

Oregano demonstrates distinct tumour-suppressive effects in the breast carcinoma model

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Abstract

Purpose There has been a considerable interest in the identification of natural plant foods for developing effective agents against cancer. Thus, the anti-tumour effects of oregano in the in vivo and in vitro breast cancer model were evaluated.

Methods Lyophilized oregano (ORE) was administered at two concentrations of 0.3 and 3 % through diet. The experiment was terminated 14 weeks after carcinogen administration. At autopsy, mammary tumours were removed and prepared for histopathological and immunohistochemical analysis. Moreover, in vitro evaluation in MCF-7 cells was carried out.

Results Low-dose ORE suppressed tumour frequency by 55.5 %, tumour incidence by 44 %, and tumour volume by 44.5 % compared to control animals. Analysis of rat tumour cells showed Ki67, VEGFR-2, CD24, and EpCAM expression decrease and caspase-3 expression increase after low-dose ORE treatment. High-dose ORE lengthened tumour latency by 12.5 days; moreover, Bcl-2, VEGFR-2, CD24, and EpCAM expression decrease and caspase-3 expression increase in carcinoma cells were observed. Histopathological analysis revealed a decrease in the ratio of high-/low-grade carcinomas in both treated groups. In vitro studies showed that ORE decreased survival and proliferation of MCF-7 cells. In ORE-treated MCF-7 cells, an increase in cells expressing sub- G_0/G_1 DNA content and an

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increase in the percentage of annexin V/PI positive MCF-7 cells were observed. In vitro, both caspase-dependent and possible non-caspase-dependent apoptotic pathways were found. The deactivation of anti-apoptotic activity of Bcl-2, a decrease in mitochondrial membrane potential, and the activation of mitochondrial apoptosis pathway were observed in the ORE-treated MCF-7 cells.

Conclusions Our results demonstrate, for the first time, a distinct tumour-suppressive effect of oregano in the breast cancer model.

Keywords Mammary carcinogenesis · Rat · Oregano · Cancer stem cells · Angiogenesis · Apoptosis · Cell proliferation · MCF-7 cells

Abbreviations

AIF	Apoptosis-inducing factor
ALDH	Aldehyde dehydrogenase
BrdU	5-Bromo-20-deoxyuridine
CSCs	Cancer stem cells
FCM	Flow cytometry analysis
HDL	High-density lipoprotein
HG	High grade
EpCAM	Epithelial cellular adhesion molecule
LDL	Low-density lipoprotein
LG	Low grade
LOQ	Limit of quantification
MCF-7	Human adenocarcinoma cell line, oestrogen receptor positive
NMU	<i>N</i> -methyl- <i>N</i> -nitrosourea
ORE 0.3/ORE 3	Experimental group with dietary administered oregano in a concentration of 0.3 and 3 %
ORE	Oregano
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VLDL	Very low-density lipoprotein

Introduction

Phytochemicals with possible therapeutic potential were identified in the early 1990s [1]. Considering that evidence from preclinical, epidemiological, and clinical trial data indicates that a plant-based diet can reduce the risk of chronic disease, there are plethora studies demonstrating that flavonoids, terpenoids, and other phytochemicals display anti-tumour and several other biological effects, e.g. anti-inflammatory, neuro-protective, anti-allergic, and anti-aggregatory effects [2]. The additive and synergistic effects of many phytochemicals present in whole plant-derived

foods could be responsible for their potent pleiotropic effects.

Epidemiologic studies revealed that a diet enriched with phenolic compounds (mainly flavonoids) and terpenoids is associated with a decreased risk of breast cancer [3, 4]. The protective attributes of phenolic compounds are due to the alteration of numerous cell signalling pathways involved in oncogenesis such as cell cycle [5], apoptosis [6], or angiogenesis [7]. Oregano (*Origanum vulgare* L., *Lamiaceae*) is an important Mediterranean herb rich in phenolic compounds and monoterpenoids with high antioxidant capacity and antimicrobial activity [8]. There are also data reporting the anti-mutagenic and anti-carcinogenic effects of oregano, thus representing an alternative for the potential prevention and/or treatment of cancer. As well, dose-dependent antioxidant effects of *O. vulgare* in intestinal cells in a chemically induced rat colon carcinogenesis have been reported [9]. The effects of *O. vulgare* on cancer prevention have been reported in other experimental studies [10–12].

Cancer stem cells (CSCs) represent small but aggressive population of cells within the tumour mass. They may be responsible for the resistance in main cancer treatment modalities—chemotherapy, radiotherapy, and immunotherapy [13–15]. Strategies aimed at efficient targeting of CSCs are becoming important for monitoring the progress of cancer therapy and for evaluating new therapeutic approaches. It has been demonstrated that the application of phytochemicals is able to interfere with a plethora of signalling systems in CSCs, e.g. with those that sustain their self-renewal capacity or proliferation [16]. Markers used to identify and characterize these cells in breast cancer phenotype are ALDH1, CD24, and CD44 [17]. ALDH1 (aldehyde dehydrogenase) is a detoxifying enzyme which has been shown to identify and promote breast cancer stem cells [18]. CD24 is a small cell surface protein molecule anchored by glycosyl-phosphatidyl-inositol and has been reported to be a promising prognostic indicator in breast cancer [19]. CD44, a multifunctional class I transmembrane glycoprotein, has been implicated in cell migration and metastases [20]. In addition, CD326—epithelial cell adhesion molecule (EpCAM)—has been identified as an additional marker of cancer-initiating cells; it is frequently expressed in breast cancer, and its expression has been associated with poor prognosis [21]. Based on recent research data, there is a general hypothesis that plant functional foods (e.g. oregano) with a mixture of different phytochemicals may be a promising tool to target CSCs population in various cancer types [22].

As the anti-tumour activity of natural plant substances is believed to be from the combination of phytochemicals present in whole foods, rather than an isolated agent [1], the objective of our study was to assess the anti-tumour effects of the mixture of phytochemicals present in oregano

in a model of chemically induced rat mammary carcinogenesis. The effect of oregano was analysed by studying oncogenesis (markers of apoptosis, proliferation, angiogenesis, and cancer stem cells) in rat mammary tumours, as well as the histopathological analysis of carcinomas and other pathophysiological effects. To understand the mechanism of action, an *in vitro* study using MCF-7 cells, analysing cytotoxicity data, parameters of apoptosis, and proliferation after oregano treatment was performed.

Materials and methods

The experiment was approved by the Ethical Commission of the Jessenius Faculty of Medicine of Comenius University (Protocol No. EK1125/2012) and by the State Veterinary and Food Administration of the Slovak Republic (Accreditation No. Ro-1759/11-221).

Animals and induction of mammary carcinogenesis, design of experiment

Sprague–Dawley female rats (Charles River Laboratories, Sulzfeld, Germany) aged 31–35 days were acclimatized to standard vivarium conditions with temperature 23 ± 2 °C, relative humidity 40–60 %, artificial regimen (L/D 12:12 h). During the experiment, the animals were fed the Ssniff® R-Z low-phytoestrogen V1354-0 diet (Soest, Germany) and drinking water *ad libitum*. Mammary carcinogenesis was induced by *N*-nitroso-*N*-methylurea (NMU, Sigma, Deisenhofen, Germany) administered intraperitoneally (single dose of 50 mg/kg body weight on average on the 42nd postnatal day). This model mimics high-risk premenopausal women [23].

Chemoprevention with oregano (*O. vulgare* L.) (Calendula, Nová Lubovňa, Slovak Republic; country/region of origin—Slovak Republic/Prešov) began 1 week before carcinogen administration and lasted until 14 weeks after NMU administration. Oregano (hulm, cut into 2-mm particles) was administered in the diet at two concentrations of 3 g/kg (0.3 %) and 30 g/kg (3 %). Animals ($n = 25$ per group) were randomly assigned into three experimental groups: (1) control group without chemoprevention; (2) chemoprevention with oregano at a concentration of 0.3 % (ORE 0.3); and (3) chemoprevention with oregano at a concentration of 3 % (ORE 3). The animals were weighed and palpated weekly in order to register the presence, number, location, and size of each palpable tumour. Food intake (per cage) during 24 h was monitored in the 7th and 13th week of the experiment (the value obtained was divided by the number of animals in the cage to determine the average food intake per animal in the relevant cage). The measurements were taken four times (twice in each week).

The oregano doses per animal and day were calculated in accordance with the amount of chow consumed. In the last (14th) week of the experiment, the animals were quickly decapitated, the blood from each animal was collected, mammary tumours were excised, and the tumour size was recorded. Macroscopic changes in selected organs (liver, spleen, kidney, stomach, intestine, and lung) were evaluated at autopsy.

Histopathological and immunohistochemical analysis of rat tumours

A tissue sample of each mammary tumour was routinely formalin fixed and paraffin embedded. The tumours were classified according to the criteria for the classification of rat mammary tumours [24]. The additional parameter—grade of invasive carcinomas—was used. Tumour samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization (solidization, cell atypia, mitotic activity index, and necrosis) were chosen according to the standard diagnostic method of classification. HG carcinomas were considered to be tumours with ≥ 2 positive criteria; LG carcinomas were tumours with ≤ 1 positive criterion [25, 26]. Serum metabolic parameters were evaluated using an Olympus AU640 (Olympus Optical, Tokyo, Japan) automatic biochemical analyser.

The paraffin block with the most representative tumour area of each mammary tumour was chosen for immunohistochemical analysis. The detection of selected proteins was carried out by indirect immunohistochemical method on whole paraffin sections, utilizing commercially available rat-specific antibodies (Santa Cruz Biotechnology, Paso Robles, CA, USA; Dako, Glostrup, Denmark; Bioss, Woburn, MA, USA; GeneTex, Irvine, CA, USA; Abcam, Cambridge, MA, USA; Boster Biological Technology, Pleasanton, CA, USA; Thermo Fisher Scientific, Rockford, IL, USA). All steps of the immunohistochemical staining were managed according to manufacturers' recommendations. After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3 % H_2O_2 in methanol for 30 min. Antigen retrieval was performed by a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the primary antibody in PBS containing 1 % BSA, for 60 min at room temperature. The primary antibodies were visualized by a secondary staining system (EnVision, Dual Link System-HRP, Cat. No. K060911, Dako North America, Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride (DAB) as a substrate. The sections were counterstained with haematoxylin, dehydrated and mounted in Canadian balsam. Negative controls included omission of primary antibody. Immunohistochemically detected antigen expression was evaluated by precise morphometric method. Sections were screened,

and digital images at magnifications of 400 \times were taken with an Olympus Evolt E-420 mounted on an Olympus BX41N microscope. Expression of VEGF, caspase-3, and ALDH1 was analysed in the cytoplasm of tumour cells. Bax and Bcl-2 are membrane-associated onco-proteins. Ki67 was detected within the nucleus. Receptors for VEGF, CD24, CD44, and EpCAM were observed in the cell membrane. Expression of proteins was quantified as the average percentage of antigen positive area in standard fields (0.5655 mm²) of tumour hot-spot areas [27]. Morphometric analysis of the digital images was performed using Quick-PHOTO MICRO software, version 3.0 (Promicra, Prague, Czech Republic). The antigen positive area was evaluated by phase analysis with standard thresholds for weak, mild, and strong intensities of immunoreactivity. The values of protein expression were compared between treated (ORE 0.3 and ORE 3) and non-treated (control) tumour cells of female rats; at least 60 images for one protein were analysed (in total 600 images for ten proteins).

Cell culture and experimental design

The human cancer cell line MCF-7 (human breast adenocarcinoma, ECACC, Porton Down, Salisbury, UK) was cultured in Dulbecco's modified Eagle's medium with glutamax-I and sodium pyruvate (GE Healthcare, Piscataway, NJ, USA) supplemented with a 10 % foetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μ g/ml) (all Invitrogen, Carlsbad, CA, USA) in an atmosphere containing 5 % CO₂ in humidified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95 % before each experiment.

MCF-7 cells (3 \times 10⁵) were seeded in Petri dishes and cultivated 24 h in a complete medium with 10 % FCS. Cells were treated with oregano extract for 24, 48, and 72 h prior to analysis. Oregano extract (Calendula, Nová Lubovňa, Slovak Republic) was prepared from haulm powder (*O. vulgare*) diluted in cultivation medium.

Cytotoxicity assay

The cytotoxic effects of oregano extract were determined using colorimetric microculture MTT assay [28]. 1 \times 10⁴ cells were plated per well in 96-well polystyrene microplates (Sarstedt, Nümbrecht, Germany) in the culture medium containing extract at final concentrations of 10–1000 μ g/ml. After 72 h of incubation, 10 μ l of MTT (5 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After an additional 4 h, during which insoluble formazan was produced, 100 μ l of 10 % sodium dodecyl sulphate was added to each well and another 12 h was allowed for the formazan to dissolve. The absorbance was measured at 540 nm using the automated uQuant™

Universal Microplate Spectrophotometer (Biotek, Winooski, VT, USA). The blank-corrected absorbance of the control wells was taken as 100 %, and the results were expressed as a percentage of the control. All experiments were performed in triplicate. For following analyses, final concentration 480 μ g/ml was used.

5-Bromo-20-deoxyuridine (BrdU) cell proliferation assay

Cell proliferation activity was directly monitored by quantification of BrdU incorporated into the genomic DNA during cell growth. DNA synthesis was assessed using colorimetric cell proliferation ELISA assay (Roche Diagnostics GmbH, Mannheim, Germany) following the vendor's protocol. Briefly, 1 \times 10⁴ cells/well in 80 μ l medium were plated in a 96-well polystyrene microplate (Sarstedt, Nümbrecht, Germany). Twenty-four hours after cell seeding different concentrations (10–1000 μ g/ml) of the extract were added. After 48 h of treatment, cells were incubated with BrdU labelling solution (10 μ M final concentration) for another 24 h at 37 °C followed by fixation and incubation with anti-BrdU peroxidase conjugate for an additional 1.5 h at room temperature. Finally, after substrate reaction, the stop solution was added (25 μ l 1 M H₂SO₄) and colour intensity was measured with multi-well microplate ELISA reader at 450 nm (reference wavelength: 690 nm). The results were expressed as a percentage of the control. All experiments were performed in triplicate.

Analysis of cell cycle

For flow cytometric analysis (FCM) of the cell cycle, floating and adherent cells were harvested together 24, 48, and 72 h after treatment (final concentration 480 μ g/ml), washed in cold PBS, fixed in cold 70 % ethanol, and kept at –20 °C overnight. Prior to analysis, cells were washed twice in PBS, resuspended in staining solution (final concentration 0.1 % Triton X-100, 0.5 mg/ml ribonuclease A and 0.025 mg/ml propidium iodide—PI), incubated in the dark at RT for 30 min, and analysed using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Cell death and phosphatidylserine externalization analysis

The Annexin V-FITC/PI double-staining method was used to analyse cell death phosphatidylserine externalization according to the manufacturer's instructions. Adherent and floating cells (1 \times 10⁵) were harvested together 24, 48, and 72 h after treatment and stained with Annexin V-FITC (BD Biosciences Pharmingen, San Diego, CA, USA) in binding buffer for 15 min, washed, stained with Propidium Iodide

for 5 min, and thereafter analysed using a BD FACSCalibur flow cytometer.

Detection of mitochondrial membrane depolarization

The changes in MMP were analysed with FCM using tetramethylrhodamine ethyl ester per chlorate (TMRE, Molecular Probes, Eugene, OR, USA). The cells were washed with PBS, resuspended in 0.1 μM of TMRE in PBS, and incubated for 30 min at RT in the dark. The cells were then washed twice with PBS, resuspended in 500 μM of the total volume, and analysed (1×10^4 cell per sample). Fluorescence was detected with 585/42 (FL-2) optical filter.

Detection of active caspase 7

The changes in caspase-7 activation were analysed with FCM using cleaved caspase-7 rabbit mAb (Cell Signaling, Danvers, MA, USA). The adherent and floating cells were harvested together 24, 48, and 72 h after treatment and stained with primary antibody and incubated for 30 min at RT. The cells were then washed once with PBS and stained with goat anti-rabbit IgG secondary antibody DyLight 488 conjugate (Thermo Scientific, Rockford, IL, USA) and incubated for 30 min at RT in the dark. The cells were then washed with PBS, resuspended in 500 μM of the total volume, and analysed (1×10^4 cell per sample). Fluorescence was detected with 530/30 BP (FL-1) optical filter.

Measurement of mitochondrial proteins dynamics (efflux, activation/deactivation)

Adherent and floating cells (5×10^5) were washed once with PBS, and thereafter samples were stained with rabbit anti-Bcl-2 polyclonal Ab FITC conjugate 1:200 (Bioss, Woburn, MA, USA) or phospho-Bcl-2 rabbit mAb Alexa Fluor 488 conjugate 1:200 (Cell Signaling) or mouse anti-AIF polyclonal Ab FITC conjugate 1:200 (Bioss) for 30 min. Additionally, the fluorescence was measured using BD FACSCalibur (FL1). Results were quantified as the ratio of the fluorescence median intensity in experimental groups compared to the particular controls.

The examinations of secondary metabolites in oregano ethanol extract

Examined oregano ethanol extract (hauhm, Calendula, Nová Lúbovňa, Slovak Republic) consisted of 44.65 mg of extractable components per millilitre. The LC–MS analyses were performed on an Agilent 1260 Infinity LC System (Agilent Technologies, Germany), equipped with a binary pump, an autosampler, a column thermostat, and a diode array detector (DAD), coupled to a

quadrupole–time-of-flight (6520 Accurate-Mass QTOF) instrument equipped with an orthogonal ESI source (Agilent Technologies, Germany). HPLC separation of oregano extract was carried out on a Kromasil C₁₈ column (4.6×150 mm, 5 μm) at 35 °C and a flow rate of 0.4 ml/min. Water (pH 3.1 with HCOOH/NH₄HCO₂) and MeCN were used as mobile phase A and B, respectively. The following gradient program was used: 10 % B (20 min), 20 % B (25 min), 60 % (50 min), 95 % (62 min), and 10 % (70 min). The ESI ion source parameters were as follows: capillary voltage: 3.5 kV, nebulizer: 40 psi (N₂), dry gas flow: 10 l/min (N₂), and dry temperature: 300 °C. The mass spectrometer was operated in an autoMS² mode where each negative ion MS scan (m/z 100–3000, average of 4 spectra) was followed by MS² scans (m/z 100–3000, average of four spectra, isolation window of 4 amu, collision energy 20 eV) of the two most intense precursor ions. Ions were excluded from analyses for 0.5 min after two MS² spectra had been acquired. Nitrogen was used as collision gas. Phenolic compounds were identified by comparing their UV and mass spectra with the literature and authentic standards when available and by measuring accurate m/z [29].

The quantitative determination of phenolic compounds in oregano extract was provided by the method of external standards. We used luteolin-7-*O*-glucoside for determination of luteolin and eriodictyol derivative, apigenin for apigenin derivatives, protocatechuic aldehyde for protocatechuic derivatives, rosmarinic acid for determination of rosmarinic acid, caffeic acid for caffeic acid, p-hydroxybenzoic acid for p-hydroxybenzoic acid. For the quantification of coumaric acid derivative, we used coumaric acid (see Table 5). Chromatographic standards were purchased from Sigma-Aldrich, Germany.

Statistical analyses

In in vivo study, data are expressed as means \pm SD or mean \pm SEM. The Mann–Whitney test, Kruskal–Wallis test, Student's *t* test, and one-way analysis of variance (ANOVA) were the statistical methods used in data evaluation. Tumour volume was calculated according to the formula: $V = \pi \times (S_1)^2 \times S_2/12$ (S_1 , S_2 are tumour diameters; $S_1 < S_2$). In fluorescence assay, ANOVA was first carried out to test for the differences between groups; comparisons between individual groups were made using a Student–Newman–Keuls multiple comparisons test. In in vitro study, data are expressed as means \pm SD. Data were analysed using ANOVA followed by the Bonferroni multiple comparisons test. Differences were considered significant when $P < 0.05$. The examinations of secondary metabolites in oregano ethanol extract were performed in triplicate. The quantitative results were calculated from calibration curves,

Table 1 Effects of oregano in *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in female Sprague–Dawley rats at the end of experiment

Group	CONT	ORE 0.3	ORE 3
Tumour-bearing animals/all animals	18/25	10/25	18/25
Tumour frequency per group*	2.96 ± 0.70	1.32 ± 0.50 ^{a,b} (−55.5 %)	2.36 ± 0.41 (−20 %)
Tumour incidence (%)	72.0	40.0 ^c (−44 %)	72.0
Tumour latency* (days)	65.33 ± 4.08	75.60 ± 5.60 (+10 days)	77.78 ± 3.15 ^c (+12.5 days)
Average tumour volume* (cm ³)	0.81 ± 0.12	0.45 ± 0.16 ^d (−44.5 %)	0.73 ± 0.17 (−10 %)
Cumulative tumour volume** (cm ³)	60.90	15.41 (−74.5 %)	42.59 (−30 %)

CONT, control group; ORE 0.3, group with administered oregano at a concentration of 3 g/kg in diet; ORE 3, group with administered oregano at a concentration of 30 g/kg in diet

* Data are expressed as means ± SEM; ** Data are expressed as a sum of volumes per group. Values in brackets are calculated as %ual deviation from the 100 % of non-influenced control group (with exception of latency). Significantly different, ^a $P < 0.02$ versus CONT, ^b $P < 0.02$ versus ORE 3, ^c $P < 0.03$ versus CONT, ^d $P < 0.05$ versus CONT

expressed as mean values and standard deviation (SD). Data analyses were conducted using GraphPad Prism, version 5.01 (GraphPad Software, La Jolla, CA, USA).

Results

Rat mammary carcinogenesis

ORE significantly inhibited mammary carcinogenesis in female rats (Table 1). In the ORE 0.3 group, an apparent decrease in tumour frequency (the most sensitive parameter of rat breast cancer model) by 55.5 % ($P = 0.016$), decrease in incidence by 44 % ($P = 0.027$), and average tumour volume by 44.5 % ($P = 0.041$) was found in comparison to control animals. The ORE 3 group showed significant lengthening of tumour latency by 12.5 days ($P = 0.021$) and a decrease in tumour frequency by 20 % ($P > 0.05$) compared to controls.

Pathology of rat tumours

The most frequently occurring lesions in all experimental groups were mixed papillary/cribriform and cribriform/papillary carcinomas, and cribriform carcinomas (Table 2). A significant decrease in the rate of poorly differentiated (high grade, HG) and well-differentiated (low grade, LG) mammary carcinomas in both treated groups was found (Table 2). A significant positive correlations between histological grade and tumour volume in low-dose group ($r = 0.75$; $P = 0.031$) and high-dose group ($r = 0.72$; $P = 0.032$) were observed.

Immunohistochemical evaluation of rat tumours

As seen in Fig. 1, immunohistochemical analysis showed significant Ki67 expression decrease by 44 % ($P = 0.0001$),

Table 2 Histopathological classification of mammary tumours and grading of carcinomas

Mammary tumours	CONT	ORE 0.3	ORE 3
<i>Malignant lesions</i>			
P,C	24	15	26
C,P	21	12	17
C	13	1	10
C,CO	7	2	1
P	2	–	3
P,C,CO	2	–	–
C,P,CO	2	–	–
P,CO,C	1	–	–
C,CO,P	1	–	–
CA-SA	–	1	–
<i>Precancerous lesion</i>			
IDP	1	2	2
Total number	74	33	59
HG/LG carcinoma	28/45	5/26*	11/46*

Dominant type in mixed tumours is the first in order

Type invasive carcinoma (C cribriform, P papillary, CO comedo), IDP intraductal proliferation, CA-SA carcinosarcoma, HG high grade, LG low grade

Significant different, * $P < 0.05$ versus CONT

VEGFR-2 expression decrease by 20 % ($P = 0.022$), and caspase-3 expression increase by 18 % ($P = 0.043$) in rat mammary carcinoma cells after low-dose oregano treatment. In the high-dose ORE group, Bcl-2 expression decreased by 30.5 % ($P = 0.0016$), VEGFR-2 expression decreased by 26.5 % ($P = 0.002$), and caspase-3 expression increased by 42 % ($P < 0.001$) versus control. No changes in Bax and VEGF expressions in treated carcinoma cells were observed compared to the control cells; however, a trend to increase Bax expression in both treated groups was seen (Fig. 1).

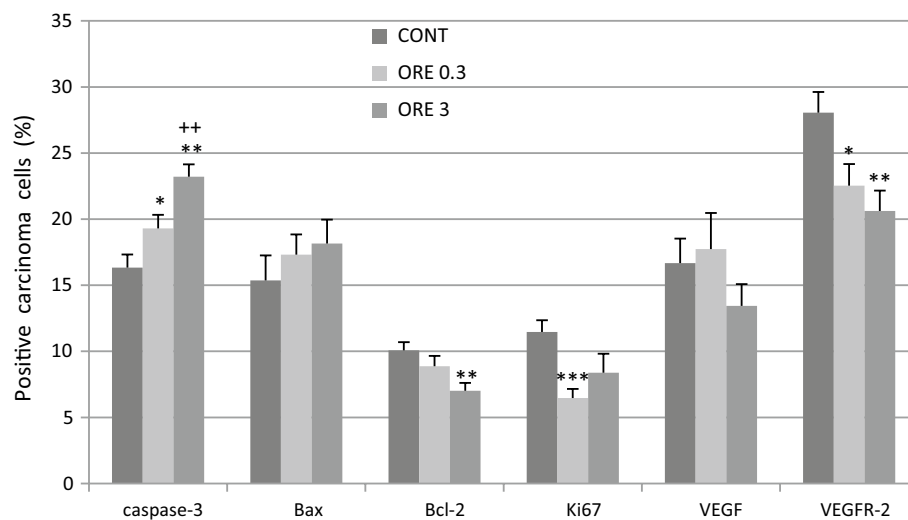


Fig. 1 Immunohistochemical evaluation of caspase-3, Bax, Bcl-2, Ki67, VEGF, and VEGFR-2 expression in rat mammary carcinoma cells after the treatment with oregano in two doses. Data are expressed as mean \pm SEM. Significantly different, * P < 0.05; ** P < 0.01; *** P < 0.001 versus CONT, ++ P < 0.01 versus ORE 0.3. Figure shows the expression of proteins quantified as the average per-

centage of antigen positive area in standard fields (0.5655 mm²) of tumour hot-spot areas. The values of protein expression were compared between treated (ORE 0.3, ORE 3) and non-treated (control) carcinoma cells of rat females; at least 60 images for one protein were analysed

The analysis of cancer stem cells parameters showed significant dose-dependent decrease in the CD24 expression by 34 % (P = 0.047) and 57 % (P < 0.001) and significant dose-independent decrease in EpCAM expression by 14 % (P = 0.002) and 10 % (P = 0.012) when compared to control carcinoma cells. The treatment with oregano did not affect CD44 and ALDH1A1 expression (Fig. 2). Representative images of caspase-3, Bax, Bcl-2, Ki67, VEGF, VEGFR-2, CD24, CD44, ALDH1A1, and EpCAM expressions in rat mammary carcinoma cells are shown in Fig. 3.

In vitro analysis in MCF-7 cells

The anti-proliferative effect of oregano extract on MCF-7 cell line was evaluated by both MTT and BrdU incorporation assays. As result showed, oregano extract decreased metabolic activity followed by significantly decreased cell survival by a dose- and time-dependent manner (Fig. 4)

Flow cytometric analysis of MCF-7 cells cell cycle progression (Table 3) after oregano extract treatment was evaluated after 24, 48, and 72 h. Significant occurrence of cells in sub- G_0/G_1 fraction (24, 48, 72 h) and consequently decrease in G_0/G_1 (24, 48, 72 h) and S (72 h) populations was observed after oregano extract treatment.

Increased population of MCF-7 cells in sub- G_0/G_1 fraction suggests induction of programmed cell death. Analysis of phosphatidyl serine externalization by annexin V/PI flow cytometry methods (Table 4) showed significant increase

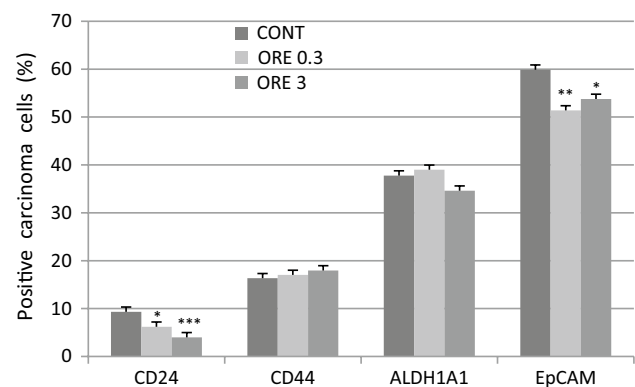
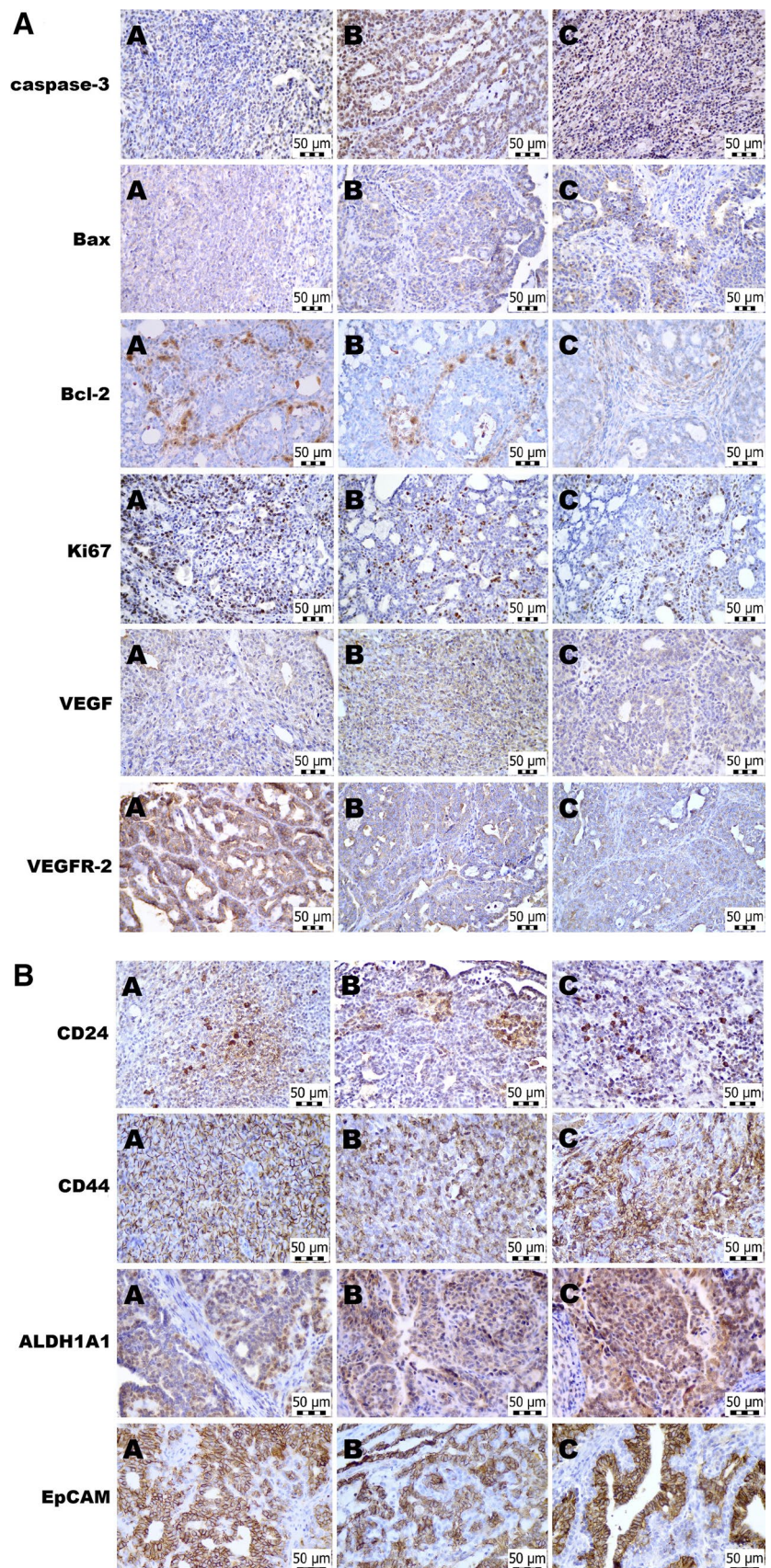


Fig. 2 Immunoexpression of cancer stem cell markers in rat breast carcinoma cells after the treatment with oregano. Data are expressed as means \pm SEM. Significantly different, * P < 0.05; ** P < 0.01; *** P < 0.001 versus CONT. The values of protein expression were compared between treated (ORE 0.3, ORE 3) and non-treated (control) carcinoma cells of rat females; at least 60 images for one protein were analysed

in annexin V-positive MCF-7 cells after 24 h. After 72 h, 49.9 % of oregano extract-treated cells showed annexin V positivity compared with untreated control.

Analysis of caspase-7 activation (Fig. 5a) after 48 and 72 h clearly demonstrated caspase-dependent form of cell death in MCF-7 cells. Otherwise, AIF analysis (Fig. 6a) after 48 h oregano extract treatment showed possible contribution of parallel non-caspase-dependent apoptotic pathways in short time after mitochondrial proteins cleavage.

Fig. 3 Representative images of caspase-3, Bax, Bcl-2, Ki67, VEGF, and VEGFR-2 (**a**) and CD24, CD44, ALDH1A1, and EpCAM (**b**) expressions in control and treated rat mammary carcinoma cells. **A** control group, **B** ORE 0.3 group, **C** ORE 3 group. Final magnifications: $\times 400$. For detection polyclonal caspase-3 antibody (Bioss, Woburn, USA), polyclonal Bcl-2 antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA), monoclonal Ki67 antibody (Dako, Glostrup, Denmark), monoclonal VEGFR-2 antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA), polyclonal CD24 antibody (GeneTex, Irvine, CA, USA) and polyclonal EpCAM antibody (Abcam, Cambridge, MA, USA) were used



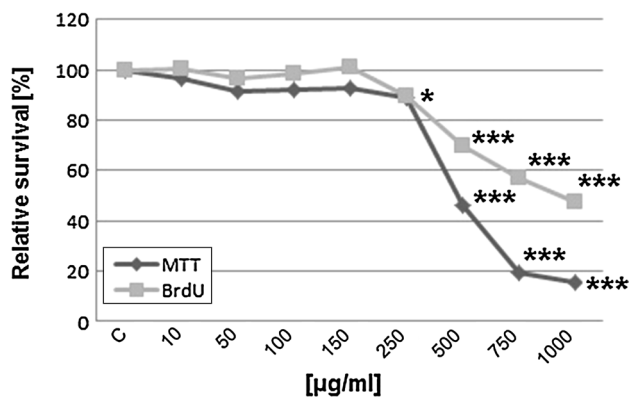


Fig. 4 Relative survival of MCF-7 cells treated with oregano extract (10–1000 µg/ml) and analysed by MTT and BrdU incorporation assays. Data were obtained from three independent experiments and significant differences were marked as * $P < 0.05$; *** $P < 0.001$ versus control cells (untreated)

Moreover, oregano extract caused time-dependent depletion of MMP in MCF-7 cells (Fig. 5b). Mitochondria damage after oregano extract treatment led to release of anti-apoptotic Bcl-2 protein (Fig. 6b). Analysis of phosphorylation status clearly showed deactivation in anti-apoptotic activity of Bcl-2 and activation of mitochondrial apoptosis pathway.

Side effects in animals

In comparison with the controls, oregano did not change serum lipid metabolism (triacylglycerols, total-, LDL-, HDL-, VLDL-cholesterol); however, slight (but significant)

increase in serum glucose by 12 % ($P < 0.01$) (ORE 0.3) and 9.5 % ($P < 0.05$) (ORE 3) was observed (data not shown). Compared to control animals, there was no difference in final body weight. Animals from both treated groups showed a significant increase in daily food intake by 3 g (ORE 0.3) and 4 g (ORE 3) when compared to controls ($P < 0.001$). The administration of oregano was well tolerated by animals (in both doses); no macroscopic changes in the selected organs (e.g. liver steatosis, hepato/splenomegaly, ovarian cysts, gastritis, apparent hematopoietic disorders) were seen.

Secondary metabolites in oregano

Using LC–MS, we identified 12 polar compounds in oregano ethanol extract. The extract was characterized by the presence of phenolic acids and flavonoids derivatives. The most abundant compounds were luteolin-7-*O*-glucuronide (1011 µg/ml), rosmarinic acid (754 µg/ml), and 4'-*O*-β-D-glucopyranosyl-3',4'-dihydroxybenzyl protocatechuate (684 µg/ml). The qualitative and quantitative analysis of ethanol extract of oregano is summarized in Table 5.

Discussion

Plants are an important source of biologically active chemicals with beneficial effects on plethora diseases, including cancer. Cancer aetiology involves multiple mechanisms, and pleiotropic effects of phytochemicals could be advantageous in treating this disease. Moreover, phytochemicals as a low-dose component of whole foods are relatively

Table 3 Flow cytometric analysis of cell cycle distribution in MCF-7 cells treated with oregano extract (in %)

Treatment	Time (h)	Sub G_0/G_1	G_0/G_1	S	G_2/M
Control		0.1 ± 0.00	70.8 ± 2.29	19.3 ± 1.35	9.9 ± 2.32
	24	14.55 ± 1.37*	54.7 ± 3.40**	18.65 ± 0.50	12.10 ± 3.30
Oregano	48	17.20 ± 1.07**	51.60 ± 1.41**	20.70 ± 1.03	10.50 ± 2.38
	72	28.40 ± 2.95***	49.40 ± 0.10***	14.00 ± 1.35*	8.2 ± 1.70

Each value is the mean ± SD of three independent experiments; significantly different, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus untreated cells (control); sub- G_0/G_1 fraction of cells identified as apoptotic population

Table 4 Annexin V/PI flow cytometry analysis of apoptosis occurrence in MCF-7 cells after oregano extract treatment (in %)

Treatment	Time (h)	An [−] /PI [−]	An ⁺ /PI [−]	An ⁺ /PI ⁺	An [−] /PI ⁺
Control		89.4 ± 0.60	1.22 ± 0.50	4.23 ± 0.62	5.16 ± 0.52
	24	59.9 ± 0.20***	20.52 ± 2.32**	13.3 ± 1.10**	6.28 ± 1.52
Oregano	48	34.3 ± 3.21***	42.25 ± 3.67***	10.11 ± 2.60*	13.35 ± 2.88**
	72	29.9 ± 1.20***	49.9 ± 3.66***	7.91 ± 2.29*	12.29 ± 2.01**

The significant differences were signed as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus untreated cells (control). An[−]/PI[−], live cells; An⁺/PI[−], early apoptotic cells; An⁺/PI⁺, late apoptotic/necrotic cells; An[−]/PI⁺, death cells

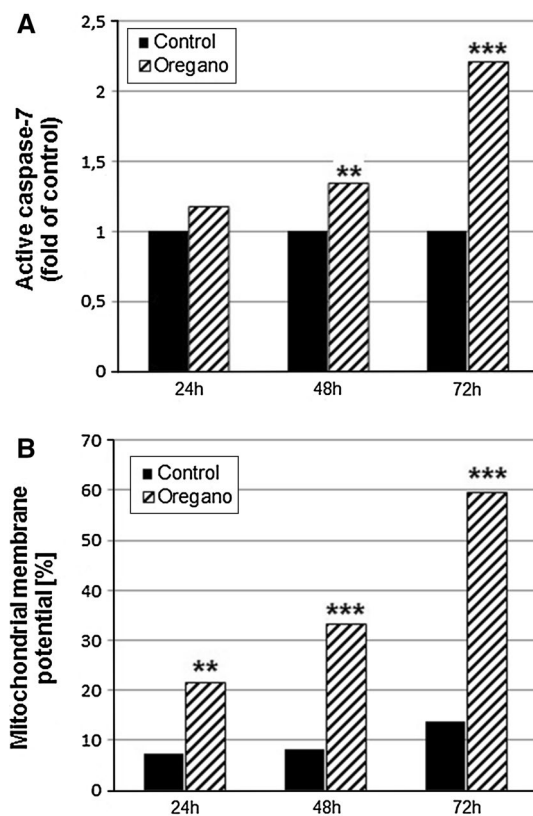


Fig. 5 Effect of oregano extract treatment on caspase-7 activation and mitochondrial membrane potential (MMP) changes. **a** Caspase-7 activation after oregano extract treatment analysed by flow cytometry. **b** MMP changes in MCF-7 cells treated by oregano extract after 24, 48, and 72 h. Results are expressed as mean values of three independent experiments. Significant different, ** $P < 0.01$; *** $P < 0.001$ versus untreated control

non-toxic with generally positive safety profile. We have found that the single phytochemical (resveratrol) cannot replace the combination of natural phytochemical mixture in whole plant foods [25, 26, 30]. As few whole plant foods have been researched in depth for their tumour-suppressive effects in carcinogenesis, we have tested anti-tumour potential of oregano in the model of breast cancer in vivo and in vitro.

We did not find any paper about the dosing of oregano administered dietary to rats published before our study. Srihari et al. [9] administered oregano extract to male rats orally, however as an individual doses to each animal in different experimental groups (20, 40, and 60 mg \times kg⁻¹ b. w. each day for 15 weeks). In our study, we have used doses of dried oregano haulm based on our previous experience using the same animal model and testing plant functional foods [25, 26]. The dose of oregano in ORE 0.3 group (0.3 % of the diet) is similar to human dosing; moreover, we have used ten times higher dose, due to supposed different

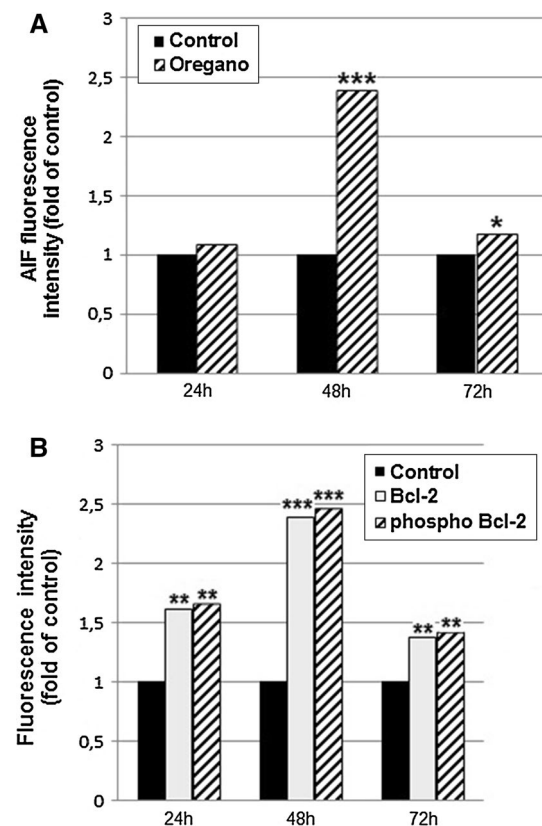


Fig. 6 Distribution and activity of pro-/anti-apoptotic mitochondrial-associated proteins after oregano extract treatment analysed by flow cytometry. **a** AIF mitochondrial efflux after 24, 48, and 72 h treatment with oregano extract in MCF-7 cells. **b** Distribution and deactivation of anti-apoptotic protein Bcl-2 after oregano extract treatment in MCF-7 cells. Results are expressed as mean values of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus untreated control

pharmacokinetics and pharmacodynamics of phytochemicals in oregano between humans and rats. We have found that the average daily dose of oregano per rat was 57.5 mg in the ORE 0.3 group and 604.2 mg in the ORE 3 group. In addition, our results clearly showed that a lower dose of oregano was more efficient than a higher dose in animals. This could be explained by excess dose leading to toxicity in the animal or increased metabolism and elimination. However, our previous experiments using the same model and dietary dosing (0.3 and 3 %) demonstrated significant dose-dependent antineoplastic effects of chlorella [25] and fruit polyphenols [26]. Thus, dosing of whole plant foods appears to be specific among mammal species, and only clinical studies will provide proper dosing regimens in humans. Importantly, we have found highly effective dose of oregano against rat mammary carcinogenesis in this study—it is 3 g of oregano per kg of the diet (0.3 %). On the other hand, it is very difficult even impossible to compare in vitro and in vivo doses in our

Table 5 Polar phenolic compounds in oregano extract, their corresponding retention times (T_R), molecular ions $[M-H]^-$ and MS^2 fragments in LC–MS analysis, and quantitative abundance of polar phenolic compounds ($\mu\text{g/ml}$)

Compound	T_R (min)	$[M-H]^-$ (m/z)	MS^2 (20 eV) (m/z)	concentration ($\mu\text{g/ml}$)* \pm SD
Protocatechuic acid ^a	9.943	153.0184	109.0262	126.4 \pm 0.2
p-Hydroxybenzoic acid ^b	19.371	137.0235	108.0183	<LOQ
Caffeic acid ^c	21.302	179.0304	135.0412	<LOQ
p-Coumaric acid derivative ^d	32.178	281.0553	163.0381 119.0436	61.5 \pm 0.7
Eriodictyol-7- <i>O</i> -glucuronide ^e	35.298	463.0860	287.0434	<LOQ
Luteolin-7- <i>O</i> -glucuronide ^e	35.751	461.0696	285.0384	1011.0 \pm 20.6
4'- <i>O</i> - β -D-glucopyranosyl-3',4'-dihydroxybenzyl protocatechuate ^a	36.567	437.1048	315.0703 153.0172	683.8 \pm 8.6
4-[[[(2',5'-dihydroxybenzoyl)oxy]methyl]phenyl- <i>O</i> - β -D-glucopyranoside ^a	37.858	421.1111	315.0705 153.0171	108.1 \pm 1.8
Luteolin-7- <i>O</i> -glucoside ^e	38.595	447.0905	285.0370	112.3 \pm 1.5
Apigenin-7- <i>O</i> -glucuronide ^f	43.783	445.0746	269.0418	221.0 \pm 6.2
Rosmarinic acid ^g	45.179	359.0750	197.0358 179.0258 161.0160 135.0367	754.8 \pm 14.1
Apigenin-7- <i>O</i> -glucoside ^f	47.733	431.0955	269.0421	195.4 \pm 4.6

* Values ($\mu\text{g/ml}$ liquid extract) are presented as mean \pm standard deviation ($n = 3$)

LOQ limit of quantification

External standards: ^a protocatechuic aldehyde, ^b p-hydroxybenzoic acid, ^c caffeic acid, ^d coumaric acid, ^e luteolin-7-*O*-glucoside, ^f apigenin, ^g rosmarinic acid

study. In many cases, both models—in vivo and in vitro—require specific dosing of tested substances due to different extrinsic conditions of cells. In our in vitro experiment, concentration range of oregano extract was taken from the paper analysing the effects of *Origanum majorana* extract on breast cancer cells [31].

We have found that ethanol oregano extract consists of 12 phenolic acids and flavonoids derivatives as previously shown [32, 33]. The mixture of phenolics present in oregano was very effective against chemically induced breast cancer—we have found significant suppression of all parameters in rat mammary carcinogenesis. Whether or not a single phytochemical could also produce the same results remains to be evaluated [1]; however, using the same experimental model, we have previously confirmed that resveratrol administered alone in lower or higher dose did not show any anti-tumour effects in female rats [30, 34], yet a mixture of carotenoids and phenolics present in *Chlorella pyrenoidosa* (concentration of 3 % in the diet) has caused significant reduction in tumour frequency and significant lengthening of tumour latency [25]. In addition, in the same model, we have similarly demonstrated significant reduction in tumour frequency and incidence, also the lengthening of latency after the administration of dark fruit peels (3 %) [26]. In our recent experiments, young barley

leaf powder (0.3 %) or cloves (1 %) demonstrated reduction in tumour frequency by 37 or 59 %, respectively, using the same rat model [Kubatka et al. in press]. We are confident that the additive and synergistic effects of phenolics present in oregano were responsible for its distinct anti-tumour activities in this in vivo study. We have consistently shown that the single phytochemical cannot replace the phytochemical mixture present in whole plant foods with more complex mechanism of action in breast carcinoma model in rats.

An emerging strategy for cancer chemotherapy is the use of drugs that induce programmed cell death, suppress proliferation, and/or disrupt angiogenesis with the expected elimination of tumour cells. Among the dietary phytochemicals of potential therapeutic effectiveness in cancer are flavonoids, terpenoids, and organosulfur compounds. As well, selected whole plant foods with a specific cocktail of low-dose phytochemicals can be used. Apoptosis is regulated by a family of cysteine proteases known as the caspases. Slee et al. [35] showed that caspase-3 is the primary caspase in this system. Moreover, Bax and Bcl-2 apoptosis regulator proteins are often investigated parameters of programmed cell death. There is evidence that isolated phytochemicals effectively trigger the activation of caspase-3 [36] or increase the Bax/Bcl-2 ratio [37] in cancer cell lines. This

study clearly demonstrates pro-apoptotic effects of high-dose oregano, which is represented by significant increase in the expression of caspase-3 and Bax/Bcl-2 ratio in rat tumour cells. In our previous experiment, the mixture of fruit peel polyphenols showed similar significant increase in caspase-3 expression and Bax/Bcl-2 pro-apoptotic ratio in rat mammary tumour cells in vivo [37]. Identically, in our experiments testing whole “green foods”, we have also found significant pro-apoptotic effects of *Chlorella pyrenoidosa* and young barley leaves in rat mammary carcinomas and MCF-7 cell lines [25; Kubatka et al. in press]. With the aim to confirm possible involvement of oregano extract in cellular changes leading to cell death on MCF-7 cell line, we performed annexin V/PI staining (detects an early stage of apoptosis and combined staining with PI detects a late stage of apoptosis or necrosis) and caspase-7 activation and parallel non-caspase-dependent apoptotic pathways analyses. These analyses significantly confirmed pro-apoptotic effects of oregano in MCF-7 cells. Moreover, our results confirmed that oregano induces apoptosis in MCF-7 cells through significant deactivation in anti-apoptotic activity of Bcl-2 and activation of mitochondrial apoptosis pathway. Finally, we have shown that the decrease in cell viability of MCF-7 cells by oregano was associated with an increase in the fraction of cells with sub- G_0/G_1 DNA content which is considered a marker of apoptotic cell death.

In addition to the pro-apoptotic effects, recent reports have demonstrated also anti-proliferative and anti-angiogenic effects of various phytochemicals [38–40]. Ki67 is considered a good tumour marker which is strictly associated with cell proliferation [41]. In this study, we have observed a significant decrease in the expression of Ki67 in rat mammary carcinoma cells after low-dose oregano treatment in comparison with the control. This result confirmed our previous results with whole dietary foods, where the mixture of fruit peel polyphenols or flavonoids from young barley leaves demonstrated significant reduction in Ki67 expression in mammary carcinoma cells in vivo and significant anti-proliferative effects in MCF-7 cells [15; Kubatka et al. in press]. Moreover, this study showed that oregano extract significantly decreased (in a dose- and time-dependent manner) metabolic activity and viability of MCF-7 cell lines in MTT assay and DNA synthesis measured by BrdU proliferation assay. In addition, oregano prevented cell cycle progression by significant decrease in G_0/G_1 and S populations' enrichment. All our in vitro results clearly demonstrated that oregano may lead to inhibition of proliferation in MCF-7 cells. Regarding angiogenesis, it is well documented that the VEGF-kinase ligand/receptor signalling plays a key role in this system. We have shown that the expression of VEGFR-2, which mediates almost all of the known cellular responses to VEGF, was significantly decreased by both doses of oregano in rat

mammary carcinomas in vivo. These results clearly suggest that further research on the benefits of wide spectrum of phytochemicals present in whole plant foods on organism is warranted.

The cancer stem cells (CSCs) hypothesis proposes that a small subset of cancer cells is responsible for the initiation, proliferation, and metastasis of tumours. An aggressive population of CSCs within the malignancy may be capable of resisting chemotherapeutic drugs, leading to relapse and multidrug resistance [42]. Although multiple putative tissue-based breast cancer stem cell markers have been suggested, the most studied surface molecules of breast cancer stem cell marker proteins are ALDH1, CD24, and CD44—all of them we have analysed in our animal study. Recently, both CD24 and CD44 have been shown as good CSCs markers in chemically induced breast cancer in rats [43]. In our in vivo study, we have also analysed EpCAM expression, a CSCs marker which overexpression has been supposed to support tumour progression [44]. We have observed that the immunorexpression of CSCs markers, CD24, and EpCAM were significantly decreased in rat mammary carcinomas after oregano treatment. Our results support the use of this model for tumour pathology and herbal testing studies focusing on CSCs. It is important to note that it is the first study analysing anti-CSCs properties of oregano in experimental breast cancer in vivo. Only few studies have evaluated in vitro antineoplastic effects of different phytochemicals (isolated or mixture) on breast CSCs markers. Wu et al. [45] showed that 6-shogaol and pterostilbene decreased the expression of CD44 in breast CSCs and promoted β -catenin phosphorylation through the inhibition of hedgehog/Akt/GSK3 β signalling, thus decreasing the protein expression of downstream c-Myc and cyclin D1 and reducing breast CSC cells. In another study, combination of six well-established pro-apoptotic phytochemicals down-regulated the expression of CD44 and other onco-static markers—PCNA, Rb, CDK4, Bcl-2, SVV in two breast cancer cell lines [46]. Due to the lack of available data from breast CSCs research analysing phytochemicals, the mechanism of anti-CSCs action of phytochemicals is not adequately understood. Our data demonstrated significant anti-neoplastic effects of oregano over CSCs in an in vivo model of breast carcinoma. The exact mechanism involved in the anti-CSCs action of oregano awaits further in vitro and in vivo validation, including analysis of other specific cell signalling pathways. Additional preclinical and clinical studies demonstrating that the effect of oregano is targeted on neoplastic cells including CSCs in breast cancer are needed.

In general, efforts to evaluate dietary intake of herbs and spices on human organism are complicated by measurement issues, such as recall bias and difficulty estimating portion size and frequency of intake. Concentrations

of herbs and spices used in food preparation often vary based on individual preference, but frequently falls within the range of 0.5–1 % [47]. Mills et al. [48] aimed to determine what advice health food store employees present to individuals seeking treatment options for breast cancer. They found a recommendation of 2 g of oregano oil per day in the complementary treatment of breast cancer. However, only clinical studies focused on the effect of oregano on breast cancer risk or treatment may give precise answer on the question what daily dose is optimal. However, eating habits of people in the Mediterranean region (as a large epidemiological sample) clearly demonstrate that the daily oregano consumption about 1–2 g is safe and evidently gives favourable effects on organism—delaying the onset of several diseases of civilization, including cancer [49].

Currently there are no sufficient data supporting the idea that plant-derived whole foods decrease the risk or prognosis of cancer disease. The results of this study showed significant anti-tumour activity of oregano in breast carcinoma model in vivo and in vitro. Moreover, this is the first study to confirm the inhibitory effect of oregano against CSCs in vivo. The further mechanistic and clinical studies exploiting the cancer preventive and therapeutic role of plant whole foods containing a mixture of phytochemicals by targeted elimination of CSCs are needed. Daily consumption of several different herbs and spices typical for Mediterranean kitchen (oregano, rosemary, thyme, sage, cloves, etc.) could have more pronounced effects on human organism compared to single herb. All these above-mentioned plant functional foods represent natural substances with supposed anti-ageing effects. We conclude that daily consumption of oregano could be beneficial for the prevention and/or treatment of breast cancer.

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Compliance with ethical standards

Conflict of interest The authors state that there are no conflicts of interest.

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