Essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits exerts an anti-tumor effect in triple-negative breast cancer cells

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Abstract. At present, the growing spread of tumor cases worldwide renders the research of new promising and selective anticancer drugs urgent. The biological action of extracts of medicinal plants or their essential oils (EOs) is an emerging field of interest, since they could comprise a rich source of phytochemicals that can prove promising. In the present study, the biological activity and mechanism of action of the EO of *Foeniculum vulgare* subsp. *piperitum* fruits (FVPEO) were investigated using MTT assays, morphological analyses and western blotting in MDA-MB231 cells, a triple-negative breast cancer cell line. The findings revealed that FVPEO could exert strong anticancer effects, causing a dose-dependent inhibition of breast cancer MDA-MB231 cell growth, accompanied with DNA condensation and fragmentation. The cytotoxic effect of FVPEO was counteracted by the addition of the antioxidant N-acetylcysteine and was associated with a marked increase in reactive oxygen species and stress-related proteins; such as manganese superoxide dismutase, c-Jun, phospho-c-Jun N-terminal kinase and nuclear factor E2-related factor 2, and the latter's transcriptional targets, Heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1 (NQO1). As evidenced by the activation of caspase-3 and fragmentation of poly(ADP-ribose) polymerase-1, which are typical apoptosis markers, FVPEO promoted apoptotic cell death accompanied with an increase in phosphorylated H2A histone family member X and the activation of the NQO1/p53 axis. In combination, the present experiments provided evidence that FVPEO could represent a reservoir of biologically active compounds suitable for both cancer prevention and treatment.

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Abbreviations: FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; EOs, essential oils; TNBC, triple-negative breast cancer

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

Introduction

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

Several parts of the plant are edible, including its leaves, stalks and seeds (fruits), which are a source of a wide range of phytochemicals such as fatty acids, phenolic compounds and flavonoids, as well as volatile compounds such as anethole, estragole and fenchone, which are major phytoconstituents found in *F. vulgare* (2,3). Most of these phytochemicals are found in essential oils (EOs), which can be found in almost any part of the plant, including the root, stem, seeds and fruits (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 diuretic, antispasmodic, analgesic, mucolytic and anti-inflammatory functions (1). The whole plant, as well as its stems, fruits, leaves and seeds are used to treat a wide range of ailments of the 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, it was used as remedy for snake bite; in addition, the infusion of its fruits was used as a carminative, while its roots have been found to possess efficient purgative properties. In certain parts of Southern Italy, the decoction of the fruits of

F. vulgare subsp. *piperitum* was used for digestive reasons, and chewing its leaves was considered a cure for mouth ulcers (9). Furthermore, it has been prescribed as a muscle relaxant, a weak diuretic, a carminative and a mild stimulant (10).

The genus *Foeniculum* is found in Italy, with only one species identified 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, based on the difference in distribution, some botanists believe that they represent two distinct species. The presence of *F. vulgare* subsp. *piperitum* has been reported in the 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 parts of *F. vulgare* subsp. *piperitum* collected in Sicily were evaluated by gas chromatography (GC) and GC-mass spectrometry (12). The results were compared with those of the EOs from the same parts of *F. vulgare* subsp. *vulgare*, collected in the same region and with those reported in the literature for other accessions of *F. vulgare* subsp. *piperitum*. The EOs of *F. vulgare* subsp. *vulgare* exhibited completely different compositions, clearly indicating the differences between the two subspecies.

A number of *in vitro* and *in vivo* studies highlighted how various extracts of *F. vulgare* possess antioxidant, anti-inflammatory, anti-mutagenic and anticancer properties. *F. vulgare* EOs exhibited anti-mutagenic effects in mice, as they 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 reported in different cancer cells, such as melanoma (14), prostate (15), lung cancer (16) and hepatocarcinoma cells (17). Ke *et al* (16) demonstrated that ethanol extract of *F. vulgare* seeds induced apoptosis in HCI-H446 and NCI-H661 lung cancer cell lines and inhibited the growth of NCI-446-derived xenografts by reducing Bcl-2 protein expression. 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 effects on Elrich ascites carcinoma-bearing mice by modulating lipid peroxidation and potentiating antioxidant defense (18).

Thus, based on the promising previous results regarding the anti-cancer potential of *F. vulgare*, the aim of the present study was to examine the possible anti-cancer action of the EOs of *F. vulgare* subsp. *piperitum* (FVPEO) in MDA-MB231 triple-negative breast cancer (TNBC) cells, demonstrating that FVPEO induces an apoptotic cell death process through the activation of the NAD(P)H quinone oxidoreductase 1 (NQO1)/p53 axis.

Materials and methods

F. vulgare subsp. *piperitum plant material and fruit EO 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). A total of 136 g *F. vulgare* subsp. *piperitum* fruits were 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. MDA-MB231 TNBC and estrogen-positive MCF7 cells were obtained from 'Istituto Scientifico Tumori' (Genoa, Italy) and cultured as monolayers in DMEM medium (cat. no. ECM0749L, Euroclone SpA) supplemented with 10% (v/v) heat-inactivated FCS (cat. no. ECS0180L; Euroclone SpA), 1% non-essential amino acids (cat. no. ECB3054D; Euroclone SpA), 2 mM glutamine (cat. no. ECB3000D, Euroclone SpA) and 1% penicillin/streptomycin solution (cat. no. ECB3001D; Euroclone SpA). Cells were plated on 96-well microplates or on 6-well cell culture plates and allowed to adhere overnight in culture medium at 37˚C in a humidified atmosphere containing 5% CO_2, followed by treatment with FVPEO or vehicle only. Media and cell culture reagents were purchased from Euroclone SpA. All other chemicals and reagents were provided by Millipore Sigma.

Cell viability assessment and morphological detection of apoptosis. In order to assess the viability of FVPEO-treated breast cancer cells, an MTT assay was performed, as previously described (19). Briefly, $8x10^3$ cells/well were plated in 200 μl DMEM in a 96-well plate and treated. At the end, 4 μl 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. Cells were then lysed in lysis buffer and absorbance was read at 570 and 690 nm using an automatic ELISA plate reader (OPSYS MR; Dynex Technologies).

In order to determine either changes in nuclear morphology or plasma membrane damage, the cells were stained with Hoechst 33342 (cat. no. H3570; Invitrogen; Thermo Fisher Scientific, Inc.), a cell permeant fluorochrome emitting blue fluorescence when bound to dsDNA and excited by ultraviolet light. For these assays, cells ($8x10^3$) were incubated with Hoechst 33342 (2.5 μg/ml medium) for 30 min, washed with PBS and suspended in culture medium prior to FVPEO treatment. Morphological changes in apoptotic cells, manifesting as chromatin condensation and fragmentation, were detected by fluorescence microscopy using an excitation wavelength of 372 nm and emission wavelength of 456 nm. All images were captured by Leica Q Fluoro Software (Leica Microsystems, Inc.). For this analysis, at least 10 fields were considered for each sample and apoptotic cells were counted in random fields at a magnification of x100.

The apoptotic cell morphology was also studied by acridine orange and ethidium bromide double staining, as reported by Liu *et al* (20).

Analysis of reactive oxygen species generation. The detection of intracellular reactive oxygen species production was

carried out using 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) staining, as previously described (21). H_2-DCFDA (cat. no. D399; Molecular Probe; Thermo Fisher Scientific, Inc.) is a non-polar dye that can easily cross the cell membrane and can be oxidized to DCFDA in the presence of reactive oxygen species (ROS) emitting green fluorescence.

After incubating the cells with FVPEO, the medium was removed and cells were incubated with 10 μM H_2-DCFDA for 30 min at 37˚C. Next, positive cells were analyzed under a Leica fluorescence microscope (Leica Microsystems, Inc.) with excitation at 485 nm and emission at 530 nm, as previously described (22).

Western blot analysis. For western blot analysis, 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, and 1 mM EDTA supplemented with phosphatase inhibitor mix (Sigma-Aldrich; Merck KGaA). Extracts were sonicated thrice and protein content was determined using the Bradford assay (Bio-Rad Laboratories, Inc.) using a BSA (Sigma-Aldrich; Merck KGaA) standard curve. Next, 30 μg/lane of protein sample were subjected to SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Manganese superoxide dismutase (MnSOD; cat. no. sc-133254; 1:200), c-Jun (cat. no. sc-1694; 1:200), phospho-c-Jun N-terminal kinases (pJNK; cat. no. sc-6254; 1:200), γ-H2A histone family member X (H2AX; cat. no. sc-517348; 1:200), p53 (cat. no. sc-126; 1:200), pro-caspase-3 (cat. no. sc-65497; 1:200) and PARP-1 (cat. no. sc-53643; 1:200) were detected using specific antibodies produced by Santa Cruz Biotechnology, Inc. The antibody for Heme oxygenase (HO-1; cat. no. orb5455; 1:1,000) was provided by Biorbyt Ltd., and that for NQO1 (cat. no. 3187S; 1:1,000) by Cell Signaling Technology, while the nuclear factor E2-related factor-2 (Nrf-2) antibody (cat. no. NBP1-32822, 1:1,000) was purchased from Novus Biologicals. Next, the nitrocellulose filters were incubated with anti-rabbit IgG (H+L) HRP conjugate (cat. no. W4011; 1:5,000; Promega) or Anti-Mouse IgG (H+L) HRP conjugate (cat. no. W4021; 1:5,000; Promega) secondary antibody for 1 h. Protein bands were detected using ECL™ Prime Western Blotting System (cat. no. GERPN2232; Cytiva), and quantified using Quantity One software 4.6.6 (Bio-Rad Laboratories, Inc.). The correct protein loading was examined using immunoblotting for γ-tubulin (cat. no. T3559; 1:2,000, Sigma-Aldrich; Merck KGaA). All blots shown are representative of at least three separate experiments.

Statistical analysis. Statistical analysis of data was performed using GraphPad Prism 5.0. software (GraphPad Software Inc.). A Student's t-test was applied to evaluate significant differences between untreated and treated samples. For the analysis of multiple groups a one-way ANOVA test was used. Data are expressed as the mean ± SD. The statistical significance threshold was set at $P<0.05$.

Results

Chemical profiling of EO of F. vulgare subsp. *piperitum*. Hydro distillation of the fruits of *F. vulgare* subsp. *piperitum* gave a pale-yellow oil. The EO composition was previously reported (12). As shown in the pie chart of Fig. 1, FVFEO was particularly enriched 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 of EO. The second most abundant class was phenylpropanoids (14.36%), typical metabolites of *F. vulgare* subsp. *vulgare* (12), with estragole (10.96%) and myristicin (3.09%) as the main products of this class of compounds. Oxygenated monoterpenes were present at a lower amount (10.78%) with fenchone (8.83%) as the principal metabolite of this class. Based on these observations, the anti-tumor potential of FVFEO was explored.

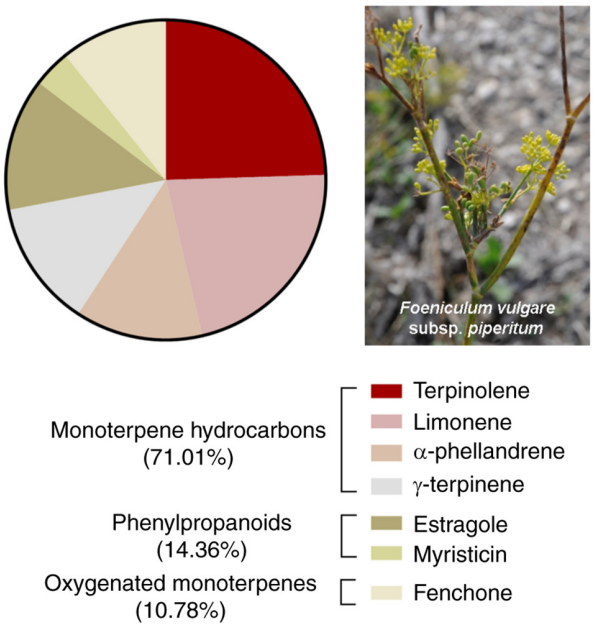

Figure 1. Chemical composition of FVPEO. The chemical profiling of the main constituents of the essential oils of *F. vulgare* subsp. *piperitum* fruits was performed as reported in the Materials and methods section.

Effects of FVPEO on the viability of breast cancer cells. To demonstrate a possible anti-proliferative effect of the EO of the fruits of *F. vulgare* subsp. *piperitum* (FVPEO), the present study focused on MDA-MB231, a very aggressive and poorly differentiated breast cancer cell line, which does not express estrogen, progesterone and human epidermal growth factor receptor 2 (HER2)/receptors (23). MDA-MB231 cells were treated with increasing concentrations of FVPEO (125-2,000 μg/ml) for various periods of time, and the viability was assessed using MTT assay, as reported in the Materials and methods. As shown in Fig. 2A, the cell survival rate displayed a marked dose- and time-dependent decrease following FVPEO treatment, as compared to the untreated control. Following 24 h of treatment the viability of MDA-MB231 cells was reduced by 20% of the control with 125 μg/ml FVPEO. Increasing the treatment dose, the viability diminished progressively and a consistent cytotoxic effect was reached at the highest concentration examined (only ~5% of viable cells with 2,000 μg/ml). The cytotoxic effect of FVPEO was further increased as treatment time increased to up to 48 h, while the viability was decreased to 15% with 500 μg/ml FVPEO.

Light microscopy findings showed that, following exposure to FVPEO, MDA-MB231 cells underwent morphological

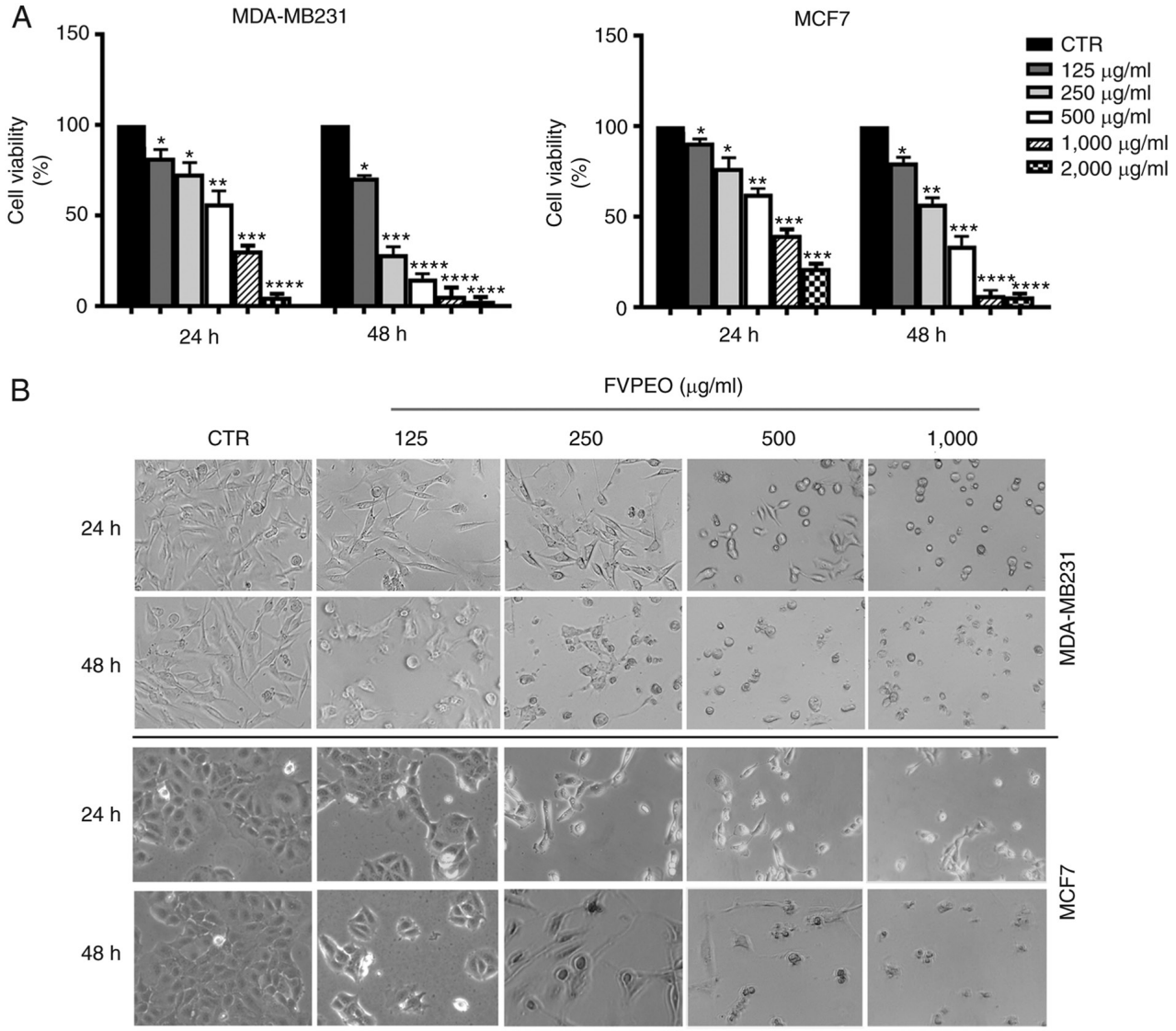

Figure 2. FVPEO induces cytotoxic effects and morphological changes in breast cancer cells. (A) Dose and time dependence of FVPEO effect on cell viability. MDA-MB231 and MCF7 cells ($8x10^3$) were incubated with various doses of FVPEO (125-2,000 μg/ml) at the indicated times. Cell viability was assessed using MTT assay and expressed as the percentage of the viable control cells. Values are presented as the mean ± SE. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control. (B) Morphological changes of MDA-MB231 and MCF7 cells treated for 24 and 48 h with different concentrations of FVPEO observed under light microscopy. The results are representative of three independent experiments. FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; CTR, control.

changes. As shown in Fig. 2B, cells treated with lower doses (125-250 μg/ml) of FVPEO appeared elongated, as compared with untreated cells. As the dose of FVPEO increased, typical morphological changes of apoptotic cells (i.e. cell shrinkage and roundness) appeared and a marked reduction in cell number was observed.

A growth inhibition effect was also observed when FVPEO was tested on MCF7 cells, an estrogen- and progesterone-positive breast cancer cell line (Fig. 2). Following incubation with FVPEO, the cell viability declined in a dose- and time-dependent manner, and cells showed clear signs of death, supporting the anti-cancer potential of FVPEO.

FVPEO-induced cytotoxic effect is counteracted by the antioxidant N-acetylcysteine (NAC) and is accompanied by ROS generation. Next, it was explored whether the cytotoxic effect of FVPEO was dependent on oxidative stress. To this end, MDA-MB231 cells were pre-incubated for 2 h with NAC, a ROS scavenger; different doses of FVPEO were then 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 notably reduced that exerted by high concentrations (500-1,000 μg/ml). To better explore these effects, the generation of ROS by H2-DCFDA, a fluorochrome that binds ROS and emits green fluorescence in its oxidized form, was also evaluated. Using this experimental approach, a clear rise in green fluorescence, indicative of ROS production in FVPEO-treated cells, was observed (Fig. 3B). The increase, which had already appeared at 30 min of incubation with 250 and 500 μg/ml FVPEO, peaked at 60 min after application. Western blot analysis was also performed to evaluate whether FVPEO treatment modified the level of MnSOD, one of the main cellular antioxidant enzymes (24). As shown in

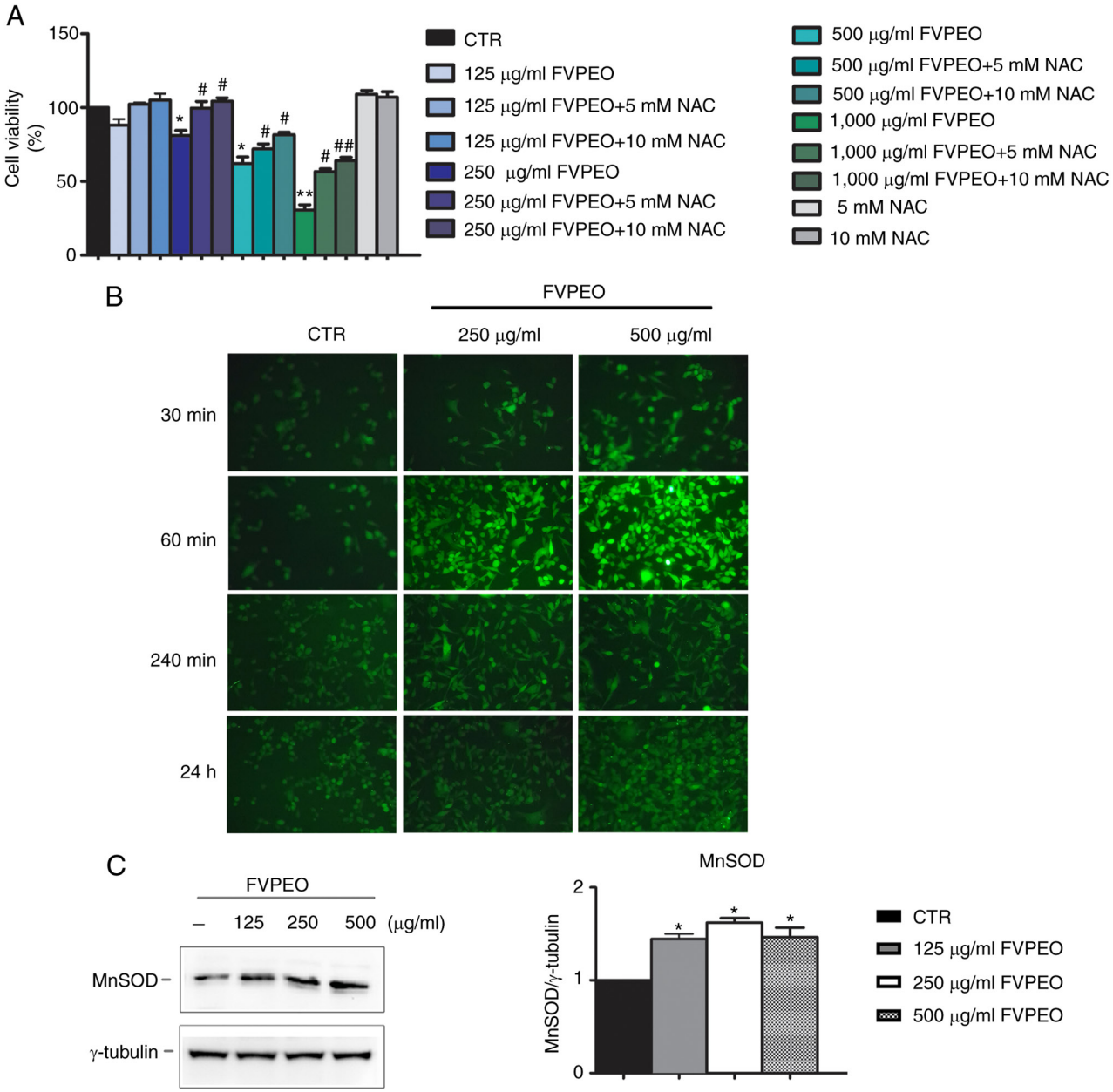

Figure 3. Protective effects of NAC against FVPEO-induced cytotoxic effects. (A) Antioxidant NAC counteracts the cytotoxic effects of FVPEO on MDA-MB231 cells. Cells ($8x10^3$) were incubated with different concentrations of FVPEO alone or in the presence of NAC. After 24 h of treatment the viability was assessed by MTT assay and expressed as a percentage of the control cells. Values are the means of three independent experiments ± SE. $^*P<0.05$ and $^{**}P<0.01$ vs. the control; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. sample treated with the corresponding FVPEO dose. (B) FVPEO treatment increased intracellular ROS, which was detected using the redox-sensitive fluorochrome H2-DCFDA. The oxidation of the fluorochrome generates green fluorescence, which was visualized using a Leica microscope equipped with a DC300F camera with a FITC filter. Representative micrographs of fluorescence microscopy were taken at a magnification of x200. (C) FVPEO treatment increased the level of the anti-oxidant enzyme MnSOD. MDA-MB231 cells ($2x10^5$) were treated for 24 h in the presence of different concentrations of FVPEO. Cell lysates were analyzed using western blotting with a specific antibody against MnSOD. The data are representative blots from three independent experiments. Densitometry analysis histogram normalized to γ-tubulin is reported. $^*P<0.05$ vs. control. NAC, N-acetylcysteine; FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; CTR, control.

Fig. 3C, an increased level of MnSOD was observed following FVPEO incubation.

FVPEO cytotoxic effect is mediated by oxidative stress and the upregulation of stress-associated proteins. In light of the observed data demonstrating ROS production in MDA-MB231-treated cells, it was explored whether the cytotoxic effect induced by FVPEO can be accompanied by the activation of stress-related proteins (Fig. 4).

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

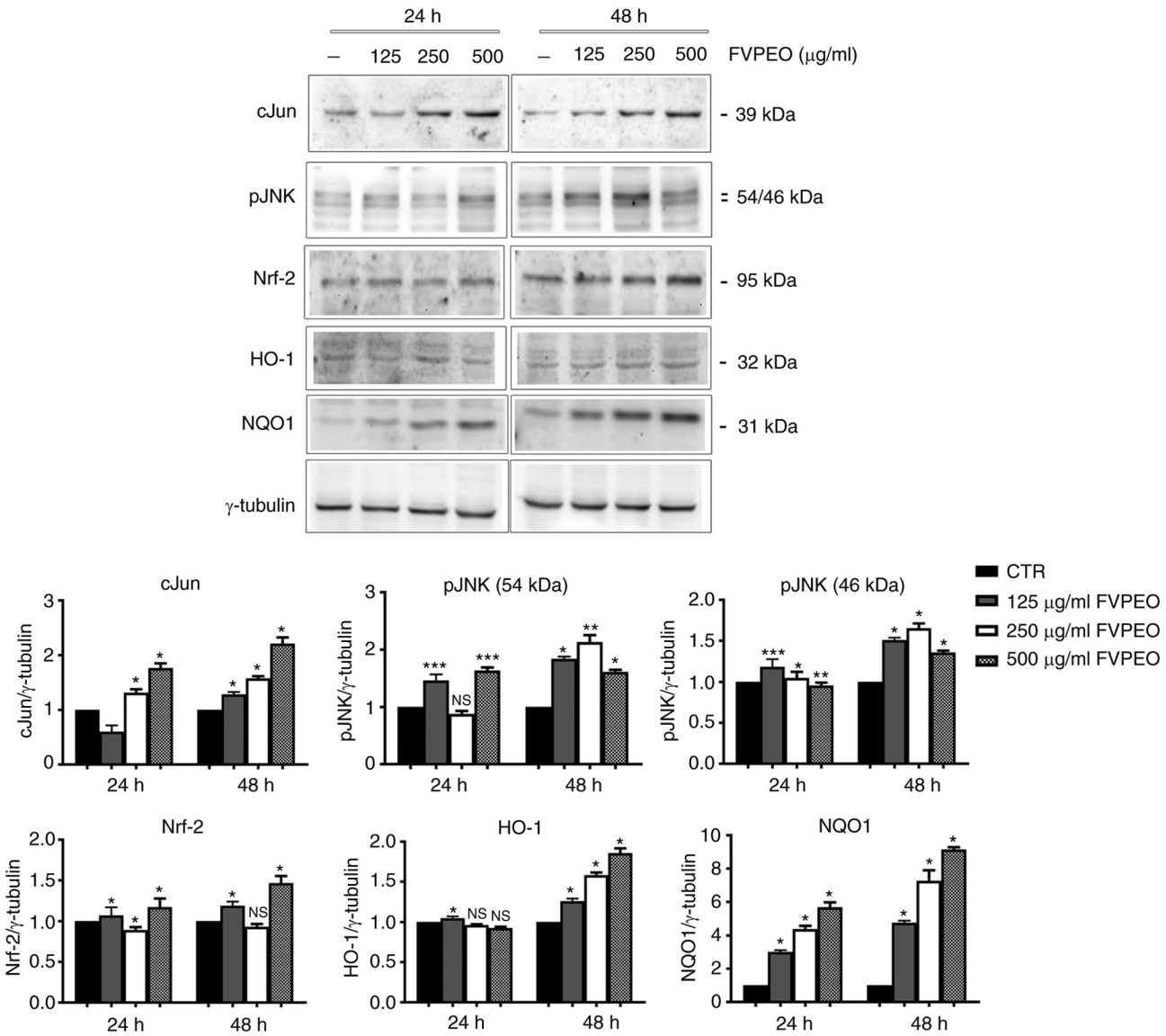

Figure 4. FVPEO upregulates the level of stress proteins and antioxidant factors. Cell lysates of MDA-MB231 cells were prepared following incubation with FVPEO for 24 and 48 h respectively. The expression of stress proteins (c-Jun and pJNK), as well as that of antioxidant factors (Nrf2, HO-1 and NQO1), were analyzed using western blotting using specific antibodies, as reported in the Materials and methods section. *P<0.05, **P<0.01, ***P<0.001 vs. control. NS, not significant; FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; CTR, control; Nrf-2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase; NQO1, NAD(P)H quinone oxidoreductase 1.

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

The involvement of stress in FVPEO-treated cells was also confirmed by the upregulation of NF-E2 p45-related factor 2 (Nrf2), 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, an increase in Nrf-2 content that was associated to an upregulation of its target genes HO-1 and NQO1 was observed in FVPEO-treated cells.

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 can identify nuclear apoptotic changes. According to fluorescence microscopy images, MDA-MB231 cells treated with FVPEO exhibited a clear nuclear fragmentation and condensation, as compared with untreated control cells (Fig. 5A). The proportion of cells with condensed and fragmented nuclei increased as the FVPEO dose increased (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 (Fig. 5C). Indeed, some FVPEO-treated cells were positive for a yellow-green acridine orange nuclear staining (early apoptotic cells), with a considerable proportion of them (~45%) showing 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

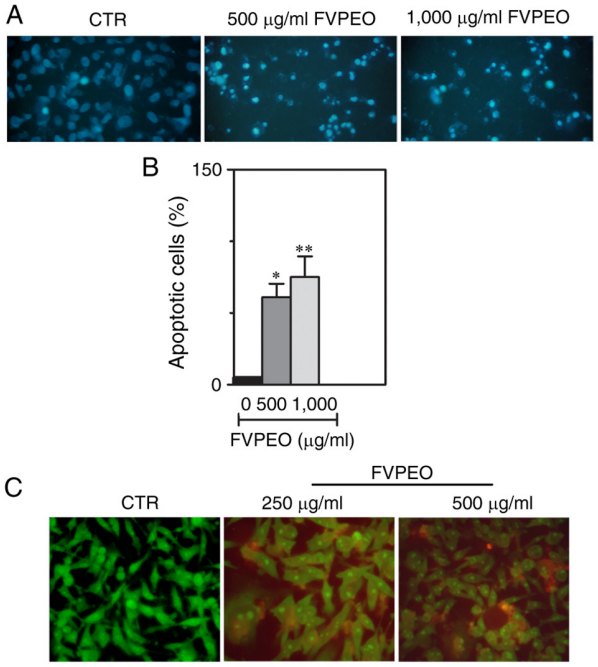

Figure 5. FVPEO induces apoptosis in MDA-MB231 cells. (A) MDA-MB231 cells ($8x10^3$) were stained with Hoechst 33342 (2.5 μg/ml medium; blue fluorescence) and then treated with different concentrations of FVPEO for 24 h. Cell morphology was visualized using a Leica DC 300F microscope with fluorescent filter for DAPI. The image shows that FVPEO treatment induced the appearance of cells with condensed and fragmented chromatin. (B) Cells with condensed and fragmented chromatin were counted in three different microscopic fields in each well (three wells for each treatment) and expressed as a percentage of the total number of cells counted in the field. Data in the figure are presented as percentage of apoptotic cells per total number of cells per field (mean of three independent experiments). $^*P<0.05$ and $^{**}P<0.01$ vs. control. (C) MDA-MB231 cells ($8x10^3$) were treated with different concentrations of FVPEO for 24 h and then stained with acridine orange/ethidium bromide dual staining as reported in the Materials and methods section. The image shows that FVPEO treatment induced the appearance of some cells that were positive for a yellow-green acridine orange nuclear staining (early apoptotic cells) and a marked percentage of cells with orange nuclear ethidium bromide staining (late apoptotic cells). FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; CTR, control.

mechanism of FVPEO-induced apoptosis, it was examined whether the observed effects and association with DNA injury can be linked 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, a form known as γH2AX (28). This event is induced by the kinases ataxia-telangiectasia mutated, ataxia-telangiectasia mutated and Rad3-related protein and DNA-dependent protein kinase, allowing the formation of H2AX phosphorylated on serine 139, which is considered a marker of DNA damage (29). The present data showed that the treatment of MDA-MB231 cells with FVPEO induced a strong phosphorylation of H2AX at Ser139 in a dose-dependent manner (Fig. 6).

Next, possible changes in the level of p53 protein, a key factor involved in the induction of apoptosis in response to DNA damage, was examined (30). As shown in Fig. 6, the level of p53 markedly increased in MDA-MB231-treated cells compared with the untreated control.

In the present study, the effect of FVPEO on caspase-3, a key mediator of apoptosis of mammalian cells, whose activation by the cleavage of procaspase-3 is responsible for chromatin condensation, was also analysed (31). These results indicated that FVPEO treatment induced a dose-dependent decrease in the inactive procaspase-3, indicating the activation of caspase-3 (Fig. 6). This suggestion was confirmed by the cleavage of PARP-1, a well-known target of caspase-3 (32), which was observed following FVPEO treatment. In combination, these data indicated that FVPEO induced caspase-dependent apoptosis triggered by DNA damage.

Discussion

Nowadays, the global increase in the number of cancer cases renders the research on specific and targeted medical therapies urgent. Particular attention has been paid on the plant kingdom as a possible bio-resource for new phytochemicals that can be used as preventative or protective compounds in cancer therapies, either alone or in combination (33-35). Indeed, since their discovery, a vast array of plant-derived phytochemicals, such as etoposide, taxol, doxorubicin, topotecan, irinotecan and camptotecin, have been identified as valuable and highly effective chemotherapeutics routinely used in clinical practice (36). Over 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 this, the present study was conducted to evaluate the possible anti-cancer properties of EOs of *F. vulgare* subsp. *piperitum* fruits (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 thus far, the aim of the present study was to investigate whether it can exert anti-proliferative effects in TNBC cells.

Breast cancer is one of the most common tumors affecting women; its incidence tends to rise with age (median age at diagnosis is 63 for breast cancer patients in United States) and it is the second leading cause of death (38,39). Based on the presence or absence of estrogen, progesterone and HER2 receptor, this tumor has been classified into three distinct subtypes with different incidence rates among women. In particular, the hormone receptor positive/Erb-B2 receptor tyrosine kinase 2 (ERBB2) negative form affects up to 70% of patients, the ERBB2 positive form affects 15-20% of cases, and the TNBC subtype, characterized by the lack of all receptors, affects ~15% of patients (40). TNBC is a particularly concerning type of breast cancer, as it is the most aggressive type with a highly invasive profile that is associated with a poor prognosis and high resistance to the most common cancer therapies, which renders its treatment challenging (41).

The present data demonstrated that FVPEO induced a marked reduction in cell viability in TBNC cells and that effect was associated with oxidative injury, as evidenced by a consistent ROS generation. As is well known, ROS are highly reactive molecules that, when produced at a physiological level in the cell, can participate in intracellular signaling functioning as redox messenger (42). However, the extensive ROS generation is dangerous for the cells, since their production gets around intracellular scavenger systems, triggering cell

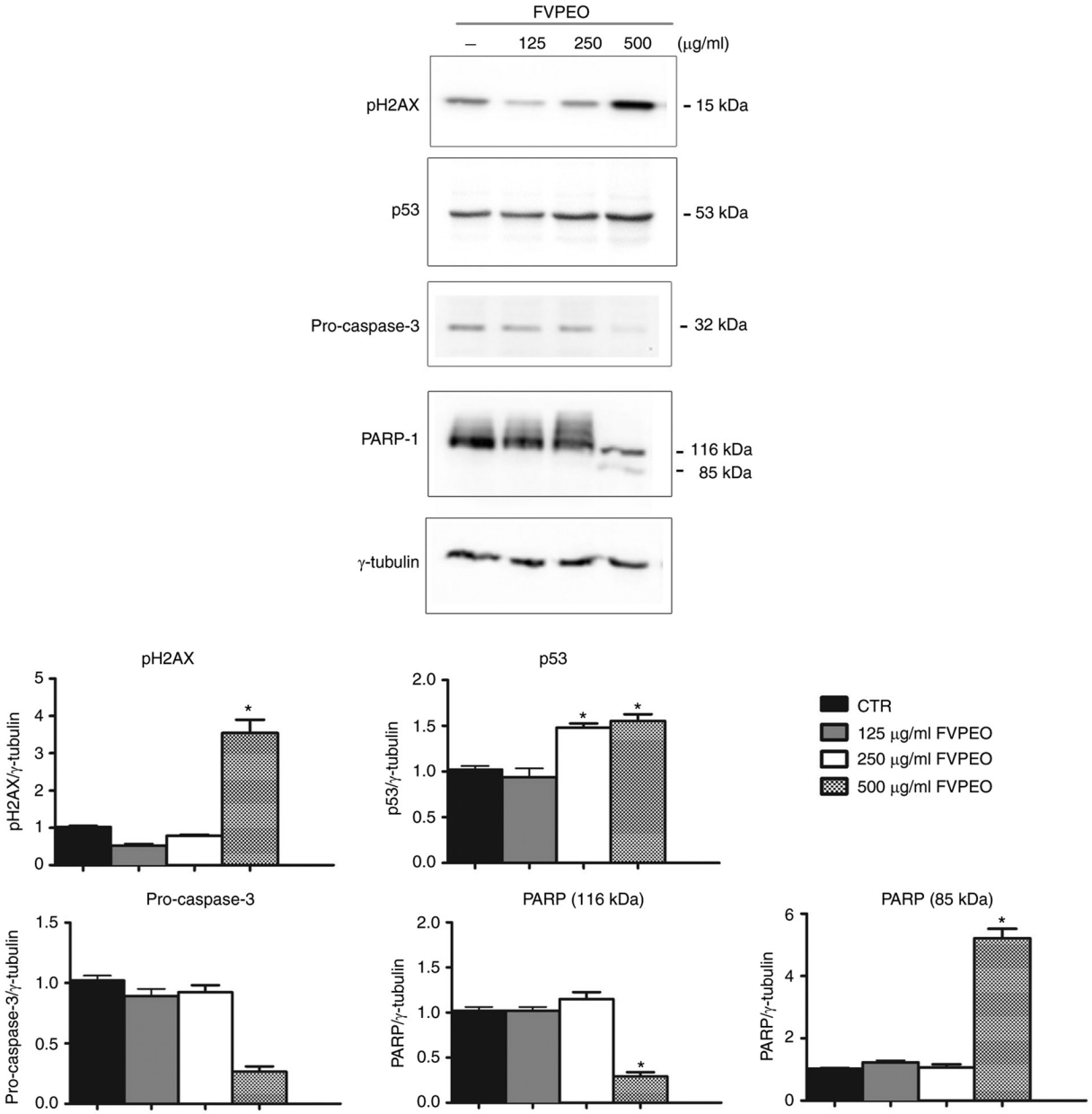

Figure 6. FVPEO effects on DNA damage markers, caspase activation and PARP-1 fragmentation. MDA-MB231 cells ($2x10^5$) were treated for 24 h in the presence of different concentrations of FVPEO. Cell lysates were then analyzed using western blotting with specific antibodies against pH2AX (γH2AX), p53, pro-caspase-3 and PARP-1, as reported in the Materials and methods section. The data are representative blots from three independent experiments. Densitometry histograms normalized to γ-tubulin are reported in the bottom panel. *P<0.05 vs. control. FVPEO, essential oil of *Foeniculum vulgare* subsp. *piperitum* fruits; CTR, control; PARP-1, Poly(ADP-ribose) polymerase-1; H2AX, γ-H2A histone family member X; pH2AX, phosphorylated H2AX.

death (43). In accordance with these observations, the present data provided evidence that FVPEO treatment induced ROS production, upsurged stress-related proteins such as c-Jun, pJNK and antioxidant defense systems represented by Nrf-2 and its targets, MnSOD and NQO1. Of note, FVPEO induced a dose-dependent effect on pJNK upregulation at 24 h. Differently, for longer times of exposure (48 h) the maximum effect of FVPEO on the level of this stress-related factor was observed with 250 μg/ml, probably because cells underwent apoptotic cell death at the higher dose.

Nrf-2 is a transcription factor that, under stress conditions, travels from the cytosol to the nucleus, where it 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 antioxidant-based response (thioredoxin, sulfiredoxin), ROS and xenobiotic detoxification (NQO1, glutathione peroxidase 2 and several glutathione S-transferases) and iron metabolism (HO-1).

In our experimental conditions, Nrf-2 upregulation induced by FVPEO treatment was associated with 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 findings seem to sustain the mode of action of FVPEO in the breast cancer cells used in the present study.

Indeed, it was demonstrated that FVPEO promoted a marked DNA condensation and fragmentation through caspase-3 activation and PARP-1 fragmentation. The cell death induced by FVPEO appears 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, apoptotic cell death was accompanied by p53 and γH2AX upregulation, two typical markers of DNA damage. In accordance with Patino-Morales data (47) the increase in the NQO1 and p53 level appeared to be closely linked to one another. 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 marked cytotoxic effect in breast cancer MCF-7 cells through cell cycle arrest, while no cytotoxicity was observed in normal human peripheral blood lymphocytes *in vitro*. The cytotoxic effect was attributed by El-Garawani to anethole and estragole, which were identified as the main constituents of these EOs.

In conclusion, the present data suggested that FVPEO exerts a marked apoptotic effect on triple-negative breast cancer cells, which appears to be correlated with ROS increase, whose level increases 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 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

ADA, ML, AM and MB conceived and designed the experiments. ML, NB and GDDA conducted all the experiments. ML, AM and ADA acquired and analyzed the data. ML, AM and ADA wrote and revised the manuscript. ML and ADA confirm the authenticity of all the raw data. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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