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Running Title: LAURICELLA et al. ANTI-CANCER EFFECTS OF THE ESSENTIAL OIL OF FOENICULUM VULGARE SUBSP. PIPERITUM

Key Words: essential oil; phytochemicals, antitumor effect; breast cancer; apoptotic cell death

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Abstract

Nowadays, the growing spread of tumor cases worldwide makes the research of new promising and selective anticancer drugs an urgent need. On this subject the interest on the biological action of extracts of medicinal plants or their essential oils is emerging since they could represent a rich source of phytochemicals that deserve particular attention and need to be investigated. In this paper we explored the biological activity and the mechanism of action of the essential oil of Foeniculum vulgare subsp. piperitum fruits (FVPEO) in MDA-MB231, a triple negative breast cancer cell line. Our analysis provided evidence that FVPEO is endowed with particularly strong anticancer capabilities, causing a dose-dependent growth inhibition of breast cancer MDA-MB231 cells accompanied to DNA condensation and fragmentation. The cytotoxic effect of FVPEO was counteracted by the addition of the antioxidant N-acetylcysteine (NAC) and was associated to a remarkable ROS increase and stress-related proteins such as MnSOD, c-Jun, pJNK, Nrf2 and its transcriptional targets (HO-1, NQO1). As evidenced by the activation of caspase-3 and fragmentation of PARP-1, typical apoptosis markers, FVPEO promoted an apoptotic cell death program that was accompanied to an increase in phosphorylated H2AX and to the activation of NQO1/p53 axis. Taken together, our analyses provided evidence that FVPEO could represent a reservoir of biologically active compounds suitable for both prevention and cancer treatment.

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The essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits exerts anti-tumor activity in triple negative breast cancer cells.

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Abbreviations: essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits (FVPEO); essential oils (Eos) the triple-negative breast cancer (TNBC).

Keywords: essential oil; phytochemicals, antitumor effect; breast cancer; apoptotic cell death

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Abstract

Nowadays, the growing spread of tumor cases worldwide makes the research of new promising and selective anticancer drugs an urgent need. On this subject the interest on the biological action of extracts of medicinal plants or their essential oils is emerging since they could represent a rich source of phytochemicals that deserve particular attention and need to be investigated. In this paper we explored the biological activity and the mechanism of action of the essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits (FVPEO) in MDA-MB231, a triple negative breast cancer cell line. Our analysis provided evidence that FVPEO is endowed with particularly strong anticancer capabilities, causing a dose-dependent growth inhibition of breast cancer MDA-MB231 cells accompanied to DNA condensation and fragmentation. The cytotoxic effect of FVPEO was counteracted by the addition of the antioxidant N-acetylcysteine (NAC) and was associated to a remarkable ROS increase and stress-related proteins such as MnSOD, c-Jun, pJNK, Nrf2 and its transcriptional targets (HO-1, NQO1). As evidenced by the activation of caspase-3 and fragmentation of PARP-1, typical apoptosis markers, FVPEO promoted an apoptotic cell death program that was accompanied to an increase in phosphorylated H2AX and to the activation of NQO1/p53 axis.

Taken together, our analyses provided evidence that FVPEO could represent a reservoir of biologically active compounds suitable for both prevention and cancer treatment.

Introduction

Foeniculum vulgare Mill. (*F. vulgare*), commonly named fennel, is one of the most widespread aromatic plants. This species belongs to the Apiaceae family and it is widely cultivated throughout different parts of world as Asia, North and South America, and in the Southern regions of Europe (1).

The plant offers many different edible parts as leaves, stalks and seeds (fruits) that represent a source of a wide range of phytochemicals like fatty acids, phenolic compounds, and flavonoids as well as volatile compounds, such as anethole, estragole, and fenchone, as major phytoconstituents (2,3). Mostly of these phytochemicals are found in essential oils (EOs) appearing practically in any part of the plant, as root, stem, seed and fruit (4).

F. vulgare fruits are commonly used as a culinary spice. The EOs from fennel are often used as flavoring agents, but also as constituents of cosmetic and pharmaceutical products (5).

F. vulgare is widely used in traditional medicine for its ability of exerting diuretic, antispasmodic, analgesic, mucolytic, and anti-inflammatory effects (1). Its stems, fruits, leaves, seeds and whole plant itself are used to treat a wide range of ailments of digestive, reproductive and respiratory systems, including abdominal pains, constipation, diarrhea, amenorrhea, fever, flatulence, arthritis, insomnia, irritable colon, liver pain, mouth ulcer, and stomachache (1,6–8). In ancient China its use found application as remedy for snake bite, and in addition the infusion of fruits was applied as carminative, while roots revealed to possess efficient purgative properties. In some parts of Southern Italy the decoction of the fruits of *F. vulgare* subsp. *piperitum* is used as digestive or, chewing its leaves is considered a cure for mouth ulcers (9). Furthermore, it has been prescribed as a muscle relaxant, a weak diuretic, carminative, and a mild stimulant (10).

The genus *Foeniculum* is present in Italy with only one species, that has been divided into two subspecies, *Foeniculum vulgare* subsp. *vulgare* Miller and *Foeniculum vulgare* subsp. *piperitum* (Ucria) Coutinho. This distinction is still a subject of debate among botanists. In fact, on the basis of the different distribution, some botanists believe that they represent two distinct species. The certain presence of *F. vulgare* subsp. *piperitum* is reported in Central-Southern Mediterranean, but outside this area the species is quite rare. Furthermore, this subspecies does not contain anethole (11) and it is often confused with a chemotype of *F. vulgare* var. *vulgare*, which presents a bitter, but different taste.

Recently, the chemical compositions of the EOs of different vegetative parts of *F. vulgare* subsp. *piperitum* collected in Sicily were evaluated by GC and GC-MS (12). The results were compared

with those of the EOs of the same vegetative parts of *F. vulgare* subsp. *vulgare*, collected in the same station and with those reported in literature for other accessions of *F. vulgare* subsp. *piperitum*. The oils of *F. vulgare* subsp. *vulgare* showed completely different compositions clearly indicating the differentiation of the two subspecies.

A number of studies performed in *in vitro* and *in vivo* models highlighted how various extracts of *F. vulgare* possess antioxidant, anti-inflammatory, anti-mutagenic and anticancer properties. *F. vulgare* EOs showed anti-mutagenic effects in mice where it reduced chromosomal aberrations induced in mouse bone marrow cells by cyclophosphamide. This effect was mediated by a reduction of oxidative stress (13). The anti-tumor activity of *F. vulgare* has been shown against different cancer cells, such as melanoma (14), prostate (15), lung cancer (16), and epatocarcinoma cells (17). Ke et al demonstrated that ethanol extract of *F. vulgare* seeds induced apoptosis in HCI-H446 and NCI-H661 lung cancer cell lines as well as inhibited the growth of NCI-446-derived xenografts by reducing Bcl-2 protein expression (16). Extracts of *F. vulgare* seeds also induced apoptosis and inhibited cell migration of hepatocarcinoma cells *in vitro* and significantly constrained the growth of HCC xenografts in nude mice by targeting survivin (17). In addition, extracts of *F. vulgare* seeds also exerted anticancer effect on Elrich ascites carcinoma-bearing mice by modulating lipid peroxidation and potentiating antioxidant defense (18).

Thus, based on the promising previous results concerning the anti-cancer potential of *F. vulgare*, we sought to examine the possible anti-cancer action of essential oil of *F. vulgare* subsp. *piperitum* (FVPEO) in triple negative MDA-MB231 breast cancer cells, demonstrating that FVPEO induces an apoptotic cell death process through the activation of NQO1/p53 axis.

Materials and Methods

F. vulgare subsp. *piperitum: plant material and fruits essential oil preparation.* Fruits of *F. vulgare* subsp. *piperitum* were collected on the southern slopes of the limestone massif of Rocca Busambra (Corleone, Palermo, Italy). Typical specimens (PAL 109709), identified by Prof. Vincenzo Ilardi, have been deposited in Herbarium Mediterraneum Panormitanum of the "Orto Botanico", Palermo, Italy. One hundred thirty-six grams (135 g) of *F. vulgare* subsp. *piperitum* fruits was hydro-distillated for 3 h using Clevenger's apparatus. The oil (yield 1.36 %) was dried with Na_2SO_4, filtered, and stored in the freezer at 20 °C, until the time of analysis. The chemical composition of FVPEO was performed as previously reported (12).

Cell cultures, reagents, and chemicals. Triple negative breast cancer MDA-MB231 cells, obtained from ''Istituto Scientifico Tumori'' (Genoa, Italy), were cultured as monolayers in DMEM medium supplemented with 10% (v/v) heat-inactivated FCS, 1% non-essential amino acids, 2mM glutamine and 1% penicillin/streptomycin solution. Cells were plated on 96 well plates or on 100-mm culture dishes, then were allowed to adhere overnight in culture medium at 37 °C in a humidified atmosphere containing 5% CO_2, before the treatment with FVPEO or vehicle only. Media as well as cell culture reagents were purchased from Euroclone. All other chemicals and reagents were provided by Sigma-Aldrich (Milan, Italy).

*Cell viability assessment and morphological detection of apoptosis***.** In order to assess cell viability of breast cancer cells exposed to FVPEO treatment, MTT test was performed as previously reported (19). For assays, cells ($8x10^3$/well) were plated in 200 μL of DMEM in a 96-well plate and exposed to the treatment. At the end, 4 μL of MTT solution (5 mg/mL in PBS) were added to the cell medium and the incubation was protracted for 2 h at 37 °C in the dark. Mitochondria dehydrogenase activity of viable cells converts MTT to formazan, which is soluble in tissue culture medium. At the end cells were lysed in lysis buffer and the absorbance was read at 570 and 690 nm by an automatic ELISA plate reader (OPSYS MR, Dynex Technologies, Chantilly, VA).
In order to determine either changes in nuclear morphology or plasma membrane damage, the cells were stained with Hoechst 33342, a cell permeant fluorochrome emitting blue fluorescence when bound to dsDNA and excited by ultraviolet light. For these assays, cells ($8x10^3$) were incubated in the presence of Hoechst 33342 (2.5 μg/mL medium) for 30 min, washed with PBS and suspended in culture medium before FVPEO treatment. Morphological detection of apoptotic cells evidenced

by chromatin condensation as well as fragmentation was analyzed by fluorescence microscopy using an excitation wavelength of 372 nm and an emission wavelength of 456 nm. All the pictures were taken by Leica Q Fluoro Software. For the analysis at least 10 fields for each sample were considered and apoptotic cells were counted in random fields using a 100x magnification.
The apoptotic cell morphology was also studied by acridine orange and ethidium bromide (AO/EB) double staining as reported by Wu et al. (20).

Analysis of reactive oxygen species generation. The detection of intracellular reactive oxygen species production was carried out by H_2-DCFDA staining as previously reported (21). H_2-DCFDA is a non-polar dye that easily crosses cell membrane and can be oxidized to DCFDA in the presence of ROS remaining entrapped into the cells and emitting green fluorescence.
After incubating the cells in the presence of FVPEO, medium was removed and cells were incubated in the presence of 10 μM H_2-DCFDA (Molecular Probe, Life Technologies, Eugene, OR, USA) for 30 min at 37 °C. Then positive cells were analyzed under a Leica fluorescence microscope with excitation at 485 nm and emission at 530 nm as previously reported (22).

Western blotting analysis. For western blotting analyses, cells were lysed in RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM EDTA supplemented with phosphatase inhibitor mix (Sigma, Dallas, TX, USA). Extracts were sonicated thrice and protein content was determined by Bradford assay using an albumin bovine serum (BSA, Sigma Aldrich) standard curve. Then, 30 μg/lane of protein sample were subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The analyses of MnSOD, c-Jun, pJNK, NQO1, γ-H2AX, p53, pro-caspase-3 and PARP-1 were performed by using specific antibodies produced by Santa Cruz Biotechnology (Santa Cruz, CA). As for as HO-1 analyses the antibody (orb5455) was provided by Biorbyt Ltd (Cambridge, United Kingdom), while Nrf-2 antibody (NBP1-32822) purchased from Novus Biologicals (Bio-Techne SRL, Milan, Italy).
Protein bands were detected by the Enhanced chemiluminescence reagent (Amersham Biosciences, GE Healthcare Life Science, Milan, Italy), and quantified by using Quantity One software (Bio-Rad, Hercules, CA). The correct protein loading was checked by immunoblotting for γ-tubulin. All the blots shown are representative of at least three separate experiments.

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Statistics. Statistical analysis of data was performed by Graphpad Prism 5.0. software (GraphPad PrismTM Software Inc, San Diego, CA). Data were expressed as means ± SD. The statistical significance threshold was set at $p<0.05$.

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Results

Chemical profiling of essential oil of F. vulgare subsp. *piperitum.*

Hydro distillation of the fruits of *F. vulgare* subsp. *piperitum* gave a pale-yellow oil. The essential oil composition was previously reported (12).

As reported in the pie chart of Figure 1, FVFEO was particularly rich in monoterpene hydrocarbons 71.01% with terpinolene (20.10%), limonene (17.84%), α-phellandrene (10.53%) and γ-terpinene (10.43%) as the main components. The second most abundant class was the phenylpropanoids, typical metabolites of *F. vulgare* subsp. *vulgare* (12), is quite poor (14.36%), with estragole (10.96%) and myristicin (3.09%) as major products. Oxygenated monoterpenes were present in lower amount (10.78%) with fenchone (8.83%) as principal metabolites of this class.

Based on these observations, we explored whether FVFEO could exert a biological activity evaluating its putative anti-tumor potential.

Effects of FVPEO on the viability of MDA-MB231 breast cancer cells

To demonstrate a possible anti-proliferative effect of the essential oil of the fruits of *F. vulgare* subsp. *piperitum* (FVPEO) we focused our study on MDA-MB231, a very aggressive and poorly differentiated breast cancer cell line, which does not express estrogen, progesterone and HER-2/receptors (23). MDA-MB231 cells were treated with increasing concentrations (within the range of 125-2000 μg/mL) of FVPEO for various periods and the viability was assessed by MTT assay, as reported in Methods. As reported in Fig.2A, the cell survival rate displayed a remarkable dose- and time-dependent decrease after FVPEO treatment compared to untreated control. After 24 h of treatment the viability of MDA-MB231 cells was reduced by 20% of control with 125 μg/mL FVPEO. Increasing the dose of treatment, the viability diminished progressively and a consistent cytotoxic effect was reached at the highest concentration examined (only about 5% of viable cells with 2000 μg/mL). The cytotoxic effect of FVPEO further increased prolonging the time of treatment up to 48 h, when the viability lowered to 15% with 500 μg/mL FVPEO.

Light microscopy observations showed that following exposure to FVPEO, MDA-MB231 cells underwent morphological changes. As shown in Fig. 2B, cells treated with lower doses (125-250 μg/mL) of FVPEO appeared elongated in comparison to untreated cells. Increasing the dose of FVPEO, the typical morphological changes of apoptotic cells, as cell shrinkage and roundness, appeared and a marked reduction of cell number was observed.

FVPEO-induced cytotoxic effect is counteracted by the antioxidant N-acetylcysteine and ~~was~~ is accompanied by ROS generation

We next examined whether the cytotoxic effect of FVPEO was dependent on oxidative stress. To this end, MDA-MB231 cells were pre-incubated for 2 h with *N*-acetylcysteine (NAC), a ROS scavenger; then different doses of FVPEO were added for another 24 h. Our data demonstrated that the addition of NAC counteracted the cytotoxic effect of FVPEO. In particular, as shown in Fig. 3A, 10 mM NAC prevented the cytotoxic effect induced by low concentrations (125-250 μg/mL) of FVPEO and importantly reduced that of high concentrations (500-1000 μg/mL) of the compound. To better explore these effects we also evaluated the generation of ROS by H2DCFDA, a fluorochrome that binds ROS and emits green fluorescence in the oxidized form. Using such an experimental approach we observed a clear rise in green fluorescence indicative of ROS production in FVPEO-treated cells (Figure 3B). The increase, that already appeared at 30 min of incubation with 250 and 500 μg/ml FVPEO, reached a maximum level at 60 min after application. Then, western blotting analyses were also performed to evaluate whether FVPEO treatment modified the level of MnSOD, one of the main cellular antioxidant enzymes (24). As shown in Fig. 3 B, an increased level of MnSOD was observed only after treatment with lower doses of FVPEO (125-250 μg/mL).

FVPEO cytotoxic effect is mediated by oxidative stress and upregulation of stress-associated proteins.

In light of the observed data demonstrating ROS production in MDA-MB231-treated cells we explored whether the cytotoxic effect induced by FVPEO can be accompanied by the activation of stress associated proteins (Figure 4).

Firstly, we analysed the level of c-Jun and phospho-JNK (pJNK). c-Jun is a member of the activating protein (AP-1) transcription factor that can be activated in response to different extracellular stimuli such as pro-inflammatory cytokines, UV radiation and many different forms of cellular stress (25). Its activation has been correlated to the signalling of c-Jun N-terminal kinases (JNKs), a family of stress mediated kinases capable of integrating many different cellular stimuli such as mitogenic signals, environmental stresses, and different apoptotic insults (26).

Our data provided evidence that FVPEO treatment caused a modest increase in pJNK, but a consistent increase in c-Jun level that was already visible at 24 h with 250 μg/mL dose and further increased for longer periods of incubation.

The involvement of stress in FVPEO-treated cells was also confirmed by the upregulation of Nrf2 (NF-E2 p45-related factor 2), a transcription factor that has been considered one of the main regulatory factors of redox homeostasis that controls a battery of detoxification and cytoprotective genes (27). In our experimental conditions, we observed in FVPEO-treated cells an increase in Nrf-2 content that was associated to an upregulation of its target genes Heme oxygenase (HO-1) and NADPH quinone oxidoreductase 1 (NQO1).

FVPEO induces apoptosis in MDA-MB231 cells

To investigate whether the loss of viability of MDA-MB231 cells under FVPEO treatment was due to the induction of apoptosis, cells were stained with Hoechst 33342, a fluorescent dye that binds to DNA and permits to identify nuclear apoptotic changes. According to fluorescence microscopy images, MDA-MB231 cells treated with FVPEO exhibited a clear nuclear fragmentation and condensation, compared with control untreated cells (Fig. 5A). The proportion of cells with condensed and fragmented nuclei increased with the dose of FVPEO treatment (Fig. 5B). Such an effect was also confirmed by acridine orange/ethidium bromide dual staining showing the presence of typical morphological features of apoptosis in MDA-MB231 treated cells (Figure 5C). Indeed, some FVPEO- treated cells resulted positive to a yellow-green acridine orange nuclear staining (early apoptotic cells) and a remarkable percentage of them showed a concentrated orange nuclear ethidium bromide staining (late apoptotic cells).

FVPEO induces a p53-dependent intrinsic apoptotic pathway in MDA-MB231 cells.

To further explore the underlying mechanism of FVPEO-induced apoptosis, we examined whether the observed effects and associated with DNA injury can be accompanied to the recruitment of DNA damage markers. When a double-strand break occurs in DNA, alteration in chromatin structure promotes the phosphorylation of the histone variant H2AX at the Ser-139 residue (28). This event is induced by the kinases ATM, ATR and DNA-PK, allowing the formation of γH2AX (H2AX phosphorylated on serine 139), that is thus considered a marker of DNA damage (29). Our data demonstrated that treatment of MDA-MB231 cells with FVPEO induced a strong phosphorylation of H2AX at Ser139 (γH2AX) in a dose-dependent manner (Fig. 6).

We next examined possible changes in the level of p53 protein, a key factor involved in the induction of apoptosis in response to DNA damage (30). The same Fig. 6 showed that the level of p53 markedly increased in MDA-MB231- treated cells compared with untreated control.

242 In our study we also analysed the effect of FVPEO on caspase-3, a key mediator of apoptosis of
243 mammalian cells whose activation by cleavage of pro-caspase is responsible for chromatin
244 condensation (31). Our results indicated that FVPEO treatment induced a dose-dependent decrease
245 of the inactive procaspase-3, indicating the activation of caspase-3 (Fig.6). This suggestion was
246 validated by the cleavage of PARP-1, a well-known target of caspase-3 (32), observed after FVPEO
247 treatment. Taken together these data indicated that FVPEO induced a caspase-dependent apoptosis
248 triggered by a DNA damage.

Discussion

Nowadays, the increasing number of cancer cases around the world makes the research of specific and targeted therapies an urgent global need at medical level. On this scenario a particular attention has been addressed on the vegetal kingdom as a possible bio-resource where to research new phytochemicals as preventative or protective compounds to apply to cancer therapies alone or in combination (33–35). Indeed, from their discovery to the cancer research application along the years a vast array of plant derived phytochemicals have been identified as etoposide, taxol, doxorubicin, topotecan, irinotecan and camptotecin that have been revealed to be valuable and highly effective chemotherapeutics routinely applied in clinical practice (36). More than three quarters of anti-cancer chemotherapeutics currently used in medicine are natural products or their analogues chemically modified with active pharmacophores to enhance their anti-tumor potential (37).

In light of these considerations, we undertook a study to evaluate the possible anti-cancer properties of essential oils of *F. vulgare* subsp. *piperitum* (FVPEO) grown on the Sicilian rural areas. Our previous studies highlighted the composition of the most abundant secondary metabolites present in FVPEO (12) and since no data are available on the biological activity of the FVPEO grown in Sicily so far, we aimed at investigating whether it can exert anti-proliferative effects in triple negative breast cancer cells.

Breast cancer is one of the most spread tumor affecting women, incidence tends to rise with age and ranks the second leading cause of death (38,39). On the basis of the presence or absence of estrogen, progesterone and human epidermal growth factor 2 (HER2) receptor, this tumor has been classified into the three distinct subtypes that present different percentage of distribution in affected women. In particular, the hormone receptor positive/ERBB2 negative form affects up to 70% of patients, the ERBB2 positive form concerns the 15%-20% of cases, and the triple-negative (TNBC) subtype, characterized by the lack of all receptors, affects about 15% of patients (40). In particular, a focal point of concern for breast cancer incidence has been posed for TNBC. It is the most aggressive breast cancer form, for which the poor prognosis, the highly invasive profile as well as the development of a remarkable resistance to the most common cancer therapies still represent the main challenges in the fight of this form of tumor (41).

Our data demonstrated that FVPEO induced a remarkable reduction of cell viability in triple negative breast cancer cells and such an effect was associated to oxidative injury as evidenced by a consistent ROS generation. As it is well known ROS are highly reactive molecules that, when

produced at physiological level in the cell, can participate to intracellular signaling functioning as redox messenger (42). However, the generation of ROS at high doses turns to be dangerous for the cells, since their production overcomes intracellular scavenger systems triggering cell death (43). In accordance with these observations, our data provided evidence that ROS production induced by FVPEO treatment up surged stress-associated proteins such as c-Jun, pJNK as well as that of antioxidant defense systems represented Nrf-2 (Nuclear factor E2-related factor 2) and its targets MnSOD and NQO1.

Nrf-2 is a transcription factor that under stress condition shuttles from cytosol to nucleus where promotes the basal and stress-inducible expression of a plethora of cytoprotective enzymes (44) involved in glutathione metabolism (components of glutamate-cysteine ligase complex), thioredoxin antioxidants based response (thioredoxin, sulfiredoxin), ROS and xenobiotic detoxification (NADPH quinone dehydrogenase 1 (NQO1); glutathione peroxidase 2 (Gpx2) and several glutathione S-transferases) and iron metabolism (Heme-oxygenase (HO-1)).

In our experimental conditions Nrf-2 upregulation induced by FVPEO treatment was associated to an increased level of HO-1 and NQO1.

NQO1 has been described as a putative anti-tumor factor (45) involved in ROS removal, so that the application of phytochemicals or plant-derived compounds to promote NQO1 upregulation has been indicated as a putative chemopreventive strategy for cancer (46). These evidences seem to sustain the mode of action of FVPEO in breast cancer cells reported in this paper. Indeed we demonstrated that FVPEO promoted a remarkable DNA condensation and fragmentation with activation of caspase-3 and PARP-1 fragmentation. The cell death induced by FVPEO seems to be correlated with ROS increase, as suggested by the observation that the effect of FVPEO on the reduction of cell viability was counteracted by the anti-oxidant NAC. In response to FVPEO-induced DNA damage the apoptotic cell death was accompanied by p53 and ɣ-H2AX upregulation, two typical markers of DNA damage. In accordance with Patino-Morales data (47) the increase of NQO1 and p53 level seemed to be tightly related each other. These authors demonstrated the existence of a tight interplay between NQO1 and p53, aimed at stabilizing p53 half-life and favoring its role in the induction of apoptotic cell demise.

Mechanistic studies by El Garawani et al. (48) reported that a combination of oils of *F. vulgare* and *P. graveolens* exerts a remarkable cytotoxic effects towards breast cancer MCF-7 cells through cell cycle arrest, while no cytotoxicity was observed on normal human peripheral blood lymphocytes in vitro. The cytotoxic effect of these essential oils was attributed by Garavani to

anethole and estragole which represent the main constituents present found in their experimental conditions**.**

In conclusion our data suggested that FVPEO exerts a marked apoptotic effect on triple negative breast cancer cells, which seems to be correlated with ROS increase, whose level overwhelms the ability of antioxidant systems, such as Nrf2, HO-1 and NQO1, to counteract them. The increase in the level of the antioxidant enzyme NQO1 could also favor p53 stabilization induced by DNA damage, thus contributing to the apoptotic cell death.

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Availability of data and materials

The data used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

A.D., M.L., A.M. and M.B. conceived and designed the experiments. M.L., N.B. and D.G. D'A. conducted all the experiments. M.L., A.M. and A.D. acquired and analyzed the data. M.L., A.M. and A.D. wrote and revised the manuscript. All authors read and approved the final version of manuscript.

Competing interests

The authors declare that they have no competing interests.

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Legends of Figures

Figure 1. Chemical composition of essential oil of *F. vulgare* subsp. *piperitum* fruits. The chemical profiling of the main constituents of essential oil of *F. vulgare* subsp. *piperitum* was performed as reported in methods.

Figure 2 Essential oil of *F. vulgare* subsp. *piperitum* fruit induced cytotoxic effects and morphological changes in MDA-MB231 breast cancer cells. (A) Dose and time dependence of FVPEO effect on cell viability**.** MDA-MB231 cells (8 x 10^3) were incubated in the presence of various doses of essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits (FVPEO) (125-2000 μg/mL) at the indicated times. Cell viability was assessed by MTT assay and expressed as the percentage of the viable control cells. Values are the means of three independent experiments ± S.E. (*) $p < 0.05$, (**) $p<0.01$, compared to the control. (B) Morphological changes of MDA-MB231 cells treated for 24 h with different concentrations of FVPEO observed under light microscopy. The results are representative of three independent experiments.

Figure 3. Protective effects of N-acetylcysteine against FVPEO-induced cytotoxic effects.
(A) The antioxidant N-acetylcysteine counteracts the cytotoxic effects of FVPEO on MDA-MB231 cells. Cells (8 x 10^3) were incubated with different concentrations of FVPEO alone or in the presence of N-acetylcysteine (NAC). After 24h of treatment the viability was assessed by MTT assay and expressed as percentage of control cells. Values are the means of three independent experiments ± S.E. (*) $p < 0.05$ compared to the control. (#) $p < 0.05$, (##) $p<0.01$ compared to FVPEO -treated sample.
(B) FVPEO treatment increased intracellular ROS detected using the redox-sensitive fluorochrome H2-DCFDA. After treatment with FVPEO per different time, the medium was replaced with 10 μM H2DCFDA solution and the incubation was protracted for 30 min at 37 °C. The oxidation of the fluorochrome generates green fluorescence, which was visualized by a Leica microscope equipped with a DC300F camera using a FITC filter. Representative micrographs of fluorescence microscopy were taken at x200 magnification. (C) FVPEO treatment increased the level of the anti-oxidant enzyme MnSOD. MDA-MB231 cells (2 x 10^5) were treated for 24 h in the presence of different concentrations of FVPEO. Then, cell lysates were analyzed using Western blotting with specific antibody directed against MnSOD. The data are representative blots of three independent

512 experiments. Densitometry analysis histogram normalized to ɣ–tubulin is reported. (*) $p < 0.05$
513 compared to the control.

514 **Figure 4. FVPEO upregulates the level of stress proteins and antioxidant factors.** Cell lysates
515 of MDA-MB231 cells were prepared after the incubation in the presence of FVPEO for 24 and 48 h
516 respectively. The expression of stress proteins (c-Jun and pJNK) as well as antioxidant factors
517 (Nrf2, HO-1 and NQO1) was analyzed by western blotting using specific antibodies as reported in
518 methods section. The equal loading of proteins was ascertained by probing the blot with ɣ–tubulin
519 antibody. The data are representative blots of three independent experiments. Densitometry analysis
520 histograms normalized to ɣ–tubulin are reported in the bottom panel. (*) $p < 0.05$, (**) $p<0.01$,
521 (***) $p<0.001$ compared to the control.

522 **Figure 5. FVPEO induces apoptosis in MDA-MB231 cells.** (A) MDA-MB231 cells (8×10^3)
523 were stained with Hoechst 33342 (2.5 mg/mL medium; blue fluorescence) and then treated with
524 different concentrations of FVPEO for 24 h. Cell morphology was visualized with a Leica DC 300F
525 microscope with fluorescent filters for DAPI. The image shows that FVPEO treatment induced the
526 appearance of cells with condensed and fragmented chromatin. (B) Cells with condensed and
527 fragmented chromatin were counted in three different microscopic fields in each well (three wells
528 for each treatment) and expressed as percentage of the total number of cells counted in the field.
529 Values reported in the figure as percentage of apoptotic cells per total number of cells per field are
530 the mean of three independent experiments. (*) $p < 0.05$, (**) $p < 0.01$, compared to the control.
531 (C) MDA-MB231 cells (8×10^3) were treated with different concentrations of FVPEO for 24 h and
532 then stained with acridine orange/ethidium bromide dual staining as reported in methods. The image
533 shows that FVPEO treatment induced the appearance of some cells positive to a yellow-green
534 acridine orange nuclear staining (early apoptotic cells) and a remarkable percentage of cells with
535 orange nuclear ethidium bromide staining (late apoptotic cells).

536 **Figure 6 FVPEO effects on DNA damage markers, caspase activation and PARP-1**
537 **fragmentation.** MDA-MB231 cells (2×10^5) were treated for 24 h in the presence of different
538 concentrations of FVPEO. Then, cell lysates were analyzed using Western blotting with specific
539 antibodies directed against phosphorylated H2AX (ɣH2AX), p53, pro-caspase-3 and PARP-1, as
540 reported in Materials and Methods. The data are representative blots of three independent

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541 experiments. Densitometry analysis histograms normalized to ɣ–tubulin are reported in the bottom
542 panel. (*) $p < 0.05$ compared to the control.

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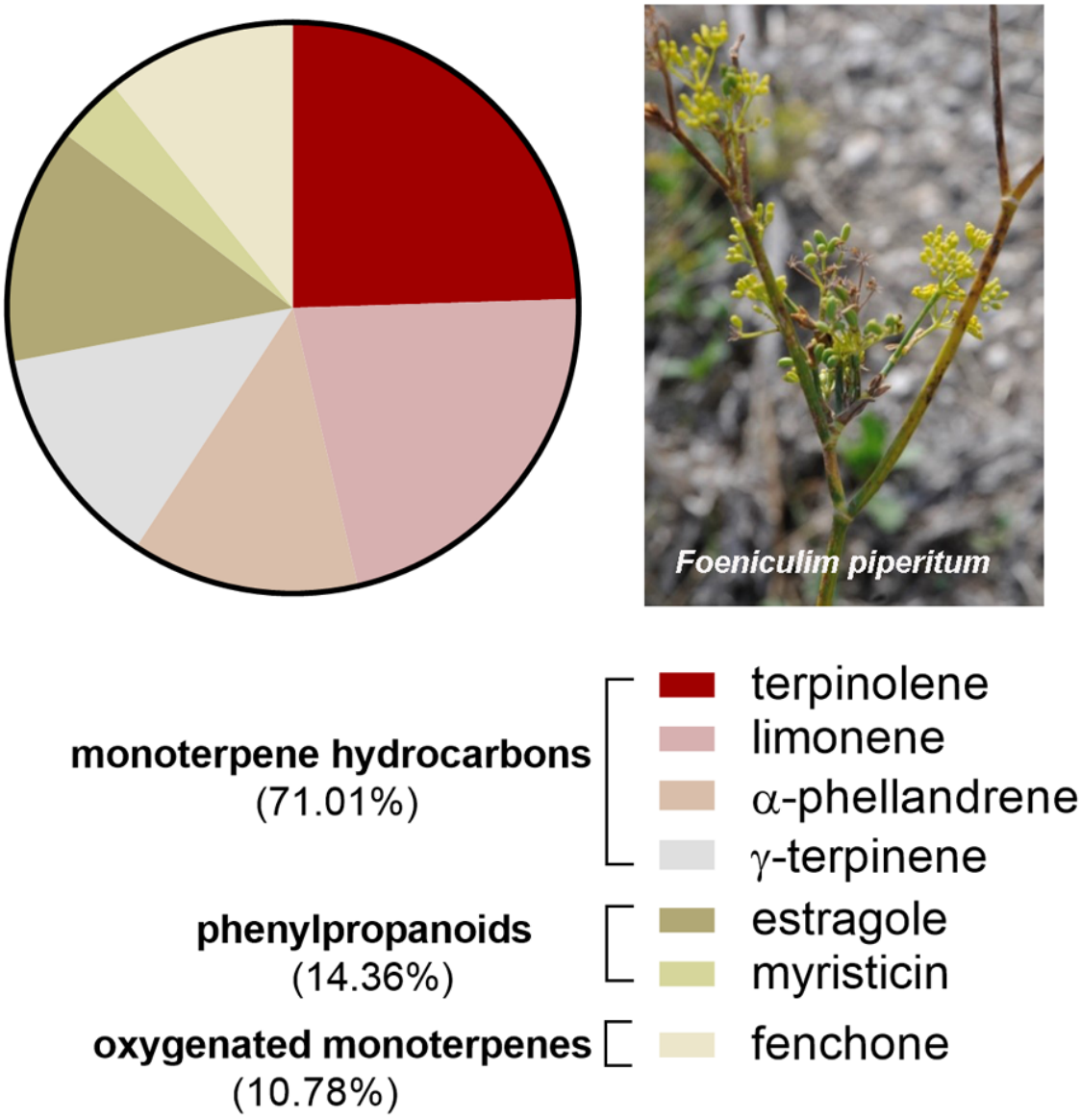

Figure 1

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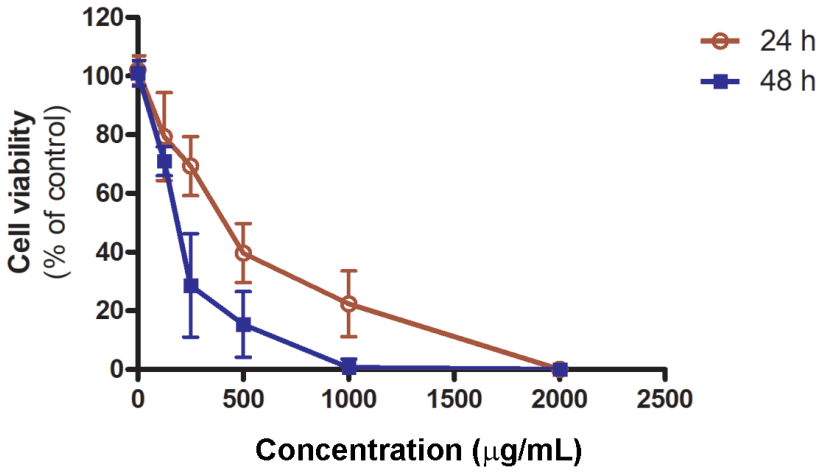
A



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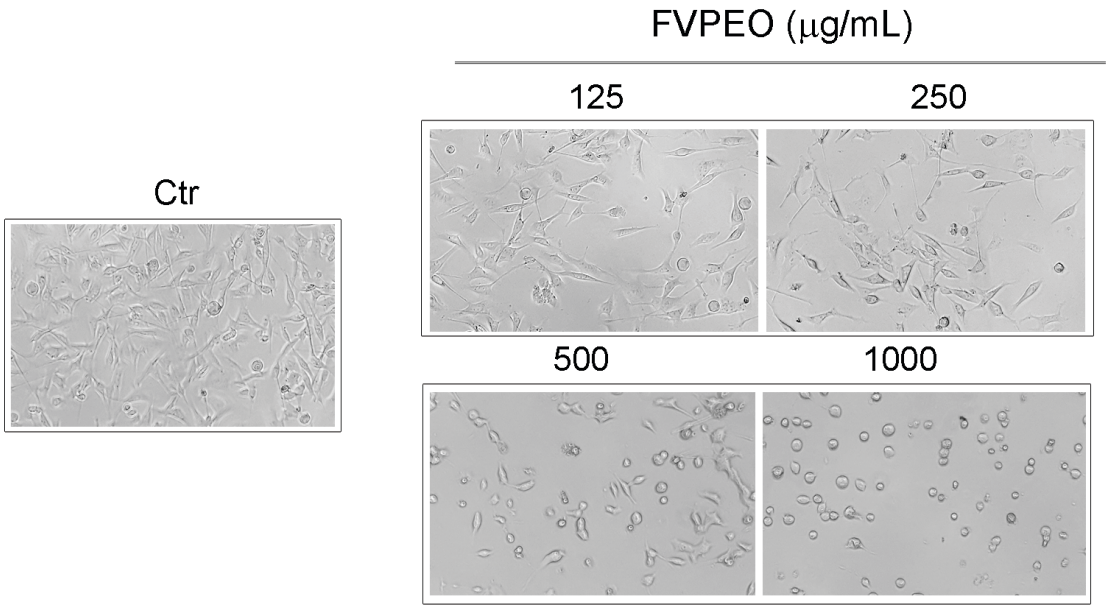

Figure 2

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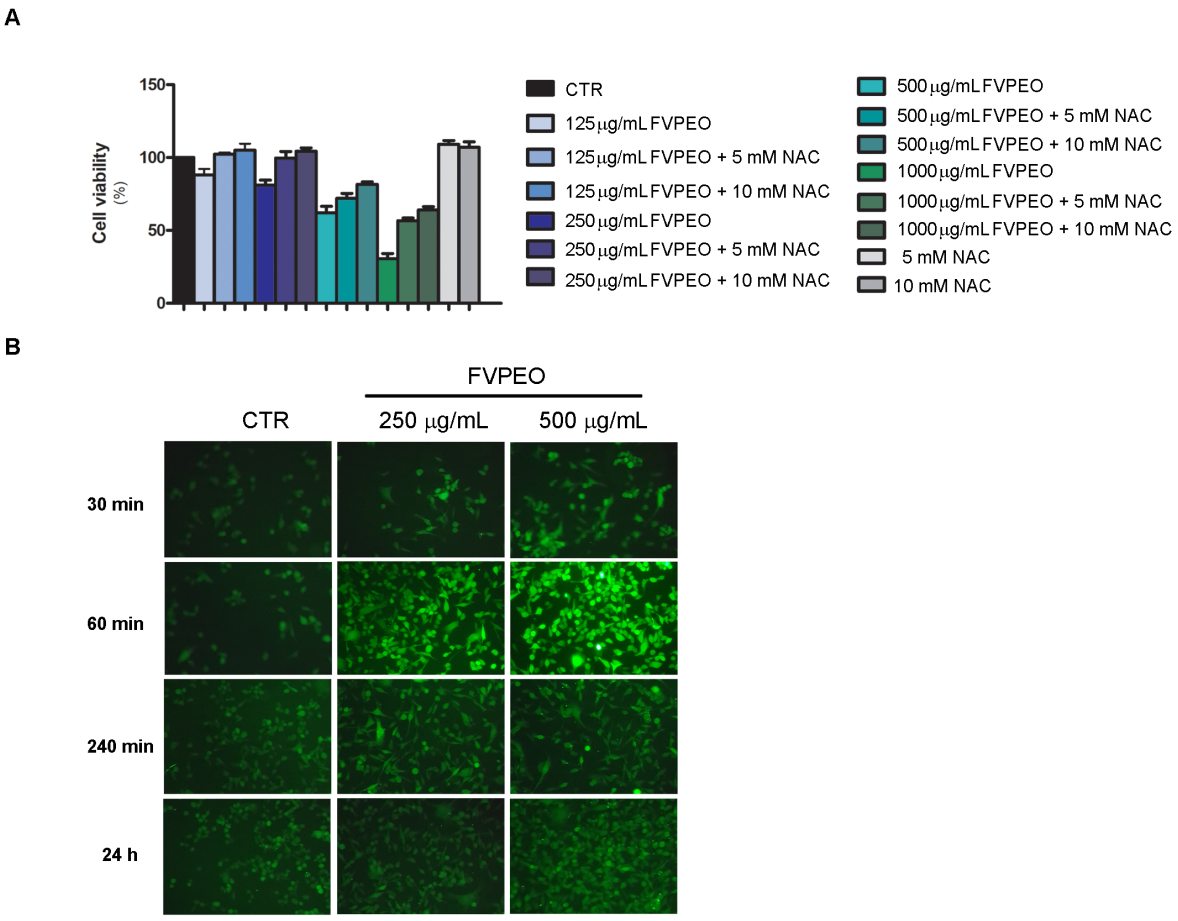

Figure 3

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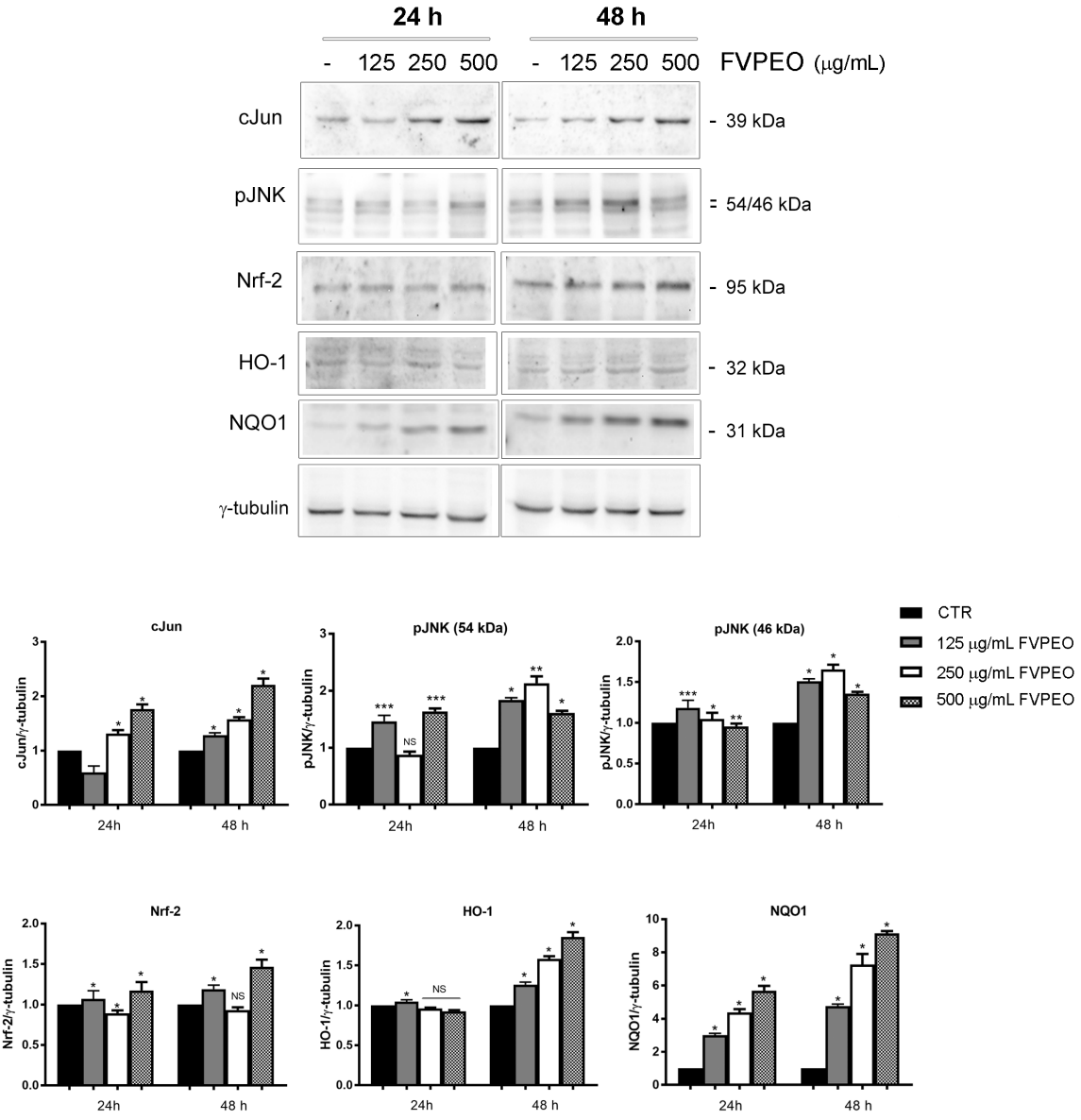

Figure 4

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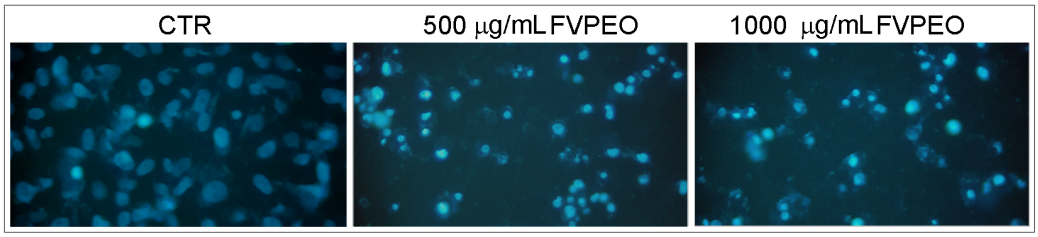
A



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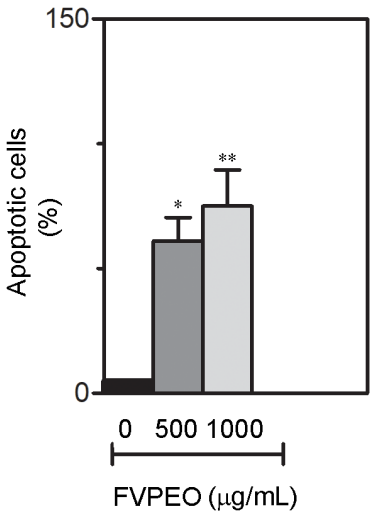

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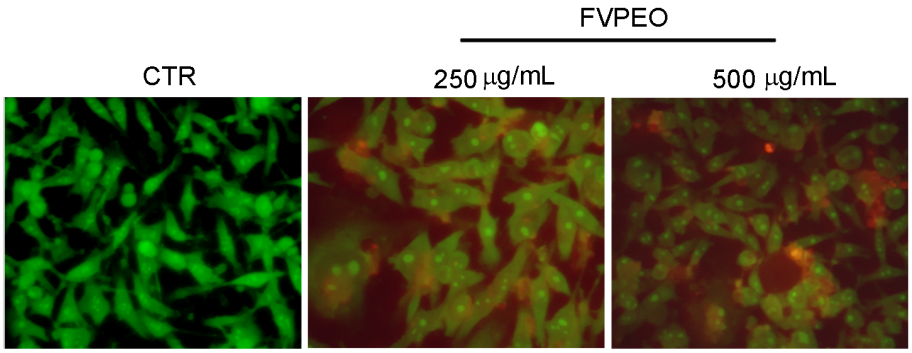

Figure 5

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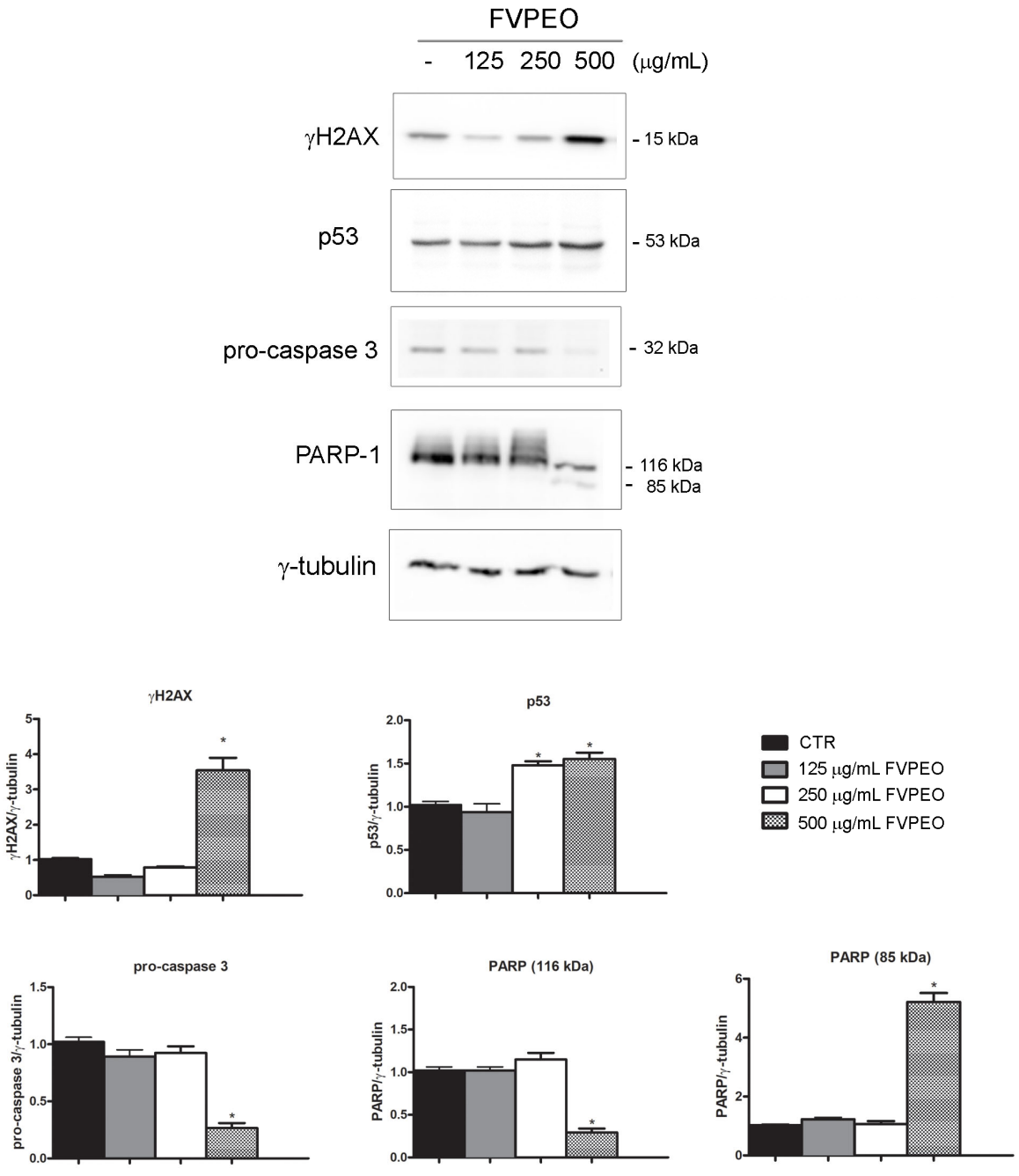

Figure 6