Basic nutritional investigation

# Antineoplastic effects of *Chlorella pyrenoidosa* in the breast cancer model

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## ARTICLE INFO

## ABSTRACT

*Objectives:* There has been considerable interest in both clinical and preclinical research about the role of phytochemicals in the reduction of risk for cancer in humans. The aim of this study was to determine the antineoplastic effects of *Chlorella pyrenoidosa* in experimental breast cancer in vivo and in vitro.
*Methods:* In this experiment, the antineoplastic effects of *C. pyrenoidosa* in the chemoprevention of *N*-methyl-*N*-nitrosourea–induced mammary carcinogenesis in female rats were evaluated. Chlorella powder was administered through diet at concentrations of 0.3% and 3%. The experiment was terminated 14 wk after carcinogen administration. At autopsy, mammary tumors were removed and prepared for histopathological and immunohistochemical analysis. In vitro cytotoxicity assay, parameters of apoptosis, and proliferation after chlorella treatment in human breast adenocarcinoma (MCF-7) cells were carried out.
*Results:* Basic parameters of experimental carcinogenesis, mechanism of action (biomarkers of apoptosis, proliferation, and angiogenesis), chosen metabolic variables, and side effects after long-term chlorella treatment in animals were assessed. Chlorella at higher concentration suppressed tumor frequency by 61% ($P < 0.02$) and lengthened tumor latency by 12.5 d ($P < 0.02$) in comparison with the controls. Immunohistochemical analysis of rat tumor cells showed caspase-7 expression increase by 73.5% ($P < 0.001$) and vascular endothelial growth factor receptor-2 expression decrease by 19% ($P = 0.07$) after chlorella treatment. In a parallel in vitro study, chlorella significantly decreased survival of MCF-7 cells in a dose-dependent manner. In chlorella-treated MCF-7 cells, a significant increase in cells having sub-$G_0/G_1$ DNA content and significant increase of early apoptotic and late apoptotic/necrotic cells after annexin V/PI staining assay were found. Decreases in mitochondrial membrane potential and increasing reactive oxygen species generation were observed in the chlorella-treated MCF-7 cells.
*Conclusions:* This study is the first report on the antineoplastic effects of *C. pyrenoidosa* in experimental breast cancer in vivo and in vitro.

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## Introduction

The aim of chemopreventive trials is to find an efficient substance that can be administered for a long period with minimum adverse effects [1]. Epidemiologic studies have consistently shown the protective effects of increased consumption of fruits, vegetables, whole grains, and other plant ingredients against the risk for developing chronic diseases such as cancer or cardiovascular disease. It is estimated that there are >5000 individual phytochemicals in plant-based foods [2]. Their identification and mechanism of action evaluation need to be resolved before we can fully understand the health benefits in humans. The phytochemicals demonstrated antiproliferative, anti-inflammatory, antiangiogenic, and pro-apoptotic effects, or the ability to reduce oxidative stress [3], and thus they are of high interest to scientists around the world and the general public. In vitro studies on different cancer cell lines proved the role of polyphenols as growth inhibitors, either by induction of G1-cell cycle arrest [4], G2/M arrest [5], or cell death [6]. Similarly, different carotenoids demonstrated G-1 arrest [7] and apoptosis [8] in various cancer cells. Some polyphenolic compounds demonstrably decreased the levels of one of the most important molecules in angiogenesis, vascular endothelial growth factor (VEGF), thereby inhibiting capillary formation [9].

*Chlorella pyrenoidosa*—freshwater algae—is an important source of different carotenoids [10]. Polyphenols in *C. pyrenoidosa* are present in lower levels. The positive effects of chlorella in terms of treatment and prevention of cancer have been reported in several in vitro experiments [11–13] and also in clinical study [14]. In one animal study, *C. pyrenoidosa* demonstrated significant chemopreventive effects in chemically induced rat hepatocarcinogenesis [15].

*C. pyrenoidosa* has not been tested in experimental mammary carcinogenesis in vivo and in vitro so far. The main aim of this study was to evaluate the preventive effects of long-term chlorella administration in a well-established model of *N*-methyl-*N*-nitrosourea (NMU)–induced mammary carcinogenesis in female rats. In rat mammary tumors, the immunohistochemical analysis of caspase-3, caspase-7, Bax, and bcl-2 proteins as the apoptotic parameters, VEGF and VEGFR-2 as parameters of angiogenesis, and finally Ki67 as a proliferation parameter after chlorella treatment were determined. Another aim of this study—a histomorphologic analysis of carcinomas—may have implications for assessment of chlorella effects on the differentiation and prognosis of the tumors. Some side effects of chlorella after long-term administration in animals were observed. To obtain more complex results, the parallel in vitro study with cytotoxicity data, parameters of apoptosis, and proliferation in MCF-7 cells after chlorella treatment was carried out.

## Materials and methods

### Animals and induction of mammary carcinogenesis, design of experiment

Female rats of the Sprague-Dawley strain (Charles River Laboratories, Sulzfeld, Germany) aged 30 to 34 d were used in the experiment. The animals were adapted to standard vivarium conditions with temperature $23°C \pm 2°C$, relative humidity 40% to 60%, and artificial regimen (light/dark 12:12 h). During the experiment, the animals were fed the Sniff diet (Soest, Germany) and drank tap water ad libitum. Mammary carcinogenesis was induced by NMU (Sigma, Deisenhofen, Germany) administered intraperitoneally in a single dose of 50 mg/kg body weight on average on the postnatal day 42.

Chemoprevention with *C. pyrenoidosa* (Green Ways, Prague, Czech Republic; country of the origin: Taiwan) began 1 wk before carcinogen administration and lasted until the end of the experiment—14 wk after NMU administration. Chlorella powder was administered in the diet at concentrations of 3 (0.3%) and 30 g/kg (3%). Animals were randomly assigned to one of three experimental groups: control group without chemoprevention; chemoprevention with chlorella at a concentration of 0.3% (CHLO 0.3); or chemoprevention with chlorella at a concentration of 3% (CHLO 3). Each group consisted of 25 animals. The animals were weighed and palpated weekly in order to register the presence, number, location, and size of each palpable tumor. Food intake per cage during 24 h was monitored during weeks 7 and 13 of the experiment (the value obtained was divided by the number of animals in the cage and thus we determined the average food intake per animal in the relevant cage). The measurements were taken four times (twice in weeks 7 and 13). The chlorella doses per animal and day were calculated in accordance with the amount of chow consumed. In the last week of the experiment (week 14), the animals were quickly decapitated, the blood from each animal was collected, mammary tumors were excised, and tumor 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 tumors

A tissue sample of each mammary tumor was routinely formalin-fixed and paraffin-embedded. The tumors were classified according to the criteria for the classification of rat mammary tumors [16]. The additional parameter—grade of invasive carcinomas—was used. Tumor 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 tumors with ≥2 positive criteria; LG carcinomas were tumors with ≤1 positive criterion. Serum lipid parameters were evaluated using an Olympus AU640 (Olympus Optical, Tokyo, Japan) automatic biochemical analyser.

The paraffin block with the most representative tumor area of each mammary tumor 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 Inc., Paso Robles, CA, USA; Dako, Glostrup, Denmark; Abcam, Cambridge, UK). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. Sections were pretreated in a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the primary antibody in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, for 60 min at room temperature. The primary antibodies were visualized by a secondary staining system (EnVision, Dua Link System-HRP, cat. No. K4061, Dako North America Inc., Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride as a substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted in Canadian balsam. Negative controls included sections where the primary antibody was omitted in the staining process. Immunohistochemically detected antigen expression was evaluated by precise morphometric method. Sections were screened and digital images of microscopic views at magnifications of ×200 were taken with an Olympus Evolt E-420 installed in an Olympus BX41N microscope. Expression of VEGF, caspase-3, and caspase-7 was analyzed in the cytoplasm of tumor cells. Ki67 was detected within the nucleus. Receptors for VEGF were observed in the cell membrane. Bcl-2 and Bax were detected as a membrane-associated oncoproteins. Expression of proteins was quantified as the average percentage of antigen-positive area in standard fields (0.5655 mm$^2$) of tumor hotspots. Morphometric analysis of the digital images was done using QuickPhoto Micro software, version 2.3 (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 only between effectively treated (CHLO 3 group) and nontreated (control group) carcinoma cells of female rats (the changes in the CHLO 0.3 group were not expected due to the main in vivo results). At least 40 images for one protein were analyzed (280 images for seven proteins).

### Antiproliferative activity: Tumor cell lines and cell proliferation assay

MCF-7 (human breast adenocarcinoma, estrogen receptor-positive) cell line was provided by Dr. M. Hajdúch (Olomouc, Czech Republic). The cells were routinely maintained in Dulbecco's modified Eagle's medium with Glutamax-I supplemented with 10% fetal calf serum, penicillin (100 IU $\times$ mL$^{-1}$), and streptomycin (100 µg $\times$ mL$^{-1}$) (all from Invitrogen, Carlsbad, CA, USA) in humidified air with 5% carbon dioxide at 37°C. Before each proliferation assay, cell viability was determined using the trypan blue exclusion method and found to be >95%.

The effects of compounds on cell proliferation were determined using colorimetric microculture assay with the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) end point [17]. Briefly, $3 \times 10^3$ cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing tested chemicals at final concentrations of 0.0012 to 1.25 mg/mL or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Fluka, Buchs, Schwitzerland) at final concentrations 100 and 300 µM, or at mutual combinations. After 72 h of incubation, 10 µL of MTT (5 mg $\times$ mL$^{-1}$) (Sigma, Germany) were added to each well. After an additional 4 h, during which

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

| Group | CONT | CHLO 0.3 | CHLO 3 |
|---|---|---|---|
| All animals/tumor-bearing animals | 24/19 | 25/20 | 25/17 |
| Tumor frequency per group* | 2.88 ± 0.60 | 2.00 ± 0.29 (−30.5%) | 1.12 ± 0.21[†] (−61%) |
| Tumor incidence (%) | 79.2 | 80 | 68 (−14%) |
| Tumor latency* (days) | 70.74 ± 3.07 | 74.90 ± 3.49 (+4 d) | 83.18 ± 3.88[†] (+12.5 d) |
| Average tumor volume* (cm$^3$) | 0.28 ± 0.06 | 0.58 ± 0.21 (+107%) | 0.20 ± 0.05 (−28.5%) |
| Cumulative tumor volume[‡] (cm$^3$) | 19.32 | 29 (+51%) | 5.60 (−71.5%) |

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

* Data are expressed as means SEM.
[†] Significantly different, $P < 0.02$ vs. CONT.
[‡] Data are expressed as a sum of volumes per group. Values in parentheses are calculated as percent deviation from the 100% of noninfluenced control group (with exception of latency).

insoluble formazan was produced, 100 µL of 10% sodium dodecyl sulfate were added to each well and another 12 h were 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.

*5-Bromo-20-deoxyuridine cell proliferation assay*

Cell proliferation activity was directly monitored by quantification of 5-bromo-20-deoxyuridine (BrdU) incorporated into the genomic DNA during cell growth. DNA synthesis was assessed using colorimetric cell proliferation enzyme-linked immunosorbent assay (ELISA; Roche Diagnostics GmbH, Mannheim, Germany) following the vendor's protocol. Briefly, 5000 cells per well in 80 µL medium were plated in 96-well polystyrene microplates (Sarstedt, Nümbrecht, Germany). Twenty-four h after cell seeding different concentrations (1.25–0.0012 mg/mL) of the compound were added. After 48 h of treatment, cells were incubated with BrdU labeling solution (10 µM 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 µL 1 M H$_2$SO$_4$) and color intensity was measured with a multi-well microplate ELISA reader at 450 nm (reference wavelength: 690 nm).

*Apoptosis analysis*

Apoptosis was analyzed using the Annexin V/propidium iodide apoptosis kit (BD Biosciences Pharmingen, San Diego, CA, USA), according to the manufacturer's recommendation. MCF-7 cells were harvested 24, 48, and 72 h after treatment and stained with Annexin V-FITC in binding buffer for 15 minutes, washed, stained with propidium Iodide for 5 min and thereafter analyzed using a BD FACSCalibur flow cytometer.

*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, washed in cold PBS, fixed in cold 70% ethanol and kept at −20°C overnight. Before 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 room temperature for 30 min, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

*Mitochondrial membrane potential assay*

The changes in mitochondrial membrane potential (MMP) were analyzed 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 room temperature in the dark. The cells were then washed twice with PBS, resuspended in 500 µM of the total volume, and analyzed (1 × 10$^4$ cell per sample). Fluorescence was detected with a 585/42 (FL-2) optical filter.

*Reactive oxygen species production measurement*

The intracellular production of reactive oxygen species (ROS) was detected with FCM analysis using dihydrorhodamine-123 (DHR-123, Fluka, Buchs, Switzerland), which reacts with intracellular hydrogen peroxide. The cells treated with chlorella, Trolox, or their combinations were harvested, washed twice in PBS, and resuspended in PBS. DHR-123 was added at a final concentration of 0.2 µM. The samples were then incubated for 15 min in the dark and after incubation samples were placed on ice. Fluorescence was detected with a

530/30 (FL-1) optical filter. Forward and side scatters were used to gate the viable populations of cells.

*Statistical analysis*

Statistical analyses were conducted using GraphPad Prism, version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). The Mann-Whitney and Kruskal-Wallis tests and one-way analysis of variance were the statistical methods used in data evaluation. $P \leq 0.05$ were considered statistically significant. Tumor volume was calculated according to the formula: $V = \pi \cdot (S_1)^2 \cdot S_2/12$ ($S_1$, $S_2$ are tumor diameters; $S_1 < S_2$).

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).

## Results

### Rat mammary carcinogenesis

Table 1 summarizes the effects of chlorella on rat mammary carcinogenesis. In this study, chlorella inhibited rat mammary carcinogenesis in a dose-dependent manner. A significant 61% decrease in tumor frequency ($P = 0.019$) was observed in the CHLO 3 group compared with the control animals. Tumor latency was significantly lengthened by 12.5 d ($P = 0.016$) in the CHLO 3 group when compared to the control group.

### Pathology of rat tumors

A histopathological classification of all mammary tumors is summarized in Table 2. The most frequently occurring lesions

**Table 2**
Histopathological classification and number of mammary tumors

| Mammary tumors* | CONT | CHLO 0.3 | CHLO 3 |
|---|---|---|---|
| Malignant lesions | | | |
| P,C | 23 | 26 | 7 |
| C | 25 | 11 | 12 |
| C,P | 9 | 5 | 6 |
| C,CO | 4 | 2 | – |
| DCIS | 4 | 2 | – |
| P | 2 | 3 | 2 |
| P,C,CO | 1 | 1 | – |
| C,P,CO | – | – | 1 |
| Precancerous lesion | | | |
| IDP | 1 | – | – |
| Total | 69 | 50 | 28 |

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

* Type: Invasive carcinoma (C, cribriform; P, papillary; CO, comedo), DCIS, ductal carcinoma in situ; IDP, intraductal proliferation. Dominant type in mixed tumors is the first in order.
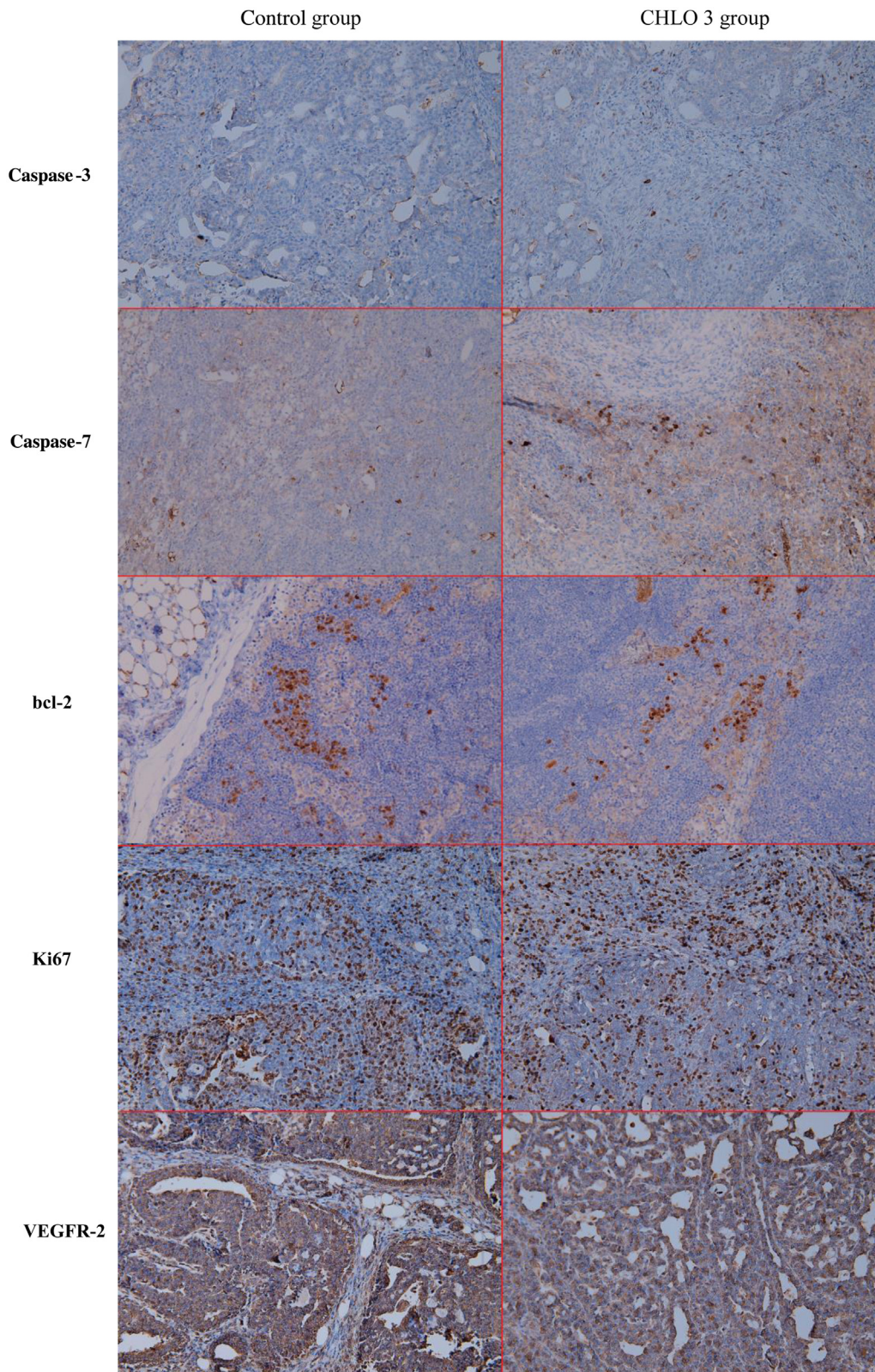
**Fig. 1.** The immunohistochemical detection of caspase-3, caspase-7, bcl-2, Ki67, and VEGFR-2 expressions in mammary tumor cells after treatment with chlorella. For detection polyclonal caspase-3 antibody (Abcam, Cambridge, UK), polyclonal caspase-7, and bcl-2 antibody (Santa Cruz , Paso Robles, CA, USA), monoclonal Ki67 antibody (Dako, Glostrup, Denmark), and monoclonal Flk-1 antibody (Santa Cruz, Paso Robles, CA, USA) were used; final magnifications: ×200. VEGFR, vascular endothelial growth factor receptor.
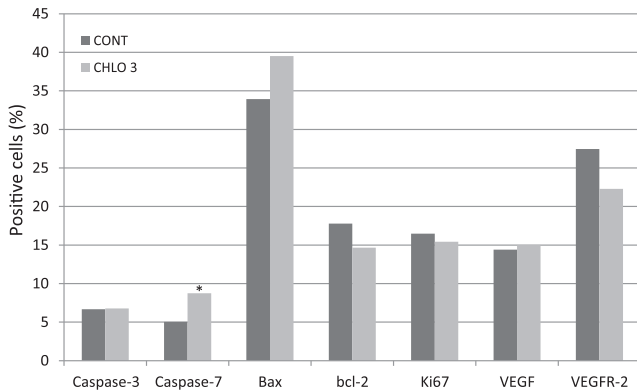
**Fig. 2.** Immunohistochemical evaluation of caspase-3, caspase-7, Bax, bcl-2, Ki67, VEGF, and VEGFR-2 expression in rat mammary tumor cells after chlorella treatment. Data are expressed as means. CONT, control group; CHLO 3, group with significant antineoplastic effects of chlorella in vivo (dosing of 30 g/kg in diet); VEGFR, vascular endothelial growth factor receptor. Significantly different, *$P < 0.001$ vs. CONT.

in all experimental groups were mixed papillary/cribriform and cribriform carcinomas. A shift in the rate of poorly differentiated (HG) and well-differentiated (LG) mammary carcinomas to a higher representation of LG lesions after treatment with chlorella was revealed after histopathological analysis (control group: 27 of 36 [HG/LG]; CHLO 0.3: 14 of 33; $P = 0.16$ versus control; CHLO 3: 10 of 18; $P = 0.52$ versus control). In the CHLO 3 group, the decrease in average tumor volume (the parameter of staging of the tumors) significantly correlated ($r = 0.74$; $P = 0.034$) with the decrease in tumor frequency (best parameter of treatment efficacy) in the same group when compared with controls.

### Immunohistochemical evaluation of rat tumors

As seen in Figures 1 and 2, immunohistochemical analysis of rat mammary tumors showed a significant increase in caspase-7 expression by 73.5% ($P = 0.0001$) in chlorella-treated tumor cells compared with control cells. A mild decrease in VEGFR-2 expression by 19% ($P = 0.07$) was found after chlorella treatment. Compared with the control tumor cells, we observed a tendency of Bax expression to increase by 16.5% ($P = 0.17$) and bcl-2 expression to decrease by 17.5% ($P = 0.34$) in treated cells. No changes were seen between treated and control cells regarding the expression of caspase-3, Ki67, and VEGF.

### Side effects in animals

The drug was well tolerated by animals, and no macroscopic changes due to chlorella administration in the selected organs (liver, kidney, stomach, intestine, and lung) were observed. Compared with the control animals, chlorella at higher doses decreased low-density lipoprotein cholesterol by 25% ($P = 0.002$), triglycerides by 20% ($P = 0.07$), and very low-density lipoprotein cholesterol by 19% ($P = 0.08$; data not shown). Compared with controls, the evaluation of final body weight gain in rats revealed a 12.5% ($P = 0.016$) increase in CHLO 3. Animals from both treated groups exhibited a tendency (not significantly different) to increased food intake. The average daily food intake per rat was $19.82 \pm 0.56$ g (control group), $20.55 \pm 0.45$ g (CHLO 0.3), and $20.95 \pm 0.51$ g (CHLO 3).

### In vitro analysis in MCF-7 cells

Chlorella significantly inhibited viability in the MCF-7 cells in a dose-dependent manner as detected by MTT assay ($P < 0.05$ to
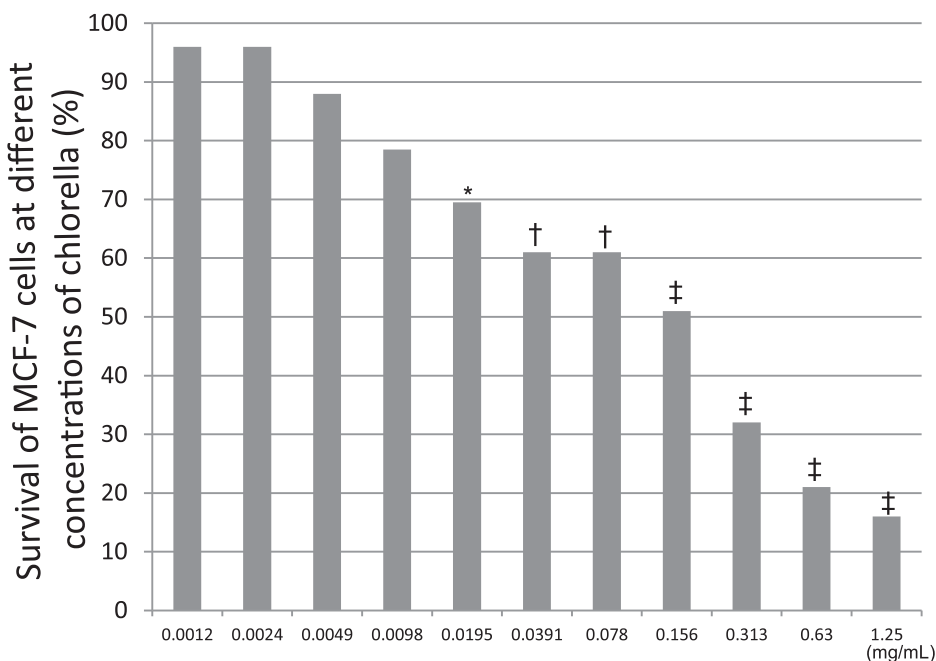


**Fig. 3.** MTT assay showing the percentage of viable MCF-7 cells after 72 h incubation with chlorella at 0.0012 to 1.25 mg/mL concentrations. MTT is a membrane permeable dye that is metabolized to dark-blue crystals of formazan by mitochondrial dehydrogenases of living cells. After lysis of the cell and solubilization of the formazan crystals, the absorbance was measured at 540 nm using the automated uQuant™ Universal Microplate Spectrophotometer (Biotek). The blank-corrected absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control. Data are expressed as means. Data were obtained from three independent replicate experiments with at least three wells per treatment group in each individual replicate. Significantly different, *$P < 0.05$ vs. control cells (untreated), †$P < 0.01$, ‡$P < 0.001$.
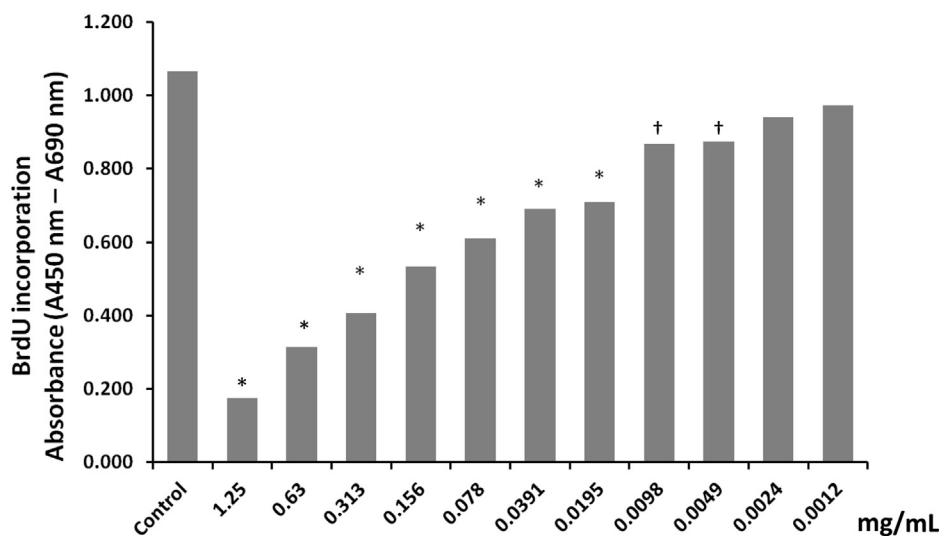
**Fig. 4.** Effects of chlorella on the proliferation of human breast cancer MCF-7 cells, as examined by BrdU incorporation. Cells were cultured as described in the Materials and methods section and then plated at the density of 5000 cells per well in 96-well plate. After 24 h of plating, cells were treated with chlorella at concentrations 0.0012 to 1.25 mg/mL for 48 h and then incubated with BrdU for another 24 h. BrdU incorporation was measured with a multiwell microplate enzyme-linked immunosorbent assay reader at 450 nm. Data were obtained from three independent replicate experiments with at least three wells per treatment group in each individual replicate after 72 h of incubation. Significantly different, $*P < 0.001$, $^{\dagger}P < 0.05$ vs control. BrdU, 5-bromo-20-deoxyuridine.

$P < 0.001$) (Fig. 3). To clarify whether the observed reduction in cell viability resulted from the suppressed cell growth, we analyzed the effects of chlorella on DNA synthesis by measuring Brdu incorporation. As shown in Figure 4, chlorella significantly decreased MCF-7 cell proliferation at a concentration of 1.25 to 0.0049 mg/mL ($P < 0.001$; $P < 0.05$).

To quantify the apoptosis in MCF-7 cells triggered by chlorella, cells were stained with Annexin V-FITC/PI. The percentage of early apoptotic cells and late apoptotic/necrotic cells significantly increased ($P < 0.05$ to $P < 0.001$) in a time-dependent manner (Table 3).

The distribution of cells in different phases of the cell cycle is shown in Table 4. After treatment with chlorella at a concentration of 159 µg/mL for 48 and 72 h, a significant increase in cells having sub-$G_0/G_1$ DNA content was found; it was accompanied by a proportional decrease in $G_0/G_1$ phase cells ($P < 0.01$; $P < 0.001$).

As shown in Figure 5, the levels of DHR-123 fluorescence in chlorella-treated cells were significantly increased ($P < 0.05$; $P < 0.01$), suggesting that generation of ROS is involved in the apoptotic process. To show that generation of ROS is a key step in the chlorella-induced apoptotic pathway, we pretreated MCF-7 cells with Trolox, a water-soluble analogue of α-tocopherol. Trolox significantly decreased ROS accumulation in a time-dependent manner ($P < 0.01$; $P < 0.001$). These effects also were associated with recovered cell viability (Fig. 6). Moreover,

chlorella caused a time-dependent depletion of mitochondrial membrane potential in MCF-7 cells ($P < 0.01$; $P < 0.001$) (Fig. 7).

## Discussion

Well-defined chemopreventive studies carried out on animal models demonstrate a high validity of antineoplastic evaluation of new drugs in preclinical tests. A large variety of plant products are commercially available as dietary supplements. However, only insufficient data about their efficacy and mechanism of action are known. Microalgae have become an important commercial source of phytochemicals, especially carotenoids, which play an important role in carcinogenesis. *C. pyrenoidosa* mostly contains all-*trans*-lutein (in an exceptionally large amount), followed by the *cis*-isomer of lutein, all-*trans*-α-carotene, zeaxanthin, *cis*-isomer of β-carotene, all-*trans*-β-carotene, *cis*-isomer of α-carotene, β-cryptoxanthin, neoxanthin, cis-isomer of neoxanthin, neochrome, auroxanthin, violaxanthin, and *cis*-isomer violaxanthin [10].

To our knowledge, this is the first study to report on the antineoplastic effects of *C. pyrenoidosa* in rat mammary carcinogenesis. We suppose that the additive/synergistic effects of carotenoids (or, to a lesser extent, polyphenols) were responsible for the high anticancer activity of chlorella observed in

**Table 3**
Chlorella-induced apoptosis in MCF-7 cells measured by flow cytometry

| Treatment | Time (h) | An$^-$/PI$^-$ | An$^+$/PI$^-$ | An$^+$/PI$^+$ |
|-----------|----------|---------------|---------------|---------------|
| Control | | 95.52 ± 1.62 | 0.75 ± 0.70 | 3.72 ± 0.15 |
| CH | 24 | 92.56 ± 0.18 | 3.25 ± 0.26 | 4.20 ± 0.43 |
| CH | 48 | 78.66 ± 0.20* | 12.87 ± 0.27* | 8.48 ± 0.24$^{\dagger}$ |
| CH | 72 | 52.15 ± 1.65* | 31.67 ± 2.50* | 16.18 ± 1.69$^{\ddagger}$ |

An$^-$/PI$^-$, live cells; An$^+$/PI$^-$, early apoptic cells; An$^+$/PI$^+$, late apoptotic/necrotic cells; CH, chlorella 156µg/mL

\* Significantly different, $P < 0.001$ vs. control.

$^{\dagger}$ Significantly different, $P < 0.05$ vs. control.

$^{\ddagger}$ Significantly different, $P < 0.01$.

**Table 4**
Flow-cytometric analysis of cell-cycle distribution in MCF-7 cells treated with chlorella (%)

| Treatment | Time (h) | sub-$G_0/G_1$ | $G_0/G_1$ | S | $G_2/M$ |
|-----------|----------|---------------|-----------|---|---------|
| Control | | 0.41 ± 0.26 | 80.22 ± 2.29 | 12.68 ± 1.90 | 6.69 ± 1.29 |
| CH | 24 | 2.70 ± 0.39 | 81.22 ± 3.36 | 9.72 ± 0.66 | 6.36 ± 0.66 |
| CH | 48 | 14.56 ± 1.97* | 70.84 ± 2.03* | 8.58 ± 0.43 | 6.02 ± 1.58 |
| CH | 72 | 16.07 ± 1.12* | 67.85 ± 3.44$^{\dagger}$ | 8.13 ± 0.45 | 7.95 ± 1.77 |

CH, chlorella 156 µg/mL; sub-$G_0/G_1$, fraction of cells identified as apoptotic population; $G_0$, resting phase; $G_1$, presynthetic phase; $G_2$, premitotic phase; S, synthesis phase; M, mitosis phase

\* Significantly different, $P < 0.001$ vs. untreated cells (control).

$^{\dagger}$ Significantly different, $P < 0.01$ vs. untreated cells (control).
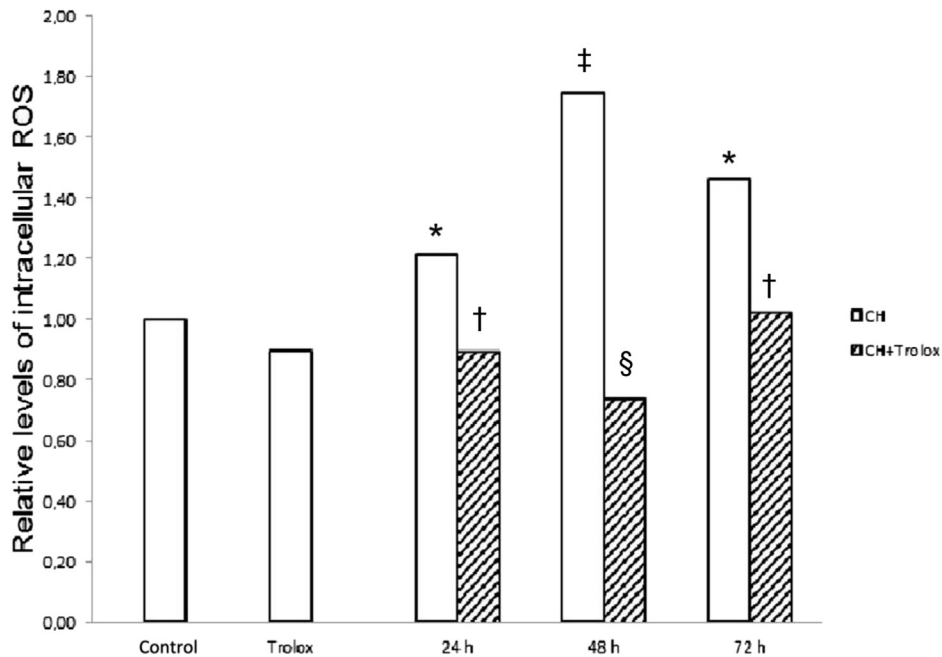
**Fig. 5.** Effect of chlorella on reactive oxygen species (ROS) production. MCF-7 cells were cultured in the absence (control) or presence of chlorella (156.0 μg/mL) or Trolox (100 μM) or at mutual combinations (striped columns) for 24, 48, and 72 h. ROS were detected by flow-cytometry analysis using dihydrorhodamine-123 (DHR-123). The cells treated with chlorella were harvested, washed twice in phosphate-buffered saline, and resuspended in phosphate-buffered saline. DHR-123 was added at a final concentration of 0.2 μM. The samples were then incubated for 15 min in the dark and after incubation samples were placed on ice. Fluorescence was detected with a 530/30 (FL-1) optical filter. Data were obtained from three independent experiments. Significantly different, $^*P < 0.05$ vs control, $^†P < 0.01$ vs. chlorella, $^‡P < 0.01$, $^§P < 0.001$.
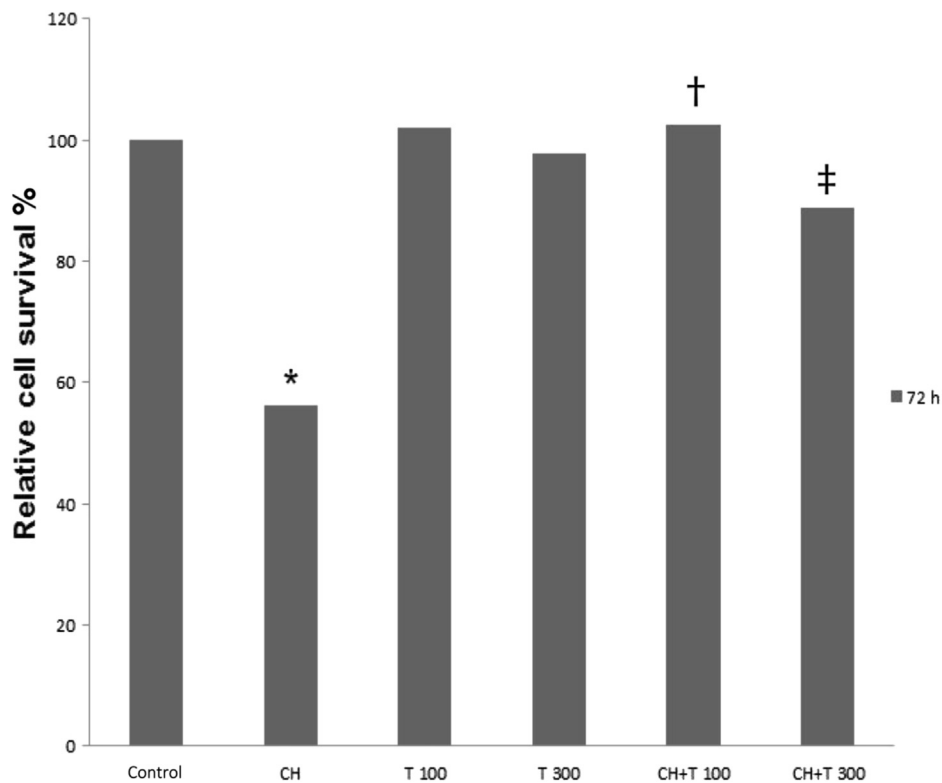


**Fig. 6.** Relative survival of MCF-7 cells treated with chlorella, Trolox, or mutual combinations as evaluated by MTT assay. Cells were cultured either with chlorella (CH, 156.0 μg/mL), Trolox at concentration 100 and 300 μM (T 100, T 300), or their combination (CH+T 100, CH+T 300) for 72 h. Data were obtained from three independent replicate experiments with at least three wells per treatment group in each individual replicate. Significantly different, $^*P < 0.001$ vs. control cells (untreated), $^†P < 0.001$, $^‡P < 0.01$ vs. chlorella.
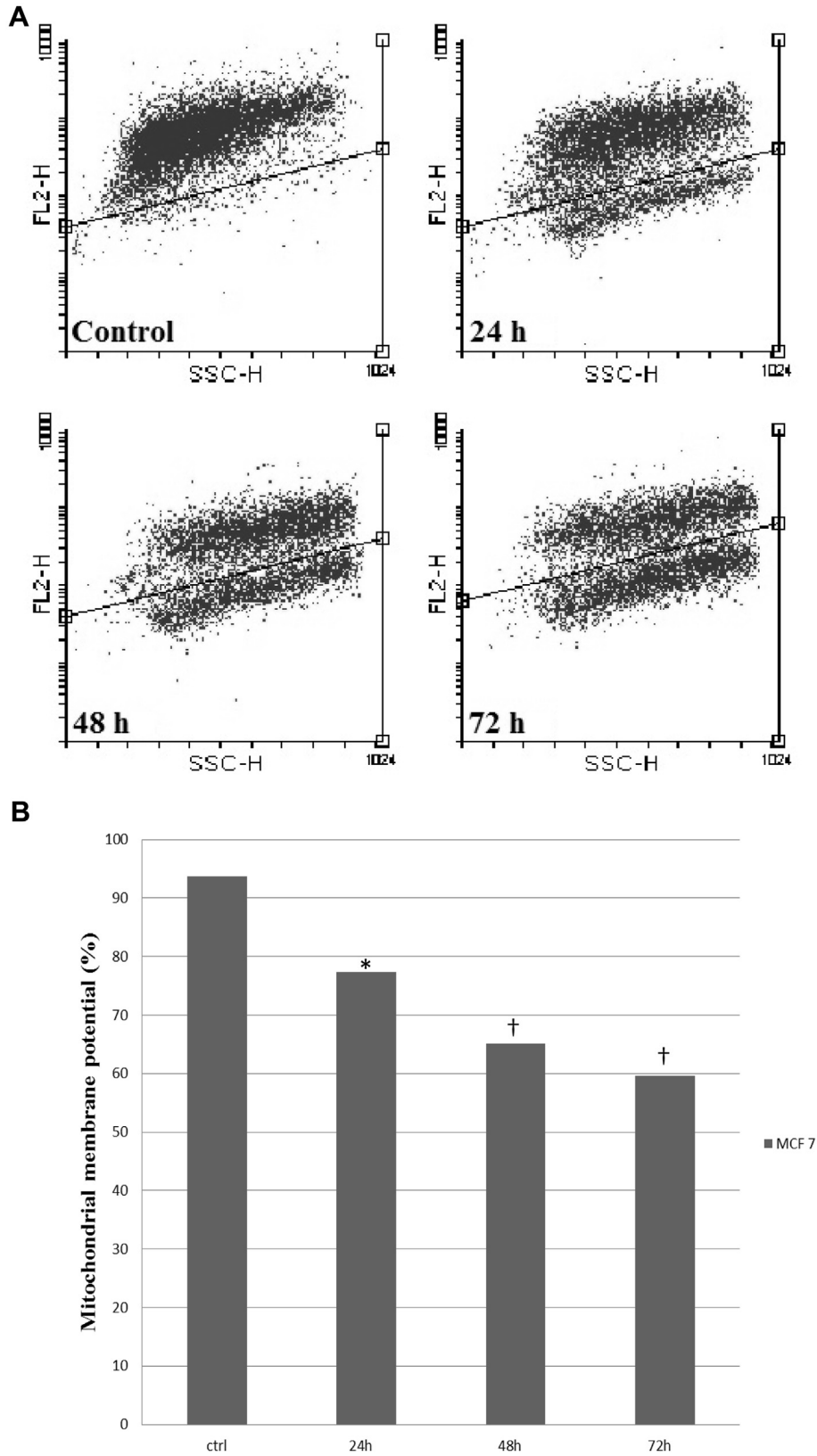
**Fig. 7.** Effect of chlorella on mitochondrial membrane potential. MCF-7 cells were cultured in the presence or absence of chlorella (156.0 μg/mL) for 24, 48, and 72 h. Fluorescence intensity was detected by flow cytometry using tetramethylrhodamine ethyl ester per chlorate (TMRE). The cells were washed with phosphate-buffered saline (PBS), resuspended in 0.1 μM of TMRE in PBS, and incubated for 30 min at room temperature in the dark. The cells were then washed twice with PBS, resuspended in 500 μM of the total volume, and analyzed ($1 \times 104$ cell per sample). Fluorescence was detected with a 585/42 (FL-2) optical filter. (A) Representative fluorescence-activated cell sorting analyses after staining with TMRE. (B) Mean of three independent experiments. Significantly different, $^{*}P < 0.001$, $^{\dagger}P < 0.01$ vs. control.

this study. Tumor frequency (considered the most sensitive parameter of rat mammary carcinogenesis) was significantly decreased by (high-dose) chlorella administration. The lower concentration of chlorella was less effective. Moreover, a histopathological analysis of mammary tumors revealed a shift from poorly to well-differentiated invasive tumors in animals from both treated groups. The improved prognosis of mammary gland tumors after chlorella treatment may have notable implications for clinical practice. The effective dose of chlorella in this experiment was 10 times higher than the maximal clinical dose (10 g); however, the animals tolerated it well. Dosing in this experiment was based on a previously described study [15] and also on our previous experience with this model, where various statins [18] or aromatase inhibitors [19] were used. In general, rats demonstrate different pharmacokinetics and pharmacodynamics of many drugs compared with humans, therefore it was necessary to use high doses of chlorella to prove its antineoplastic effect in this experiment (the average daily dose of chlorella per rat was 61.75 mg in the CHLO 0.3 group and 628.5 mg in the CHLO 3 group). For this reason, the dosing of chlorella in oncology patients will be based on the results from clinical research in the future.

We have assumed that the observed tumor-suppressive effect of chlorella is a consequence of pro-apoptotic, antiproliferative, antiangiogenic, and other antineoplastic activities of different phytochemicals in rat mammary tumor cells. Sequential activation of caspases plays a key role in the execution phase of apoptosis. Caspase-3 and caspase-7 have been implicated as "effector" caspases associated with the initiation of the cell death. For example, in one study, two carotenoids—neoxanthin and fucoxanthin—induced apoptosis in PC-3 human prostate cancer cells through activation of caspase-3 and poly(ADP)-ribose polymerase [8]. In our study, a significant increase in caspase-7 expression in rat mammary tumor cells after treatment with chlorella was observed. However, we did not find a correlation in the expression of two apoptosis-executioner proteases in our study. The fundamental functional differences between caspase-3 and caspase-7 in terms of their ability to cleave multiple natural substrates is probably brought about by other cell-signaling pathways and could be related to alternative, caspase-3–independent pathways in apoptosis [20,21]. The pro-apoptotic effect of chlorella in this study was also indicated by the pro-apoptotic shift in Bax/bcl-2 expression in treated tumor cells. Our in vitro study demonstrated that the decrease in cell viability by chlorella 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. Apoptosis was also confirmed by annexin V/PI staining. The precise mechanism of chlorella apoptotic effect has not been elucidated yet. Several studies have reported that apoptosis involves a disruption of mitochondrial membrane integrity and the depolarization of mitochondrial membrane potential is a characteristic feature of apoptosis [22]. Our results confirmed that chlorella induces apoptosis in MCF-7 cells through the disruption of mitochondrial membrane potential. ROS are regarded as the byproducts of normal cellular oxidative processes. On the other hand, ROS levels can increase dramatically on environmental or chemical stress (e.g., presence of cytotoxic agent). Moreover, the association between the mitochondrial dysfunctions and ROS production is still debated. It is known that overproduction of ROS can promote apoptosis [23]. Our results showed that chlorella treatment significantly stimulated ROS generation in MCF-7 cells. To confirm the role of ROS in chlorella-induced cell death, MCF-7 cells were pretreated with antioxidant Trolox.

Compared with Chlorella treatment only, Trolox pretreatment caused a reduction in ROS levels and significantly rescued chlorella-induced MCF-7 cytotoxicity. These results indicate that ROS can be crucial in the induction of chlorella-induced apoptosis.

Ki67 is a cancer antigen that is found in growing, dividing cells but is absent in the resting phase of cell growth. This characteristic makes Ki67 a good tumor marker; e.g., it was used in a recent clinical study [24] for differentiation between paclitaxel- and docetaxel-sensitive breast cancers. The antiproliferative activity of chlorella carotenoids on human colon cancer cells in MTT assay has been evaluated [12]. The carotenoids (violaxanthin, antheraxanthin, zeaxanthin, and lutein) were separated from marine *Chlorella ellipsoidea* and freshwater *Chlorella vulgaris*. Both extracts inhibited HCT116 cell growth and proliferation in a dose-dependent manner. Similarly, in our parallel in vitro study, *C. pyrenoidosa* significantly decreased (in a dose-dependent manner) survival of human breast adenocarcinoma cell lines in MTT assay. The results from in vitro studies are usually confronted with in vivo experiments. Some of them have shown apparent antiproliferative effects of different phytochemicals, demonstrated by decreased Ki67 expression, in various animal cancer models [25–27]. In our study, we observed only a slight 2% decrease in the expression of Ki67 in mammary carcinoma cells from the CHLO 3 group compared with control cells. Our study pointed to in vitro antiproliferative effects of *C. pyrenoidosa*, but this result was not confirmed in vivo.

The VEGF-kinase ligand/receptor signaling system plays a key role in both vasculogenesis and angiogenesis. VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF [28]. Chlorella in this study decreased VEGFR-2 levels in tumor cells by 19% compared with controls, and this result points to mild antiangiogenic effects of the drug. VEGF as well as other growth factors, for example, fibroblast growth factor-b and transforming growth factor-α, are potentially important targets of antiangiogenic therapy.

## Conclusion

The results of this experiment showed significant tumor-suppressive effects of *C. pyrenoidosa* in experimental breast cancer in rats. Histopathological analysis of tumors pointed to a better prognosis of carcinomas after chlorella treatment. The results obtained suggested pro-apoptotic and antiangiogenic effects of chlorella in rat mammary tumor cells. Additionally, treatment with chlorella was accompanied by positive effects on plasma lipid metabolism in rats. In vitro analysis demonstrated pro-apoptotic and antiproliferative effects of chlorella in human breast adenocarcinoma cells. Despite the fact that epidemiologic studies have not provided sufficient evidence about the tumor-preventive effects of various phytopharmaceuticals/phytochemicals, strictly defined in vivo experiments may be a way to prove their antineoplastic activities in organism.

## Acknowledgments

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