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ORIGINAL ARTICLE

Cytotoxic effects of two extracts from garlic (*Allium sativum* L.) cultivars on the human squamous carcinoma cell line SCC-15

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KEYWORDS

Garlic;
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Abstract Garlic (*Allium sativum* L., Alliaceae) has acquired a reputation as a therapeutic agent and herbal remedy to prevent and treat several pathologies. The aim of the present study was to determine the effect of two *Allium sativum* L. cultivars, Harnaś and Morado, on reactive oxygen species (ROS) production, viability and apoptotic process in human squamous carcinoma cell line SCC-15. The experiments were conducted on SCC-15 cell line exposed to increasing concentrations of garlic extracts of 0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL. After the experiments, ROS formation, caspase-3 activity and neutral red uptake were measured in the cells, and in a collected medium lactate dehydrogenase (LDH) release was measured. The Spanish cultivar Morado has demonstrated higher potential to stimulate ROS production in SCC-15 cells after a short time period (6 h) than the Polish cultivar Harnaś. However, the Polish cultivar Harnaś manifested more prolonged potential to stimulate ROS production in SCC-15 cells. Both studied garlic extracts induced cytotoxicity on SCC-15 cell line which was probably ROS-dependent. We also determined

Abbreviations: Caspase, cysteine-aspartic acid protease; DMSO, dimethyl sulfoxide; DPPH$^{\cdot}$, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; PBS, phosphate-buffered saline; ROS, reactive oxygen species; LDH, lactate dehydrogenase

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that in SCC-15 cells high concentrations of studied extracts did not cause activation of caspase-3 which suggested caspase-independent or necrotic cell death.

1. Introduction

Garlic (*Allium sativum* L., Alliaceae) has acquired a reputation as a therapeutic agent and herbal remedy to prevent and treat several pathologies, including microbial infections, allergy, hypertension, hypercholesterolemia, diabetes, atherosclerosis and cancer (Bhandari, 2012). Health properties of garlic depend on its bioactive compounds, especially the organosulfur compounds which include diallyl trisulfide, s-allylcysteine, vinyldithiines, allylpropyl disulfide, ajoene and allicin (Bhandari, 2012). In addition to those compounds, garlic is also characterized by phenolic compounds, which have interesting pharmacological properties (Beato et al., 2011; Matysiak et al., 2015). High polyphenolic content, a number of natural antioxidants, and many different bioactive compounds can directly and indirectly enhance the expression of antioxidant enzymes, which protect normal cells (Chen et al., 2013; Piątkowska et al., 2015).

Reactive oxygen species (ROS) is a commonly used term that includes not only oxygen radicals (superoxide and hydroxyl) but also some non-radical derivatives of molecular oxygen (O_2) such as hydrogen peroxide (H_2O_2) which can diffuse into the nucleus and attack DNA, thereby contributing to genetic instability (Halliwell, 1999). The overproduction of ROS disrupts physiological cellular homeostasis and results in apoptosis via the activation of the mitochondrial pathway. It has been proven that high consumption of foods rich in natural antioxidants or foods which cause increased production of antioxidant enzymes, significantly reduces the risk of several types of cancer, including colon, breast, prostate and bladder cancers (Carmen Valadez-Vega et al., 2013). It has been proven that garlic extracts and components obtained from garlic bulbs prevent oxidative modification of DNA, proteins and lipids by scavenging ROS, increasing the expression of cellular antioxidant enzymes and enhancing glutathione levels inside normal cells (Amagase, 2006; Bhandari, 2012). As mentioned above, it is well known that in normal cells garlic increases ROS metabolizing enzymes. The mechanism of its action depends on slight stimulation of ROS production in cells (Wang et al., 2012). In contrast, many studies have also demonstrated that cancer cells exhibit an increased level of ROS, which is the effect of high metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxygenases and thymidine phosphorylase (Liou and Storz, 2010; Pelicano et al., 2004). That property makes them especially vulnerable to an additional increase in the amount of ROS. For this reason, a slight stimulation of ROS production which is beneficial for normal cells can be fatal for cancer cells. Up to date only several papers show that in cancer cell lines garlic extracts can cause ROS-dependent cell death (Avci et al., 2010; Choi and Park, 2012; Delshad et al., 2010; Yang et al., 2009).

Apoptosis is a physiologically programed mechanism, by which cells die. It is characterized by chromatin condensation, cell membrane blebbing, and DNA fragmentation. It is well known that increased intracellular ROS level triggers apoptosis by activating mitochondrial-dependent intrinsic apoptotic pathway and usually leads to activation of caspase-3. Caspase-3-dependent apoptosis, is dependent on the concentration, time of exposure and the composition of the plant extract. Garlic extract increases caspase-3 activity in the human cancer cell lines, such as hepatic (HepG2), colon (Caco-2), prostate (PC-3), and breast (MCF-7) (Bagul et al., 2015). However, authors show that in each of studied cell lines different concentrations of garlic extracts stimulated caspase-3 activity. Moreover, it has also been demonstrated that garlic extracts or garlic components such as allicin or gallic acid cause a caspase-independent cell death (De Martino et al., 2016; Ji et al., 2009; Park et al., 2005).

Squamous cell carcinomas (SCC) encompass at least 90% of all oral malignancies and The World Health Organization expects a worldwide rising oral squamous cell carcinomas (OSCC) incidence (Massano et al., 2006). According to some statistical data analysis smokers and alcohol drinkers are the group of high risk of this disease (Leite and Koifman, 1998; Ribeiro et al., 2003). However, OSCC also frequently occurs in Western societies in nonsmokers and nondrinkers. OSCC implies quite significant mortality and morbidity rates, and in spite of the vast amount of research and the advances accomplished in the field of oncology and surgery, the mortality rates remain unchanged (La Vecchia et al., 2004; Massano et al., 2006).

Therefore the aim of the present study was to determine the involvement of ROS production in the viability and apoptosis in human tongue squamous carcinoma (SCC-15) cell line after exposure to two *Allium sativum* L. cultivars, Harnaś and Morado.

2. Materials and methods

2.1. Reagents

Trypsin, penicillin, streptomycin, neutral red solution, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, hydrocortisone, Hoechst 33342, calcein AM, sodium pyruvate, sodium bicarbonate, fetal bovine serum (FBS), N-acetyl-L-cysteine (NAC), staurosporine, Ac-DEVD-CHO (caspase-3 inhibitor), hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The DMEM/F12 (1:1) medium was purchased from ATCC (Manassas, VA, USA). Caspase-3 substrate (Ac-DEVD-pNA) was purchased from Calbiochem (Merck Corporation, Darmstadt, Germany). The LDH-based cytotoxicity detection kit was purchased from Roche Applied Science (Mannheim,

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Germany). H_2DCFDA, Hoechst 33342, calcein AM and staurosporine stock solutions were prepared by dissolving the compounds in DMSO. Plant extracts were dissolved in ethanol. The final concentration of ethanol in the culture medium was always 0.1%.

2.2. Preparation of *Allium sativum* L. bulb extracts

Polish and Spanish garlic cultivars were kindly donated by Krzysztof Markiewicz from Markie-Pol company (Dąbrówka Wielka, Poland). Aqueous extracts from raw garlic were prepared according to a previously described procedure (Lemar et al., 2002). 10 g of peeled garlic cloves were mixed with 100 ml distilled water and crushed using a blender on an ice bath. Afterward the extracts were incubated for 30 min at 4 °C and centrifuged for 10 min at 3900*g*. Collected supernatants were passed through a 0.22 μm filter (Merck Millipore) to sterilize the extract. The extracts were stored in aliquots at −20 °C.

2.3. Cell culture with plant extract treatment

The human tongue squamous carcinoma cell line SCC-15 (ATCC CRL-1623) was obtained from American Type Culture Collection (ATCC, distributors: LGC Standards, Łomianki, Poland). SCC-15 cells were maintained in DMEM/F12 1:1 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate supplemented with 400 ng/mL hydrocortisone, and 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded in 96-well culture plates (Costar, St. Louis, MO, USA) at a density of 8×10^3 (for the 6 h treatment), 6×10^3 (for the 24 h treatment) or 4×10^3 (for the 48 h treatment) per well and initially cultured before the experiment for 24 h. Subsequently, the medium was changed to a fresh one with rising concentrations of extracts from two garlic cultivars (*Allium sativum* L.), the Polish cultivar Harnaś and the Spanish cultivar Morado (0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL). Additionally, after 24 h the addition of 10 μM (NAC) ROS scavenger was used to confirm the involvement of garlic extract-stimulated ROS production and to determine ROS-dependent cytotoxicity.

2.4. Measurement of reactive oxygen species formation in SCC-15

The fluorogenic dye H_2DCFDA was used to detect intracellular ROS. After diffusion into the cell, H_2DCFDA was deacetylated by cellular esterases into a non-fluorescent compound that was subsequently oxidized by ROS into 2′,7′-dichlorofluor escein (DCF) (Gomes et al., 2005). To determine the ability of the obtained extracts to induce ROS production in human squamous carcinoma cells, 10 μM H_2DCFDA was applied. The cells were incubated in H_2DCFDA in serum-free DMEM/F12 (1:1) medium for 45 min before the treatment with extracts. After 6, 24 or 48 h of incubation of the cells with plant extracts (0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL), (5% CO_2 at 37 °C), the culture medium was replaced with a fresh DMEM/F12 (1:1) medium to remove extracellular residual DCF and plant extracts to reduce the fluorescence background. As is recommended (Szychowski and Wójtowicz, 2016), we examined whether plant extracts without cells affected the fluorescence of the H_2DCFDA. Cells treated with 0.3% hydrogen peroxide (H_2O_2) was used as a positive control. DCF fluorescence was measured using a microplate reader (FilterMax F5) at maximum excitation and emission spectra of 485 nm and 535 nm, respectively.

2.5. LDH release cytotoxicity assay

The cytotoxicity detection kit is a colorimetric assay for the quantification of cell death and cell lysis based on the release of LDH from the cytosol of damaged cells into the surrounding medium. An increase in the amount of dead or plasma membrane-damaged cells results in an increase in LDH activity in the culture medium. After 6, 24 and 48 h of treatment with two extracts of garlic cultivars (0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL), 100 μL of culture supernatants were collected and incubated in the reaction mixture from the kit. After 30 min, the reaction was stopped by adding 1 N HCl, and the absorbance at a wavelength of 490 nm was measured using the FilterMax F5 Multi-Mode microplate reader (Molecular Devices, Corp., Sunnyvale, CA, USA).

2.6. Neutral red uptake cytotoxicity assay

The number of viable cells in experimental condition was evaluated using neutral red uptake test. This method is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Neutral Red Uptake cytotoxicity assay is commonly used to study the viability of *in vitro* cultured primary cells as well as cell lines of diverse origin (Repetto et al., 2008). After 6, 24 and 48 h of exposure to two extracts of garlic cultivars (0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL) the culture medium was removed and the cells were incubated for 2 h in 100 μL DMEM/F12 (1:1) containing 1% FBS and 10% neutral red. Each well was washed with 150 μL PBS and incubated with 100 μL of acidified ethanol solution (50% ethanol, 1% acetic acid, 49% H_2O) for 5 min at room temperature, on a rotating platform. The absorbance was measured at a wavelength of 540 nm using FilterMax F5 Multi-Mode microplate reader (Molecular Devices, Corp., Sunnyvale, CA, USA).

2.7. Caspase-3 activity

Caspase-3 activity was used as a marker of cell apoptosis and was assessed according to Nicholson et al. (1995). After 6, 24 and 48 h experiments with two extracts of garlic cultivars (0.062, 0.125, 0.250, 0.500 and 1.000 mg/mL), the medium was removed and cultured SCC-15 cells were lyzed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT) in 10 °C for 10 min. The lysates were incubated with the caspase-3 substrate Ac-DEVD-pNA at 37 °C. Cells treated with 1 μM staurosporine were used as a positive control. 10 μM of highly specific, potent inhibitor of caspase-3 Ac-DEVD-CHO was used to control caspase-3 assay. After 30 min, the absorbance of the lysates at 405 nm was measured using a microplate

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reader (FilterMax F5 Multi-Mode microplate reader). The amount of the colorimetric product was continuously monitored for 120 min. The data were analyzed using Multi-Mode Analysis software (Molecular Devices, Corp., Sunnyvale, CA, USA) and were normalized to the absorbance in vehicle-treated cells.

2.8. Cell staining

Hoechst 33342 and calcein AM staining was performed to measure apoptotic bodies formation and intracellular esterase activity in SCC-15 cell cultures 24 h after an initial treatment with 0.250 mg/mL of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado. Hoechst 33342 bounds to the DNA fragments and the apoptotic bodies formed, emits blue fluorescence. The living cells show esterase activities that are visualized by calcein AM as green-fluorescent light. Therefore, this staining is used to show the apoptosis, metabolism and cell viability (Szychowski et al., 2015). To block esterase activity present in the growth media, the cells were washed with PBS. The cells grown on glass cover slips were then incubated in 10 μM Hoechst 33342 and 4 μM calcein AM in PBS at 37 °C in an atmosphere of 5%/CO_2/95% for 10 min. A fluorescence microscope (LSM 700, ZEISS) was used to visualize the stained cells.

2.9. Statistical analysis

The data are presented as mean ± SEM of four independent experiments. Each treatment was repeated eight times ($N = 8$) and measured in quadruplicate; thus, the total number of replicates was 32. The average of the quadruplicate samples was used for statistical analyses. Statistical analysis was performed on the original results. Considering the different data from the measurement of fluorescence or absorbance, the results were presented as percentage of controls. The data were analyzed via one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ vs. the control cultures.

3. Results

3.1. Effect of extracts from garlic (*Allium sativum* L.) cultivars on ROS formation in SCC-15 cell line

After 6 h of exposure of SCC-15 cells to the extract from the Polish garlic (*Allium sativum* L.) cultivar Harnaś, ROS formation significantly increased compared to control in concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL (the increase compared to control by 53.38, 161.81, 255.52, and 240.99%, respectively). A similar effect was observed in cells treated with the extract from the Spanish cultivar Morado, in concentrations of 0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL (the increase compared to control by 72.48, 179.79, 207.35, 208.93, and 217.39%, respectively) (Fig. 1A).

After 24 h of exposure of SCC-15 cells to the extract from the Polish garlic cultivar Harnaś, ROS formation increased significantly compared to control in concentrations of 0.250, 0.500, and 1.000 mg/mL (increase compared to control by 236.71, 220.05, and 170.99%, respectively). In cells treated with the extract from the Spanish cultivar Morado in the concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL, and the increase in ROS formation was observed as compared to the control cells by 95.67, 53.60, 40.82, and 83.62%, respectively (Fig. 1B).

After 48 h of exposure of SCC-15 cells to the extract from the Polish garlic cultivar Harnaś, ROS formation still remained at a high level compared to the control cells in concentrations of 0.250, 0.500, and 1.000 mg/mL (the increase compared to control by 177.66, 150.22, and 157.05%, respectively). However, in the cells treated with the extract from the Spanish cultivar Harnaś, only the highest concentration (1.000 mg/mL) increased ROS formation compared to the control cells by 55.48% (Fig. 1C).

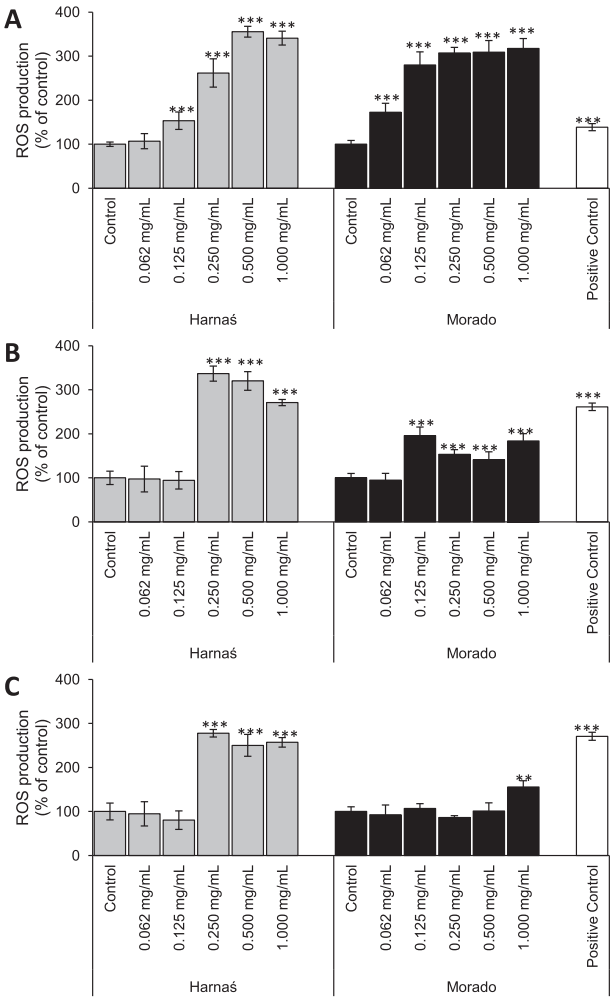

Figure 1 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on ROS formation in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). Hydrogen peroxide treated cells were used as positive control. The data are expressed as mean ± SEM of four independent experiments, each of which consisted of eight replicates per treatment group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control.

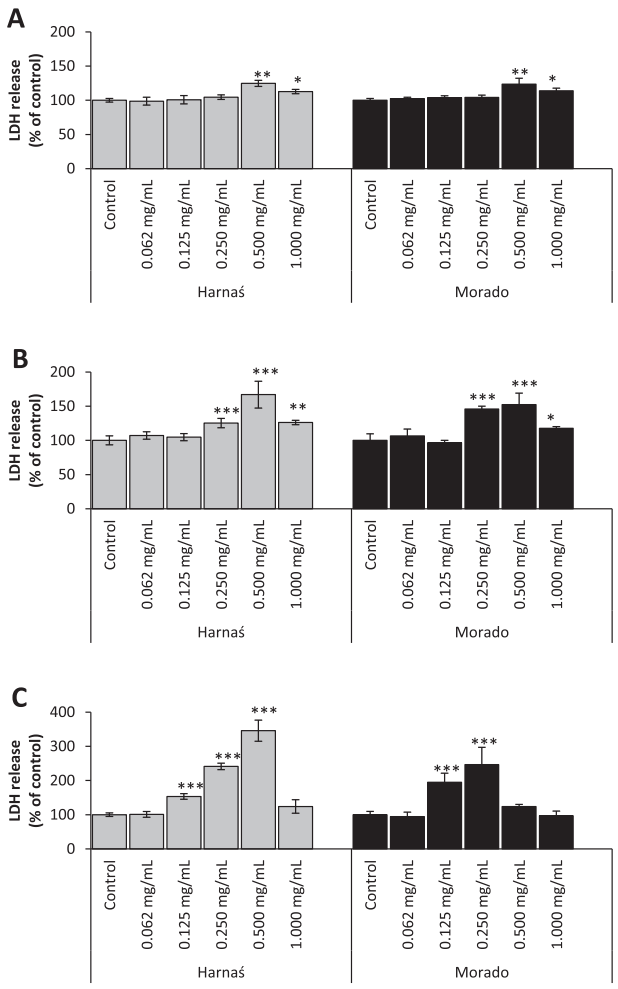

Figure 2 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the LDH release in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). The data are expressed as mean ± SEM of four independent experiments, each of which consisted of eight replicates per treatment group. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control.

Additionally, the separated experiment showed that there were no interactions between plant extracts and H_2DCFDA substrate in DMEM/F12 medium.

3.2. Effect of extracts from Allium sativum L. cultivars on the lactate dehydrogenase release in SCC-15 cell line

In SCC-15 cells, after 6 h of exposure to the extract from the Polish garlic (*Allium sativum* L.) cultivar Harnaś, LDH release increased compared to control in concentrations of 0.500, and 1.000 mg/mL (the increase compared to control by 24.65, and 12.72%, respectively). A similar effect was observed in cells treated with the extract from the Spanish cultivar Morado in concentrations of 0.500, and 1.000 mg/mL (the increase compared to control by 23.42 and 13.97%, respectively) (Fig. 2A).

After 24 h of exposure to 0.250, 0.500 and 1.000 mg/mL of the extract from the Polish garlic cultivar Harnaś, LDH release was increased compared to vehicle control (increase by 25.27, 66.89, and 26.13%, respectively). Similarly, 0.250, 0.500 and 1.000 mg/mL of the extract from the Spanish garlic cultivar Morado caused the increase in LDH release compared to the vehicle control (the increase by 45.74, 52.43, and 17.59%, respectively). (Fig. 2B).

After 48 h of exposure to 0.125, 0.250, and 0.500 mg/mL of the extract from the Polish garlic cultivar Harnaś, the LDH release was increased compared to vehicle control (the increase by 53.34, 141.21, and 245.73%, respectively). Similarly, 0.125, 0.250, and 0.500 mg/mL of the extract from the Spanish garlic cultivar Morado caused the increase in LDH release compared to the vehicle control (the increase by 95.03, 146.43, and 23.90%, respectively). (Fig. 2C).

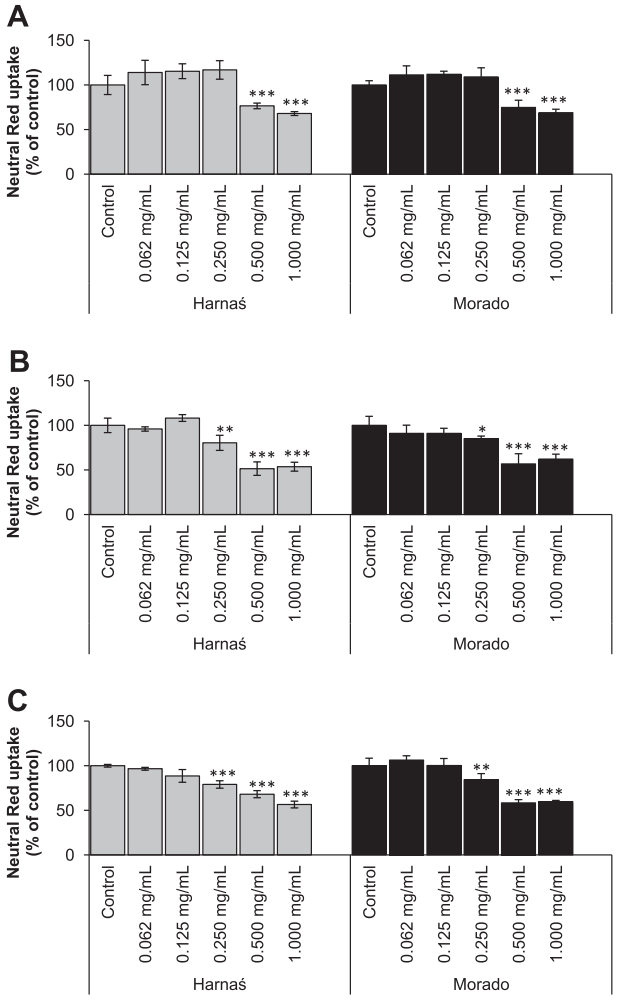

Figure 3 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the Neutral Red uptake in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). The data are expressed as mean ± SEM of four independent experiments, each of which consisted of eight replicates per treatment group. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control.

3.3. Effect of extracts from Allium sativum L. cultivars on the Neutral Red uptake in SCC-15 cell line

Neutral Red assay revealed that after 6 h of exposure only the highest concentrations of studied extracts affect the viability of SCC-15 cells. The extract from the Polish garlic cultivar Harnaś in the concentrations of 0.500 and 1.000 mg/mL, decreased the uptake of neutral red compared with vehicle control by 23.43, and 32.05%, respectively. Similar effects were observed in case of the exposure to 0.500 and 1.000 mg/mL extracts from the Spanish garlic cultivar Morado. Neutral Red uptake decreased compared to the vehicle control by 25.29, and 31.07%, respectively (Fig. 3A).

After 24 h a decrease in cell viability was induced by 0.250, 0.500 and 1.000 mg/mL of the extract from the Polish garlic cultivar Harnaś, the uptake of Neutral Red decreased compared with vehicle control by 19.61, 48.53, and 46.32%, respectively. Similar effects were observed in case of the exposure to 0.250, 0.500 and 1.000 mg/mL of the extracts from the Spanish garlic cultivar Morado. Neutral Red uptake decreased compared to the vehicle control by 14.94, 43.26, and 37.91%, respectively (Fig. 3B).

After 48 h of exposure of SCC-15 cells to 0.250, 0.500 and 1.000 mg/mL of the extract from the Polish garlic cultivar Harnaś, the uptake of Neutral Red decreased compared with vehicle control by 21.02, 31.89, and 43.50%, respectively. Similar effects were observed in exposure to 0.250, 0.500 and 1.000 mg/mL of the extract from the Spanish garlic cultivar Morado. Neutral red uptake decreased compared to the vehicle control by 15.79, 41.68, and 40.61%, respectively (Fig. 3C).

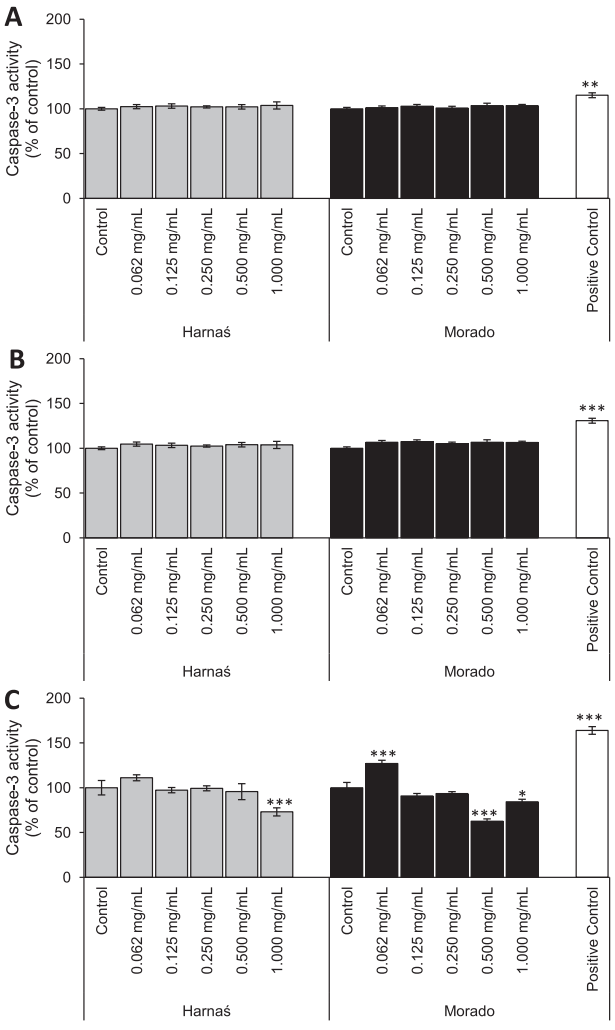

Figure 4 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the caspase-3 activity in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). Staurosporine treated cells were used as positive control. The data are expressed as mean ± SEM of four independent experiments, each of which consisted of eight replicates per treatment group. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control.

3.4. Effect of extracts from Allium sativum L. cultivars on the caspase-3 activity in SCC-15 cell line

Assay for caspase-3 activity revealed that after 6 and 24 h of exposure to both studied garlic extracts activity of caspase-3 was not affected (Fig. 4A, B).

After a 48 h treatment of SCC-15 cells with garlic extract of Polish cultivar Harnaś, 1.000 mg/mL of the extract decreased caspase-3 activity compared with the vehicle control (the decrease by 26.98%). In cells exposed to the extract from the Spanish cultivar Morado, 0.062 mg/mL, extract increased caspase-3 activity compared to control by 27.07%. However, the extracts in the concentration of 0.500 and 1.000 mg/mL caused the reduction of caspase-3 activity compared with the control by 37.50 and 15.62%, respectively (Fig. 4C).

3.5. Effect of extracts from Allium sativum L. cultivars in co-treatment with ROS scavenger NAC

After 24 h of exposure the SCC-15 cells to 0.250 mg/mL of extracts from the Polish cultivar Harnaś or the Spanish cultivar Morado the effects on the ROS production, the LDH release and Neutral Red uptake was reduced by ROS scavenger (NAC) (Fig. 5A–C). We did not observed any effect of studied garlic extracts on caspase-3 activity with or without ROS scavenger (Fig. 5D).

3.6. Effect of garlic extracts on Hoechst 33342 and calcein AM staining in SCC-15 cell cultures

To ascertain whether apoptosis was induced and to assess the viability of the cells, the human squamous carcinoma cells were stained with Hoechst 33342 and calcein AM. The apoptotic bodies appeared as bright blue fragmented nuclei that showed condensed chromatin, which is typical for apoptotic cells. Living cells had a light green-fluorescent cytoplasm. In the control cultures containing the vehicle or control with 10 μM NAC, healthy cells with intact nuclei and green-fluorescence cytoplasm were predominant (Fig. 6A–D respectively). The lack of apoptotic bodies, the lack of reduction of green fluorescence, and the reduction of cell number were observed after 24 h of the exposure to 0.250 mg/mL of the extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado (Fig. 6E, F and I, J respectively). Co-treatment with 10 μM NAC, and 0.250 mg/mL of Polish cultivar Harnaś

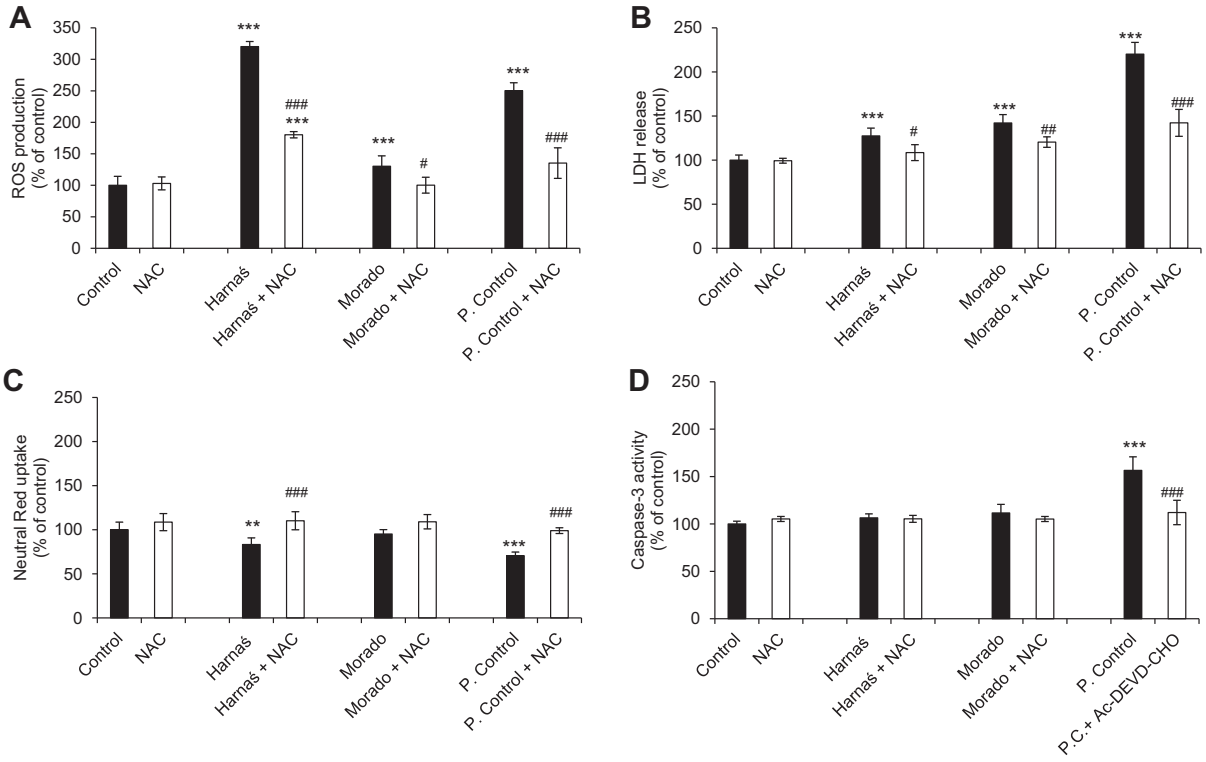

Figure 5 Effect of 0.250 mg/mL of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado with/without ROS scavenger (NAC) on the ROS production (A), LDH release (B), Neutral Red uptake (C) and caspase-3 activity (D) in SCC-15 cells after 24 h of exposure. Black bars represent cells treated with plant extracts or tool compounds alone. White bars represents cells in co-treatment with plant extracts and NAC. Hydrogen peroxide treated cells were used as positive control for ROS, LDH, Neutral Red measurements. Staurosporine treated cells were used as positive control for caspase-3 activity. 10 μM of Ac-DEVD-CHO potent inhibitor of caspase-3 was used as control in caspase-3 assay. The data are expressed as mean ± SEM of four independent experiments, each of which consisted of eight replicates per treatment group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. the cells without NAC.

or 0.250 mg/mL of Spanish cultivar Morado showed the increase in cell number and did not affect the DNA fragmentation or cell viability (Fig. 6G, H and K, L respectively).

4. Discussion

Our study for the first time examined the impact of extracts from two cultivars of garlic (*Allium sativum* L.): the Polish cultivar Harnaś and the Spanish cultivar Morado on human squamous carcinoma (SCC-15) cell line.

Since the production of ROS is considered one of the principal mechanisms of cytotoxicity, the first stage of the conducted research was to investigate the impact of extracts from two garlic cultivars on ROS production in SCC-15 cell line. The obtained results showed that both plant extracts caused a significant increase in ROS production. ROS production increased after 6 h of exposure to 0.125 mg/mL extract from the Harnaś cultivar and 0.062 mg/mL extract from the Morado cultivar. After 24 and 48 h, the ability to generate high level of ROS remained only for the Harnaś cultivar, but decreased in the case of the Morado cultivar. Up to date several papers show that in cancer cell lines such as human colon adenocarcinoma (Ht29), human leukemia (U937), human colon cancer cell line (Colo 205), and mouse chronic myelocytic leukemia (32Dp210), garlic extracts caused ROS-dependent cell death (Avci et al., 2010; Choi and Park, 2012; Delshad et al., 2010; Yang et al., 2009). On the other hand, several studies reported antioxidant properties of different garlic cultivar extracts (Narendhirakannan and Rajeswari, 2010; Othman et al., 2011). In the above mentioned papers authors used 2.2-diphenyl-1-picrylhydrazyl (DPPH•) assay to determine free radical scavenger properties of plant extracts in *in vitro* cell-free model. That model provided important data but should be considered in comparison with a cell culture model. Generally, it is believed that contents of phenolic compounds are able to scavenge endogenous cellular ROS. However, in living cells those compounds can also exhibit pro-oxidant action and in that way increase expression of ROS metabolising enzymes and protect cells for a longer period of time against ROS. Those properties of compounds from garlic extracts may play an important role in cancer therapy by activating apoptotic processes where the basal level of ROS is already very high (Hadi et al., 2000; Spencer et al., 2007). A study conducted on the SH-SY5Y cell line showed that *Allium sativum* extract itself induced ROS production but in co-administration with 6-hydroxydopamine (6-OHDA) protected cells from ROS induced by 6-OHDA (Kohda et al., 2013). Furthermore, the protective effect of garlic extracts was

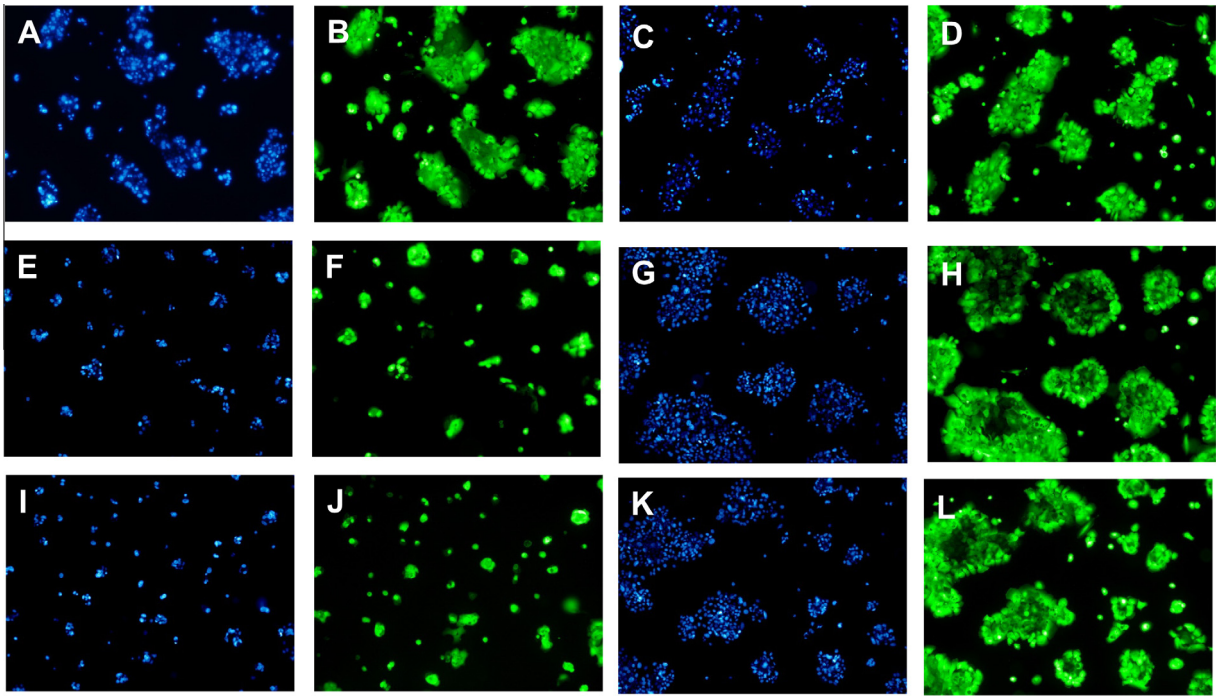

Figure 6 Effect of 0.250 mg/mL of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on Hoechst 33342 and calcein AM staining in cultures of SCC-15 cells examined 24 h post-treatment. (A) Control cells stained with Hoechst 33342; (B) Control cells stained with calcein AM; (C) Cells treated with 10 μM of NAC stained with Hoechst 33342; (D) Cells treated with 10 μM of NAC stained with calcein AM; (E) Cells treated with 0.250 mg/mL of extracts from the Polish cultivar Harnaś stained with Hoechst 33342; (F) Cells treated with 0.250 mg/mL of extracts from the Polish cultivar Harnaś stained with calcein AM; (G) Cells treated with NAC and extracts from the Polish cultivar Harnaś stained with Hoechst 33342; (H) Cells treated with NAC and extracts from the Polish cultivar Harnaś stained with calcein AM; (I) Cells treated with 0.250 mg/mL of extracts from the Spanish cultivar Morado stained with Hoechst 33342; (J) Cells treated with 0.250 mg/mL of extracts from the Spanish cultivar Morado stained with calcein AM; (K) Cells treated with NAC and extracts from the Spanish cultivar Morado stained with Hoechst 33342; (L) Cells treated with NAC and extracts from the Spanish cultivar Morado stained with calcein AM; Cells with light-colored cytoplasm were identified as live cells. Cells with bright, fragmented nuclei containing condensed chromatin were identified as apoptotic. Photomicrographs are shown at 200× magnification.

accompanied by activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway and the increase in mRNAs of heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1. The two enzymes are important in the cellular antioxidant system. Those results indicated that garlic extracts protected cells from ROS damage by not only capturing ROS directly but also activating the cellular antioxidant system by stimulating antioxidant gene expression via the Nrf2-ARE pathway (Kohda et al., 2013). Similar results are described by Peng et al. (2002) where garlic extracts protected rat pheochromocytoma (PC12) cell line by amyloid-β peptide ROS-dependent apoptosis. For now, mechanisms of the protective action of garlic extracts in normal cells are well documented in different cell culture models (Colín-González et al., 2012; Shouk et al., 2014).

To determine the type of cell death caused by garlic extracts, two different methods for detection of cytotoxicity and apoptosis were used. Our results showed that one of the most popular methods used for detection of cytotoxicity, measured by LDH release indicated that after 6 and 24 h both garlic extracts in range from 0.250 mg/mL to 1.000 mg/mL are cytotoxic. Interestingly, after 48 h garlic extracts stimulated LDH release from the lower concentration (0.125 mg/mL), while the highest concentrations of garlic extracts did not cause cytotoxic effects. The phenomenon can be explain by the fact that the highest concentrations of extracts can quickly reduce cell numbers, which was confirmed by the pictures from calcein AM and Hoechst 33342 staining. Moreover, LDH released to a culture medium by high cytotoxic concentrations of plant extracts can quickly be decomposed, which is well documented (Riss and Moravec, 2004). Another explanation, is that a high concentration of plant extracts inhibits LDH activity. For now, some studies showed that garlic extracts alone compared to control also caused increased LDH release (Ide and Lau, 2001, 1999).

The second assay for determination of cytotoxicity or proliferation (Neutral Red uptake) revealed that indeed the cells' number was decreased, which was a result of high cytotoxic properties of garlic extracts in cancer SCC-15 cells. Results similar to ours were obtained by Siegers et al. (1999) where garlic extracts inhibited cancer proliferation measured by Neutral Red assay in a concentration of 0.330 mg/mL on HepG2 cells, and 0.480 mg/mL on Caco-2 cells (Siegers et al., 1999). Due to different sensitivity of the method used, neutral red assay showed that cytotoxicity started from higher concentrations than in LDH release assay.

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As was mentioned in introduction the increased intracellular ROS level triggers apoptosis by activating mitochondrial-dependent intrinsic apoptotic pathway and usually leads to activation of caspase-3. Our results showed that both studied garlic extracts did not stimulate caspase-3 activity after 6 and 24 h of exposure. Lack of apoptosis was confirmed by Hoechst 33342 staining which did not shown apoptotic body formation. Furthermore, our results showed that only one (0.062 mg/mL) concentration of extract from the Spanish garlic cultivar Morado caused an increase in activity of caspase-3. Similar results were obtained by Su et al. (2006) who showed that concentrations of 0.0005–0.002 mg/mL of garlic extracts caused caspase-3 dependent apoptosis in Colo 205 cell line (Su et al., 2006). Moreover, Kim et al. (2012), showed that 100 μg/mL (0.1 mg/mL) of hexane extracts of garlic cloves induce apoptosis through the generation of reactive oxygen species and activation of caspase-3, caspase-8, and caspase-9 in Hep3B cells after 48 h of exposure (Kim et al., 2012). In our study the highest concentrations of extracts of both studied garlic cultivars significantly decreased caspase-3 activity. This decrease in caspase-3 activity is probably a result of high cytotoxicity of the studied extracts on human squamous carcinoma cells and reduced cell number. Similar results were reported by Avci et al. (2010) where chronic myelocytic leukemia cells (32Dp210) exposed to 0.4% garlic extract died by apoptosis, but in high concentration extracts (1%) cells died in a non-apoptotic way. Furthermore it has been proven that a range of 0.030–0.100 mg/mL of allicin, which is a major component of garlic extract, induced caspase-independent apoptosis in human gastric carcinoma cell line (Park et al., 2005). In our experiments high concentrations of garlic extract definitely contains a lot of allicin, which could be the explanation of the lack of caspase-3 activity. Similar to our results, De Martino et al. (2016) showed that the aqueous garlic extract, supplemented with copper, enhanced anti-proliferative and a caspase-independent apoptotic activity in HepG2 cancer cell line (De Martino et al., 2016).

To confirm the involvement of ROS in the garlic-induced cytotoxicity we performed experiments with NAC ROS scavenger. As previous studies indicated, ROS-dependent cell death is postponed in time (Delshad et al., 2010). Therefore for studies of mechanism of cytotoxicity and apoptosis we chose a 24-h time period. In our experiments the addition of 10 μM NAC reduced garlic-stimulated ROS production. Moreover, NAC protects SCC-15 cells by cytotoxic effects of both studied garlic extracts, which supports hypothesis that garlic extracts cause ROS-dependent cytotoxicity.

5. Conclusion

We have found that the garlic Polish cultivar Harnaś manifested more prolonged potential to stimulate ROS production in human squamous carcinoma (SCC-15) cells. Both studied garlic extracts stimulated ROS-dependent cytotoxicity on cell line SCC-15. We have determined that the highest concentrations of the studied extracts did not cause activation of caspase-3 characteristic for apoptosis. Furthermore, our results showed that the cytotoxicity caused by studied concentrations of both garlic extracts in cells SCC15 is mainly ROS-dependent.

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