7) caffeic acid.

In the presence of 1mM H<sub>2</sub>O<sub>2</sub>, the emitted fluorescence intensity was significantly increased (Figure 3). Conversely, a progressive decrease in the quantity of produced ROS was observed in cells treated with increasing concentrations of OTE to reach an almost complete protection against ROS at highest concentrations. On the other hand, no significant effect on ROS production were noticed with 100 $\mu$ M tyrosol, oleuropein and protocatechuic acid, while hydroxytyrosol, apigenin and luteolin significantly reduced the amount of ROS produced during cell incubation. Thus, the anti-radical effect of olive tree extract could be the result of the antioxidant activity (synergistic action) of these phenolic compounds, although the determination of the role of other compounds (not identified at this stage) is required to confirm these observations.

### ***In vitro* anti-angiogenic effect on tube formation.**

Endothelial cells grown on an extracellular matrix spontaneously form a capillary network in the presence

of growth factors present in the fetal calf serum. This *in vitro* test allow to determine the capacity of a molecule or substance to inhibit the formation of capillary structures. It is considered to be close to *in vivo* tests and to require the proliferation, migration and degradation of the basement membrane of endothelial cells. The photographs taken after a 16h of endothelial cells incubation on Matrigel at 37 °C and 5% CO<sub>2</sub> in the presence of increasing concentrations of olive tree extract and phenolic compounds (100 µM) are presented in figure 4.



**Figure 4.** Representative photographs of the network structure formed by endothelial cells (HSkMEC) on Matrigel in the presence of increasing concentrations of olive tree extract and pure phenolic compounds used at 100 µM. Control cells are incubated without Matrigel.

Photographs of figure 4 show that endothelial cells grew in complete medium containing 2% FCS as a continuous monolayer (figure 4 control cells) whereas they form capillary structures when laid down on Matrigel. This capacity to form capillary structure was severely impaired in the presence of olive tree extract, suggesting its dose-dependent anti-angiogenic effect. The OTE effect has been observed at concentration



2.5% to be complete at concentration 7.5%. Indeed, referring to the antiproliferative capacity of OTE, the anti-angiogenic activity could be due to a direct cytotoxic effect of OTE on HSkMEC cells. However, the results of the cytotoxicity indicate that the olive tree extract diluted at concentration 2.5 does not affect the proliferation and viability of HSkMEC cells, whereas the anti-angiogenic effect was evident at this concentration. Therefore the anti-angiogenic effect of pure molecules identified as major phenolic compounds present in olive tree extract was investigated in the same conditions (figure 4). Among these pure compounds hydroxytyrosol, luteolin, apigenin and to a lesser extent caffeic acid at a 100 $\mu$ M concentration elicited a marked inhibitory effect on capillary network formation of HSkMEC cells. On the other hand, tyrosol, oleuropein and protocatechuic acid moderately affect the organization of endothelial cells. Thus, we may conclude that the synergistic action of these molecules on endothelial cells might account for the OTE anti-angiogenic effect.

Indeed, the ability of endothelial cells to organize themselves into three-dimensional space to form new capillaries proceeded through cell migration, proliferation and vessel stabilization governed by the secretion of chemotactile factors (called pro- angiogenic factors). Several proteins are involved to support angiogenesis phenomena, such as VEGF factors (A, B and C isoforms), angiopoietin (1 and 2), HIF and the VEGF receptor-2. The expression rate of genes coding for these pro- angiogenic factors was, therefore, measured using quantitative real time RT-PCR (Figure 5) and summarized in table 4.

Analytical data plotted on figure 5 were consistent with observation of micro-capillary formation on Matrigel. Basically, the expression of pro-angiogenic genes was

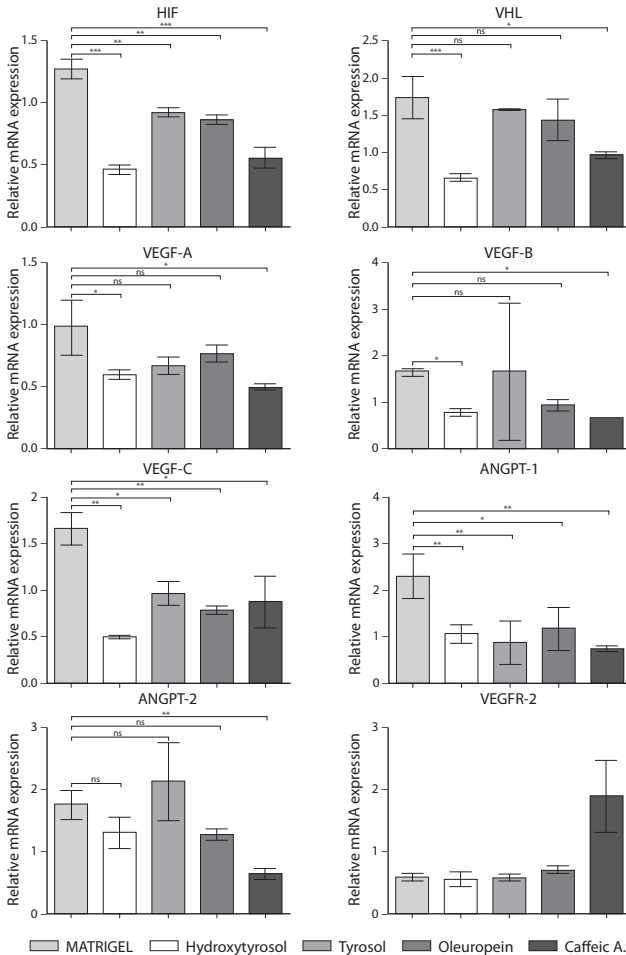
significantly affected in the presence of hydroxytyrosol which was the molecule having the highest inhibitory effect on microtubule formation, and to a lesser extent by caffeic acid which is a weaker microtubule disruptor. The two modest inhibitors of microtubule formation tyrosol and oleuropein did not affect the expression of VEGF A and B. It is noteworthy that none of these compounds modify the expression of the VEGF receptor-2 which binds VEGF-A. VHL is a tumor suppressor which binds to HIF-1 $\alpha$  to allow its degradation by the proteasome. Its expression is regulated by HIF-1 $\alpha$  through an HRE element present in its promoter: thus HIF could promote its own degradation by the induction of VHL gene expression (Luczak *et al.*, 2011, Karhausen *et al.*, 2005). This is consistent with the co-regulation observed in HSkMEC cells exposed to phenolic compounds and presented in figure 5 and table 4.

**Table 4. Summary of anti-angiogenic effect of the major polyphenols present in olive tree extract. (+), Inhibitory effect of angiogenesis; (-), No effect.**

Molecule (100 $\mu$ M)	HIF	VHL	VEGF-A	VEGF-B	VEGF-C	ANGPT-1	ANGPT-2	VEGFR-2
Hydroxytyrosol	+++	+++	+	+	++	++	-	-
Tyrosol	++	-	-	-	+	++	-	-
Oleuropein	++	-	-	-	++	+	-	-
Caffeic acid	+++	+	+	+	+	++	++	-

All together the lower expression of VEGFs, angiotensin and HIF-1 $\alpha$  can explain the anti-angiogenic of active phenolic compounds present in the olive tree extract, individually and/or synergistically.

Figure 5. Effect of phenolic compounds in olive tree extract on the expression of pro- angiogenic agents. HIF Hypoxia Inducible Factor-1 $\alpha$ ; VHL protein of Von Hippel- Lindau; VEGF (A, B and C), Vascular Endothelial Growth Factor; ANGPT (1 and 2), Angiopoietin; VEGFR-2, cell receptor for VEGF proteins. Values are expressed as mean  $\pm$  SD (n = 3). \*\*\* ( $P < 0.001$ ), \*\* ( $P < 0.01$ ) \* ( $P < 0.05$ ); ns, not significant difference.



## DISCUSSION

The olive tree extract tested in this study, displayed potent cytotoxic activity against human cancer cells through the activation of necrotic and/or apoptotic pathways. This is consistent with several previous studies have reported that crude extracts from olive leaves and phenolic compounds demonstrate high cytotoxic activity against various cancer cell lines derived from blood, colon and breast cancers (Fu *et al.*, 2010; Hashim *et al.*, 2008; Abaza *et al.*, 2007). This cytotoxic activity was, particularly, remarkable in HER-2 positive breast cancer cells. Olive tree extract reduces the overexpression of HER-2 and decreases resistance to trastuzumab, an anti-HER-2 monoclonal antibody used clinically for breast cancer treatment (Menendez *et al.*, 2007). Independently, Taamalli *et al.*, (2012) have shown that the inhibition of breast cancer cells viability was depended on the polyphenol content in an olive leaves extract.

As expected our olive tree extract is rich in hydroxytyrosol and its secoiridoid derivatives (oleacein and oleocanthal), and in flavones such as luteolin and apigenin. Therefore, the individual effect of these compounds to explain the cytotoxicity toward cancer cells of this crude extract deserves more attention in future studies. Interestingly the whole OTE effect might not result from the abundance of a single compound, but rather from the synergistic interaction between different compounds including those that are not already identified. In this respect, authors have recently pointed out that food polyphenols who exert their pharmacological effects through synergistic interactions involve the implication of multiple targets (Efferth and Koch, 2011; Wagner, 2011).

Thus, the effects of molecules abundant in OTE like hydroxytyrosol (50 µg.ml<sup>-1</sup>) and oleuropein (200 µg.ml<sup>-1</sup>)

were demonstrated in MCF-7 cells (Han *et al.*, 2009), and confirmed *in vitro* on a variety of human cancer cell lines together with apigenin (Kim *et al.*, 2011; Bulotta *et al.*, 2011; Fabiani *et al.*, 2006, 2002), and *in vivo* in tumor-bearing mice (Hamdi and Castellon, 2005). Moreover, many epidemiological studies suggest a potential correlation between the consumption of olive products (olive fruits and olive oil) and a low incidence of breast cancer in Mediterranean area, suggesting a protective effect of olive compounds against cell proliferation e.g. cancer (Corona *et al.*, 2007). Thus, we can conclude that the OTE's cytotoxic effect is mainly due to these phenolic compounds.

ROS play a crucial role in tumorigenesis as suggested by Sabharwal and Schumacker (2014) who reported that signaling pathway(s) triggered by ROS contributes to proliferation and tumor survival of many cancers. These authors propose that improving mitochondria potential redox is a promising target for future cancer therapies. In accordance with Nardi *et al.*, (2014), we reported herein that olive tree extract and some of its phenolic components showed elevated antioxidant activity in HT-29 cells, giving them a central role in the fight against carcinogenic risks. Likewise, Deiana *et al.* (1999) reported that hydroxytyrosol reduced biochemical effects of peroxynitrite, such as the deamination of adenine and guanine in certain cell lines. ROS generated during oxidative stress may be directly involved in DNA damage and/or in the intrinsic apoptotic pathway induction (mitochondria- dependent). Thus, the intracellular accumulation of ROS may be involved in the partial disruption of the mitochondrial membrane causing the release of mitochondrial material (cytochrome-c, pro-apoptotic factors) in the cytosol, finally leading to the activation of the caspase cascade (Eloy *et al.*, 2012). Moreover, cellular ROS production by different

metabolic pathways has been associated with cell aging phenomenon call the "radical" hypothesis of aging (Barouki, 2006). Thus, we can postulate the antioxidant activity of OTE and these phenolic compounds has a double effect, i) the elimination of carcinogenic ROS and ii) the prevention of cell aging.

Angiogenesis is a critical process implicated in the proliferation and dissemination of cancer cells by metastasis. The fast and limitless multiplication of cancer cell requires an important and incremental supply in oxygen and nutrients through the blood circulation. The spreading of circulating cancer cells is highly facilitated by the density of blood vessels and the cell aggressiveness enabling the metastasis formation.

Angiogenesis is under the control of multiple regulation pathways, among them are the vascular endothelial growth factors which stimulate vasculogenesis and angiogenesis (Jiang *et al.*, 2000) and facilitate the formation of new capillary vessels in association with angiopoietins reviewed by Rak *et al.*, 2000. The transcription of VEGF is stimulated by Hypoxia Inducing Factor 1- $\alpha$  present in the cytosol as a heterodimer with VHL allowing its degradation by the proteasome. The key role of VEGFs was also highlighted by a clinical report which positively correlated polymorphism in the VEGF-A gene (-1154 A/G, -2578 C/A and -460 T/C) and the lower susceptibility to develop breast cancer in Moroccan individuals (Rahoui *et al.*, 2014).

Data reported herein clearly demonstrate the inhibitory effect of olive tree extract and of some of its phenolic components (hydroxytyrosol, apigenin, luteolin and caffeic acid) on the formation of new capillaries from human endothelial cells. This inhibition was consistent

with previous observations made on various human cells, animal tumors and *in silico* (Ambasta *et al.*, 2015; Simon Silvan *et al.*, 2013; Fortes *et al.*, 2012; Gacche *et al.*, 2011; Engelmann *et al.* 2002). Two mechanisms could explain the anti-angiogenic effect of olive tree extract and phenolic compounds: a severe restriction of cell proliferation reducing the number of endothelial cells required for building the capillary structure and a lack of pro-angiogenic factors production thus restricting cell migration and assembly. Clearly, hydroxytyrosol oleuropein and caffeic acid had no effect on cell proliferation, but are potent down-regulator of HIF, VEGF and angiopoietin suggesting their direct anti-angiogenic effect. Additionally, Kim *et al.* (2011) had shown that apigenin has an anti-angiogenesis effect through a down-regulation of VEGF and MMP-8 release in hepatocellular carcinoma cells. Therefore, OTE's effect is the result of the cumulative effect of individual phenolic compounds reducing the cell supply and thus restricting the tumor growth.

On the other hand, an excessive angiogenesis ensues from progression of inflammatory process in synovium, and leads to pannus proliferation and rheumatoid arthritis symptoms complication. Angiogenesis is a major contributor in the development and maintain of inflammation in rheumatoid arthritis (Semerano *et al.*, 2011), and a correlation between rheumatoid arthritis progression and VEGF level has been observed in patient with RA (Sone *et al.*, 2001). Thus, the inhibition of angiogenesis could be a potential therapeutic target for the treatment of rheumatoid arthritis (Ghanam *et al.*, 2015). However, the VEGF-dependent antiangiogenic effect of olive tree polyphenols, in both cancer and rheumatoid arthritis diseases, need to be confirmed in future *in vivo* studies.

## CONCLUSION

The cellular assays have demonstrated the effectiveness of phenolic olive tree extract as an antioxidant in cells exposed to oxidative stress. This extract has also demonstrated a strong VEGF-dependent antiangiogenic activity. Indeed, ROS elimination, apoptosis and/or necrosis activation and angiogenesis inhibition make this extract an excellent agent to prevent cancer diseases. The characterization of bioactive molecules in this extract, as well as the contribution of each to the global biological activity, could provide the chemical basis for the development of new anticancer drugs. Further studies are needed to extent the antiangiogenic activity of phenolic compounds derived from studied olive tree extract (hydroxytyrosol, oleacein and oleocanthal) in case of angiogenesis-dependent diseases.

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