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"Olive oil phenols as potential source of chemopreventive and therapeutic agents against bladder cancer"

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List of abbreviation

BC	Breast Cancer
BCa	Bladder Cancer
CRC	Colorectal Cancer
EA	Elenolic Acid
ECM	Extracellular Matrix
EGCG	Epygallocathechingallate
EGFR	Epidhermal Growth Factor Receptor
EMT	Epithelial To Mesenchimal Transition
EPIC	European Prospective Investigation Into Cancer And Nutrition
ER	Estrogen Receptor
ERK	Extracellular-Signal-Regulated Kinases
EVOO	Extra Virgin Olive Oil
EVOO-E	Extra Virgin Olive Oil Extract
HGF	Hepatocyte Growth Factor
HPEA-EDA	Dialdehydic Form Of Elenolic Acid Linked To Tyrosol
HT	Hidroxytyrosol
LDL	Low Density Lipoprotein
MDR	Multi Drug Resistance
MMP	Matrix Metalloproteinase
MUFA	Monounsaturated Fatty Acid
OLE	Oleuropein
PARP	Poly (ADP-Ribose) Polymerase
P-gp	P-Glycoprotein
PUFA	Poly Unsaturated Fatty Acid
ROS	Reactive Oxygen Species
TIMP	Tissue Inhibitors Of Metalloproteinases
TY	Tyrosol

Table of contents

FIGURE INDEX

ABSTRACT

The consumption of extra virgin olive oil (EVOO), a common dietary habit of the mediterranean people, seems to be related to a lower incidence of certain types of cancer including bladder neoplasm. Metastases are the major cause of bladder cancer-related deaths and targeting cell motility has been proposed as a therapeutic strategy to prevent cancer spread. This study aim to investigate the potential anti-metastatic and antiproliferative effect of total phenols extracted from EVOO against human transitional bladder carcinoma cell lines. Our results show that EVOO extract can significantly inhibit the proliferation and motility of T24 bladder cells in a dose-dependent manner. Furthermore the enzymatic activity of MMP-2 was inhibited at non-toxic EVOO extract doses in T24 cells. The qRT-PCR revealed a decrease of the MMP-2 expression, and a simultaneous increase of the TIMPs gene expression. The cell cycle analysis of two bladder cancer cell lines (T24 and 5637) after EVOO-E treatment show a marked growth arrest prior to mitosis in the G2/M phase for both the cell lines with the subsequent induction of apoptosis only in T24 cells. Finally we evaluated the ability of EVOOE to chemosensitize T24 to the cytotoxic action of common used chemoterapics. Interestingly the simultaneous treatment of Paclitaxel and EVOOE strongly increase the apoptotic cell death at every of the tested concentration if compared to the drug alone.

Our results may support the epidemiological evidences which link olive oil consumption to health benefits and may represent a starting point for the development of new anticancer strategies.

1 INTRODUCTION

1.1 Olive oil chemical composition

Extra virgin olive oil (EVOO) is a food produced from the first and second pressings of the olive fruit by the coldpressing method [1] without the use of chemicals, and it is composed of a glycerol fraction (which account for about 95–99% of the olive fruit) and a non-glycerol or unsaponificable fraction (0.4–5% of the olive fruit) which contains phenolic compounds [2]. The saponificable fraction is composed mainly of triacylglycerols (triglycerides or fats) and contains small quantities of free fatty acids (FFA), glycerol, phosphatides, pigments, flavor compounds, sterols, and microscopic bits of olive. Triacylglycerols act as energy reserve for plants and animals. Chemically speaking, these are molecules derived from the natural esterification of three fatty acid molecules with a glycerol molecule. The fatty acids forming part of the triacylglycerol molecule can be saturated or unsaturated and are represented in the Table 1.

The oleic acid which account for about 70-80% is the most represented monounsaturated lipid molecule (MUFA), followed by the polyunsaturated (PUFA) linoleic acid 10 % and palmitic and stearic acid (saturated). The high content of MUFA and PUFA is distinctive of EVOO and is partially responsible for the healthy properties of this food because most of the unsaturated fatty acid cannot be synthesized and so are contemplate as essential dietary. Initially, the beneficial health effects of EVOO were attributed to the glycerol fraction with its high concentration of MUFAs[3]. However, several seed oils (including rapeseed, sunflower and soybean) containing high quantities of MUFAs are ineffective in beneficially altering chronic disease risk factors. Therefore, a substantial number of scientific works examining the biological actions of olive

oil phenolic compounds in the unsaponificable fraction have been conducted. In recent years most of the healthy properties of EVOO are attributed to the polyphenolic fraction characterized by the presence of unique molecules that cannot be found in other biological matrix.

1.2   *Olive oil phenolics*

The total phenol content of olive oil has been reported to have a wide variability ranging between 100 and 800 mg/Kg. Variation in the phenolic profile exists among different virgin olive oils due to numerous factors, including the Olea cultivar and the agronomic and technological conditions of oil production [4, 5] The phenolic composition of EVOO is heterogeneous, with about 36 structurally distinct phenolic compounds identified [6]. Different groups of phenolic molecules can be found in virgin olive oils, such as phenolic acids, flavonoids,phenolic alcohols, , hydroxy-isochromans, lignans and secoiridoids (Figure 1). One of the first class of phenols studied in EVOO were phenolic acids, this is an eterogenous combination of molecules that include several different acids like gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, syringic, p- and o-coumaric, ferulic and cinnamic . Another interesting family of chemical compounds found in EVOO are lignans , which include (+)-1-acetoxypinoresinol and (+)-1-pinoresinol. These chemical compounds are mainly located in the woody portion of the seed and in the olive pulp and are released in EVOO during the extraction process.

Flavonoids are secondary plant metabolites constituted by planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. During the past decade, an increasing number of publications on the health beneficial effects of

flavonoids have appeared, such those related to cancer and coronary heart diseases[7-10].

Phenolic alcohols are principally represented by hydroxytyrosol (HT) (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) and tyrosol (TY)that are identical except for the presence of extra hydroxyl group in the *meta* position. Phenolics alchols concentration is high variable due to different factor and is generally low in freshly pressed oils, but this increases during oil storage, as a consequence of the hydrolysis of Oleuropein (OLE). The secoiridoids belongs to a specific group of coumarin-like compounds, abundant in Oleaceae. This class of compounds found only in the family of Olearaceae plants are well represented by Oleuropein which is an ester of hydroxytyrosol (3,4-DHPEA) and the elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of Oleaceae).

In latest years one of the most studied secoiridoid was the dialdehydic form of deacetoxy-ligstroside aglycon better known as Oleocanthal. This particular molecule gain great interest after the findings of Beauchamp and coworkers [11] that the dialdehydic form of deacetoxy-ligstroside aglycon share a similar structure of the anti-inflammatory drug Ibuprofen. In this work they demonstrated the ability of Oleocanthal to inhibit the cyclooxygenase enzymes COX-1 and COX-2 and they suggested this mechanism as the key of the beneficial effect exerted by the the Mediterranean diet characterized by a regular intake of this compound.

Now, after 10 years since the Beauchamp work was published a lots of evidences were collected about this molecule and other EVOO phenolics

compound and all data seems to point out like the nutraceutical properties of this food are due to a synergism between them.

1.3   *Bioavailability of olive oil phenolic compounds in humans*

The knowledge of phenols metabolism after ingestion has been the subject of study by the scientific community due to the high intake of this molecules, especially in mediterranean area. Using an average total phenol content of 400 mg/kg, and given a daily intake of 25-30ml of EVOO an estimate of the amount of olive oil phenolics consumed per person each day is about 10 mg. [12]. Most of the studies regarding the bioavailability of olive oil phenolics focused the attention on three major class of compounds: hydroxytyrosol, tyrosol, and oleuropein. Data obtained from different works showed how these molecules are absorbed in a dose-dependent manner[13, 14]. Tuck and coworker[15] demonstrated, using an animal model, an increase of bioavailability of HT and TY of about 20% when administered as an olive oil solution compared to an aqueous one. They suggested that they olive oil matrix, due the high content of antioxidants, may protect the breakdown of phenolics in the g.i. tract prior to absorption The mechanism olive oil phenolic compounds are absorbed remains unclear, however many studies postulated that the different polarities of the various phenolics maybe involved in the absorption of these compounds[16]. Tyrosol and hydroxytyrosol are polar molecules and their absorption has been demonstrated to occur via passive diffusion[17]; unfortunately, oleuropein and ligstroside-aglycones are bigger and less polar than HT and currently there is no data available on their mechanism of absorption. It is known that polyphenols metabolism usually undergo phase I and phase II reaction, in which they are hydrolysed and

conjugated into their sulphated, methylated and glucuronidated forms to be absorbed[18]. This process is really important, because polyphenols in its free form are undetectable or in a very low concentration in biological matrix, Many studies focused their attention also on phenolics excretion. Vissers et al [16] reported a content of hydroxytyrosol and tyrosol in urine of about 5–16% of the total ingested while in a study by Miro-Cases and colleagues[14] the rate of excretion of HT administered was about 24%. Further study performed by Visioli and colleagues[19] showed and high variability in hydroxytyrosol and tyrosol excretion between 60% and 20% of the total ingested by human subjects. Collectively these findings demonstrate an high variability in human absorption and excretion of EVOO polyphenols probably due to individual differences. As most of the obtained data is only based on three molecules, more investigation about bioavailability and urinary excretion of other EVOO bioactive molecules are needed. It's important to notice that the concentrations of EVVO phenols in biological fluids, after their ingestion, are too low to explain the biological activities obtained *in vitro* and *in vivo* models at higher doses/concentrations.

Many hypothesis have been postulated to explain discrepancies between, beneficial effects observed in clinical trial related to polyphenols content of olive oil and their poor bioavailability[20, 21]. Tuck et al[22] hypothesized that conjugated metabolites of phenols are also bioactive, they found that the 3-O-glucuronide HT conjugate had an antioxidant activity five fold higher compared to hydroxytyrosol. Most of EVOO phenols pharmacokinetic studies have focused the attention on some specific compounds such as TY and HT. Miró-Casas et al.[14] reported the presence of conjugated forms of HT in

plasma, this evidence is in a good agreement with other data that correlate the intake of EVOO with a lower oxidation status of LDL [20, 21]. Several reports demonstrated that phenolic compounds and their metabolites after olive oil ingestion are incorporated in lipoproteins and this may explain their protecting antioxidant effects[20]. Globally none of these hypotheses has been fully corroborated experimentally.

1.4 *Antioxidant activity of EVOO phenolic compound and chemopreventive effect on cancer.*

The exposure to genotoxic substances of different origin (environmental or endogenously produced) is one of the leading causes of carcinogenesis[23]. Reactive oxygen species (ROS) are continuously produced in all aerobic organisms and therefore constitute the most important genotoxic endogenous agent, this is the main reason that correlate oxidative stress with various disease in particularly cancer[24]. Various experiments conducted *in vitro* on different cellular model demonstrated the potent antioxidant activity of EVOO phenols suggesting a potential role of these chemicals in the chemopreventive ability of olive oil, however the protective role of EVOO phenols against DNA oxidative damage need to be clarified .

Hillestrom and coworker[25] demonstrated that after the ingestion of phenol rich olive oil there is no significant variation in the excretion of etheno-DNA adducts, moreover Machowetz et al. established how the consumption of EVOO with different phenolic content cannot affect the oxidation products of guanine in the urine[26]. However, on other hand there are lot of work that support the protective role of olive oil phenols against DNA damage. Fabiani et al [27] demonstrated how different phenolic compounds, obtained from olive

mill wastewater, can reduce H_2O_2 induced DNA damage in HL60 promyelocytic leukemia cells, the same results was achieved by Warleta et al, in this work HT decreases the intracellular reactive oxygen species (ROS) level in MCF10A cells but not in MCF7 or MDA-MB-231 cells while very high amounts of tyrosol is needed to decrease the ROS level in MCF10A cells[28]. Collectively all the data obtained from *in vitro* models seems to support protective role of phenol molecules against oxidative damage. It's of note that the concentration used in most of the scientific work previously cited is easily reachable with a normal intake of olive oil, at least in the enteric tract[29], taken together this findings suggest that EVOO phenols may prevent, in part, the first step of carcinogenesis *in vivo.*

1.5 Antitumor effects of olive oil phenols.

In addition to the antioxidant activity there is a wide amount of scientific literature focused on exploring the antitumor potential of EVOO phenols. Several studies demonstrated that different molecular mechanism are involved in the inhibition of proliferation or induction of apoptosis; relevant data about the most studied tumor types are reviewed in the following section.

1.5.1 Leukemia .

The first study of EVOO phenols involving a leukemia cellular model was conducted by Della Ragione et al [30] about fifteen years ago, they demonstrated that HT is able to stop cell cycle progression and inhibit proliferation in HL60 cell line. The observed effect was due to the release of cytochrome C and subsequent induction of apoptosis by Caspase 3. In this work they also suggest an effect phenotype-dependent because the same inhibition was not seen in two colorectal cancer cell line, in contrast with

more recent studies[31]. One of the most interesting finding of the Della Ragione work was that the antiproliferative effects require the presence of two ortho-hidroxyl groups in the phenyl ring of the molecule since the treatment of the cells with TY didn't exert any effect on cell proliferation similarly to what obtained in the above cited Warleta work. In the past years Fabiani and coworkers has extensively studied the effect olive oil phenols in particular HT against different Leukemia models[32-37], they proposed a "multi level" action of EVOO phenols where they modulate different signaling pathways involved in proliferation and apoptosis. The authors proposed the generation of ROS in culture media by HT as a mechanism of action. The oxidative stress produced by high concentration of HT trigger cells to apoptosis. These conclusion are supported by many evidence, since the removal of extracellular H_2O_2 by catalase induced a decrease of apoptotic cells in a dose dependent manner. In this work they clarify that HT can have a double distinct action : antioxidant at low concentration in a range of 5-15 µM and pro-oxidant at greater concentration up to 100 µM. They observed an antiproliferative activity of HT, on different cell lines directly correlated to the amount of H_2O_2 in the medium. The supposed mechanism in the formation of H_2O_2 is the auto oxidation of HT by O_2 and the subsequent formation of superoxide ions, protons and a semiquinone.

In the light of these findings we need to revise a lot of the results obtained from *in vitro* experiments that don't take in account hydrogen peroxide production. This finding provide a new perspective in the study of this kind of molecules.

1.5.2   Colorectal cancer

Dietary habits has been strictly associated with colorectal cancer (CRC) risk and Mediterranean countries shows lower prevalence of this neoplasm if compared with other western countries. One of the hallmark of the Mediterranean diet (MD) is the high consumption of olive oil and this could be a key factor in reducing the onset of CRC. Hashim et al[38, 39] demonstrated that EVOO phenols can inhibit proliferation of colon cancer cells both *in vitro* and *in vivo* using different experimental approach. One of the mechanism of action proposed by Guichard and coworkers [40], to explain the block of the proliferation by tyrosol on HT29 cells, involve the extracellular signal-regulated kinase (ERK) 1/2 and Akt/PKB pro-survival factors by altering their phosphorylation status. The same results was achieved by Corona et al [41], using Caco-2 cells they found a rapid inhibition of ERK 1/2 phosphorylation and a subsequent reduction of cyclin D1 and COX-2 expression. Cardeno et al investigated the relation between antiproliferative effect of Oleuropein and HT and the expression of HIF 1α (hypoxia inducible factor) on HT29 cells. After 24 hours of exposure OLE produced a significant downregulation of HIF 1α with a subsequent upregulation of P53 protein espression[42]. In more recent years the work of Di Francesco et al[43] evaluated the epigenetic change behind the exposure of colorectal cancer cells to HT. Interestingly they found an up regulation of *CNR1* gene − encoding for type 1 cannabinoid receptor (CB1) which is known as a tumor suppressor gene. After exposing the cells to EVOO phenol extract or HT they found a reduced CpG methylation of the promoter of CNR1 gene consistent with the antiproliferative effect seen. In the same work

similar epigenetic alteration were observed *in vivo* in the colon of rats fed for 10 days with EVOO supplementation. It's of great interest to note that many of the study conducted on colorectal cancer were made using an *in vivo* experimental model and as above mentioned the obtained data were comparable to those obtained *in vitro,* thus highlighting the potential role of this class of compound in the prevention of this kind of neoplasm.

1.5.3   Breast cancer

The incidence of breast cancer (BC) in the Mediterranean area is much lower if compared to all the world and it is the main reasons for the growing body of scientific literature about the relation between BC and nutraceuticals[44, 45]. One of the first evidence of the antiproliferative properties of EVOO phenols against a BC cellular model was obtained by Han et al[46] in this work they demonstrated that Oleuropein and Hidroxytirosol were able to block cell cycle progression at G1 stage in MCF7 breast cancer cell, with a subsequent apoptosis activation. Interesting several studies investigated the ability of EVOO phenols to interfere with ER signaling[47-53], it's well known that estrogens promote the proliferation of ER-positive breast cancer, therefore the ER receptor could be an important pharmacological target in this disease. The aromatic ring contained in OLE and HT could compete with the estrogens (which share similar structure) for the estrogen receptor binding sites, this is a common feature of other phytoestrogen like quercetin and genestein. Other than canonical activation of ER signaling Sirianni et al[54] demonstrated that OLE and HT could block BC cell proliferation trough the inhibition of ERK1/2 activation. In a recent study Akl et al[55] investigated the intracellular mechanism responsible of the antiproliferative

effect of Oleocanthal against a BC experimental model. The treatment of three different BC cell lines produced a dose-dependent inhibition of HGF-induced cell migration, invasion and a block of cell cycle progression in the G1/S phase. Moreover, They found that (-)-oleocanthal treatment effects were mediated via inhibition of HGF-induced c-Met activation and its downstream mitogenic signaling pathways with a subsequent block of EMT and reduction in cellular motility. In this work they also showed an inhibition of tumor growth in an orthotopic model of breast cancer in athymic nude mice after treatment with (-)-oleocanthal.

Despite the large amount of scientific work investigating the role of EVOO polyphenols in various tumor model, surprisingly there are no literature data about the interaction between these molecules and bladder neoplasia.

1.6   *Bladder cancer and nutrition: is there a relationship?*

Bladder cancer (BCa) is one of the most common cancer in Western countries and the first cause of death among urinary malignancies. BCa incidence and mortality rates vary substantially across countries due in part to different risk factors. In 2007, a comprehensive review by the World Cancer Research Fund and the American Institute for Cancer Research concluded that established risk factors for bladder cancer include tobacco consumption, infection with Schistosoma haematobium, and both occupational and environmental exposures to carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons and arsenic in drinking water[56]. The role of nutraceuticals like protecting factor in the developing of BCa remain still unclear but there are many data that seems to link a regular consumption of fruit and vegetables with a lower incidence of urothelial neoplasia. A recent study in the multiethnic

cohort showed how the intake of vegetables and some related micronutrients such as vitamins A, C, E and carotenoids were inversely associated with bladder cancer risk only in women[57]. Ros et al found an association between higher plasma carotenoids concentrations and a lower incidence of bladder cancer suggesting that specific compounds in fruit and vegetables may have protective a protective role towards bladder cancer carcinogenesis[58]. Moreover, data of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, found an inverse association between the dietary intake of flavanols and lignans and the risk of bladder cancer[7]. Despite the above mentioned data, collectively, there is a lack of clear epidemiological evidence that link consumption of polyphenols reach food and BCa, even if is well know that these molecules are excreted trough urine in a dose dependent manner[59]. Dietary studies are commonly limited in their conclusions because of heterogeneity of the population studied, variations in lifestyles and the complexity of diet, with different components of the food exerting potential chemopreventive effects, but also containing carcinogens derived from environmental pollution. However, the weak epidemiological evidences indicating a protective role of the diet phytochemicals against BCa are well supported by different biological findings. It was demonstrated that the urine of the BCa patients exhibit high levels of 8-hydroxydeoxyguanosine which is a marker of oxidative stress excreted in urine upon DNA repair, in this work the authors concluded that genotoxic damage induced by oxidative stress may be an important pathological feature of bladder cancer patients[60].This finding is of great interests if taken together with the above mentioned Tuck et al work that demonstrated how the conjugate form of EVOO phenols excreted in urine

retain all of the antioxidant properties of the ingested compounds. Collectively the above reviewed data seems to support the thesis that polyphenols rich food like can act like a protecting factor against BCa but more investigation about bioavailability and urinary excretion of these bioactive molecules are needed.

2 Aims

2.1 To nvestigate the ability of EVOO phenols to modulate biological process related to bladder cancer cell motility

The consumption of extra virgin olive oil (EVOO), a common dietary habit of the mediterranean people, seems to be related to a lower incidence of certain types of cancer including bladder neoplasm[61]. Metastases are the major cause of bladder cancer-related deaths and targeting cell motility has been proposed as a therapeutic strategy to prevent cancer spread[62]. The first part of this study aim at investigate the potential anti-metastatic effect of total phenols extracted from EVOO against the human transitional bladder carcinoma cell line T24 using an *in vitro* approach.

2.2 To investigate the antiproliferative activity of EVOO phenols and its ability to chemosensitize bladder cancer cells to chemotherapics.

In the second part we will focus our attention on investigating the antiproliferative activity of EVOO-E and its ability to improve the cytotoxic properties of commonly used chemoterapics in the same experimental condition described above (2.1).

Even if the correlation between bladder cancer and diet has been a topic of interest of many epidemiological studies, most of them were focused on the role of nutraceutical as a possible chemopreventive for this disease. To date there is a small amount of research about the role that nutraceutical can play once the cancer is identified. The data about the interaction between antioxidant of vegetal origin and chemotherapy are controversial and opposing[63-65]. In our opinion this topic is really important as patients are increasingly searching for ways to improve in a safe manner the common pharmacological approach.

3   Materials and methods

3.1   Extraction of the phenolic fraction.

EVOO-E were obtained according to previously published methods [66] with some modification, from 30 different lots of an endemic monocultivar olive oil (*Olea europaea* L. var. Itrana) within one month after production. Fifty milliliters of each oil sample were extracted with 150 mL of CH_3CN/H_2O (70:30 v/v) (Sigma–Aldrich, St. Louis, MO, USA). A defatting with *n*-hexane was performed to completely remove the lipid fraction. Aliquots of the raw hydrophilic extract were dried by speedvac and stored at -80°C until use. The extract was redissolved in DMSO (Sigma–Aldrich, St. Louis, MO, USA) at a stock concentration of 10 mg/mL immediately before performing experiments.

3.2   HPLC analysis of the EVOO extract.

The HPLC analysis of the extract was carried out with a Waters apparatus, equipped with a 600 F pump and pump controller models, a Rheodyne injection valve with a 50 µl loop, a Symmetry 300 column (C18 reverse phase, 4.6x250 mm, 5 µm particle size, thermostated at 25°C, with a 3.9x20 mm guard column of the same material matrix) and a Waters 2996 Photodiode array detector. The elution was performed at a flow rate of 1 mL/min, with solvent A being 2% acetic acid and solvent B being methanol. The gradient consisted of an initial 5% B for 5 min, to 50% B in 35 min, 100% B in 10 min, 100% B for 10 min. Samples were filtered through a 0.45 µm cellulose syringe filter before injection. Elution was monitored at 280 nm and peak identification was obtained by comparing retention times and spectral characteristics with those of authentic standards.

3.3 Cell cultures.

T-24 ,5637 cells (human urinary bladder carcinoma) were obtained from Cell Lines Service (CLS, Eppelheim, Germany) and cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. All media and sera for cell culture were purchased from Gibco (Grand Island, NY, USA) and were endotoxin-free. Human urothelial fibroblast were collected according to previously published methods [67] and kindly provided by Dr. Elena De Falco (Department of Medical-surgical Sciences and Biotechnologies, "Sapienza" University of Rome) and cultured as above described for T24 cells. HT1197 were obtained from ICLC (ICLC, Genova, Italy) and cultured in MEM medium supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin

3.4 Cytotoxicity assays.

Cell viability was assessed by Trypan blue (Sigma–Aldrich, St. Louis, MO, USA) exclusion assay and using the dye [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Promega Corporation, Madison, WI). The assay is based on the ability of living cells to convert MTT into an insoluble purple-coloured formazan, whose amount is proportional to the number of living cells. Cells seeded in 96-well plates at a density of 5000 cells/well were exposed to EVOO-E at a concentration ranging between 2.5 and 100 µg/mL. After 24 hours, cells were treated with 20 µl of a 5 mg/mL solution of MTT in phosphate buffered saline (Gibco, Grand Island, NY, USA) and incubated at 37°C for 4 hours. After discarding the medium, the formazan was extracted with DMSO, and absorbance read at 570 nm with a reference at 690 nm.

3.5 Colony forming ability

The cells were plated at density of 200 per 10-cm dishes and exposed to various concentration of EVOO-E. Cells were grown for 14 days with a media renewal every two days. After two weeks the cells were fixed with ethanol, and stained with Giemsa to detect colonies.

3.6 Cell adhesion assay.

Cell adhesion assay was performed as previously described [68]. Briefly, the cells (1×10^5/well) were resuspended in culture medium containing different concentrations of EVOO-E and were plated into 96-well plates coated with fibronectin (Becton–Dickinson, Bedford, MA). After incubation in 5% CO_2 at 37°C for 6 hours, the cells were washed thrice with PBS to remove the non-adherent cells. The attached cells were analyzed by MTT assay.

3.7 Migration assay.

The migration assay was performed through the chemotactic directional migration by using a 24-well transwell insert (8 μm pore size, EMD Millipore, Temecula, CA, USA). The cells (10^4 cells/well in serum free DMEM) were placed in the upper chamber and treated in the absence or in the presence of EVOO-E (2.5, 5 and 10 µg/mL) while the lower chamber was filled with DMEM supplemented with 10% FBS as a chemoattractant. After 24 hours of incubation, a cotton swab was used to remove the non-migrated cells in the upper chamber then the filters were individually stained with 2% crystal violet and the migrated cells adherent to the underside of the filter were examined, counted and photographed under a microscope at x200 magnification.

3.8 Invasion assay.

The invasion assay was performed by using Matrigel (BD Biosciences, Bedford, MA, USA)-coated transwell cell culture chambers (8 μm pore size, EMD Millipore, Temecula, CA, USA) as described previously. The cells (10^4 cells/0.4 mL) were cultured for 24 hours in serum-free DMEM medium and then placed in the upper chamber of the transwell insert (5×10^4 cells/well) and treated with 0, 2.5, 5 and 10 μg/mL of EVOO-E. The lower chamber was filled with DMEM supplemented with 10% of FBS and the corresponding concentration of extract used in the upper side. The cells were incubated for 24 hours at 37°C in a humidified atmosphere with 95% air and 5% CO_2. At the end of incubation, cells were stained as above described.

3.9 Gelatin zymography.

The activities of MMP-2 and MMP-9 were measured after exposure to various concentrations of EVOO-E. The cells (4×10^5 cells/well) were plated in 6-well tissue culture plates and incubated in DMEM supplemented with 10% of serum. Before reaching 80% of confluence the cells were washed twice with PBS and incubated in serum free medium with or without 2.5, and 10 μg/mL of extract for 24 hours. An appropriate volume of conditioned medium, adjusted for vital cell number [69], was loaded on a 9% sodium dodecyl sulfate-polyacrylamide gel under non-reducing conditions. After electrophoresis, the gel was soaked in 2.5% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) twice for a total of 60 min at 25°C, then it was incubated in substrate buffer (50 mM Tris, pH 7.6, 10 mM $CaCl_2$, 50 mM NaCl and 1μM $ZnCl_2$) at 37°C for 18 hours. Positive controls were achieved using recombinant human MMP-2 and MMP-9 (Calbiochem, San Diego, CA, USA). Bands corresponding to activity of MMP-2

and -9 were visualized by negative staining using 0.3% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid as described elsewhere [69]. Quantification of the data from the band density was performed by NIH ImageJ software.

3.10 Quantitative real-time PCR of EMT gene expression.

Cells (1×10^6 cells/well) were placed in 6-well plates and incubated with various concentrations of EVOO-E. Cells were collected and total RNA was extracted from each treatment as previously described. RNA samples were reverse-transcribed at 42°C with High Capacity cDNA Reverse Transcription Kit for 30 min according to the protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers sequences used were listed in Table 2.

Applied Biosystems 7500 Real-Time PCR system was used for each assay in triplicate and expression fold-changes were derived using the comparative CT (threshold cycle) method [70, 71] using 18S as reference gene.

3.11 Cell cycle analysis

Briefly cells were harvested and seeded into 25 cm^2 flasks at a density of 3×10^5 cells 48 hr before treatment with olive oil phenolic extract at various concentrations (0, 10, 20, 40 µg/ml) in triplicate. Media containing the olive oil phenolic extract was added and the cells incubated for 24 hr at 37°C/5% CO_2. Cells were then washed with PBS and harvested by the addition of 1 ml trypsin EDTA and incubation at 37°C for up to 5 min. Two millilitres of serum-containing medium was added to inactivate the trypsin. At this stage, a cell and viability count was carried out using a haemocytometer and trypan blue dye. The cells were transferred to polypropylene tubes (Becton Dickinson) and

centrifuged at 258g for 3 min. The supernatant was carefully discarded and the pellet re-suspended in 200 µl of ice cold PBS and 2 ml of 70% ethanol/30% PBS. Cells were incubated on ice for 30 min, centrifuged at 258g for 3 min, the supernatant carefully discarded, and the cells re-suspended in 800 µl of ice cold PBS, 100 µl of RNase A (1 mg/ml) (Sigma Chemicals), and 100 µl of propidium iodide (400 µg/ml) (Sigma Chemicals). The cells were then incubated at 37°C for 30 min before being analysed. Samples were processed on a Accuri C6 flow cytometer (Becton Dickinson Immunocytometry Systems, UK) equipped with a laser (excitation wave length = 488 nm), and the fluorescence emission spectra of propidium iodide was collected at 585 nm (designated FL2), using C6 Software (Becton Dickinson). A total of 10,000 events were captured. These emission spectra were analysed subsequently for DNA content using ModFit LT Software (Verity Software House, Topsham, ME). Each experiment was run in duplicate and the complete data set is the mean of 3 independent experiments. The complete dataset was analysed using ANOVA carried out in SPSS for Windows.

3.12 Immunofluorescent staining of F-actin and β-Tubulin.

For immunofluorescent labeling, after EVOOE treatment cells were simultaneously fixed and permeabilized by brief (1 min) treatment with 0.25% Triton X-100 and 0.5% paraformaldehyde in cytoskeleton buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, and 5 mM glucose, pH 6.1) and then postfixed with 1% paraformaldehyde for 10 min, washed with PBS, and reduced in 1 mM sodium borohydride, to reduce autofluorescence. Coverslips were then incubated for 40 min with 10 µl of 1:200 diluted primary mouse antiβ-Tubulin antibody droplets, or with 1:200 TRITC-conjugated phalloidin

(Cell Signaling Technology). The sample preincubated with primary antibody where washed three times with PBS, and incubated with the secondary goat anti mouse FITC conjugated antibodies for 40 min. After an additional three washes with PBS, the nucleus were counterstained with DAPI and the coverslips were mounted on glass slides in Elvanol (Mowiol 4-88, Hoechst, Frankfurt, Germany).

3.13 Western blot analysis

The cells (2×10^6) were seeded in a six-well plate. After 24hours, cells were treated with different concentrations of EVOO extract for 24. Cells and culture medium were then harvested, washed twice with PBS, and finally lysed into RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, and 0.1% sodium dodecyl sulfate; SDS), containing a protease-inhibitor . After 20 minutes on ice, lysates were centrifuged at 20,000 × g for 10 minutes. Protein concentration was determined with the Bradford method. The lysates (20 µg of the total protein) were resolved on 8-12% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto PVDF membranes. Membranes were blocked for 1 hour with 5% (BSA), dissolved in TBS solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). Membranes were then successively incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-PARP diluted 1:2000 in TBST (TBS with 0.1% Tween 20) with 5% BSA and mouse procaspase 3 and rabbit procaspase 9 diluted 1:500 in TBST. After washing in TBST solution, the secondary antibody (antirabbit -mouse IgG horseradish peroxidase (HRP)-conjugated antibodies Cell Signaling Technology), diluted in TBST/5% BSA, was added for 50 minutes at room

temperature. signals were visualized by ECL reagent according to the manufacture instructions.

3.14 **Statistics**.

Each assay was replicated at least four times, and statistical significance was determined using Graphpad Prism 4 statistical software package (Graphpad, San Diego, CA, USA). Data were expressed as mean ± SEM. Comparison of the groups was made by one-way ANOVA followed by Bonferroni's post hoc test. Statistical significance was defined as $P < 0.05$.

4 RESULTS AND DISCUSSION

4.1   EVOO-E chromatographic analysis.

After the extraction process from the different EVOO samples we performed identification of the main phenolic compounds in EVOOE by HPLC-DAD. Chromatographic analyses   revealed no major differences in phenolic content of the various lots (see Table 3) which were characterized by a large amount of secoiridoids such as dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA) and dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), and of lignans such as pinoresinol. As regards the remaining compounds, TY and HT were the most abundant, whereas the remain was represented by simple phenolics such as vanillic acid, vanillin, *p*-coumaric acid, ferulic acid. For our aims we extracted the phenolic fraction from thirty different lots of an endemic monocultivar olive oil (*Olea europaea* L. var. Itrana) within one month after production in order to obtain a EVOO fraction rich in complex phenols. These results are in good agreement with literature data which report chromatographic profiles strictly similar to what obtained in our experimental conditions [72-74]. Furthermore, it is noteworthy that the different lots of olive oil analyzed present a low content of simple phenols such as TY and HT (Figure 3). These two compounds typically derive from the degradation of more complex molecules, such as secoiridoids and lignans, during prolonged storage or due to sub-optimal extraction conditions to limit simple phenols TY and HT (high temperature, crushing, malaxation, prolonged contact with waste waters, etc…) [75]. Hence our data indicate that the analyzed samples were obtained by correct production procedures giving rise to a high quality EVOO.

4.2   EVOO-E inhibits T24 migration and invasion

Prior to perform cell motility related experiment we evaluated the effect of different concentrations of EVOO-E on cell viability. In our experimental conditions EVOO-E was found to affect T24 cell viability after 24 hours of treatment at concentrations greater than 10 µg/mL in a dose dependent-manner (Figure 4). Notably, at the same concentrations fibroblasts viability was not affected. Despite their different histologic origin than T24 we decided to use fibroblast as a non–cancer control because of their marked motility. These results suggested that EVOOE selectively affects urothelial cancer cells, thus indicating that the cytotoxic potential is not attributable to an unspecific   effect when compared to the control both in T24 and in fibroblasts. As regards cell migration and invasion, data indicate that fibroblasts were not inhibited by the phenolic extract at the tested concentrations (Figure 4). Contrary to what observed for fibroblasts, the migration assay showed that the phenolic extract had a significant inhibitory effect on T24 cell motility. After 24 hours of treatment with the same doses of EVOO-E used above the percent of migrated cells decrease from $70 \pm 2\%$ to $10 \pm 3\%$, respectively (Figure 4). Furthermore, the penetration of the Matrigel-coated filter by T24 cells was substantially inhibited in the presence of EVOO-E in a dose dependent manner. The percent of invading cells after exposure to EVOO-E concentration ranging between 2.5 to 10 µg/mL drastically decrease from $53 \pm 2\%$ to $12 \pm 1\%$ respectively (Figure 4). It is of note, the observed inhibition occur at subtoxic doses of EVOO-E as shown by viability experiments.

Figure 5 reports the invasion index, which is the ratio between the number of invading cells and the number of migrating cells in the same experimental

conditions, and allows to determine which is the reciprocal contribution of the two phenomena [76]. The statistical analysis of the data showed a significant difference between T24 migrating and invading cells (P<0.001) at EVOO-E concentration of 2.5 and 5 µg/mL (Figure 5). Data indicate that at 2.5 and 5 µg/mL of EVOO-E the percent of migrating vs invading T24 cells was significantly different than untreated (P<0.001), with an invasion index of 0.72 and 0.67, respectively.

4.3 *EVOO extract attenuates the enzymatic activities of MMP-2 in T24 cells*

Due to the observed biological effects exerted towards cell motility we decided to focus our attention on evaluating the activity of two proteins mainly involved in cell motility process. The matrix metalloproteinases MMP-2 and -9, also known as the gelatinases, have been long recognized as major contributors to the proteolytic degradation of extracellular matrix during tumor invasion. In order to determine the effect of EVOO-E on the proteolytic activity, we investigated whether the extract could affect the secretion of gelatinases.

After 18 hours of treatment, the conditioned media of cells grown in the presence or absence of EVOOE were subjected to gelatin zymography to analyze MMP-2 and MMP-9 activities. Matrix metalloproteinases (MMPs), a family of Zn^{2+}-dependent endogenous proteases, are able to degrade various components of the extracellular matrix. In the extracellular domain, the activity of these proteases is tightly regulated by inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). It has been postulated that TIMPs act as tumor suppressor genes due to their anti-metalloproteinase activity and their protective role on the extracellular matrix [77]. The imbalance between MMPs and TIMPs may be an indicator for cancer prognosis [78]. Most data published

on MMP-2 and MMP-9 seem to link their role to aggressive behavior of different cancer types (Table 4). Indeed, MMP-2 overexpression is found to correlate with poor survival in different type of cancers [79-82]. The results shown in Figure 7 indicate that EVOOE significantly reduced only MMP-2 activity in a dose-dependent manner, with an inhibition of about 48% at 2.5 µg/mL and of 90% at 10 µg/mL (P <0,001). Moreover the exposure to EVOO-E did not affect the MMPs activities in fibroblasts conditioned medium, with no significant differences between control and treated samples. Indeed, enzymatic activity analysis reveals a significant decrease specifically for MMP-2 expression.

4.4 EVOOE alters the levels of gene expression associated with EMT only in T24 cells.

To better understand the molecular events behind the alteration of cell motility we decided to perform qRT-PCR of genes related to EMT. The epithelial-mesenchymal transition (EMT) is a multistep process in which epithelial cells lose their epithelial phenotype and acquire mesenchymal characteristics, such as motility and invasive properties. Numerous in vitro and in vivo studies suggest that the EMT is associated with cancer cell invasion and metastasis in various malignancies, including bladder cancer[83]. Despite their epithelial origin, T24 cells are a useful tool to assess a possible reversion of EMT due to the high expression level of mesenchymal genes such as MMP2, N-Cadherin, and β-Catenin. As shown in Figure 6, EVOO-E induced a tenfold reduction of MMP-2 gene expression in T24 cells at both concentrations without affecting MMP-9 mRNA expression (Figure 6). Moreover the treatment with subtoxic doses of EVOOE also induced a strong down regulation of Vimentin and β-

Catenin. In the same experimental conditions we also observed a significant increase of the levels of TIMP-1 and TIMP-2. The treatment of fibroblasts with EVOO-E did not gene expression levels at the tested concentrations (Figure 6). The above data show an upregulation of TIMP-1 and TIMP-2 which are endogenous tissue inhibitors of metalloproteinases. TIMP-1 has been shown to regulate MMP-2 activity and numerous evidences have indicated a correlation between elevated TIMP-1 levels and diminished MMP-2 activity and cell invasiveness[84-86]. These inhibitory effects of EVOO-E on T24 cells are not due to the cytotoxic effect of polyphenols because the viability of these cancer cells was not affected in the range of concentration tested (Figure 4).

We also evaluate the gene expression levels of cadherins. E-Cadherin is considered an active suppressor of invasion and growth of many epithelial cells. Recent studies indicate that cancer cells have up-regulated N-Cadherin in addition to loss of E-Cadherin, this change is called "cadherin switch" and is one of the hallmark of EMT[87]. The data obtained showed a marked decrease in the level of N-Cadherin gene expression with a subsequent up-regulation of E-Cadherin after the treatment with EVOO-E. Taken together all the data collected with qRT-PCR demonstrated how the treatment with EVOO-E is able to revert EMT restoring a less invasive phenotype in T24 bladder cancer cells. Interestingly the gene expression analysis revealed also a strong down-regulation of β-catenin expression which is a transcription factor of the Wnt signaling pathway and is involved in the regulation of cell adhesion. Furthermore these data indicate that EVOO-E mechanism of action is in part due to a regulation of gene expression, particularly for those proteins involved in migration and invasion.

4.5   EVOOE inhibits cell growth and clonogenical survival in a dose dependent manner .

In the second section of this study we decided to evaluate the ability of EVOOE to inhibit cell proliferation, for this purpose we added other two bladder cancer cell lines to our experimental model. Initially the effect of EVOOE on cell viability was assessed by trypan blue exclusion assay and the obtained dose response curves are shown in Figure 8. After 24 hours of exposure to various amount of extract, T24 cell viability was not affected at concentrations up to 10 µg/mL whereas it decreased in a dose dependent manner at higher concentrations with an IC50 of 32 ± 3 µg/mL.

The viability of 5637 cells was also inhibited in a dose depend manner with an IC50 of 55 ± 7 µg/mL. On the contrary HT1197 viability was only slightly affected at highest concentration of EVOOE. Human primary fibroblasts viability seems to be not altered by the cytotoxic properties of the phenols extract. The different susceptibility to polyphenols extract among the tested cells is probably ascribable to different biological factors such as gene mutational status. After this set of experiments we decided to exclude the HT1197 from next experiments due to the high concentration of EVOOE needed (more than 100 µg/mL) to block cell proliferation. Even if there is a wide amount of scientific literature that studied cytotoxic effect of extracts of various origin at very high concentration, we decided to perform our experiments using a rather low concentration of EVOOE, more representative of what can be reached *in vivo* with a phenol rich MD.

After this first set of experiments we also decided to compare the cytotoxic properties of three selected components of the phenolic mixture versus the

whole extract (EVOOE), the obtained dose response curves are shown in the figure 8. As expected, despite identification of several active chemical compounds in EVOOE, none of the isolated polyphenols demonstrated a biological activity higher than the whole oil extract (Figure 8). This finding is in line with data of Cardeno and coworkers [20] who demonstrated that oleuropein and hydroxytyrosol have anticancer activity on HT-29 human colon cancer cells only at concentrations higher than 400 and 200 µM, respectively. The higher anticancer activity of the whole extract compared to the purified polyphenol could be potentially attributed to a synergistic action among the various components of the extract. Based on these preliminary results, the whole phenolic extract was used for the subsequent experiments instead of the isolated chemical compounds. We also examined the effect of EVOO-E subtoxic doses on the clonogenic survival of T24 and 5637 cells. This assay determines the ability of a cell to proliferate indefinitely, retaining its reproductive ability to form a large colony or a clone. Interestingly, the data (Figure 9) showed a significative decrease in the ability of both cell lines to form colonies starting at a dose of 5 µg/mL with a marked inhibition of clonogenic activity at 10 µg/mL.

4.6   EVOOE blocks the cell cycle progression at G2/M stage

To better understand the mechanism underlying cell growth suppression by EVOOE, the cell cycle progression was investigated. T24 and 5637 cells were treated with increasing doses of EVOO-E, and cell cycle distribution analysis was then performed after 24 hours of exposure. As shown in Figure 10, G2/M population of EVOOE-treated T24 cells increased , from 11.1% of the control to 27.1% at EVOO-E doses of 20 and 40 µg/ml respectively. The treatment also

induced a marked decrease of G0/G1 population in a dose dependent manner with a subsequent increase of sub-G1 particles. This result revealed that EVOOE treatment caused marked accumulation of a G2/M population in T24 cells that lead to an accumulation of sub G1 fraction probably indicating and induction of apoptosis. The cell cycle analysis of 5637 after EVOOE treatment revealed a similar behavior to what observed for T24 with a bigger increase of G2/M population at 40 µg/ml (27.1% of T24 versus 44 %of 5637) and the absence of sub-G1 fraction. The data obtained from cell cycle analysis show a similar mechanism of action of EVOO-E in fact both the cell lines tested were subject to growth arrest prior to mitosis despite their different mutational status. An hypothesis we made to explain this similar response to the polyphenols extract action is that EVOO-E can interfere with the cytoskeleton remodeling and the subsequent mitotic process. During all of our experiments we always notice a change in cell morphology. After the treatment with greater concentration of EVOOE (20-40 µg/ml) the cells acquire a rounded morphology. In order to verify the alteration of the cytoskeleton we performed Immuno fluorescence experiments using Phalloidin (that bind to F-Actin) and an antibody against β-Tubulin . As it can be clearly visible from the Figure 11 the treatment induce a change in cell morphology with a marked variation of cytoskeleton network arrangement. This observation is in line with the findings of Hamdi et al[88] that demonstrated how Oleuropein treatment disrupts the organization of actin filaments altering cancer cells shape and their ability to proliferate.

4.7 EVOOE induce apoptosis only in T24

Despite the cell cycle is blocked in the G2/M phase in both the cell lines, cell cycle analysis experiment revealed only in T24 cell, an accumulation of particles (sub G1) with a small content of DNA that usually indicate apoptosis(Figure 12). In order to confirm the above hypothesis we performed Western blot analysis of PARP and pro-Caspase 3-9. EVOO-E was found to induce apoptotic cell death only in T24 cells in a dose dependent manner. Therefore apoptosis was observed at 24 h, as indicated by the cleavage of PARP. Proteolytic cleavage of specific proteins such as PARP has been shown to occur in cells exposed to a number of apoptotic stimuli[89-91]. Western blot analysis of the cleavage of PARP showed a decrease in the full-size Mw 116,000 fragment and an increase in the cleaved fragment within 24 h after the bladder tumor cells were treated with 20 µg/ml or 40 µg/ml of EVOOE (Figure 13). Western blot analysis of procaspase 3 and 9 also showed a marked decrease of their protein expression at higher doses of polyphenols extract indeed cleavage that triggers the activation of various enzymes to initiate apoptotic cell death. Conversely to what observed for T24, the treatment of 5637 cells does not induce apoptosis as it is clearly visible from the Figure 13. This different behavior between the two cell lines tested, in response to EVOO-E treatment, can be explained by different reasons such as different mutational status in key genes involved in cell proliferation regulation(Table 5). The induction of programmed cell death only in T24 can also explain the different susceptibility to the cytotoxic action of EVOOE showed in the firsts experiments (figure 3).

4.8   *Evaluation of cytotoxic effect of conventional chemoterapics in association with EVOOE*.

In the last part of this study we wanted to evaluate if the simultaneous treatment of T24 cells with commonly used chemotherapics and sub toxic doses of EVOOE can improve the cytotoxic properties of the drugs. We chose to use for all the experiments a concentration of EVOOE of 10 µg/ml which is not toxic, as previously described (see Figure 4) but can strongly affect gene expression (Figure 6). Prior to each experiments we performed cytotoxicity assay using only the chemoterapics in order to asses the IC50 (data not shown) and the right doses of drugs to use in the next experiments.

4.8.1   *EVOOE does not alter Etoposide cytotoxicity*

Etoposide is a semisynthetic derivative of podophyllotoxin, a substance extracted from the mandrake root *Podophyllum peltatum*. Having potent antineoplastic properties, etoposide binds to, and inhibits, topoisomerase II and impairs its function in ligating cleaved DNA molecules, resulting in the accumulation of single- or double-strand DNA breaks, the inhibition of DNA replication and transcription, and apoptotic cell death. Etoposide acts primarily in the G2 and S phases of the cell cycle. Etoposide is approved by the Food and Drug Administration (FDA) to treat refractory testicular cancer and small cell lung cancer. It has also been useful for other types of cancer, including non-small cell lung cancer, leukemia (acute myelocytic leukemia),uterine carcinoma, myeloblastoma, mycosis fungoides , neuroblastoma , and prostate cancer. After the determination of drugs IC50, we treated for 48 hours, T24 cells with different doses of Etoposide in association with 10 µg/ml of EVOOE. As it is clearly visible from the Figure 14 the cotreatment does not improve the

cytotoxicity of the drug and there with no significant difference between the concentration tested.

4.8.2 EVOOE decreases Mitomycin C toxicity

Mitomycin is a methylazirinopyrroloindoledione antineoplastic antibiotic isolated from the bacterium Streptomyces caespitosus and other Streptomyces bacterial species. Mitomycin C generates oxygen radicals, alkylates DNA, and produces interstrand DNA cross-links, thereby inhibiting DNA synthesis. Preferentially toxic to hypoxic cells, mitomycin C also inhibits RNA and protein synthesis at high concentrations. In the chemotherapy of bladder cancer, togheter with the baciluss of Calmette Guerin, this drug is often used[92]. The treatment consistS in an intravesical instillation of a 1 mg/mL solution of Mitomycin retained for two hours inside the bladder. To evaluate the effect on cell viability of the simultaneous exposure to the drug and EVOO-E we treated for 48 hours T24 cells with different doses of Mitomycin with and without 10 µg/ml of phenolic extract. The data showed in Figure 14 panel b demonstrated that, at any concentration tested, the simultaneous treatment reduced the drug cytotoxicity. At a drug concentration of 100 µg/ml the cell viability increased from about 15% when exposed to Mitomycin alone to 50% of the cotreatment, and similar behavior was observed at all of the concentration tested. The data showed show that EVOO-E reduced Mitomycin antiproliferative ability. These results are surprising only in part, indeed it is know that one of the Mitomycin C mechanism of action is a ROS-dependent activation of apoptotic cell death[93], probably the antioxidant properties of EVOOE can interfere with the ROS production induced by the drug, thus reducing its citoxicity. Even if this is the first study about the interaction of EVOO phenols and Mitomycin there are

46

many reports [94-96] that show how different phenolic molecules extracted from various biological matrix can reduce Mitomycin genotoxic damage due to their antioxidant activity.

4.8.3 EVOOE strongly increases Paclitaxel induced apoptosis.

Paclitaxel is a mitotic inhibitor isolated from the bark of the Pacific yew tree, *Taxus brevifolia* and named it taxol. Later it was discovered that endophytic fungi in the bark synthesize paclitaxel that belongs to the family of taxoid wich are diterpenoids with a structure based on the taxane skeleton. This antineoplastic agent is indicated for the treatment of advanced carcinoma of the ovary, and other various cancers including bladder cancer. Paclitaxel is a antimicrotubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization[97]. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for basic vital process and cell replication. Paclitaxel effect is due to its ability to interfere with the normal function of microtubule growth. Whereas drugs like colchicine cause the depolymerization of microtubules *in vivo*, paclitaxel arrests their function by having the opposite effect; it hyper-stabilizes their structure destroying the cytoskeleton flexibility. Specifically, paclitaxel binds to the β subunit of tubulin. Tubulin is the "building block" of microtubules, and the binding of paclitaxel locks these building blocks in place. The resulting microtubule/paclitaxel complex does not have the ability to disassemble.

To perform our experiments, first we determined the drug dose response curve and then we treated we treated for 48 hours T24 cells with three different concentrations of Paclitaxel alone or in association with 10 µg/mL of EVOOE.

The obtained data are show in Figure 14 panel C. Interestingly, at every tested concentration the addition of EVOO-E strongly increases the drug cytotoxicity. When we treated the cells with 5 nM of Paclitaxel the cell viability decreased from 75% of the drug alone to about 25% of the association treatment, and similar response was observed at 20 and 100 nM. Moreover the data analysis showed that exposure to 100 nM of Paclitaxel exert the same cytotoxicity of 5 nM of the same drug if used in association with EVOOE. Collectively the data demonstrates, that combination of EVOOE and paclitaxel possesses a higher antiproliferative activity *in vitro* compared with each of them alone. In order to evaluate the mechanism behind the increase cell death we performed western blot to asses PARP cleavage after the same treatments of the above experiment. As it is clearly visible from the Figure 15, the simultaneous treatment of Paclitaxel and EVOOE strongly increase the apoptotic cell death at every of the tested concentration, with a marked accumulation of the 85 kDa cleaved fragment of PARP. To our knowledge this the first report that show how EVOO phenols drastically improve the activity of Paclitaxel and more experiments are needed to clarify the mechanism behind this synergism. One of the hypothesis we made, that need to be investigated, is the involvement of MDR protein such as P-Glycoprotein in the chemosensitization induced by EVOOE. There are many reports that show how different polyphenol molecules (catechin from green tea or tannin from grape) exert their chemosensitizing activity reverting MDR expression[98]. Another interesting evidence supporting our hypothesis is the recent work of Xia et al[99] demonstrating that aberrant expression of the MDR1-encoded P-glycoprotein (P-gp) is associated with alteration in Wnt signaling. In this work the authors show how β-catenin knocking down f

completely   abrogates MDR1 transcription and expression resulting in a complete reversal of P-gp-dependent efflux function. The above findings are of great interest because in our EMT gene expression experiment we also found a strong downregulation of β-catenin (Figure 6) therefore this can be a good starting point for further experiments.

5 Conclusion

It is widely known that the polyphenols contained in various foods exert beneficial effects on health due to their antioxidant, anticancer and anti-inflammatory properties [100]. There is a wide body of literature which studies the effect of single polyphenols such as TY, EGCG, quercetin, resveratrol, etc., whereas less efforts have been devoted to the elucidation of complex matrices extracted from foods. In recent years our group started to study the biological effects of complex matrices extracted from different foods rich in polyphenols and antioxidants [101]. In the present work, our first aim was to evaluate the effects of total polyphenols extracted from a locally produced EVOO against cancer cells ability to spread. The anticancer effect of olive oil polyphenols is well known, however, for the first time we demonstrated that EVOO-E exerts an antiproliferative effect on bladder cancer cells *in vitro* and furthermore we demonstrated that olive oil polyphenols are able to inhibit the metastatic potential of T24 by inhibiting cell migration and invasion through a downregulation of MMP-2 expression. Also, it is of great interest to highlight that the cytotoxic effects observed were specific for T24 cells, a cell line widely used as a model for bladder carcinogenesis [102-104] and known to be highly invasive and aggressive, whereas no effect was evidenced in normal human fibroblasts.

It is well known that tumor metastasis occurs in many steps including vessel formation, cell attachment, adhesion, invasion, migration and proliferation, and these events are regulated by extremely complex mechanisms. Our findings indicate that EVOO-E is able to drastically reduce *in vitro* invasion and migration of T24 bladder cancer cells interfering with EMT gene expression. Interestingly we found that the exposure to low concentration of EVOO-E, easy

51

to achieve *in vivo*, can revert the epithelial to mesenchymal transition in our cellular model, giving a less invasive phenotype mainly targeting metalloproteinases. Numerous reports have shown that the inhibition of MMPs expression and/or enzymatic activities can be used as an early target for preventing metastasis[105, 106]. MMP-2 is involved in the invasive metastatic potential of tumor cells not only through the degradation of ECM [107] but also by the regulation of cell motility by interacting with integrines and guiding the direction of cell migration [108]. Our results show that at subtoxic doses, EVOOE inhibits MMP-2 activity and subsequently affects the process of migration and invasion. Notably, both phenomena can be attributed to the lower activity of MMP-2. Indeed, gene expression analysis reveals a significant decrease of MMP-2 expression and this result is in good agreement with what observed for the enzymatic activity analysis.

We notice and upregulation of TIMP-1 and TIMP-2 which are endogenous tissue inhibitors of metalloproteinases. TIMP-1 has been shown to regulate MMP-2 activity and numerous evidences have indicated a correlation between elevated TIMP-1 levels and diminished MMP-2 activity and cell invasiveness[84-86]. These inhibitory effects of EVOO-E on T24 cells are not due to the cytotoxic effect of polyphenols since the viability of these cancer cells was not affected in the range of concentration tested (Figure 4). We also performed qRT-PCR on HT1197 transitional bladder cell line which derives from a grade 4 transitional cell carcinoma of the bladder. We observed an effect strictly similar to what evidenced in T24 cells, *i.e.* a strong down-regulation of MMP-2 expression and a negligible effect on MMP-9 (data not shown). These data indicate that EVOOE exerts its effects on MMP-2 expression independently of

the cell line used. Furthermore these data indicate that EVOOE mechanism of action is in part due to a regulation of gene expression, particularly for those proteins involved in migration and invasion. This findings are well supported by the gene expression analysis of EMT related protein that show how the EVOO-E treatment revert this phenotypical change that is a hallmark of different type of cancer cells. In the second part of this study we clarify how the olive oil phenolics exert their antiproliferative activity inducing a block of the cell cycle progression at G2/M stage probably due to interaction with the cytoskeleton. Interestingly we found a different cellular response in EVOO-E ability to induce cell death. We found the activation of apoptosis only in T24 and therefore an improvement of cell toxicity. This finding is not surprising to our point of view because of the different biology of the cell lines tested, but highlights the need of more and different experimental models to better understand olive oil phenols mechanism of action. In the last section of this work we evaluated the ability of low concentration of EVOO-E to improve the activity of different chemotherapics used in the common clinical practice. It is of great interest to underline that the current adjuvant therapy in the management of BCa consist in bladder instillation of alchilant agents [92] that otherwise could exert unfavourable effects [109]. Usually from an analytical point of view, different statistical analyses could be performed to test for synergistic, additive, or sub-additive interactions of combined treatments. These methods include isobologram analyses[110], mean inactivation dose[111] or the median effect principle[112], all of them require the generation of big data amount using a broad range of concentrations of different treatments. In this study we were able to use a simple approach based on the comparison of viability since one of

the two treatments given in co-association is always not toxic (EVOO-E). As expected we obtained different results based on the drug tested. Our data demonstrated that simultaneous treatment with Mitomycin and EVOO-E markedly decrease the cytotoxicity of the drug probably due to the antoxidant activity of the phenol molecules contained in the extract. It is well known that many molecules of vegetal origin can interfere with drug therapy. For example the interaction between garlic and Coumadin is well documented[113], but to our knowledge there are no data about customized diet plan to take before and after Mytomycin treatment even if there are controversial data about the role of antioxidants supplements during chemotherapy[63-65]. Probably, a good intake of EVOO-E after the chemotherapy can be helpful in two different manner because it can counteract the toxic residual of alchilating agent, exerting at the same time, the above mentioned anticancer properties. Conversely to what observed for Mitomycin, the addition of EVOO-E to the Paclitaxel treatment lead to a strong increase of the drug toxicity with a marked induction of apoptotic cell death. These data are of big interest because the dose of EVOO-E used for this experiment is not toxic to the cells themselves, and not induce the cytoskeletal alteration observed at higher concentration. In our opinion the cytotoxicity enhancing of Paclitaxel maybe due the observed alternation of gene expression (occuring at low doses of phenolic extract) that chemosensitize the cells by altering the signaling pathways involved in cell proliferation. Collectively our findings suggest that the potential adjuvant properties of EVOO phenols are related to the class of drugs used according to previously published data that point out how the adjunct of high doses of antioxidant to chemotherapy does not always improve the pharmacological treatments[114].

It is important to highlight that even if the mechanism of action of many chemotherapics and also radiotherapy is based in part on the production of free radicals[64, 65] (in that case the intake of antioxidant is not suitable) there are also cytostatic drugs that do not work through oxidative stress mechanism like paclitaxel, vinca alkaloids, anthracycline and many others that can be improved by the use of antioxidant rich supplements[63]. The results obtained in this work suggest that EVOO-E may have efficacy, if delivered directly to the bladder, either alone or in combination with specific therapeutic agents for the treatment of bladder cancer. Moreover, the experimental evidence collected support the growing body of epidemiological data which link olive oil consumption to lower incidence of BCa confirming the bioactivity of natural compounds and their potential use in cancer therapy. Collectively, EVOO-E has multiple anti-metastatic effects and has the potential to be developed as a non-toxic adjuvant for bladder cancer treatment.

6 Figures

Fatty acid	Description
Oleic Acid (C18:1)	monounsaturated omega-9 fatty acid. It makes up 55 to 83% of olive oil.
Linoleic Acid (C18:2)	polyunsaturated omega-6 fatty acid that makes up about 3.5 to 21% of olive oil
Palmitic Acid (C16:0)	saturated fatty acid that makes up 7.5 to 20% of olive oil.
Stearic Acid (C18:0)	saturated fatty acid that makes up 0.5 to 5% of olive oil
Linolenic Acid (C18:3)	polyunsaturated omega-3 fatty acid that makes up 0 to 1,5% of olive oil

Table 1 Fatty acids composition of a common extra virgin olive oil.

Polyunsaturated fatty acids (PUFAs) with 18 carbon (C18) atoms such as linoleic (18:2 ω-6), and α-linolenic (18:3 ω-3) are known as essential fatty acids (EFAs) in human nutrition. These fatty acids, although regarded as an indispensable component for cell structure and development and function, cannot be synthesized by the human body. The intake of PUFA is necessary through diet, and should account for only 6–8% of calories from fat.
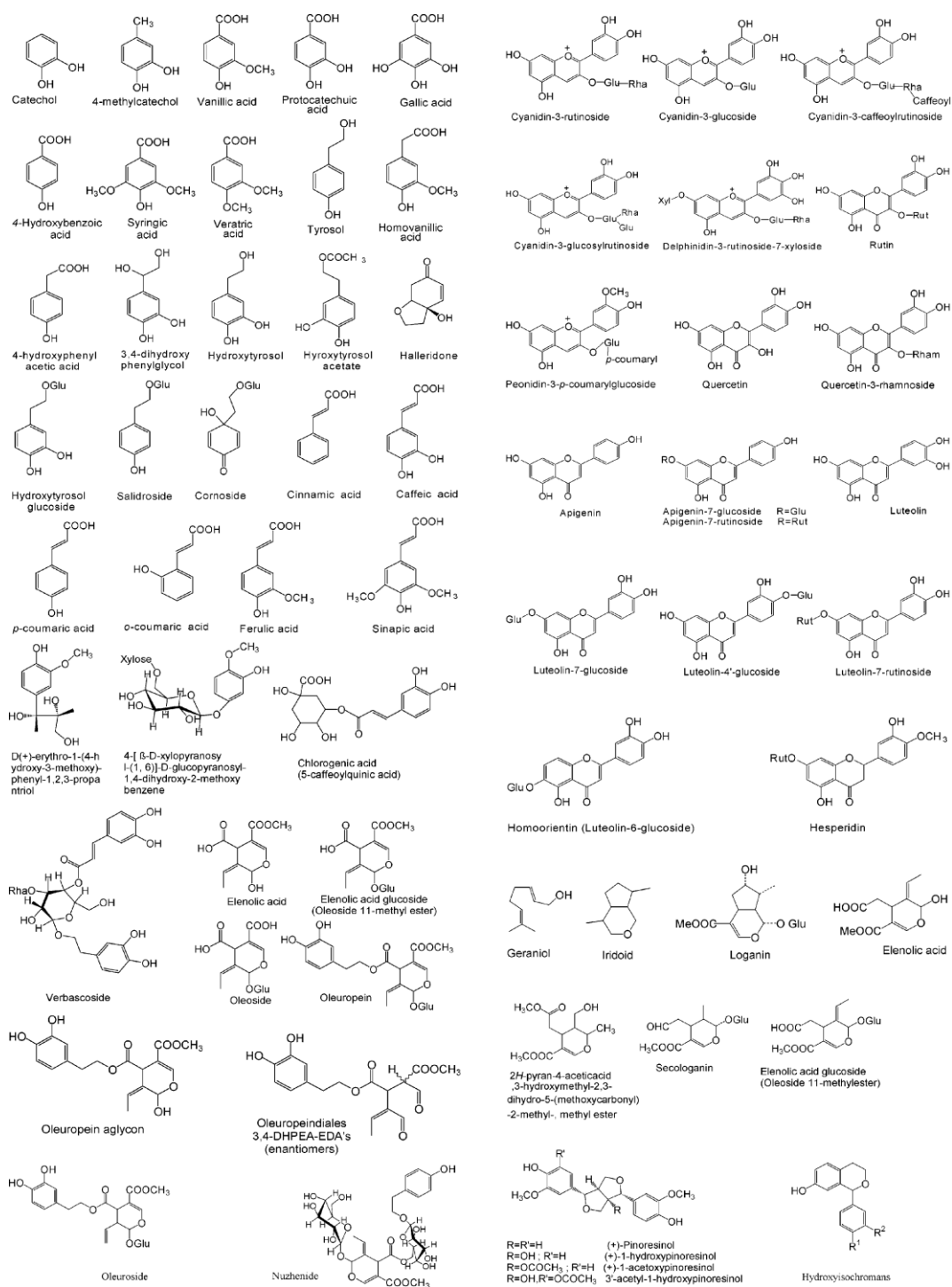
Figure 1 Biophenols and related compounds identified in olive or olive mill waste.

Iridoids are monoterpenes (C10) arising from the folding of geraniol (114), characterized by a bicyclic fused ring system (six-membered heterocyclic ring fused to cyclopentane ring). Secoiridoids are obtained through opening of the five-membered ring of iridoids.
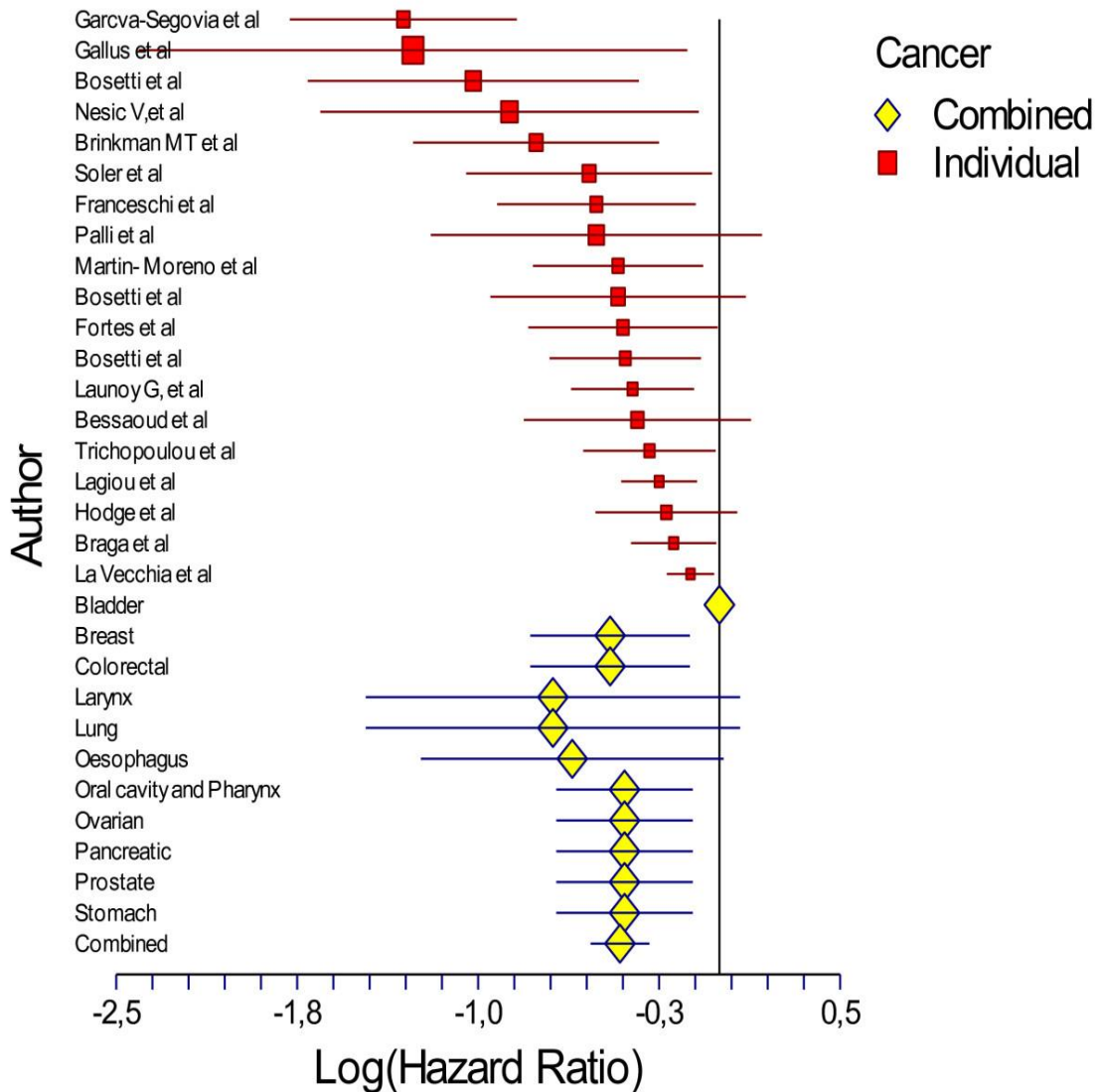
Figure 2 Forest plot of studies that evaluated the association between olive oil intake and cancer development

Data are presented as log Odds Ratios and the corresponding 95%CI. The highest category of olive oil consumption was associated with lower odds of having any type of cancer if compared with the lowest (log odds ratio = -0.41, 95%CI -0.53, -0.29, Cohran's Q = 47.52, p = 0.0002, I-sq = 62%); Moreover, olive oil consumption was associated with lower odds of developing breast cancer (logOR = -0,45 95%CI -0.78 to -0.12), and a cancer of the digestive system (logOR = -0,36 95%CI -0.50 to -0.21), compared with the lowest intake (Psaltopoulou et al.) [115]

MMP2	Reverse: GCTTGCGAGGGAAGAAGTTG	
	Forward: CCCCAGACAGGTGATCTTGAC	
MMP9	Reverse: AGGTTGGATACATCACTGCATTA	
	Forward: CGCTGGGCTTAGATCATTCC	
TIMP1	Reverse: GGCTTGGAACCCTTTATACATC	
	Forward: GGGACACCAGAAGTCAACCA	
TIMP2	Reverse: TCACAGCCAGTACCTGGTCGCA	
	Forward: TCTCAGGCCCTTTGAACATC	
18S	Reverse: CCATCCAATCGGTAGTAGCG	
	Forward: TCTCAGGCCCTTTGAACATC	
E-Caderin	Reverse: TCTTTGACCACCGCTCTCCT	
	Forward: AGTGCCAACTGGACCATTCA	
N-Caderin	Reverse: TGCAGATCGGACCGGATACT	
	Forward: TGGGAATCCGACGAATGG	
β-Catenin	Reverse: ACGCAAAGGTGCATGATTTG	
	Forward: CCCACTGGCCTCTGATAAAGG	

Table 2 List of primers used in qRT-PCR experiments and their sequences
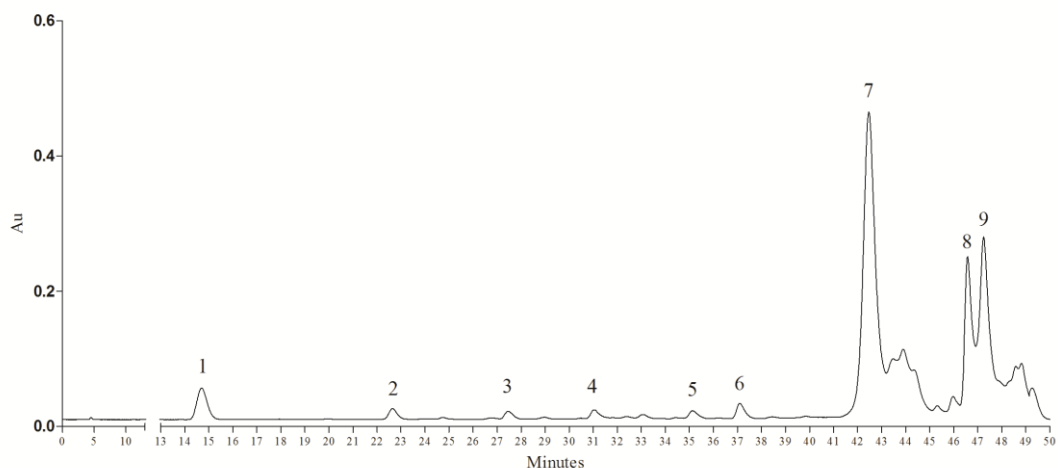
Figure 3 High-performance liquid chromatograms of the phenolic fraction of a representative virgin olive oil extract. Elution was monitored at 280 nm and peak identification was obtained by comparing retention times and spectral characteristics with those of authentic standards. Peaks corresponded to (1) hydroxytyrosol, (2) tyrosol, (3) vanillic acid, (4) vanillin, (5) p-coumaric acid, (6) ferulic acid, (7) dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), (8) dialdehydic form of elenolic acid linked to tyrosol (p-HPEA-EDA), and (9) pinoresinol. (Coccia et al). [116]

Peak number	Compound name	Area %
1	Hydroxytyrosol	7,5 ± 2
2	Tyrosol	4,2 ± 0,5
3	Vanillic Acid	4 ± 0,3
4	Vanillin	3,8 ± 0,5
5	P-coumaric Acid,	4,2 ± 0,5
6	Ferulic Acid	4,8 ± 0,5
	(3,4-DHPEA-EDA)	
7	Dialdehydic Form Of Elenolic Acid Linked To Hydroxytyrosol	26,3 ± 2
	(P-hpea-eda)	
8	Dialdehydic Form Of Elenolic Acid Linked To Tyrosol	16,7 ± 2
9	Pinoresinol	19,9 ± 2
	Other	8,6 ± 3

Table 3 Average composition of EVOO phenol extract.
 Chromatographic analyses was performed on 30 sample of EVOO extract obtained from different olive oil lots. The comparison between the peak area revealed no major differences in phenolic content of the various lots, which were characterized by a large amount of secoiridoids (7,8) and lignans such as pinoresinol
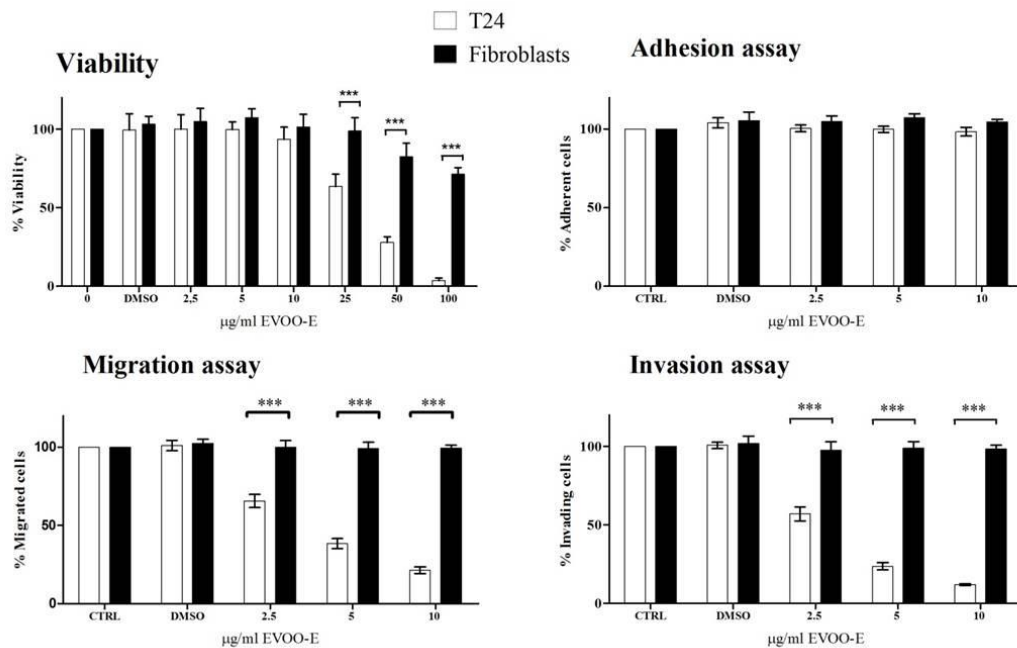
Figure 4 Effects of EVOO-E treatment on cell viability, adhesion and motility.
After 24 hours of exposure to various concentration of EVOO-E (0-100 µg/ml). EVOO-E significantly inhibits the viability of T24 bladder cancer cells. The effect on cell adhesion, migration and invasion was evaluated at subtoxic concentration of EVOO-E after 24 hours of exposure. As shown in the figure the cell adhesion assay indicates that there are no significant differences between treated cells when compared to the control both in T24 and in fibroblasts. As regards cell migration and invasion, data indicate that fibroblasts were not inhibited by the phenolic extract at the tested concentrations,while the phenolic extract had a significant inhibitory effect on T24 cell motility. After 24 hours of treatment with the same doses of EVOO-E used above, the percent of migrated cells decrease from 70 ± 2% to 10 ± 3%, respectively. Furthermore, the penetration of the Matrigel-coated filter by T24 cells (invasion assay) was substantially inhibited in the presence of EVOO-E in a dose dependent manner.The percent of invading cells after exposure to EVOO-E concentration ranging between 2.5 to 10 µg/mL drastically decrease from 53 ± 2% to 12 ± 1% respectively (Fig.3C).The data are presented as means ±SD. *** P<0.001 vs. control (Coccia et al)[116].

Invasion index
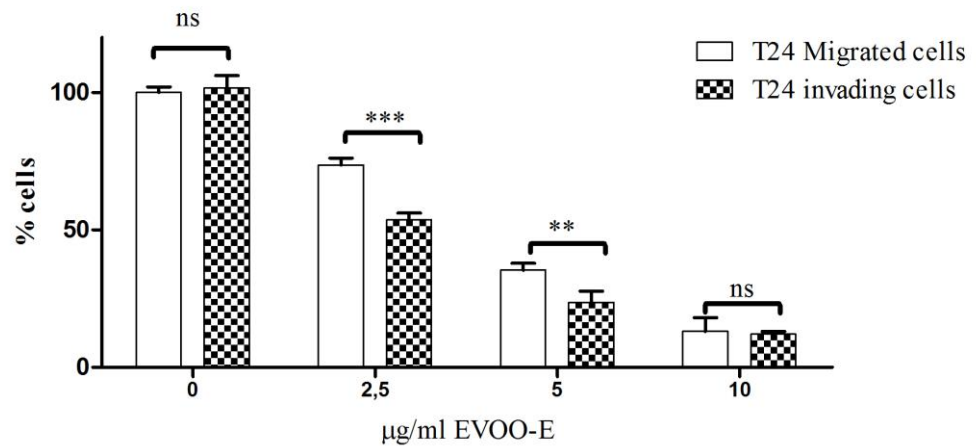
EVOO-E (µg/ml)	0	2,5	5	10
Invasion index	1,01 ± 0,03	0,72 ± 0,06	0,67 ± 0,05	0,96 ± 0,04

Figure 5 Invasion index

To evaluate the differences between migration and invasion on T24 bladder cells we compare the data of this two assays obtained in the same experimental condition. We assessed invasive potential by calculating an invasion index, which is the ratio of percentage invasion through the Matrigel-coated filters relative to migration through the uncoated filters of test cells over that of the control counterparts. The evaluation of the invasion index, whose value is significantly lower than 1, indicates that the inhibition of invasion is not only due to an altered cell motility, but also due to a limited ability to degrade ECM.The data are presented as means ±SD. *** P<0.001 T24 migrated cells vs. T24 invaded cells

Author	Number of patient	Sample source	Detection method	Conclusion proposed
Margulies et al[117]	55	Urine	ELISA, Western blot	MMP-2 levels increased in cancer
Gohji et al[118]	233	Serum	ELISA	High MMP2 in advanced disease
Hamasaki et al [86]		T24 cell line	RT-PCR, EIA	MMP2/TIMP2 imbalance suggest progression
Kanayama et al[119]	41	Frozen tissue	RT-PCR	MMP-2 & TIMP-2 associated with stage
Furukawa et al[120]		UCT1 and 2 lines	RT-PCR	MMP-2 higher in invasive UCT-1 line implants
Kanda et al[121]	61	Frozen tissue	Zymography	MMP-2 associated with grade,stage and result
Vasala et al [122]	54	Paraffin/tissue	Immunoistochemistry	MMP-2 associated with stage and worse survival
Sumi et al[123]	20	Paraffin/tissue	Immunoistochemistry	MMP-2 associated with grade,stage and result

Table 4 Scentific data about the MMP2 and TIMP2 association to aggressive behaviour of bladder cancer
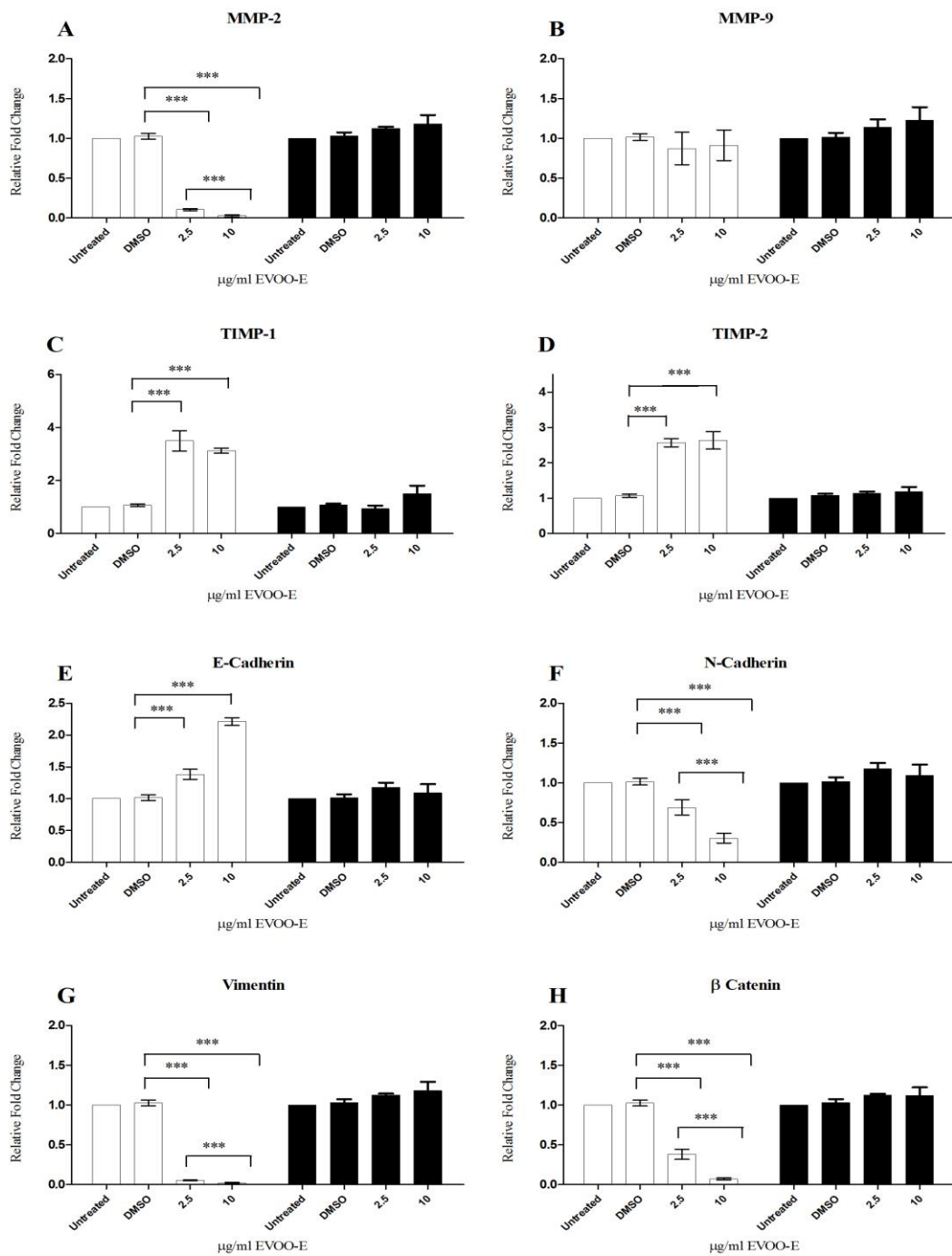
Figure 6 Quantitative real-time PCR of EMT related genes
The data obtained show a marked decrease in the gene expression level of N-Cadherin, MMP2, Vimentin and β-Catenin with a subsequent up-regulation of E-Cadherin, TIMP1 and TIMP2 after the treatment with EVOO-E. The data are presented as means ±SD.
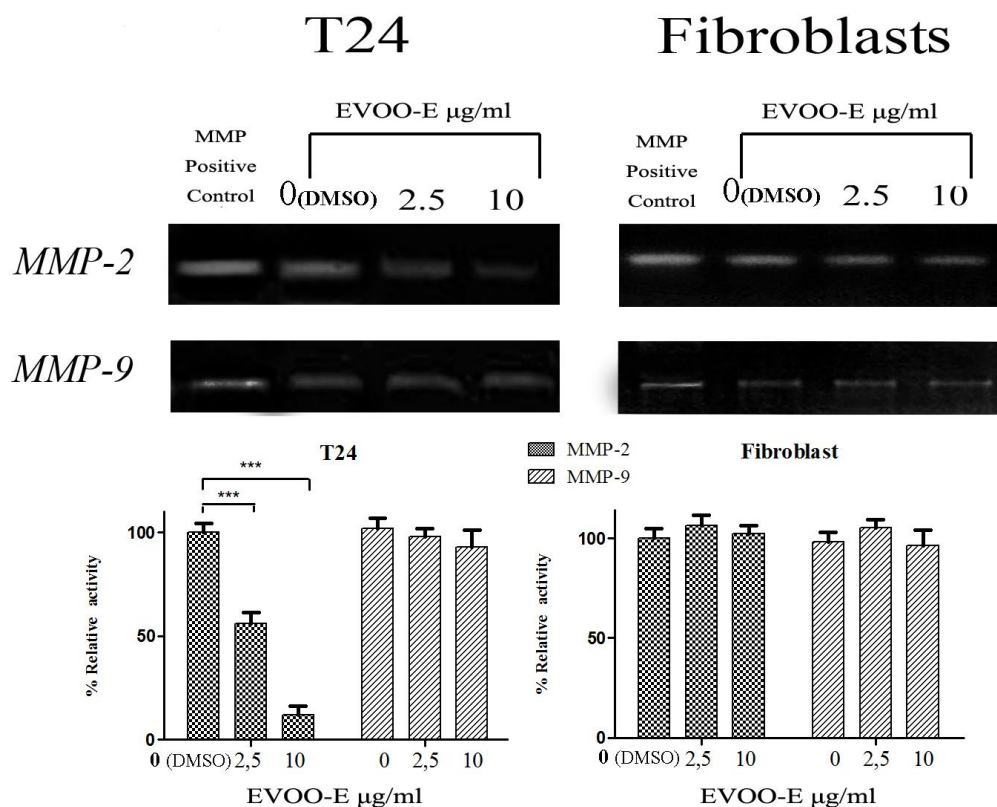
Figure 7 Gel zymography analysis of MMP-2 and MMP-9 activity in serum free conditioned medium after exposure to EVOO-E.

To achieve MMPs positive controls were used   commercially available recombinant MMP-2 (72 kDa) and MMP-9 (92 kDa). The densitometric analysis show a significantly reduction (*P<0.001)* of enzymatic MMP-2 activity in a dose dependent manner. The data are presented as means ±SD. *** *P<0.001* vs. control. (Coccia et al).

ATCC® No.	Name	Primary Site	Tissue Tumor Source	Histology	Mutant Gene	Zygosity
HTB-4™	T-24	urinary bladder	primary	carcinoma	HRAS	homozygous
					TP53	homozygous
HTB-9™	5637	urinary bladder	primary	carcinoma	TP53	heterozygous
					RB1	homozygous
					TP53	homozygous
CRL-1473™	HT-1197	urinary bladder	primary	carcinoma	NRAS	heterozygous
					PIK3CA	heterozygous

Table 5 Gene mutation status of bladder cancer cell lines used in the present work

Although some significant heterogeneity and complexity between these cell lines their genomic profiles exhibited a similar pattern to Urinary Bladder cancer. 5637 well represent the E2F3/RB1 pathway due to amplification of 6p22.3, concomitant with loss of one copy of RB1 and mutation of the remaining copy. The T24 belongs to the alternative pathway of FGFR3/CCND1 by presenting mutated HRAS and over-represented CCND1. These cell lines cover the more frequent subtypes of Bladder cancer and are reliable models that can be used, as a group, in preclinical studies[124]. The HT1197 where chose for their high degree of epithelial differentiation.
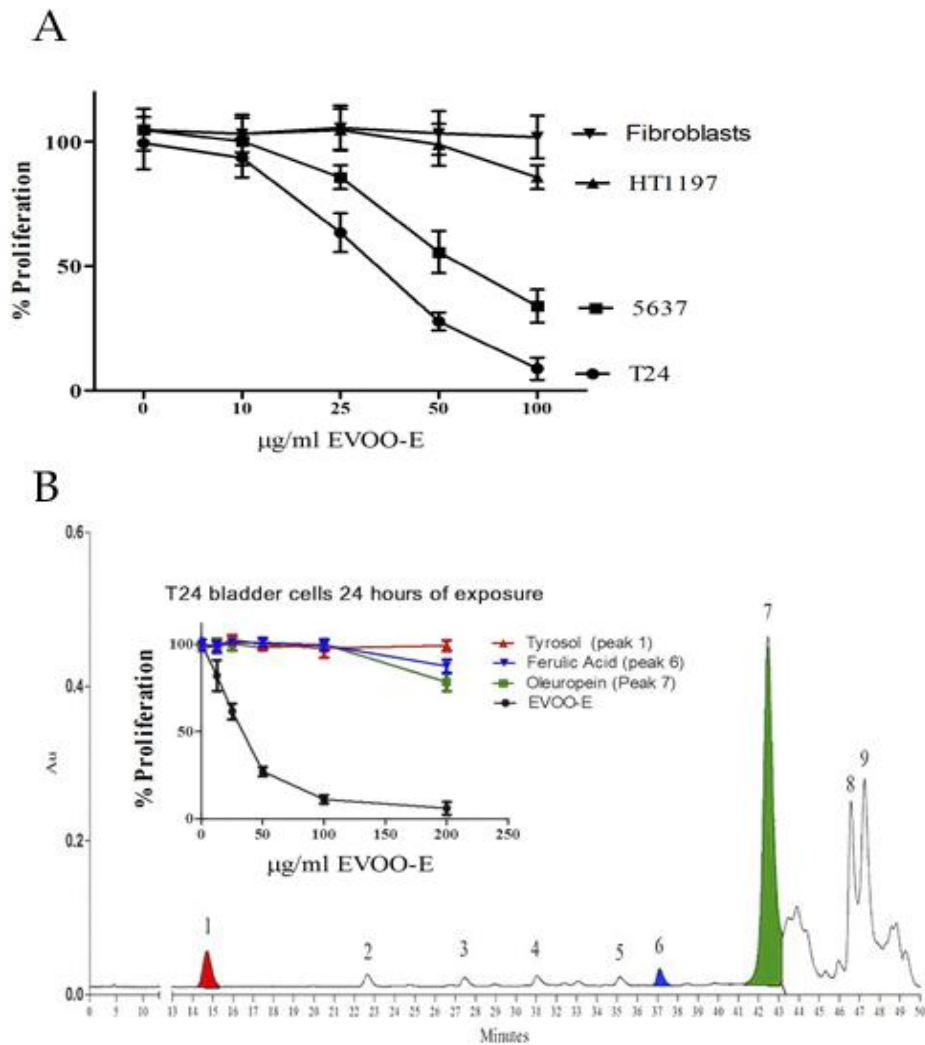
Figure 8 EVOO-E affect cells viability in a dose dependent manner
Panel A show the different susceptibility to polyphenols extract among the
tested cells. T24 were the most affected by the cytotoxic action of EVOO-E with
an IC50 of 32 ± 3 μg/mL while the 5637 retain an IC50 of 55 ± 3 μg/mL.
HT1197 were only slightly affected at the highest concentration of phenolic
extract tested. The comparison between dose response curve obtained from
three selected component of the phenolic mixture versus the whole extract
(EVOO-E), is shown in panel B. As expected, despite identification of several
active chemical compounds in EVOO-E, none of the isolated polyphenols
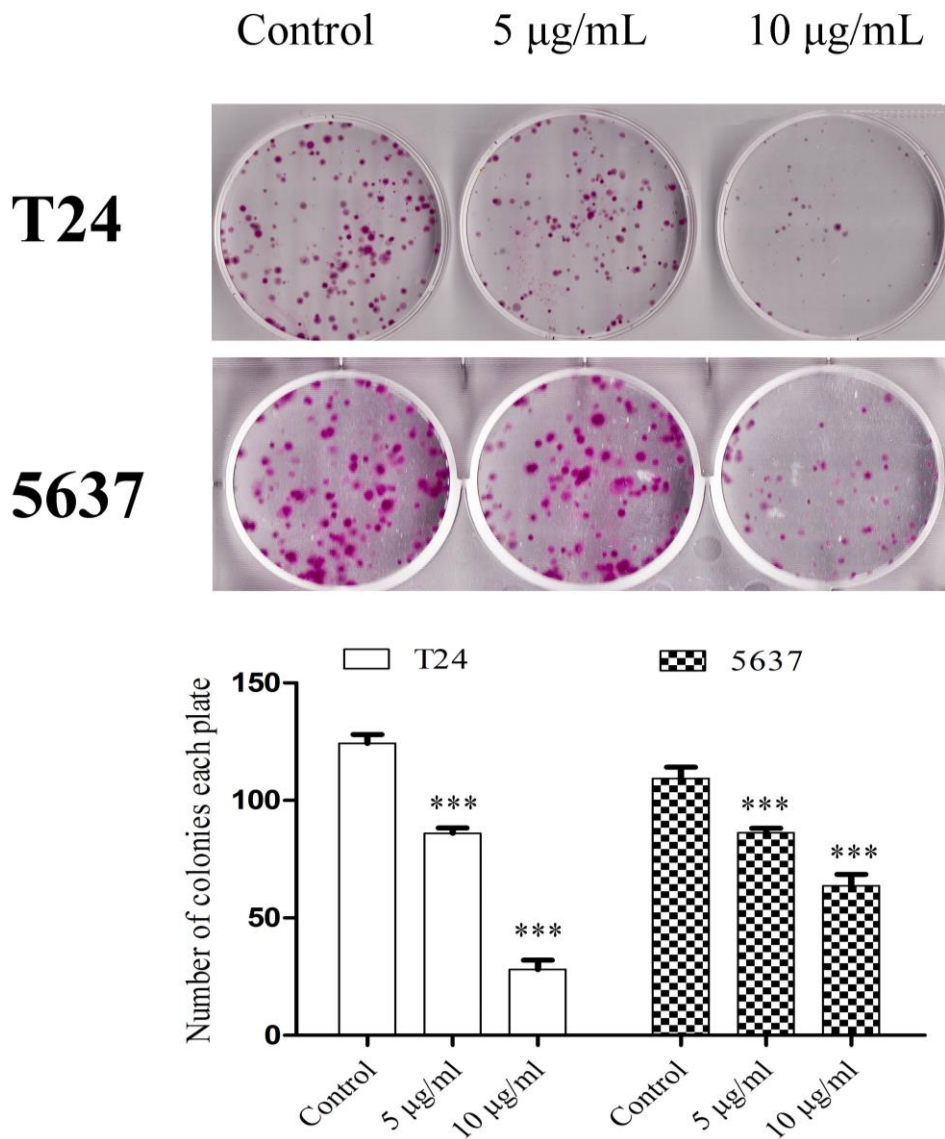demonstrated a biological activity higher than the whole oil extract.

Figure 9 Clonogenical survival assay
After 14 days of exposure to low doses of EVOO-E the colony forming ability of both the cell lines tested markedly decrease. In particular T24 were the most affected cells by the inhibitory action of phenolic extract starting   at a dose of 5 ug/mL with a marked inhibition of clonogenic activity at 10 ug/mL.

	Control	10 µg/ml	20 µg/ml	40 µg/ml
T24				
5637				

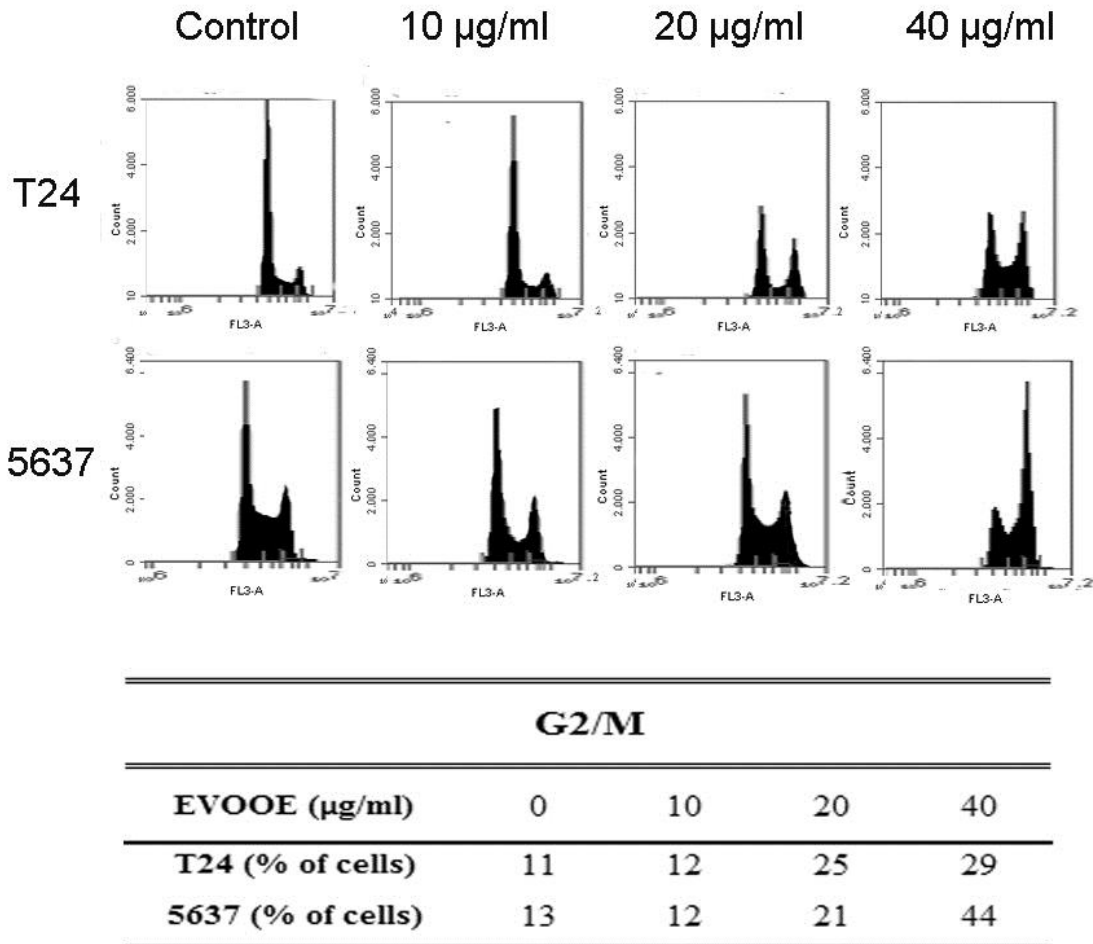| G2/M				
EVOOE (µg/ml)	0	10	20	40
T24 (% of cells)	11	12	25	29
5637 (% of cells)	13	12	21	44

Figure 10 EVOO-E blocks the cell cycle progression at G2/M stage
Cell growth suppression by EVOO-E, was investigated by analyzing the cell cycle progression. T24 and 5637 cells were treated with increasing doses of EVOO-E, and cell cycle distribution analysis was then performed after 24 hours of exposure. G2/M population of EVOO-E-treated T24 cells was increased , from 11.1% of the control to 27.1% at 20 and 40 µg/ml respectively. The treatment also induced a marked decrease of G0/G1 population in a dose dependent manner. The cell cycle analysis of 5637 after EVOO-E treatment revealed a similar behavior to what observed for T24 with a bigger increase of G2/M population at 40 µg/ml (27.1% of T24 versus 44% of 5637.
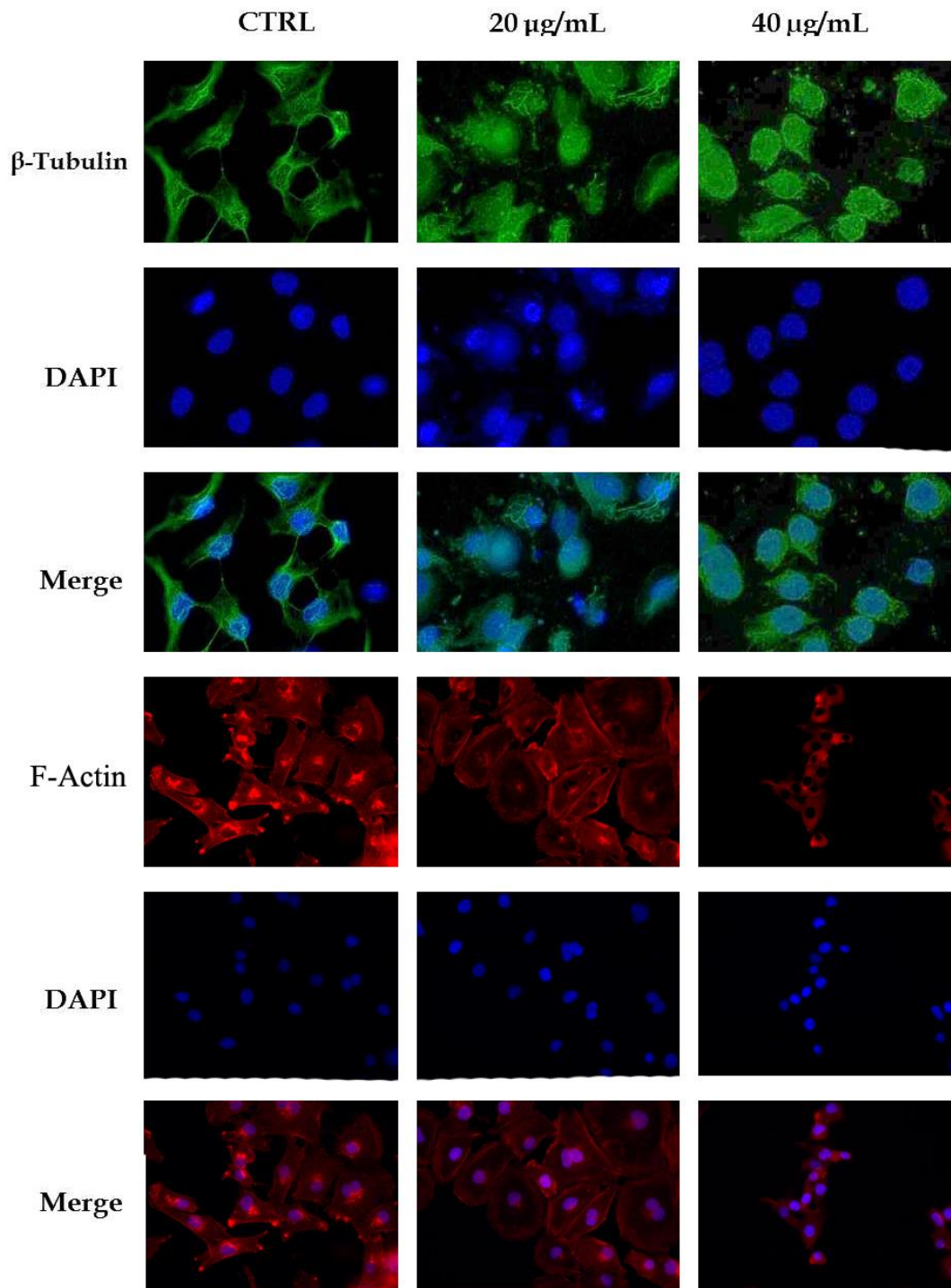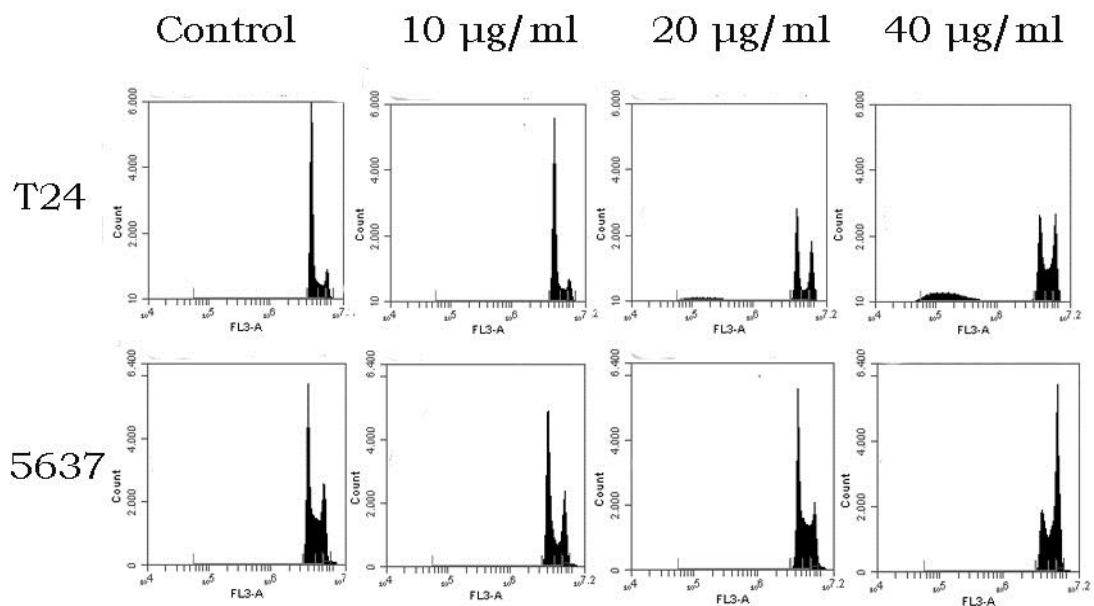
Figure 11 Immunofluorescent staining of F-actin and β-Tubulin.
Immunofluorescence was performed after treating the cells for 24 hours with different doses of EVOO-E, for this purpose we used an antibody against β-Tubulin (green) and phalloidin marked with TRITC (red) against F-actin.

	Control	10 µg/ml	20 µg/ml	40 µg/ml

sub G1				
E VO OE (µg/ml)	0	10	20	40
T 24 (% of cells)	6	8	18	28
5637 (% of cells)	3	2	4	7

Figure 12 Cell cycle analysis of sub G1
The cell cycle analysis after EVOO-E treatment revealed only in T24 cell, and accumulation of particles (sub G1) with a small content of DNA that usually indicate apoptosis. The percent of sub G1 events in T24 increase from about 6% of non treated cells to 28% after the exposure for 24 hours to 40
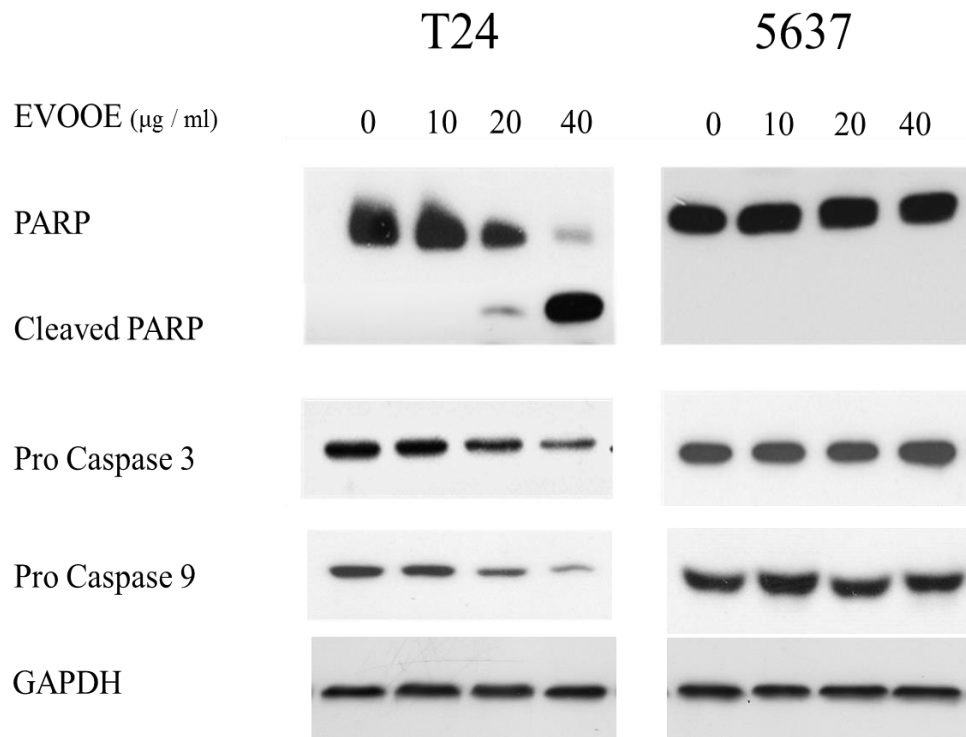
Figure 13 Western blot analysis of apoptosis related protein
Apoptosis was observed at 24 h, as indicated by the degradation of PARP.Western blot analysis of the cleavage of PARP showed a decrease in the full-size Mr 116,000 fragment and an increase in the Mr 85,000 cleaved fragment within 24 h after the bladder tumor cells were treated with 20 µg/ml or 40 µg/ml of EVOO-E. Western blot analysis of procaspase 3-9 also showed a marked diminished expression consistent with the apoptosis induction.
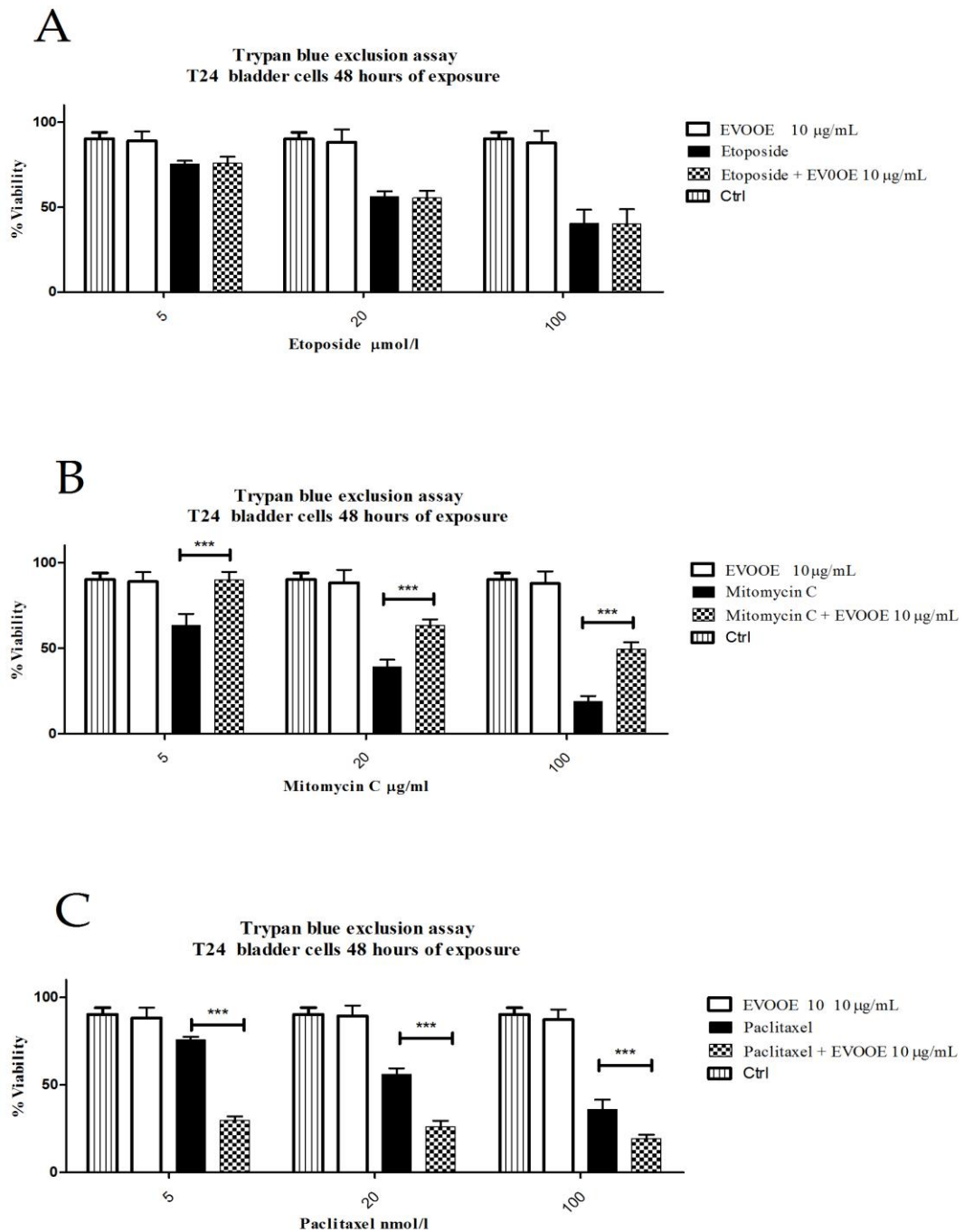
Figure 14 Evaluation of cytotoxic effect of conventional chemotherapics in association with EVOO-E.

The data show the viability after the simultaneous treatment of T24 cells with commonly used chemotherapics and EVOO-E. We choose to use for all the experiments a concentration of EVOO-E of 10 μg/ml which is non toxic (white bar).

Figure 15   EVOO-E strongly increases Paclitaxel induced apoptosis.
The simultaneous treatment of Paclitaxel and EVOO-E strongly increases the apoptotic cell death at any of the tested concentration, with a marked accumulation of the 85 kDa cleaved fragment of PARP. To our knowledge this the first report that show how EVOO phenols drastically improve the activity of Paclitaxel.

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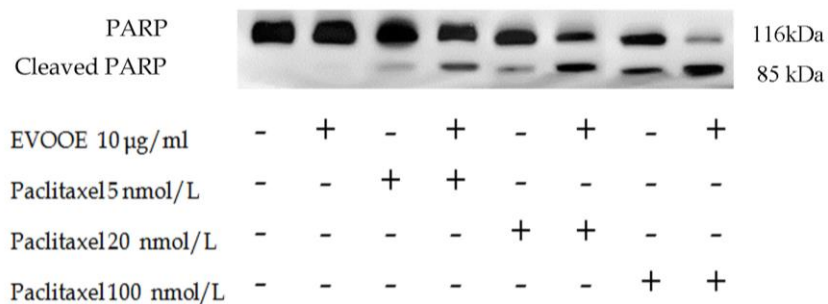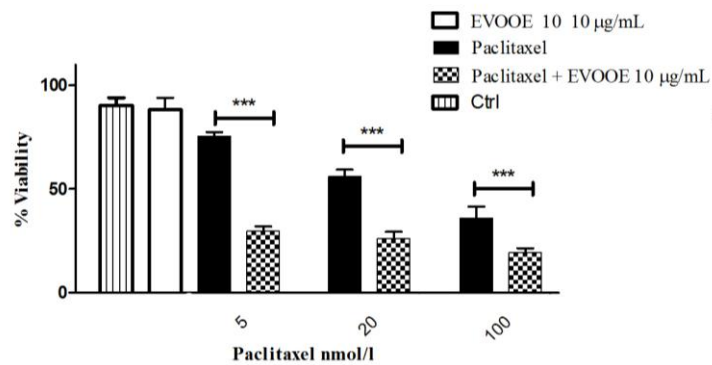2.   Casaburi I, Puoci F, Chimento A, Sirianni R, Ruggiero C, Avena P, Pezzi V: **Potential of olive oil phenols as chemopreventive and therapeutic agents against cancer: A review of in vitro studies**. *Molecular Nutrition & Food Research* 2013, **57**(1):71-83.

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