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Evaluation of cytotoxic activity and anticancer potential of indigenous Rosemary (*Rosmarinus officinalis* L.) and Oregano (*Origanum vulgare* L.) dry extracts on MG-63 bone osteosarcoma human cell line

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Abstract

We aimed to investigate the cytotoxic activity of indigenous Rosemary and Oregano freeze-dried extracts upon MG-63 osteosarcoma human cell line. We have determined the influence of analyzed dry extracts on cell morphology, cell survival and cell proliferation. The evaluation of dry extracts effect upon cell proliferation and viability was assessed by means of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. For cytotoxicity evaluation, Live & Dead and lactate dehydrogenase assays have been used. To further investigate the potential anticancer effect, we have studied the influence of dry extracts upon cells, by means of caspase-3/7 assay and proliferation cell nuclear antigen (PCNA) expression. Cells were incubated with extracts in the following concentration range (100–700 µg/mL) for 24 hours. According to our results, both dry extracts have shown cytotoxic effects by means of all used methods. Bone osteosarcoma cells viability significantly decreased with increasing concentration of analyzed extracts (beyond 300 µg/mL for Rosemary dry extract and only at 700 µg/mL for Oregano dry extract). According to our results, apoptosis is one of the main mechanisms involved in the cytotoxic properties of analyzed extracts. Moreover, Rosemary extract has also shown decreased expression of PCNA, when compared to control (untreated cells). Both extracts were standardized in phenolic compounds (being a rich source of flavones and phenolcarboxylic acids), so we assume that these are the main constituents involved in the cytotoxic effect. Still, further preclinical studies are needed to confirm the antitumor properties and to go deeply in the molecular mechanisms involved.

Keywords: Rosemary leaves, rosmarinic acid, osteosarcoma, polyphenols, apoptosis.

Introduction

Cancer is a leading cause of death and an important barrier to increasing life expectancy all over the world. According to the *World Health Organization* (WHO) for 2019, female breast cancer was the most commonly diagnosed cancer followed by lung, prostate, colorectal and stomach cancers [1]. It is well known that due to metastasis from other malignancies, skeletal bones can host numerous types of cancer, although osteosarcoma, chondrosarcoma and Ewing sarcoma arise in bone itself [2]. Although they account for less than 1% of diagnosed cancers each year, they are associated with significant mortality [2, 3]. Among risk factors associated with bone cancers, genetic factors [*p53* oncogene mutation, translocation in chromosomes 11 and 22, retinoblastoma (*Rb*) oncogene alteration], growth factors (tall stature, Paget disease, high birth weight) along with environmental factors (radiation therapy, pollution, orthopedic surgery followed by prosthetic implant) are of great importance [2, 4].

Among bone cancers, osteosarcoma is the most common cancer in adolescence, and it is associated with malignant mesenchymal cells. Its peak incidence is in the second decade of life, most prominent between 10–30 years [3]. It usually affects long bones, such as proximal tibia (15%), distal femur (30%) and proximal humerus (15%) [4].

The histological hallmark of osteosarcoma is the development of malignant osteoid, due to proliferation of abnormal osteoblasts, which further leads to suppression of bone marrow, metastasis, and increased pressure in the bone (which represents the main cause of pain, swelling and fracture) [4].

The molecular pathology of osteosarcoma include: (*i*) alteration of signal transduction pathways [receptor activator of nuclear factor-kappa B ligand (RANKL), Wnt/*β*-catenin, Notch, phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), bone morphogenetic protein (BMP)], (*ii*) downstream kinase pathways [integrin-linked kinase, mitogen-activated protein kinase (MAPK) cascades], (*iii*) alterations in insulin-like growth factor type 1 receptor (IGF-1R) and ErbB2 receptor [5, 6], (*iv*) altered expression of micro-ribonucleic acid (mi-RNA) (miR-135b, miR-150, miR-542-5p, miR-652) [7], (*v*) up-regulation of oncogene genes [*c-myc*, *c-Fos*, tripartite motif containing 14 (*TRIM14*)], (*vi*) epigenetic mechanisms (histone modification or nucleosome remodeling), (*vii*) inflammation [increased levels of interleukin (IL)-6, IL-17, IL-34, transforming

growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α)] [8] and increased levels of osteoprotegerin (OPG) [9]. Tumor growth and progression are assisted by growth factors and angiogenic enzymes [vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGF-R)] [10].

Osteosarcoma treatment usually involves surgery and radiation therapy, chemotherapy (with Cisplatin, Doxorubicin, high dose of Methotrexate with Leucovorin rescue and Ifosfamide with or without Etoposide) or immunotherapy [9]. Although chemotherapy has increased overall survival to 60–75%, survival rates have remained the same for the last 20–30 years. Besides, chemotherapy often leads to serious side effects (ototoxicity, nephrotoxicity, or cardiotoxicity) [9]. Moreover, osteosarcoma is characterized by refractoriness to treatment [10–13], since tumors only respond to high doses of chemotherapy and rapidly acquire resistance [2, 9].

Having in mind the previous mentioned aspects, the use of natural compounds, as antitumor agents, for osteosarcoma treatment has become an area of great interest recently, since several reports have demonstrated the beneficial role of bioactive compounds, such as phenolic compounds (curcumin, resveratrol, apigenin, baicalin, etc.), thioderivatives (sulforaphane) or triterpenic compounds (raddeanin) as antitumor agents [11, 14, 15].

Medicinal plants of the *Lamiaceae* family are an important source of natural compounds with antibacterial, antiviral, antifungal, antioxidant, sedative, and antitumor effects [16, 17]. Among *Lamiaceae* species, Rosemary (*Rosmarinus officinalis* L.) and Oregano (*Origanum vulgare* L.) are extensively studied due to their complex chemical composition and pharmacological effects. Oregano aerial parts are an important source of essential oil (rich in terpinene-4-ol, α-pinene, borneol, thymol, carvacrol, limonene, etc.) [18] and phenolic compounds (gentisic acid, chlorogenic acid, *p*-coumaric acid, rosmarinic acid, quercitrin, rutin, luteolin) [19], with antitumor [20], antioxidant [21], anti-inflammatory [22] and antibacterial properties [21].

Regarding Rosemary, its leaves are an important source of essential oil (rich in 1,8-cineole, camphor, borneol, *p*-cymene-7-ol, etc.), flavones (hesperidin, diosmin, isoscutellarein 7-O-glucoside, etc.), phenolcarboxylic acids (caffeic acid, chlorogenic acid, rosmarinic acid) and diterpenes (carnosol, carnosic acid, rosmanol) [23]. Rosemary leaves are known for their hepatoprotective, anti-inflammatory, antidiabetic, antimicrobial and neuroprotective effects [23–25].

Aim

Taking into consideration the scientific literature, the aim of our paper was the evaluation of *in vitro* cytotoxic activity and potential antitumor effects of indigenous Rosemary and Oregano dry extracts (which were previously characterized in our studies) [26, 27] on MG-63 bone osteosarcoma human cell line.

Our purposes were: (*i*) the evaluation of cell morphology, (*ii*) cell survival (by means of Live & Dead assay), (*iii*) cytotoxicity [lactate dehydrogenase (LDH) assay], (*iv*) cell proliferation {3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay}, (*v*) evaluation of proliferating cell nuclear antigen (PCNA) expression, and (*vi*) evaluation of apoptosis by means of caspase-3/7 assay.

Materials and Methods

Dry extracts

Oregano (*Origani extractum* – OE) and Rosemary (*Rosmarini extractum* – RE) freeze-dried extracts were obtained using 50% ethanol as previously described [26, 27]. Both extracts were standardized in phenolic compounds (flavones and phenolcarboxylic acids), by means of spectrophotometric and high-performance liquid chromatography (HPLC) assays [26, 27].

Preparation of samples

RE and OE dry extracts (0.0100 g) were dissolved in 10 mL Dulbecco's Modified Eagle's Medium (DMEM) (stock solution). Stock solutions were further used for obtaining the working dilutions (100 μg/mL, 300 μg/mL, 500 μg/mL, and 700 μg/mL).

Cell line and culture

MG-63 osteosarcoma human cell line was obtained from American Type Culture Collection (ATCC, Manassas, USA) and maintained in DMEM (Thermo Fisher Scientific, China) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin, at 37°C, in a humified atmosphere with 5% carbon dioxide (CO_2).

Assessment of cell morphology

Prior to evaluation of *in vitro* cytotoxic activity of OE and RE, we determined the influence of analyzed extracts on MG-63 cell line morphology and density. For this purpose, MG-63 cells were seeded in 24-well plates at an initial density of 1×10^4 cells/cm^2. After 24 hours of culture, the medium was replaced with the appropriate treatments (100–700 μg/mL OE/RE) for 24 hours. All experiments were performed against a control that contained untreated cells grown in DMEM. Cell were observed using a Carl Zeiss Axio phase contrast microscope (with AxioCam ERc 5s camera) (Carl Zeiss, Germany).

LDH assay

LDH assay is a common method used for evaluation of cellular toxicity, since LDH release is considered as an early event of necrosis. LDH is a cytosolic enzyme that leaks out of the cell in case of cell damage. The principle behind the assay is that LDH released into the medium catalyzes the conversion of lactate to pyruvate and converts nicotinamide adenine dinucleotide oxidized form (NAD^+) into NAD reduced form (NADH). Then, NADH transforms the yellow tetrazolium salt 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) into a red formazan. The color change is proportional to the amount of LDH released and was measured at an absorbance of 490–520 nm [28].

LDH assay was performed according to Legrand *et al.* (1992), using a commercial kit (TOX-7, Sigma-Aldrich, Germany) [29]. Briefly, at the end of the experimental time (after 24 hours incubation of MG-63 cells with OE and RE dry extracts 100–700 μg/mL), 100 μL of culture medium was treated with a mixture of LDH assay substrate, LDH assay cofactor (NADH, H^+) and LDH assay dye

solution (tetrazolium salt) (prepared according to the commercial kit). The mixture was incubated at room temperature, in the dark, for 30 minutes. The enzymatic reaction was ended by addition of 30 μL 1N hydrochloric acid. The absorbance was measured at λ 490 nm using a Multimode Microplate reader (Thermo Scientific Appliskan).

Assessment of cell survival by means of Live & Dead assay

Live & Dead assay is a quick two-color assay, which is used to determine the viability of cells based on plasma membrane integrity and esterase activity in live cells. The esterase substrate Calcein-AM stains live cells in green, while Ethidium homodimer I (EthD-1) stains dead cells in red (excitation/emission 495 nm/635 nm) [30]. The Live & Dead kit (Invitrogen™ Molecular Probes™) was used to perform this assay. Briefly, after 24 hours of cell incubation with analyzed dry extracts, the culture medium was removed and the monolayers were washed (three times) with phosphate-buffered saline (PBS) and then stained for 30 minutes at room temperature and darkness, with a fresh solution of Calcein-AM (2 mM) and EthD-1 (4 mM). For highlighting live and dead cells, all samples were analyzed by fluorescence microscopy using an Olympus IX71 fluorescence microscope and Cell F Imaging software for image capture and edit.

Assessment of cell proliferation by means of MTT assay

The MTT assay is based on the reduction of the tetrazolium salt to its formazan (which is violet colored) only in the mitochondria of living cells, due to mitochondrial dehydrogenases activity [31, 32]. Briefly, after 24 hours of culture, the medium was removed and incubated for three hours with a 1 mg/mL fresh MTT solution, at 37°C, allowing cells to form formazan crystals, which were further solubilized in isopropanol. The absorbance was measured at λ 550 nm using a Multimode microplate reader (Thermo Scientific Appliskan) [31].

PCNA evaluation using Western blot analysis

Western blot analysis is a widely used analytical technique, which is performed to detect specific proteins, that bind to a certain antibody [33, 34]. Western blot analysis implies (*i*) protein extraction and equal loading of proteins, (*ii*) separation of proteins by molecular weight, (*iii*) electrophoretic transfer (blotting) and (*iv*) antibody probing [34]. For our experiment, first we prepared Western blot samples by protein extraction using specialized cell lysis buffers [radioimmunoprecipitation assay (RIPA) or urea/thiourea buffers] and protease/phosphatase inhibitors (P8340 and P2850, Sigma-Aldrich, Germany). Since there must be an equal concentration of proteins per Western blot sample, we conducted a Bradford assay. Briefly, 250 μL of Bradford reagent (a mixture of Coomassie Brilliant Blue G-250 and 85% phosphoric acid) was added to 5 μL sample proteins and the change of color was recorded at λ 595 nm after 60 minutes [enzyme-linked immunosorbent assay (ELISA) Thermo Scientific Appliskan plate reader]. For proteins quantification, we have used a specific calibration curve obtained using Bovine Serum Albumin (BSA) (concentration range 0.1–1.4 mg/mL, n=5, R^2>0.999). The separation of proteins by molecular weight was performed using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), using two different sized sieves (a stacking gel and a resolving gel). The cells lysis was mixed with Laemmli discontinuous buffer system (in a ratio of 5:1) and 30 μL of this mixture was bought to the electrophoresis gel. The electrophoretic migration was performed at 140 mA. Afterwards, blotting consisted in the electrophoretic transfer of gel contents (by means of Mini Trans-Blot, Bio-Rad Laboratories) on a suitable nitrocellulose membrane (GE Healthcare Life Sciences), using a transfer sandwich with a modified electrode buffer (Towbin buffer – 25 mM Tris; 192 mM Glycine; 20% methanol; pH 8.3). The transfer was performed at 160 mA, for two hours. The transfer was verified by membrane Ponceau Red staining protocol for five minutes. Prior to antibody probing, membranes were incubated with a blocking buffer (which included BSA) for one hour. The nitrocellulose membranes were processed using a Western breeze kit; the incubation was performed first with the primary antibody, then the secondary antibody coupled with alkaline phosphatase was added. For chromogenic detection, we have used an alkaline phosphatase detection kit [which included 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitrotetrazolium blue chloride (NBT)] (Sigma-Aldrich, Germany).

Assessment of apoptosis by means of caspase-3/7 assay

The induction of apoptosis was evaluated by means of the caspase-3/7 assay using a CellEvent™ caspase-3/7 Green detection reagent (Invitrogen C10423). This reagent is a fluorogenic substrate for activated caspase-3/7 with absorption/emission maxima of 502–503 nm. The reagent consists of a four amino acid peptide (DEVD), which is conjugated to a nucleic acid binding dye. The conjugated dye is non-fluorescent until cleaved from the peptide and bound to deoxyribonucleic acid (DNA) [35]. Briefly, cells were seeded in 24-well plates at an initial density of 1×10^4 cells/cm^2. After 24 hours, the culture medium was replaced with the appropriate treatments (100 μg/mL, 300 μg/mL, 500 μg/mL, 700 μg/mL OE/RE extracts). After 24 hours, cells were washed with PBS and treated with 5 μL detection reagent for 30 minutes, at 37°C. Cells were observed by fluorescence microscopy using an Olympus IX71 fluorescence microscope and Cell F Imaging software for image capture and edit.

Statistical analysis

All analyses were carried out in triplicate. Results are presented as mean ± standard deviation (SD) and were statistically analyzed using GraphPad Prism 6 software (GraphPad Prism, USA), using Student's t-test. A value of p<0.05 was considered the threshold for a statistically significant difference.

Results

Assessment of cell morphology

Osteosarcoma MG-63 cells, without RE and with OE treatment (Figure 1a) showed a polygonal shape, which is considered as a normal cell growth phenomenon.

Regarding the influence of dry extracts upon MG-63

cells morphology and density, our results have not shown notable differences between untreated cells and cells treated with 100 µg/mL and 300 µg/mL OE. Osteoblasts still had their typical morphology, polygonal and elongated with lightly morphological changes (Figure 1, b and c). However, when cells were treated with concentrations of 500 µg/mL and 700 µg/mL OE, obvious morphological changes were observed. The cells rounded up and showed reduction in number and numerous vacuoles (Figure 1, d and e).

Regarding cells treated with RE, for 300 µg/mL, 500 µg/mL and 700 µg/mL concentrations, cells became slightly round, with weak interactions between cells (compared to the control – Figure 1a), probably due to cell death (Figure 2, a–c).

The changes that we have observed when treating osteosarcoma cells with OE (for 500 µg/mL and 700 µg/mL concentrations) and RE (for 300 µg/mL, 500 µg/mL, and 700 µg/mL concentrations) are typically seen in apoptosis, so we assume that analyzed dry extracts had induced apoptotic cell death.

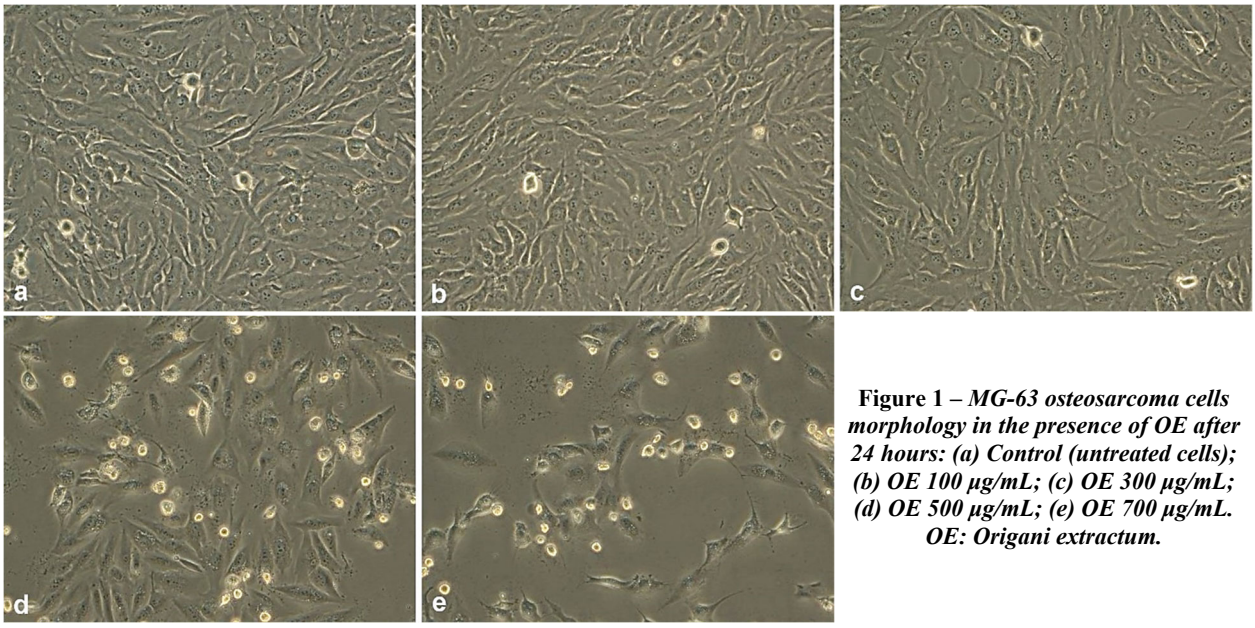


Figure 1 – ***MG-63 osteosarcoma cells morphology in the presence of OE after 24 hours: (a) Control (untreated cells); (b) OE 100 µg/mL; (c) OE 300 µg/mL; (d) OE 500 µg/mL; (e) OE 700 µg/mL. OE: Origani extractum.***

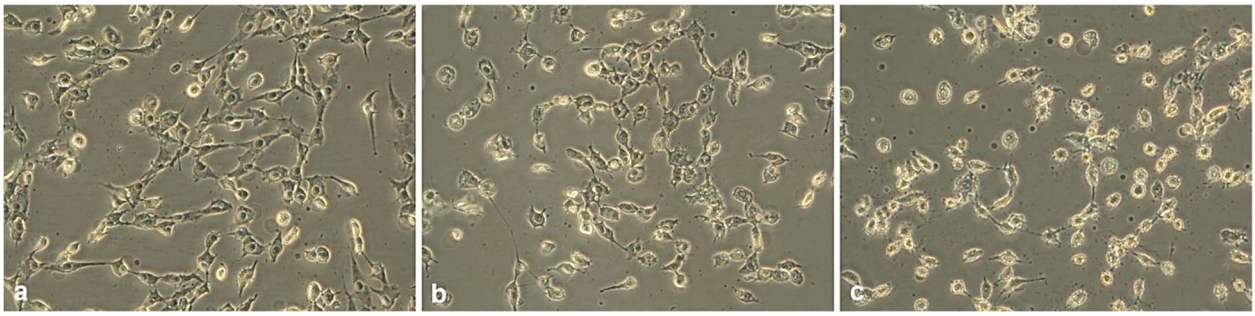


Figure 2 – ***MG-63 osteosarcoma cells morphology in the presence of RE after 24 hours: (a) RE 300 µg/mL; (b) RE 500 µg/mL; (c) RE 700 µg/mL. RE: Rosmarini extractum.***

LDH assay

To better characterize the effect of RE and OE, bone osteosarcoma cells were subjected to LDH cytotoxicity assay. LDH enzyme leakage from the cytosol of damaged cells into the medium, accompanied by an increase in absorbance is known to be an indicator of cell death, due to membrane damage.

Treatment of osteosarcoma cells with analyzed dry extracts showed a dose dependently increase in LDH release. As seen in Figure 3a, after 24-hour treatment of MG-63 cancer cells with 700 µg/mL OE, we have found a significant release (**p<0.01) of LDH in the culture medium compared to the control. However, for the other OE concentrations (100 µg/mL, 300 µg/mL, and 500 µg/mL) we have not found significant differences compared to untreated cells.

When osteosarcoma cells were treated with RE, a significant LDH release (***p<0.001) into the medium (compared to the control) was observed for concentrations beyond 300 µg/mL (Figure 3b).

Assessment of cell survival by means of Live & Dead assay

Microscopic observations performed using Calcein-AM and EthD-1 confirmed the cytotoxic effects of analyzed dry extracts at different concentrations. In bone osteosarcoma untreated cells, with active esterases, there is a visible strong cytosolic green fluorescence of Calcein (Figures 4a and 5a).

Our results have shown that cells viability treated with OE and RE dry extracts is dose dependent. As seen in Figure 4, we have not found a significant cytotoxic effect

when cells were treated with OE at concentrations of 100 µg/mL, 300 µg/mL, and 500 µg/mL (Figure 4, b–d). However, cells incubated for 24 hours with OE at 700 µg/mL concentration, had lower cytoplasmatic esterase activity, thus the decreased green fluorescence of Calcein was observed and early apoptotic cells appeared as red in color in the image (Figure 4e).

When cells were incubated with RE dry extract, a significant decrease in green fluorescence of Calcein was observed at concentrations beyond 300 µg/mL (Figure 5, c–e), which agrees with our previous results (LDH assay).

For both analyzed extracts, apoptotic cells displayed specific features such as cell shrinkage and nuclear condensation.

Assessment of cell proliferation (MTT assay)

The MTT assay was performed to determine the anti-proliferative effects of OE and RE dry extracts upon MG-63 bone osteosarcoma cell line. As shown in Figure 6a, the results of the MTT assay revealed that osteosarcoma cells are sensitive to OE and the effect was significant at 500 µg/mL (**p<0.01) and 700 µg/mL (***p<0.001) concentrations, as compared to the control group.

Regarding our MTT results, these are correlated with the Live & Dead assay, since after 24 hours incubation of cells with RE dry extract, a significant decrease in cells viability was observed for MG-63 cells treated with high concentrations (300–700 µg/mL) of the extract (Figure 6b).

PCNA assay (Western blotting)

To examine the molecular mechanisms and underlying changes in cell cycle patterns, we investigated the effects of analyzed dry extracts upon the expression of PCNA in MG-63 osteosarcoma cell line.

As shown in Figure 7, PCNA expression for osteoblasts treated with OE dry extract at all analyzed concentrations, was similar to that of untreated cells. Nevertheless, our results regarding cells treated with RE dry extract (for 500 µg/mL and 700 µg/mL concentrations) have shown a significant reduction of PCNA expression, most likely due to perturbation of cell cycle (Figure 7).

Assessment of apoptosis by means of caspase-3/7 assay

Regarding the influence of analyzed dry extracts upon caspase-3/7 activation, we have noticed a dose-dependent effect, starting at 300 µg/mL concentration for both extracts (Figure 8, a–d; Figure 9, a–d), although the activation was much pronounced for osteoblasts treated with RE (Figure 9, a–d).

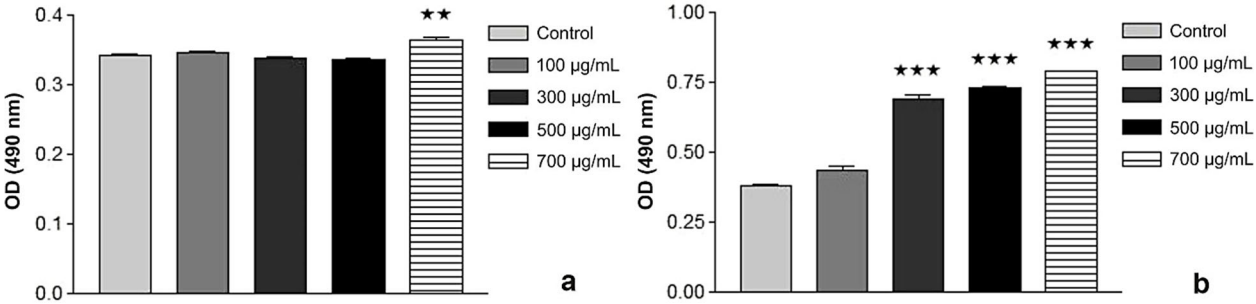


Figure 3 – ***Evaluation of dry extracts cytotoxicity by means of LDH assay: (a) OE; (b) RE. Results are mean ± SD (n=3): **p<0.01; ***p<0.001 (significant differences compared to control). LDH: Lactate dehydrogenase; OD: Optical density; OE: Origani extractum; RE: Rosmarini extractum; SD: Standard deviation.***

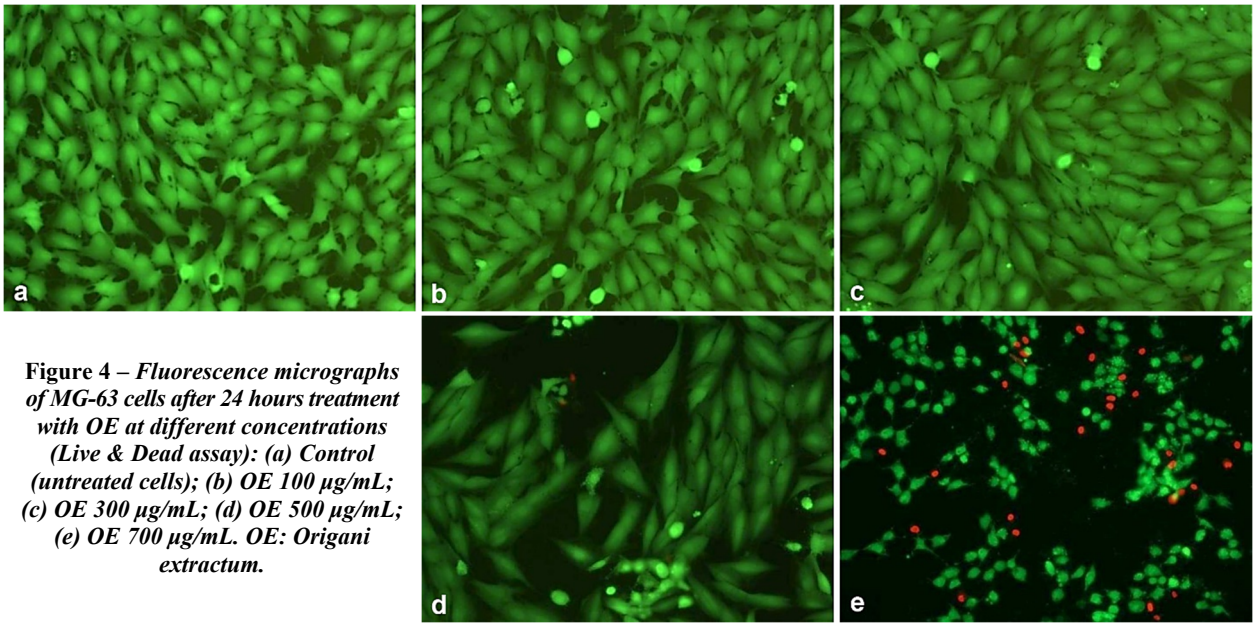


Figure 4 – ***Fluorescence micrographs of MG-63 cells after 24 hours treatment with OE at different concentrations (Live & Dead assay): (a) Control (untreated cells); (b) OE 100 µg/mL; (c) OE 300 µg/mL; (d) OE 500 µg/mL; (e) OE 700 µg/mL. OE: Origani extractum.***

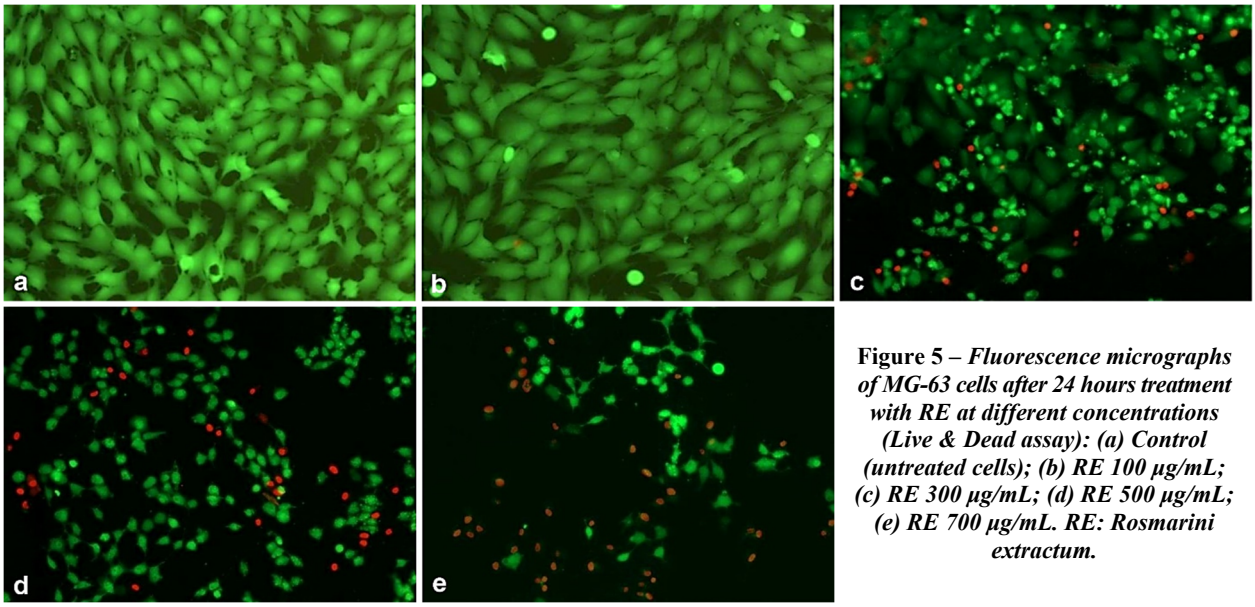


Figure 5 – ***Fluorescence micrographs of MG-63 cells after 24 hours treatment with RE at different concentrations (Live & Dead assay): (a) Control (untreated cells); (b) RE 100 µg/mL; (c) RE 300 µg/mL; (d) RE 500 µg/mL; (e) RE 700 µg/mL. RE: Rosmarini extractum.***

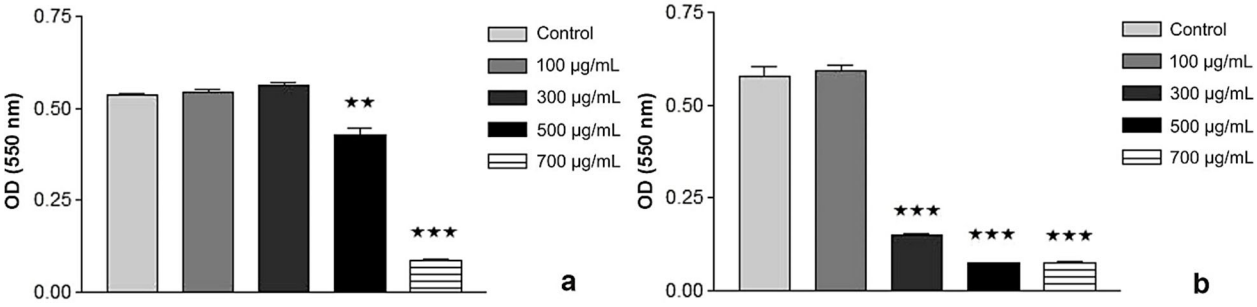
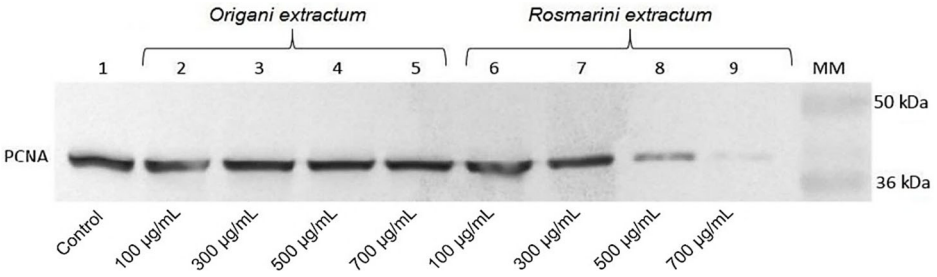


Figure 6 – ***Results of MTT assay after 24 hours incubation with MG-63 cells: (a) OE; (b) RE. Results are mean ± SD (n=3): **p<0.01; ***p<0.001 (significant differences compared to control). MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; OD: Optical density; OE: Origani extractum; RE: Rosmarini extractum; SD: Standard deviation.***

Figure 7 – ***PCNA expression for MG-63 cells treated with OE and RE dry extracts. MM: Molecular mass marker; OE: Origani extractum; PCNA: Proliferating cell nuclear antigen; RE: Rosmarini extractum.***



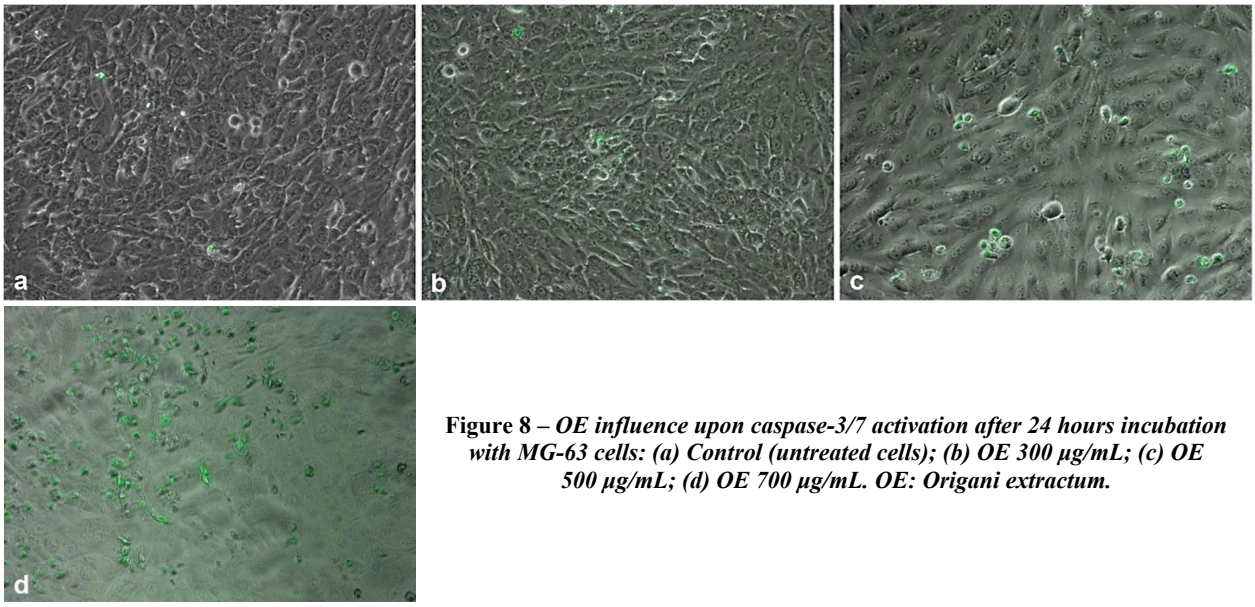


Figure 8 – ***OE influence upon caspase-3/7 activation after 24 hours incubation with MG-63 cells: (a) Control (untreated cells); (b) OE 300 µg/mL; (c) OE 500 µg/mL; (d) OE 700 µg/mL. OE: Origani extractum.***

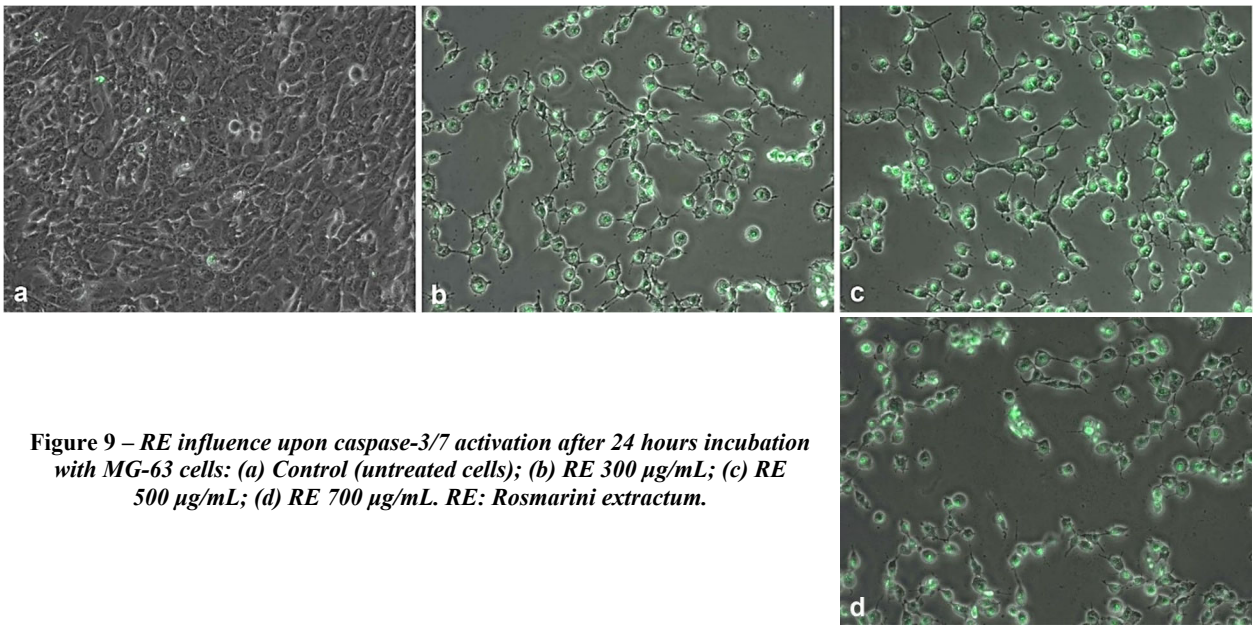


Figure 9 – ***RE influence upon caspase-3/7 activation after 24 hours incubation with MG-63 cells: (a) Control (untreated cells); (b) RE 300 µg/mL; (c) RE 500 µg/mL; (d) RE 700 µg/mL. RE: Rosmarini extractum.***

Discussions

Our results have shown cytotoxic effects and promising antitumor potential for both RE and OE on human osteosarcoma MG-63 cell line. The observed effects were dose-dependent and much pronounced for RE. According to our previous published results, both extracts are a rich source of phenolic compounds (flavones and phenolcarboxylic acids) [26, 27], so we assume that polyphenols are the main substances involved in the observed cytotoxic effects. OE contains 4.21 g% flavones, 52.75 g% phenolcarboxylic acids and 27.29 g% total phenolic content [27], while RE contains 3.22 g% flavones, 34.30 g% phenolcarboxylic acids and 31.86 g% total polyphenols [26]. By means of HPLC analysis, we have determined the content of rosmarinic acid (5.76 g% for OE and 3.29 g% for RE) [26, 27].

The role of phenolic compounds (flavones, phenolcarboxylic acids, ellagitannins, condensed tannins, curcumin, stilbenes, gingerols, etc.) in cancer prevention and therapy has become an area of great interest recently, since many studies revealed a positive correlation between the consumption of fruits and vegetables (rich in polyphenols) and a decrease in cancer's incidence [36, 37]. Mostly, *in vitro* and pre-clinical studies demonstrated that phenolic compounds influence all cancer cell hallmarks (cell morphology, resisting cell death, proliferative signaling, evading growth suppression, replicative immortality and metastasis) [37].

According to our results, both analyzed dry extracts had a significant influence upon osteosarcoma cells morphology (mainly RE at concentrations beyond 300 µg/mL). It is well known that cell morphology is an important factor of carcinogenesis and metastasis, since cell structure is heterogenous and changes due to different factors (stress, morbid state). Cancer cells usually have a lower stiffness due to disorganization of cytoskeletal structures and rearrangement of the microfilament system [38]. Moreover, according to Sapudom *et al.*, collagen fiber diameter represents a key factor for regulation of cluster formation and invasion. Besides a positive correlation was found between the invasive phenotype of cancer cells (leading to metastasis) and topological/mechanical features of cells [39]. Regarding osteosarcoma cells, Holenstein *et al.* have demonstrated a direct relationship between the metastatic potential and *in vitro* mechanical properties of osteosarcoma cells. Their results have shown a significant decrease in cell contractility and a higher stiffness with increasing metastatic potential [40].

We have obtained promising results regarding the cytotoxic effects of RE and OE upon osteosarcoma cells by means of MTT and LDH assays. MTT assay is a colorimetric method, widely used for evaluation of cell viability and *in vitro* cytotoxic effects of new drugs or plants/mushroom extracts [41]. Although MTT is one of the most popular assays involved in cell viability evaluation, it has several limitations (*i*) lack of sensitivity (compared to fluorescent methods), (*ii*) chemical interference (with plant extracts, polyphenols) and (*iii*) tetrazolium reduction reflects cell metabolism and not cell number [41].

Having in mind these limitations, we continued our research with other assays (LDH, Live & Dead) to confirm the antitumor potential of RE and OE. It is well known that LDH plays a key role in the Warburg effect and is also linked to tumor growth and invasion. According to previous reports LDHA is highly expressed in tumors while LDHB isoform is usually associated with non-malignant tissues. LDHA is upregulated in many types of cancer (breast and lung carcinomas, pancreatic cancer, oral squamous cell carcinoma) [42]. LDH down-regulation is associated with a decrease in cancer progression and also a reactivation of the mitochondrial function [43]. It is well known that LDH increase in cancer is a consequence of tissue destruction due to neoplastic growth; furthermore, LDH is a strong predictor of cancer survival among patients with severe types of cancer [43]. Our results are similar to other authors, that found a significant increase of LDH in osteosarcoma cell lines (MG-63, U2OS). LDH knockdown significantly inhibited migration and metastasis of bone cancer. According to Li *et al.*, LDH represents a prognostic marker for tumor recurrence [43].

Rosmarinic acid is one of the most important phenolic compounds identified and quantified by us in both extracts. Several mechanisms have been proposed for its antitumor

properties: (*i*) impaired tumor formation and development; (*ii*) reduced lipid peroxidation by products; (*iii*) increased apoptotic protein expression [B-cell lymphoma 2 (BCL-2) protein family]; (*iv*) cell cycle arrest through modulation of histone deacetylases (HDACs) expression; (*v*) modulation of different signal pathways or transcription factors involved in carcinogenesis [signal transducer and activator of transcription 3 (STAT3), c-Jun N-terminal kinase (JNK) pathway]; (*vi*) reduced TNF-α, cyclooxygenase-2 (COX-2) and IL-6/IL-8 levels involved in inflammation; (*vii*) modulation of p65 expression [44]; (*viii*) inhibition of certain protein kinases [microtubule affinity-regulating kinase 4 (MARK4)] [45]; (*ix*) suppression of epithelial to mesenchymal transition (EMT); and (*x*) up-regulation of miR-506 [46].

We also assume that other phenolic compounds (caffeic acid, chlorogenic acid and ferulic acid), which have been identified by others in Oregano aerial parts and Rosemary leaves [19, 23], play a major role in the observed cytotoxic effects. According to previous research, the above-mentioned compounds have shown antiproliferative effects towards breast, liver, and leukemia cells through activation of nuclear factor-kappa B (NF-κB) pathway, enhancement of oxidative DNA damage and apoptosis in the presence of other chemotherapeutic agents/radiation or through inhibition of angiogenesis [47]. According to Wang *et al.*, ferulic acid significantly descend osteosarcoma cells viability by promoting apoptosis (through activation of caspase-3 and apoptosis regulator – Bax proteins), induced cell cycle arrest and down-regulated the expression of cell-cycle related proteins [cyclin-dependent kinase (CDK)2, CDK4 and CDK6] [48]. According to Sapio *et al.*, chlorogenic acid inhibits cell viability, induces cell cycle arrest and apoptosis in different human osteosarcoma cell lines (U2OS, Saos-2 and MG-63) [49].

Several phenolic compounds, mainly flavones, which are found in Oregano and Rosemary aerial parts [19, 23] might also be responsible for the overall cytotoxic effects. Recent research revealed that quercetin induces apoptosis in breast cancer (MCF-7) cells *via* increase of Bax-2 expression and caspase activation and down-regulation of PI3K/Akt/mTOR/STAT3 pathway. Moreover, quercetin reduced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), which is required for tumor progression and maintenance [50]. Besides, luteolin has shown promising antitumor potential on different cancer cell lines (HepG2, MCF-7) through increased Bax/Bcl-2 ratio, release of reactive oxygen species (ROS) or inhibition of NF-κB pathway [50]. Furthermore, hesperidin exerts antitumor effects by attenuating mitochondrial potential, inducing DNA fragmentation and cytochrome c expression or over-expression of Fas (a death receptor on the surface of cells that leads to programmed cell death) [50].

Our results (MTT, LDH and Live & Dead assays) revealed a stronger cytotoxic effect for RE, although the flavones and phenolcarboxylic acids contents were lower compared to OE. So, we suppose that other compounds (which were not identified by us), mainly terpenic or volatile constituents are also involved in the overall effects. Carnosol, a diterpenic compound, was found to decrease cell viability of human osteosarcoma MG-63 cell line through induction of ROS production and autophagy [51]. According to Cheng *et al.*, rosmanol, another terpenic compound found in Rosemary leaves, have *in vitro* cytotoxic effects, on COLO 205 human colorectal adenocarcinoma cells, through promotion of apoptosis (increased expression of caspase-3 and caspase-9 and overexpression of Fas receptor) [52].

In addition, recent studies have demonstrated that major constituents of Rosemary and Oregano essential oils (carvacrol, 1,8-cineole, camphor or α-pinene) have shown cytotoxic effects by various mechanisms [53, 54].

Our results agree with other reports that confirmed the cytotoxic effect of Rosemary leaves extracts on different cancer cell lines [AGS – human gastric carcinoma, KYSE 30 – human esophageal squamous cell carcinoma, HT-29 – colon adenocarcinoma; (GBM) U87 MG – glioblastoma] [55–57]. Other authors reported cytotoxic activity of *O. vulgare* essential oil and its main constituents (carvacrol, thymol, limonene) on hepatocellular carcinoma cell line (HepG2) [20]. The anticancer activity of different Oregano extracts (ethanolic, hydroalcoholic, aqueous, apolar, etc.) on several cancer cell lines (LoVo – colorectal, CEM – T-cell acute lymphoblastic leukemia, MDA – human breast adeno-carcinoma, MCF-7 – breast cell line, A549 – lung cancer, etc.) was also confirmed [58].

To further investigate the possible mechanisms involved in OE and RE cytotoxicity upon MG-63 cells, we have studied the influence of analyzed extracts upon the PCNA expression and assessment of apoptosis by means of caspase-3/7 assay.

It is well known that PCNA is a protein that is involved in the metabolism of nucleic acid. PCNA is involved in DNA replication, DNA excision repair, cell cycle control, chromatin assembly and RNA transcription [59]. Its expression is correlated with tumor cell differentiation, tumor size and metastasis [59]. Besides, recent reports have demonstrated that PCNA is overexpressed in a wide range of tumors (gliomas, prostate, breast, or cervical cancer) [59]. Moreover, its concentration fluctuates during the cell cycle, with an increase during G1/S phase and a significant decrease at the beginning of G2 phase and in quiescent cells; the protein is not detected during the M phase. PCNA represents an important cell proliferation marker; increased PCNA expression being involved in cancer progression and poor prognosis [59, 60]. Regarding our extracts, a significant reduction in PCNA expression (compared to control cells) was only seen for cells treated with high concentrations of RE dry extract (500 μg/mL and 700 μg/mL). So, we assume that cell cycle arrest (probably induced by rosmarinic acid, chlorogenic acid and ferulic acid) [44, 48] is one of the mechanisms involved in RE cytotoxic properties. Instead for OR extract, most likely other mechanisms are involved, probably apoptosis, modulation of different signal pathways or DNA fragmentation [50].

Our results regarding the evaluation of apoptosis by means of caspase-3/7 assay have clearly demonstrated that both extracts induce dose-dependent apoptotic effects. Caspase protease family is a key regulator of apoptosis, which leads to DNA fragmentation, nuclear condensation, and membrane blebbing [36]. The mammalian caspases are divided into three groups according to their role: executioner caspases (caspase-3 and caspase-7), initiator caspases (caspase-2, -8, - 9, -10) and inflammatory caspases (caspase-1, -4, - 5, - 11, - 12) [36]. Caspase-3 and caspase-7 are of great importance for cancer therapy,

due to their involvement in cleaving different regulatory proteins that silent the functions of the cell and are involved in apoptosis. In addition to inducing apoptosis, caspase-3 activation is involved in cleaving other substrates [Rat sarcoma (Ras) guanosine triphosphatase (GTP-ase)], which in turn sensitizes tumor cells to apoptosis [36].

Polyphenols are unique molecules which can regulate cell apoptosis, which represents an important aspect in cancer treatment. Several preclinical and clinical studies have shown that phenolic compounds (quercetin, ferulic acid, chlorogenic acid, etc.) are involved in all apoptosis mechanisms: (*i*) the intrinsic pathway (that starts within the cell and is mediated by Bax proteins, release of cytochrome c which further activates caspase-3 and caspase-9), (*ii*) the extrinsic pathway (that involves binding of death ligands to transmembrane death receptors with further caspase-8 activation), and (*iii*) the perforin–granzyme pathway [50, 61].

Although our results regarding the potential antitumor effects of RE and OE are encouraging, another point to be considered is that *in vitro* studies do not consider the metabolic transformations of phenolic compounds and their physiological concentrations. On the other hand, polyphenols bioavailability is influenced by their chemical structure, interaction with food components, gastrointestinal absorption, first pass metabolism, enterohepatic circulation, or interaction with gut microbiota [62, 63]. Furthermore, the biological activities of some polyphenols may be also mediated by their metabolites, which are only produced *in vivo* [64].

Conclusions

Overall, analyzed dry extracts from indigenous Oregano aerial parts and Rosemary leaves have shown cytotoxic effects and promising antitumor potential upon MG-63 human osteosarcoma cell line. The cytotoxic effect was dose dependent. OE has shown cytotoxic effects at the highest concentration (700 μg/mL), while for RE this effect was seen starting with 300 μg/mL concentration. We assume that the observed effects are the consequence of apoptosis (both extracts were involved in caspase-3/7 activation) and decreased expression of PCNA (only for RE dry extract). Further preclinical studies are needed to confirm the antitumor effects and to go deeply in the molecular mechanisms involved.

Conflict of interests

The authors declare no conflict of interests.

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