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Rozprawa doktorska stanowiąca cykl publikacji pt.

Mechanizm działania ekstraktów z czosnku zwyczajnego (*Allium sativum* L.) ze szczególnym uwzględnieniem roli substancji polifenolowych i receptora aktywowanego proliferatorami peroksysomów gamma (PPARγ) w warunkach *in vitro*

Promotor
prof. dr hab. Konrad Szychowski

Rzeszów 2025

Pragnę wyrazić serdeczne podziękowania mojemu Promotorowi,
Panu ***Profesorowi Konradowi Szychowskiemu,*** *za nieocenione wsparcie merytoryczne okazywane na każdym etapie przygotowywania niniejszej rozprawy.*

Dziękuję za okazaną cierpliwość, życzliwość, a także za gotowość do dzielenia się wiedzą i doświadczeniem. Szczególnie cenię wspierającą postawę, która wielokrotnie stanowiła dla mnie źródło motywacji i inspiracji w trakcie realizacji pracy badawczej.

Spis treści

1. Wykaz stosowanych skrótów

SKRÓT	TŁUMACZENIE PL	TŁUMACZENIE EN
AKT	kinaza białkowa B	*protein kinase B*
CAT	katalaza	*catalase*
H_2DCFDA	dichlorodihydrofluoresceina diacetat	*dichlorodihydrofluorescein diacetate*
JAK2	kinaza janusowa 2	*janus kinase 2*
LC3A	mikrocząsteczkowe białko związane z autofagią 1A	*microtubule-associated proteins 1A/1B light chain 3A*
LDH	dehydrogenaza mleczanowa	*lactate dehydrogenase*
MMP	metaloproteinazy macierzy	*matrix metalloproteinase*
NF-κB	jądrowy czynnik kappa B komórek B	*nuclear factor kappa-light-chain-enhancer of activated B cells*
NRF2	jądrowy czynnik erytroidalny 2 związany z czynnikiem antyoksydacyjnym	*nuclear factor erythroid 2–related factor 2*
PI3K	kinaza 3-fosfatydyloinozytolu	*phosphoinositide 3-kinase*
PPARγ	receptor aktywowany przez proliferatory peroksysomów gamma	*peroxisome proliferator-activated receptor gamma*
ROS	reaktywne formy tlenu	*reactive oxygen species*
SOD1	dysmutaza ponadtlenkowa 1	*superoxide dismutase 1*
STAT3	przekaźnik sygnału i aktywator transkrypcji 3	*signal transducer and activator of transcription 3*
VEGF	czynnik wzrostu śródbłonka naczyniowego	*vascular endothelial growth factor*

2. Analiza bibliometryczna cyklu prac stanowiących rozprawę doktorską

1. K. A. Szychowski, **U. E. Binduga**, K. Rybczyńska-Tkaczyk, M. L. Leja, J. Gmiński. Cytotoxic effects of two extracts from garlic (*Allium sativum* L.) cultivars on the human squamous carcinoma cell line SCC-15. Saudi Journal of Biological Sciences, 2018, 25 (8): 1703-1712

   (IF_{2018}=2,280; Liczba punktów MNiSW= 25 (100) pkt.*)

2. **U. E. Binduga**, A. Kopeć, J. Skoczylas, K. A. Szychowski. Comparison of the Cytotoxic Mechanisms of Different Garlic (*Allium sativum* L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. International Journal of Molecular Sciences, 2025, 26 (1): 387

   (IF_{2023}=4,900; Liczba punktów MNiSW=140 pkt.)

3. **U. E. Binduga**, K. A. Szychowski, Current state of knowledge on the anticancer properties of polyphenolic compounds from garlic (*Allium sativum* L.) – **publikacja w recenzji**

   (IF_{2023}= - ; Liczba punktów MNiSW= - pkt.)

Sumaryczna wartość IF prac składających się na rozprawę doktorską: 7,180
Sumaryczna ilość punktów MNiSW prac składających się na rozprawę doktorską: 165 (240)* pkt. Praca w recenzji nie została uwzględniona w zestawieniu.
Przedstawione wartości wskaźnika Impact Factor (IF) odpowiadają dacie opublikowania pracy lub bazują na najnowszym, dostępnym wskaźniku IF dla danego czasopisma.

*W 2018 roku obowiązująca skala punktowa miała maksymalną wartość 50 pkt, obecnie czasopismo Saudi J Biol Sci. posiada 100 pkt

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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;
SCC-15;
Allium sativum;
Cytotoxicity;
ROS;
Apoptosis

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', 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,

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

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).

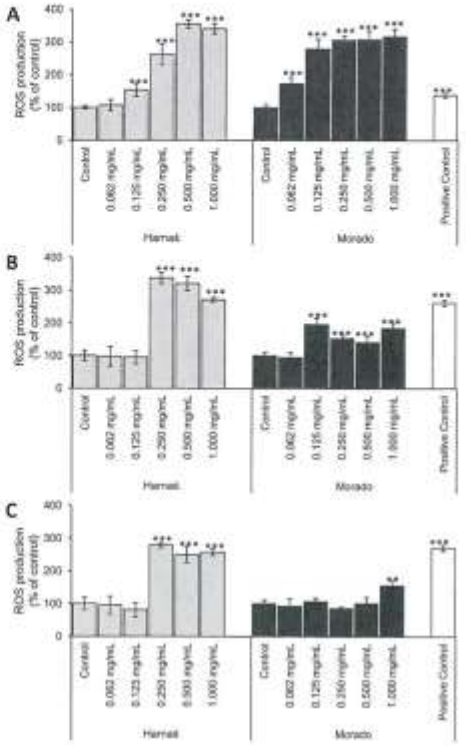

Figure 1 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on ROS formation in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). Hydrogen peroxide treated cells were used 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.

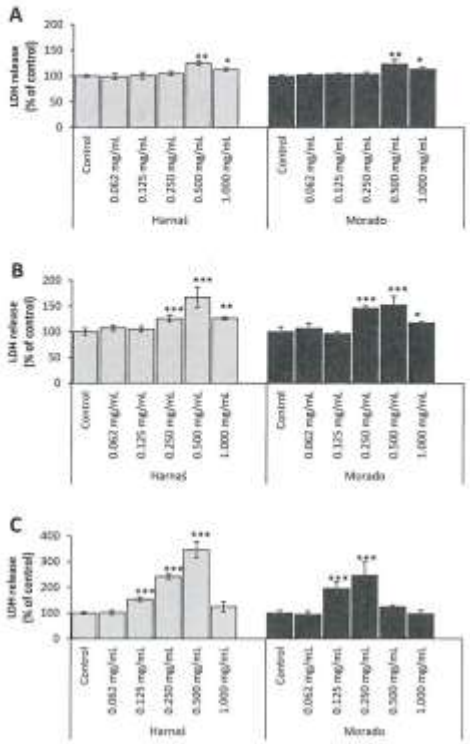

Figure 2 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the LDH release in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). The data are expressed as mean ± SEM of 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).

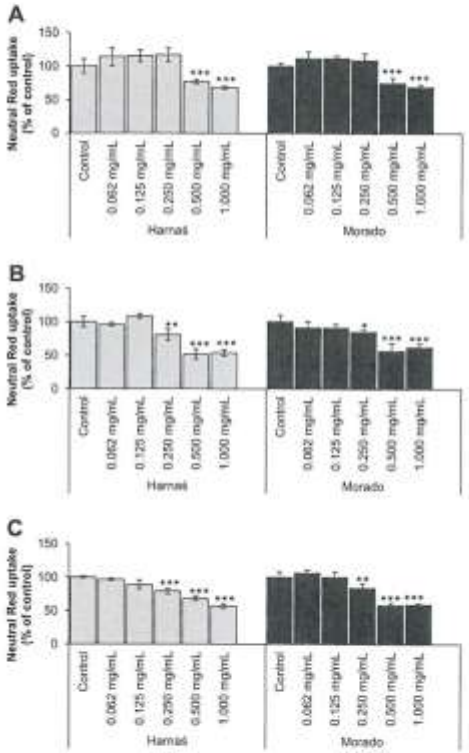

Figure 3 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the Neutral Red uptake in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). The data are expressed as mean ± 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).

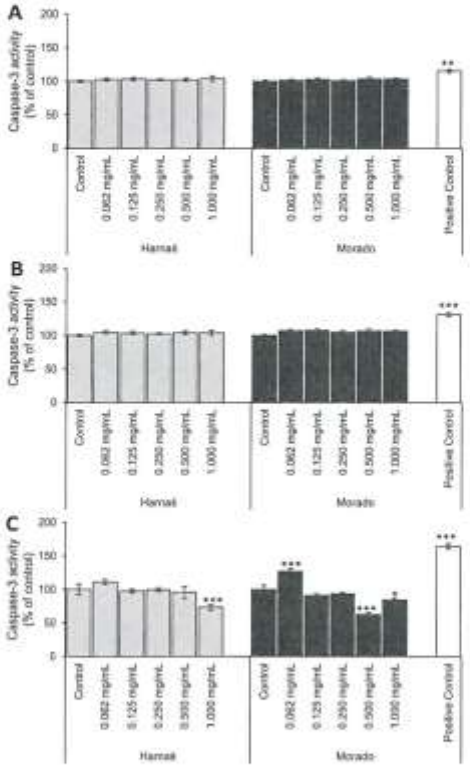

Figure 4 Effect of increasing concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on the caspase-3 activity in SCC-15 cells after 6 h (A), 24 h (B) and 48 h (C). Staurosporine treated cells were 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

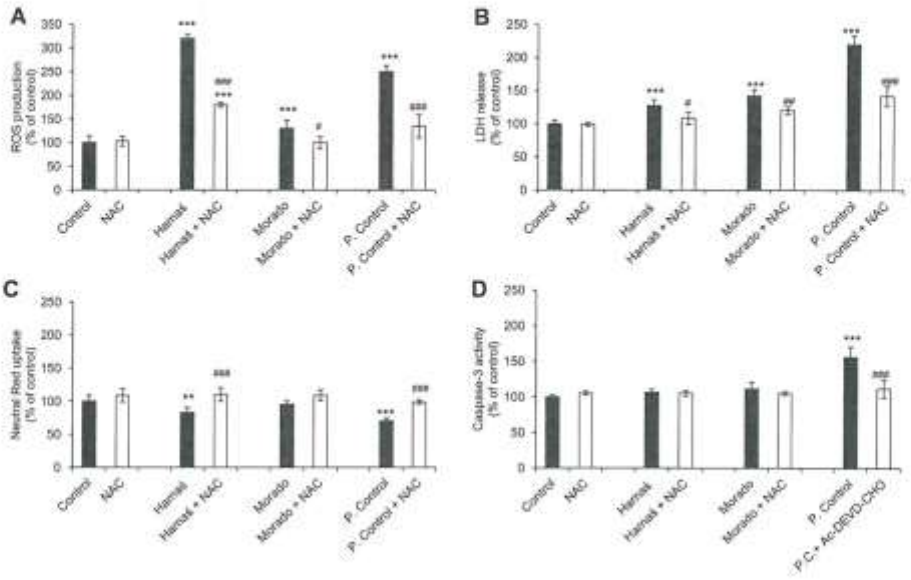

Figure 5 Effect of 0.250 mg/mL of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado with/without ROS scavenger (NAC) on the ROS production (A), LDH release (B), Neutral Red uptake (C) and caspase-3 activity (D) in SCC-15 cells after 24 h of exposure. Black bars represent cells 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 ($DPPH^{\cdot}$) 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

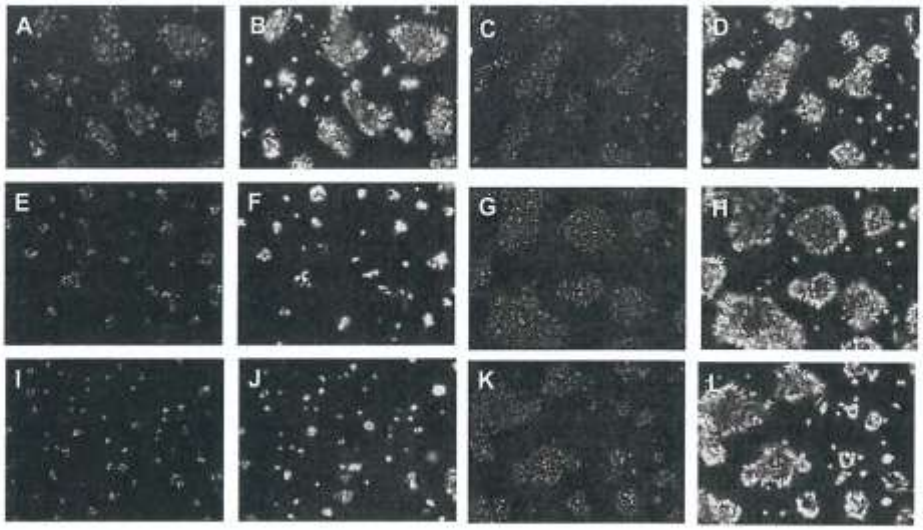

Figure 6 Effect of 0.250 mg/mL of extracts from the Polish cultivar Harnaś and the Spanish cultivar Morado on Hoechst 33342 and calcein AM staining in cultures of SCC-15 cells examined 24 h post-treatment. (A) Control cells stained with Hoechst 33342; (B) Control cells stained with calcein AM; (C) Cells 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 (Colin-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.

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.

Acknowledgments

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Publikacja 2

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Article

Comparison of the Cytotoxic Mechanisms of Different Garlic (*Allium sativum* L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma

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Abstract: Garlic (*Allium sativum* L.) is one of the oldest known useful plants, valued for thousands of years. This plant contains many biologically active compounds, including polyphenols, sterols, cysteine-sulfoxides, carbohydrates, proteins, and amino acids. The aim of our study was to compare the antioxidant potential, cytotoxicity, and apoptosis induction properties of four garlic cultivars—Harnaś, Ornak, Violeta, and Morado—in human squamous carcinoma (SCC-15) cells, colon adenocarcinoma (CACO-2) cells, and normal fibroblasts (BJ). Additionally, we investigated the mRNA and protein expression of peroxisome proliferator-activated receptor gamma (PPARγ), microtubule-associated protein 1 light chain 3 (LC3A), superoxide dismutase 1 (SOD1), and catalase (CAT) after treatment with the studied garlic extracts. Our study demonstrated that high ROS production was correlated with the strong toxicity of the garlic extracts. All studied extracts produced a lesser increase in ROS in normal BJ fibroblasts and were less toxic to these cells. The expression patterns of PPARγ, LC3A, SOD1, and CAT, along with chromatographic analysis, suggest differing mechanisms among the garlic cultivars. The highest levels of catechin, a known PPARγ agonist, were detected in the Harnaś (3.892 μg/mL) and Ornak (3.189 μg/mL) cultivars. A high catechin content was correlated with similar changes in PPARγ and related SOD1 and LC3A. Our findings showed the health-promoting and anticancer properties of garlic. However, we could not definitively identify which polyphenol or how it is involved in PPARγ activation. Further studies are required to elucidate the role of PPARγ in the mechanism of action of garlic extracts.

Keywords: *Allium sativum* L.; toxicity; PPARγ; apoptosis; catechin

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1. Introduction

Garlic (*Allium sativum* L.) is one of the oldest plants known to man, having been used for thousands of years. Currently, it is cultivated in most countries within the temperate zone [1]. Garlic, a member of the Amaryllidaceae family, is included in the group of natural food products known as 'superfoods'. This term reflects not only its value as a spice but also its wide range of health-promoting effects [2]. Garlic contains various minerals, including vitamins C and B6, as well as numerous biologically active substances [3]. Its health benefits result from bioactive compounds such as polyphenols, sterols, cysteine sulfoxides,

carbohydrates, proteins, and amino acids [4]. These compounds are responsible for garlic's potent antibacterial properties against both Gram-positive and Gram-negative bacteria, including antibiotic-resistant strains [5]. Garlic also exhibits antiviral [6], antifungal [7], and, according to some studies, selective cytotoxic properties against cancer cell lines [8]. Moreover, recent studies show that garlic extracts reduced the progression of tumours in animal models and suppressed cancer cell growth [9].

Reactive oxygen species (ROS) are natural by-products of cellular oxidative metabolism [10]. In normal cells, ROS have numerous physiological and pathological functions. However, cancer cells possess a fragile ROS balance and are highly sensitive to disturbances, a phenomenon utilised in cancer therapeutic strategies [11]. Garlic polyphenols are a major group of substances responsible for the antioxidant and health-promoting properties of this plant [12]. In normal cells, garlic polyphenols can directly affect ROS levels or indirectly influence the production of antioxidant enzymes, resulting in long-term beneficial effects [13]. Conversely, in cancerous cells, garlic extracts have been shown to induce toxicity through ROS-dependent pathways [14].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a master regulator of adipogenic differentiation and cell metabolism [15]. However, PPARγ and its ligands are also involved in cell differentiation, autophagy, antioxidant enzyme production, and apoptotic cell death [16]. Numerous studies have shown that PPARγ is essential for the proper activation of inflammatory processes and the regulation of antioxidant enzymes such as superoxide dismutase 1 (SOD1) and catalase (CAT) [16,17]. Moreover, both SOD1 and CAT are reportedly influenced by garlic extracts [17,18]. Similarly, reports suggest that biologically active compounds in garlic induce autophagic cell death involving LC3 protein in the human liver cancer (HepG2) cell line [19], a process that can also be partially regulated by PPARγ. To date, only a few studies have indicated that aged garlic extract, alliin (a garlic organosulfur compound), or garlic-derived diallyl disulfide may affect PPARγ molecular pathways in different cell culture models [20–22]. However, there is a lack of studies examining the role of PPARγ in apoptosis and toxicity after exposure of cancer cell lines to whole garlic extract, as well as comparisons with normal cell lines as controls. According to literature data, polyphenols that can be detected in garlic, such as, inter alia, catechin, quercetin, and kaempferol, can be full or partial PPARγ agonists. Therefore, it is likely that the tested extracts will activate the mentioned receptor.

The aim of our study was to compare the antioxidant potential, cytotoxicity, and apoptosis-inducing properties of four garlic cultivars in the human squamous carcinoma (SCC-15) cell line, colon adenocarcinoma (CACO-2) cell line, and normal fibroblast (BJ) cell line. Due to different growth and climate conditions, in our study the two garlic cultivars from Poland (Harnaś, Ornak) and two garlic cultivars from Spain (Violeta and Morado) were selected for analysis. We also evaluated the expression of selected genes and proteins associated with cell metabolism, free radical neutralisation, and the processes of autophagy and apoptosis.

2. Results

2.1. ROS Levels in Studied Cell Lines

Our results showed that the Harnaś garlic extract significantly increased ROS levels in BJ cells at concentrations of 0.250, 0.500, and 1.000 mg/mL, with increases of 29.8%, 184.8%, and 243.6%, respectively, compared with the control (Figure 1A). The strongest increase in ROS levels following Harnaś cultivar treatment was observed in CACO-2 cells at all tested concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL), with significant increases of 330.5%, 498.5%, 505.3%, 1566.2%, and 1255.7%, respectively, compared with the control (Figure 1E). In SCC-15 cells, a significant increase in ROS levels was observed at

concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL, although it was not as pronounced as in the CACO-2 line, with increases of 30.6%, 40.0%, 168.9%, and 355.8%, respectively, compared with the control (Figure 1I).

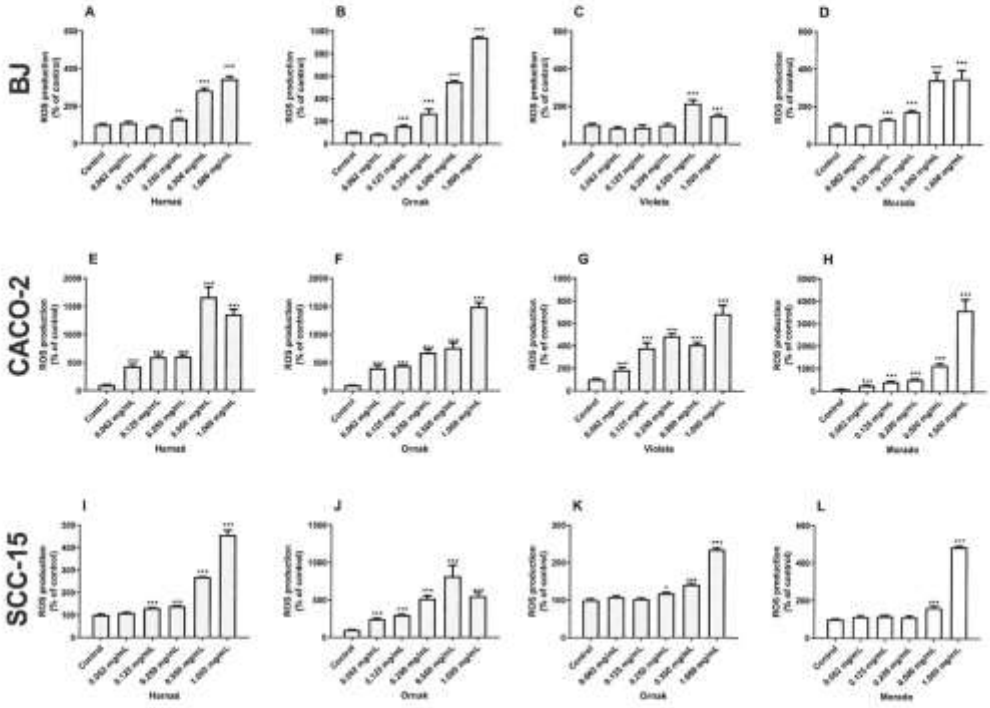

Figure 1. Effect of increasing concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on ROS production in (**A**–**D**) BJ, (**E**–**H**) CACO-2, and (**I**–**L**) SCC-15 cells after 24 h of exposure. Results are presented as mean ± SD. Statistically significant differences from the control are marked as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

In the BJ cell line, the Ornak garlic cultivar at concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL induced a significant increase in ROS production relative to the control group by 54.2%, 170.2%, 450.4%, and 841.2%, respectively (Figure 1B). In the CACO-2 cell line, the Ornak garlic cultivar significantly increased ROS production at concentrations of 0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL by 302.4%, 343.5%, 677.6%, 666.3%, and 1393.4%, respectively, compared with the control group (Figure 1F). A similar significant increase in ROS levels was observed in the SCC-15 cell line across the concentration range of 0.062 to 1.000 mg/mL, with increases of 144.8%, 197.3%, 418.9%, 715.5%, and 450.1%, respectively, compared with the control group (Figure 1J).

The Violeta garlic cultivar significantly increased ROS levels in the BJ cell line only at the two highest concentrations (0.500 and 1.000 mg/mL), with increases of 114.5% and 49.3%, respectively, compared with the control group (Figure 1C). In the CACO-2 cell line, the Violeta cultivar enhanced ROS production across the concentration range of 0.062 to 1.000 mg/mL, with significant increases of 85.9%, 279.3%, 387.5%, 308.6%, and 586.3%, respectively, compared with the control group (Figure 1G). In the SCC-15 cell line, the Violeta garlic extract increased ROS production at concentrations of 0.250, 0.500, and

1.000 mg/mL, with significant increases of 41.5%, 135.0%, and 387.5%, respectively, compared with the control group (Figure 1K).

The extract of the Morado garlic cultivar enhanced ROS production in BJ cells at concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL, with significant increases of 34.5%, 75.2%, 245.2%, and 250.6%, respectively, compared with the control (Figure 1D). In the CACO-2 cell line, the extract caused a significant increase in ROS production at all tested concentrations (0.062 to 1.000 mg/mL), with increases of 166.3%, 337.1%, 439.1%, 1067.0%, and 33520.3%, respectively, compared with the control group (Figure 1H). In the SCC-15 cell line, the Morado cultivar extract significantly increased ROS production at the highest concentrations of 0.500 and 1.000 mg/mL, with increases of 64.3% and 388.8%, respectively, compared with the control group (Figure 1L).

2.2. Level of LDH Released

The experiments showed that the Harnaś garlic cultivar significantly increased LDH release from BJ cells only at the highest concentration (1.000 mg/mL), with an increase of 16.0% compared with the control (Figure 2A). Interestingly, in the CACO-2 cell line, this garlic variety did not enhance LDH release at any tested concentration (Figure 2E). In the SCC-15 cell line, the Harnaś cultivar significantly increased LDH release at concentrations of 0.062, 0.125, and 0.250 mg/mL (40.2%, 102.3%, and 28.4%, respectively), while at 1.000 mg/mL, a decrease in LDH release was observed (7.7%) compared with the control (Figure 2I).

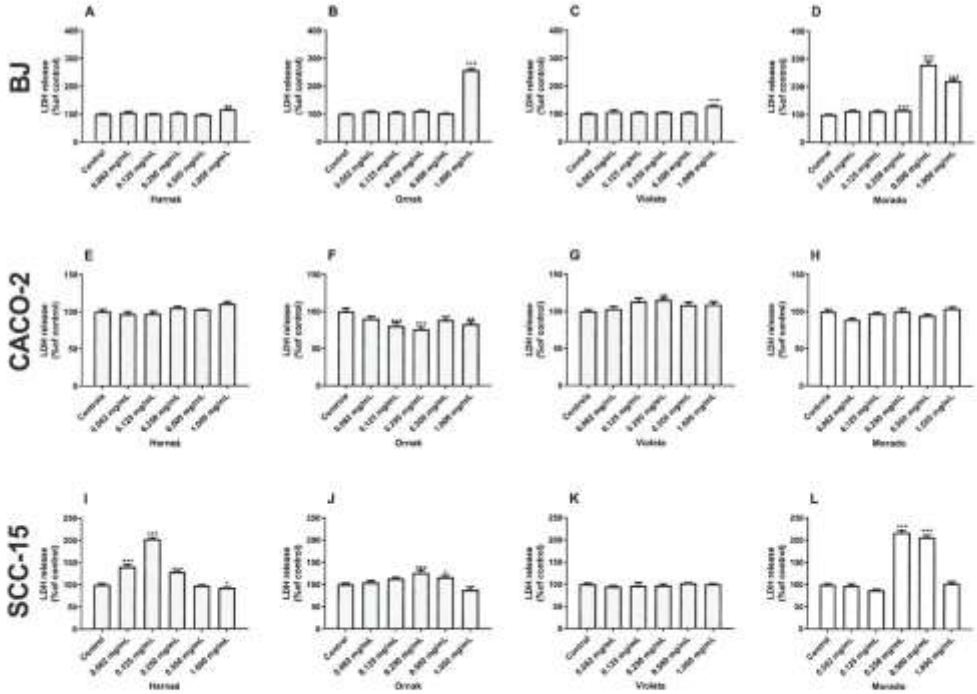

Figure 2. Effect of increasing concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on LDH release in (**A**–**D**) BJ, (**E**–**H**) CACO-2, and (**I**–**L**) SCC-15 cells after 24 h of exposure. Results are presented as mean ± SD. Statistically significant differences from the control are marked as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

For the Ornak garlic cultivar, LDH release in BJ cells increased only at the highest concentration (1.000 mg/mL), with a rise of 157.1% compared with the control (Figure 2B). Interestingly, in the CACO-2 cell line, the tested garlic extract caused a significant decrease in LDH release at concentrations of 0.125, 0.250, and 1.000 mg/mL, with reductions of 19.63%, 23.9%, and 17.7%, respectively, compared with the control (Figure 2F). In the SCC-15 cell line, the extract significantly increased LDH release at concentrations of 0.250 and 0.500 mg/mL, with rises of 25.9% and 16.1%, respectively, compared with the control (Figure 2J).

The Violeta garlic cultivar, like the Harnaś and Ornak cultivars, significantly increased LDH release in the BJ cell line only at the highest concentration (1.000 mg/mL), with an increase of 27.6% compared with the control group (Figure 2C). In the CACO-2 cell line, a concentration of 0.250 mg/mL increased LDH release by 15.3% compared with the control (Figure 2G). The Violeta garlic extract did not alter LDH release in SCC-15 cells (Figure 2K).

For the Morado garlic cultivar extract in the BJ cell line, a significant increase in LDH release was observed at concentrations of 0.250, 0.500, and 1.000 mg/mL, with increases of 75.2%, 245.2%, and 250.6%, respectively, compared with the control (Figure 2D). In the CACO-2 cell line, no changes in LDH release were observed following administration of the Morado extract (Figure 2H). In the SCC-15 cell line, the Morado extract at a concentration of 0.125 mg/mL reduced LDH release by 12.3% compared with the control group, while concentrations of 0.250 and 0.500 mg/mL increased LDH release by 117.7% and 106.6%, respectively, compared with the control (Figure 2L).

2.3. Resazurin Reduction Assay

The resazurin reduction test showed that the Harnaś garlic cultivar extract significantly increased resazurin reduction in BJ cells at a concentration of 0.062 mg/mL by 22.0% compared with the control group. However, at the highest concentration (1.000 mg/mL), a decrease in resazurin reduction of 50.2% was observed compared with the control group (Figure 3A). In CACO-2 cells, only the highest concentration of the Harnaś garlic extract (1.000 mg/mL) significantly reduced resazurin reduction by 25.9% compared with the control (Figure 3E). In SCC-15 cells, the Harnaś garlic extract reduced resazurin reduction at concentrations of 0.500 and 1.000 mg/mL by 19.7% and 89.6%, respectively, compared with the control (Figure 3I).

Garlic extract of the Ornak cultivar significantly decreased the level of resazurin reduction in the BJ cell line at concentrations of 0.125, 0.250, 0.500, and 1.000 mg/mL by 14.0%, 50.6%, 36.0%, and 97.8%, respectively, compared with the control (Figure 3B). Interestingly, in the CACO-2 cell line, the Ornak garlic extract at concentrations of 0.062, 0.125, 0.250, and 0.500 mg/mL increased the level of resazurin reduction by 48.2%, 55.2%, 58.1%, and 109.9%, respectively, compared with the control group (Figure 3F). In the SCC-15 cell line, all tested concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of the Ornak garlic extract caused a decrease in resazurin reduction by 25.2%, 59.4%, 74.8%, 75.8%, and 75.4%, respectively, compared with the control (Figure 3J).

Our experiments show that the Violeta garlic cultivar, after 24 h of exposure in the BJ cell line, significantly reduced resazurin reduction only at a concentration of 1.000 mg/mL by 52.4% compared with the control (Figure 3B). In the CACO-2 cell line, treatment with the Violeta cultivar extract did not result in any changes in resazurin reduction (Figure 3G). In the SCC-15 cell line, the Violeta extract reduced resazurin reduction at concentrations of 0.500 and 1.000 mg/mL by 41.6% and 75.3%, respectively, compared with the control (Figure 3K).

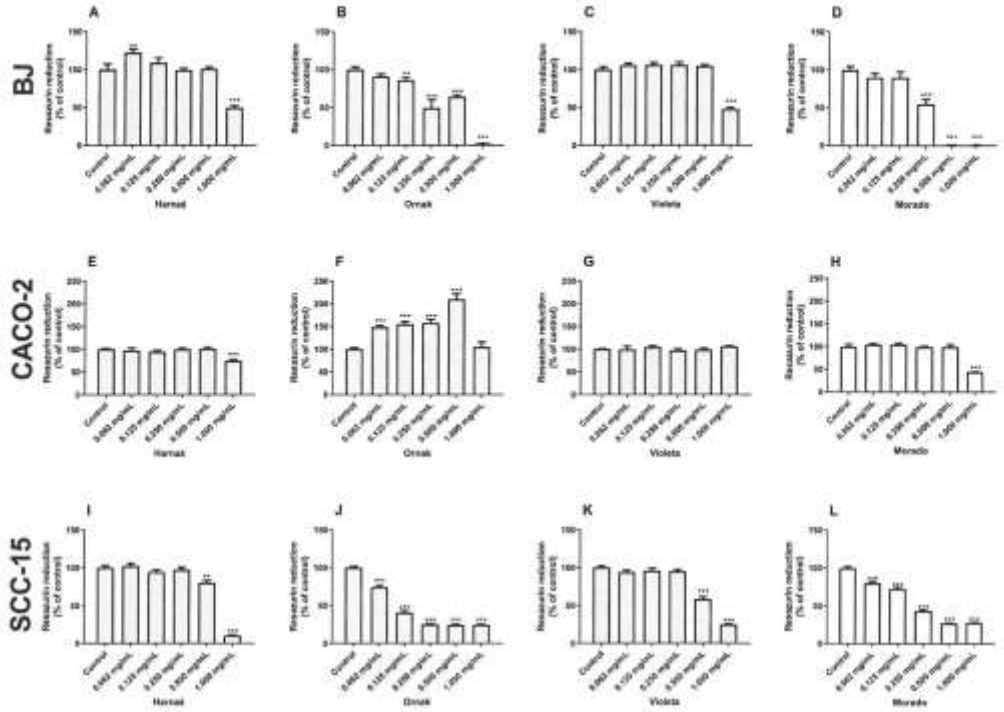

Figure 3. Effect of increasing concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on the level of resazurin reduction in (**A**–**D**) BJ, (**E**–**H**) CACO-2, and (**I**–**L**) SCC-15 cells after 24 h of exposure. Results are presented as mean ± SD. Statistically significant differences from the control are marked as ** $p < 0.01$ and *** $p < 0.001$.

The Morado garlic cultivar significantly decreased resazurin reduction in the BJ cell line at concentrations of 0.250, 0.500, and 1.000 mg/mL by 45.3%, 99.1%, and 99.0%, respectively, compared with the control (Figure 3D). In the CACO-2 cell line, the Morado cultivar extract decreased resazurin reduction only at the highest concentration (1.000 mg/mL) by 55.4% compared with the control (Figure 3H). In the SCC-15 cell line, all tested concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of the extract decreased resazurin reduction by 19.8%, 27.2%, 56.6%, 72.9%, and 72.0%, respectively, compared with the control (Figure 3L).

2.4. Caspase-3 Activity

The experiments showed that after 24 h of exposure, the Harnaś garlic extract did not affect caspase-3 activity in the BJ cell line (Figure 4A). Similarly, in the CACO-2 and SCC-15 cell lines, the Harnaś garlic extract did not alter caspase-3 activity (Figure 4E,I).

The Ornak garlic extract did not affect caspase-3 activity in the BJ and CACO-2 cell lines (Figure 4B,F). However, the extract significantly increased caspase-3 activity at all tested concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) in the SCC-15 cell line, with increases of 36.6%, 41.7%, 40.6%, 48.1%, and 35.9%, respectively, compared with the control (Figure 4J).

Our experiments showed that the Violeta garlic extract decreased caspase-3 activity at concentrations of 0.500 and 1.000 mg/mL by 27.5% and 47.9%, respectively, compared with the control (Figure 4C). In the CACO-2 cell line, the Violeta garlic extract did not

cause changes in caspase-3 activity (Figure 4G). Interestingly, in the SCC-15 cell line, a concentration of 0.500 mg/mL increased caspase-3 activity by 10.4% compared with the control, whereas a concentration of 1.000 mg/mL reduced caspase-3 activity by 17.2% (Figure 4K).

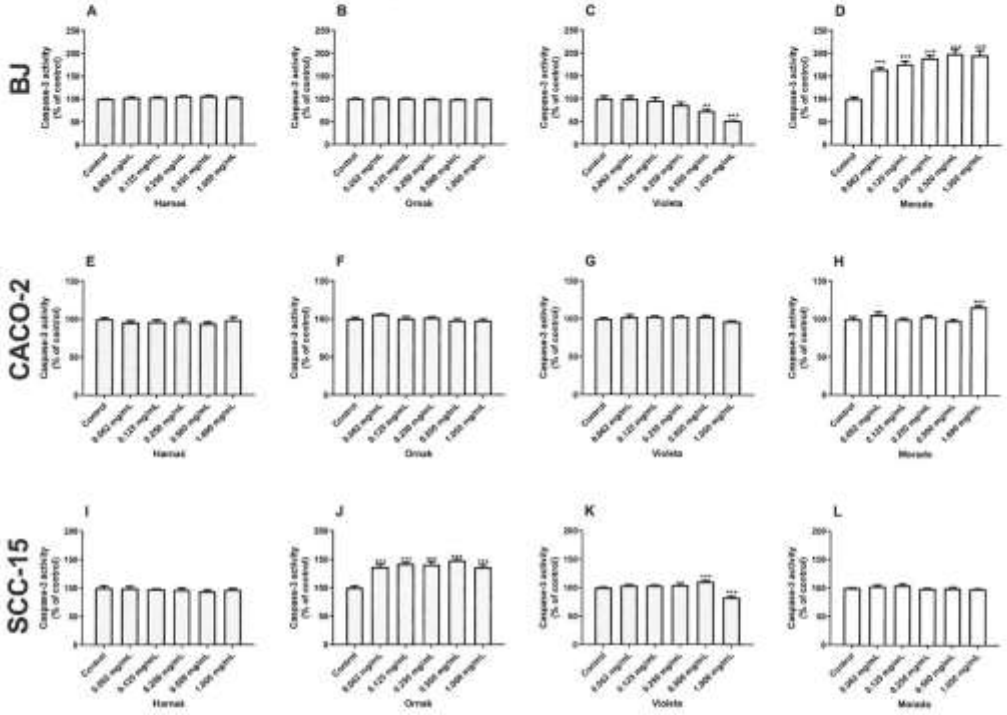

Figure 4. Effect of increasing concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on caspase-3 activity in (**A**–**D**) BJ, (**E**–**H**) CACO-2, and (**I**–**L**) SCC-15 cells after 24 h of exposure. Results are presented as mean ± SD. Statistically significant differences from the control are marked as ** $p < 0.01$ and *** $p < 0.001$.

The Morado garlic extract increased caspase-3 activity in the BJ cell line at all tested concentrations (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL) by 65.5%, 77.4%, 90.9%, 100.0%, and 96.7%, respectively, compared with the control (Figure 4D). In the CACO-2 cell line, the Morado garlic extract increased caspase-3 activity only at a concentration of 1.000 mg/mL, with a rise of 16.0% compared with the control (Figure 4H). The tested extract did not affect caspase-3 activity in the SCC-15 cell line (Figure 4L).

2.5. Gene Expression

2.5.1. PPARγ and LC3A mRNA Expression in BJ, CACO-2, and SCC-15 Cell Lines

Our experiments showed that after 6 h of exposure of BJ cells to 0.250 mg/mL of garlic extracts, the Harnaś and Ornak cultivars significantly decreased *PPARγ* mRNA expression by 44.1% and 65.4%, respectively, compared with the control. Interestingly, the Morado garlic extract significantly increased *PPARγ* mRNA expression by 41.5% compared with the control group (Figure 5A). In the CACO-2 cell line, all tested extracts reduced *PPARγ* mRNA expression: Harnaś by 20.0%, Ornak by 15.3%, Violeta by 23.2%, and Morado by

34.8%, compared with the control (Figure 5B). In the SCC-15 cell line, the Harnaś and Ornak cultivars significantly reduced *PPARγ* mRNA expression by 28.9% and 72.3%, respectively, compared with the control (Figure 5C).

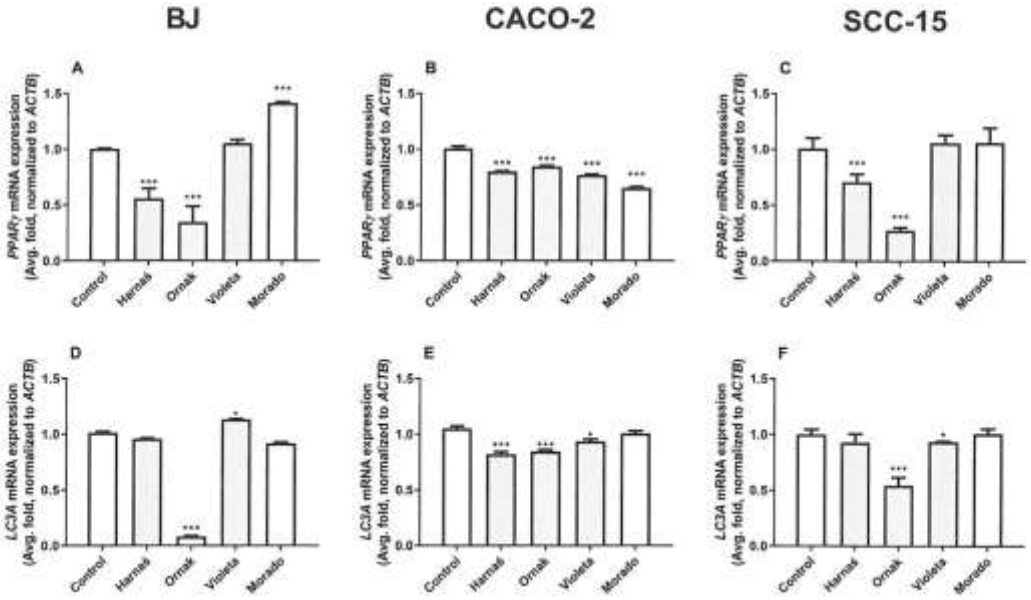

Figure 5. Effect of 0.250 mg/mL of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on mRNA expression of (**A**–**C**) *PPARγ* and (**D**–**F**) *LC3A* in (**A**,**D**) BJ, (**B**,**E**) CACO-2, and (**C**,**F**) SCC-15 cells after 6 h of exposure. *ACTB* was used as the reference gene. Results are presented as mean ± SD. Statistically significant differences from the control are marked as * $p < 0.05$ and *** $p < 0.001$.

The experiments also showed that after 6 h of exposure of BJ cells to 0.250 mg/mL of the Ornak garlic extract, *LC3A* mRNA expression was significantly decreased by 91.6% compared with the control. However, the Violeta cultivar extract slightly but statistically significantly increased *LC3A* mRNA expression by 13.5% compared with the control group (Figure 5D). In the CACO-2 cell line, exposure to 0.250 mg/mL of Harnaś, Ornak, and Violeta garlic extracts resulted in reduced *LC3A* mRNA expression, with decreases of 17.8%, 15.4%, and 6.7%, respectively, compared with the control (Figure 5E). In the SCC-15 cell line, Ornak and Violeta garlic extracts decreased *LC3A* mRNA expression by 45.3% and 6.4%, respectively, compared with the control (Figure 5F).

2.5.2. CAT and SOD1 mRNA Expression in BJ, CACO-2, and SCC-15 Cell Lines

Our experiments showed that after 6 h of exposure of BJ cells to 0.250 mg/mL of the tested extracts, the Ornak and Morado cultivars significantly decreased *CAT* mRNA expression by 70.8% and 28.4%, respectively, compared with the control (Figure 6A). In the CACO-2 cell line at the same concentration (0.250 mg/mL), extracts from the Harnaś and Violeta cultivars significantly increased *CAT* mRNA expression by 23.8% and 22.2%, respectively, compared with the control (Figure 6B). Interestingly, in the SCC-15 cell line, the Ornak cultivar extract significantly decreased *CAT* mRNA expression by 65.7%, whereas the Violeta cultivar extract increased *CAT* mRNA expression by 22.57% compared with the control (Figure 6C).

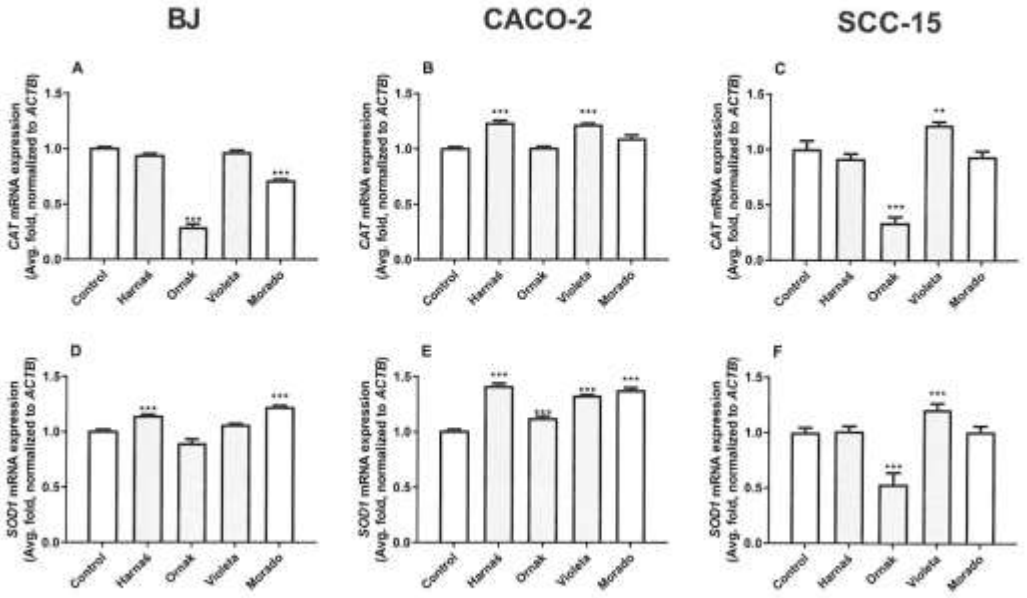

Figure 6. Effect of 0.250 mg/mL of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on mRNA expression of (**A**–**C**) *CAT* and (**D**–**F**) *SOD1* in (**A**,**D**) BJ, (**B**,**E**) CACO-2, and (**C**,**F**) SCC-15 cells after 6 h of exposure. *ACTB* was used as the reference gene. Results are presented as mean ± SD. Statistically significant differences from the control are marked as ** $p < 0.01$ and *** $p < 0.001$.

For *SOD1* mRNA expression in the BJ cell line, exposure to 0.250 mg/mL of the tested extracts showed that only the Harnaś and Morado cultivars significantly increased *SOD1* mRNA expression, by 14.6% and 22.6%, respectively, compared with the control (Figure 6D). In the CACO-2 cell line, all tested extracts at 0.250 mg/mL increased *SOD1* mRNA expression, with increases of 41.7% (Harnaś), 12.5% (Ornak), 32.9% (Violeta), and 37.8% (Morado), compared with the control (Figure 6E). In the SCC-15 cell line, the Ornak garlic extract significantly decreased *SOD1* mRNA expression by 46.3% compared with the control, while the Violeta garlic extract increased *SOD1* mRNA expression by 21.1% compared with the control (Figure 6F).

2.6. Protein Expression

2.6.1. PPARγ and LC3A Protein Expression in BJ, CACO-2, and SCC-15 Cell Lines

Our experiments showed that extracts from the Harnaś and Ornak garlic cultivars significantly increased LC3A protein expression in BJ cells by 150.5 pg/mL and 134.2 pg/mL, respectively, compared with the control. Conversely, extracts from the Violeta and Morado cultivars significantly decreased LC3A protein expression in BJ cells by 201.3 pg/mL and 122 pg/mL, respectively, compared with the control (Figure 7A). In the CACO-2 cell line, extracts from the Ornak, Violeta, and Morado cultivars reduced LC3A protein expression by 36.63 pg/mL, 111.2 pg/mL, and 85.4 pg/mL, respectively, compared with the control (Figure 7B). In the SCC-15 cell line, all tested garlic varieties strongly reduced LC3A protein expression: by 1856.0 pg/mL (Harnaś), 2039.5 pg/mL (Ornak), 2298.0 pg/mL (Violeta), and 2019.2 pg/mL (Morado), compared with the control (Figure 7C).

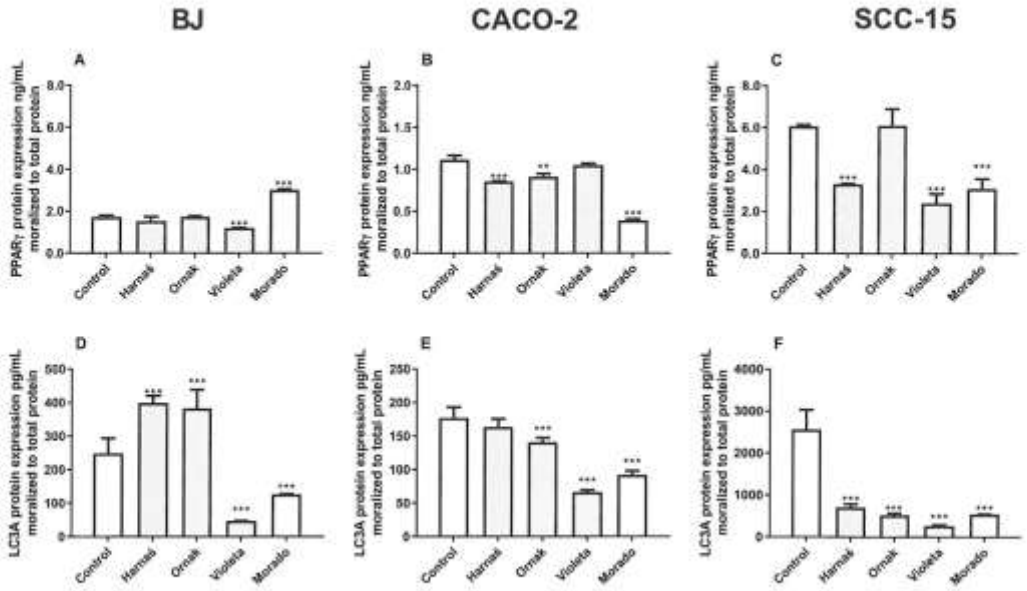

Figure 7. Effect of 0.250 mg/mL of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on protein expression of (**A**–**C**) PPARγ and (**D**–**F**) LC3A in (**A**,**D**) BJ, (**B**,**E**) CACO-2, and (**C**,**F**) SCC-15 cells after 6 h of exposure. Protein levels were normalised to total protein. Results are presented as mean ± SD. Statistically significant differences from the control are marked as ** $p < 0.01$ and *** $p < 0.001$.

After 24 h of exposure to the tested extracts in the BJ cell line, only the Violeta cultivar reduced PPARγ protein expression by 0.52 ng/mL compared with the control. Conversely, the Morado cultivar increased PPARγ protein expression by 1.3 ng/mL compared with the control (Figure 7D). In the CACO-2 cell line, a reduction in PPARγ protein expression was observed in the Harnaś, Ornak, and Morado groups, with decreases of 0.3 ng/mL, 0.2 ng/mL, and 0.7 ng/mL, respectively, compared with the control (Figure 7E). In the SCC-15 cell line, PPARγ protein expression decreased in groups treated with Harnaś, Violeta, and Morado garlic extracts, with reductions of 2.8 ng/mL, 3.7 ng/mL, and 3.0 ng/mL, respectively, compared with the control (Figure 7F).

2.6.2. CAT and SOD1 Protein Expression in BJ, CACO-2, and SCC-15 Cell Lines

Our experiments showed that after 24 h of exposure of BJ cells to the tested extracts, the Ornak, Violeta, and Morado cultivars significantly increased CAT protein expression by 8.1 ng/mL, 3.6 ng/mL, and 5.3 ng/mL, respectively, compared with the control (Figure 8A). In the CACO-2 cell line, extracts from the Harnaś and Morado cultivars decreased CAT protein expression by 7.5 ng/mL and 12.1 ng/mL, respectively, compared with the control (Figure 8B). In the SCC-15 cell line, extracts from the Harnaś and Ornak cultivars increased CAT protein expression by 5.4 ng/mL and 11.1 ng/mL, respectively, compared with the control (Figure 8C).

Our experiments also showed that all tested garlic extracts (Harnaś, Ornak, Violeta, and Morado) significantly increased SOD1 protein expression in the BJ cell line after 24 h of exposure, with increases of 22.4 pg/mL, 81.4 pg/mL, 81.5 pg/mL, and 128.9 pg/mL, respectively, compared with the control (Figure 8D). In the CACO-2 cell line, the Harnaś garlic extract decreased SOD1 protein expression by 56.06 pg/mL compared with the

control (Figure 8E). The remaining garlic extracts increased SOD1 protein expression, with increases of 47.6 pg/mL (Ornak), 152.8 pg/mL (Violeta), and 167.3 pg/mL (Morado) compared with the control (Figure 8E). Similarly, in the SCC-15 cell line, the Ornak, Violeta, and Morado garlic extracts increased SOD1 protein expression by 82.4 pg/mL, 58.6 pg/mL, and 91.1 pg/mL, respectively, compared with the control (Figure 8F).

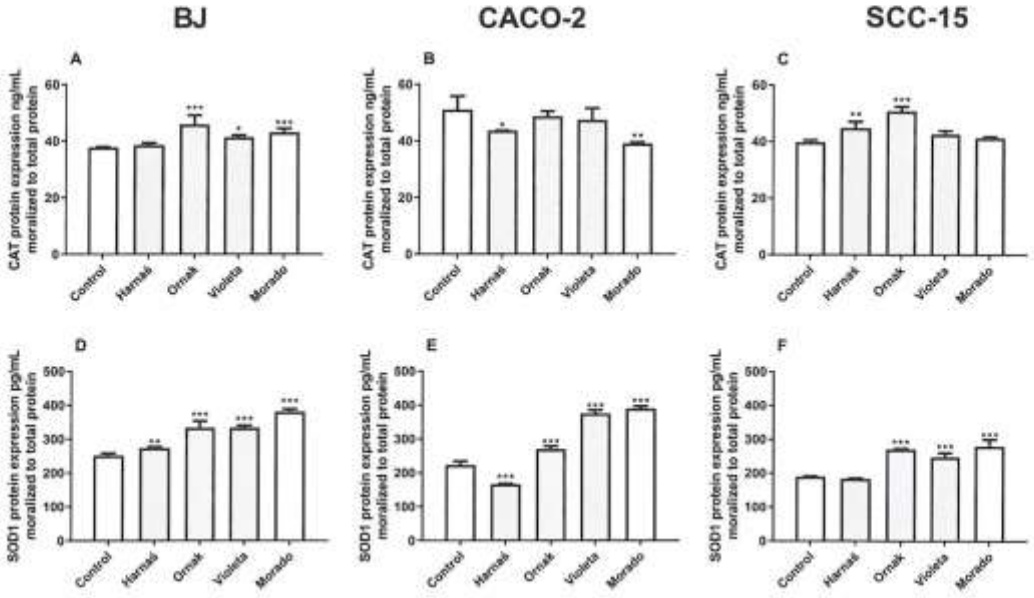

Figure 8. Effect of 0.250 mg/mL of extracts from garlic cultivars Harnaś, Ornak, Violeta, and Morado on protein expression of (**A**–**C**) CAT and (**D**–**F**) SOD1 in (**A**,**D**) BJ, (**B**,**E**) CACO-2, and (**C**,**F**) SCC-15 cells after 6 h of exposure. Protein levels were normalised to total protein. Results are presented as mean ± SD. Statistically significant differences from the control are marked as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2.7. Chromatographic Analysis of Studied Garlic Extracts

The results of the chromatographic analysis of the polyphenolic compounds are summarised in Table 1. The analysis revealed that the Harnaś cultivar is rich in apigenin, catechin, ferulic acid, isoharmentin, sinapic acid, and vanillic acid. The Ornak cultivar was rich in 4-hydroxybenzoic acid. The Violeta cultivar contained high levels of acacetin, epicatechin, hesperidin, hispidulin, myricetin, and naringenin. Finally, the Morado cultivar was the richest in kaempferol, luteolin, and rutin. In the studied extracts, quercetin and rosmarinic acid were not detected (Table 1). Statistical analysis revealed that the garlic cultivar extracts did not differ significantly in terms of the levels of 4-hydroxybenzoic acid, apigenin, epicatechin, hispidulin, kaempferol, luteolin, and naringenin. Among the cultivars, the Violeta cultivar exhibited the most distinct profile compared with the other tested cultivars.

Table 1. Results of quantitative analysis of polyphenolic compounds (presented as μg/mL± SD) in extracts obtained from garlic cultivars Harnaś, Ornak, Violeta, and Morado. Compounds identified in the extracts are listed. nd indicates compounds that were not detected. The highest values are bolded. The same letters (a, b, or c) indicate groups that do not differ statistically significantly at $p < 0.05$. If necessary, the conversion to mg/kg is given by the formula: μg/mL × (volume of solution in L)/(mass of sample in g) = mg/kg. Volume of solution = 100 mL, mass of sample = 10 g.

Compounds	Harnaś [μg/mL]	Ornak [μg/mL]	Violeta [μg/mL]	Morado [μg/mL]
4-hydroxybenzoic acid	0.181 ± 0.107 ^{a}	**0.399 ± 0.208 ^{a}**	0.185 ± 0.001 ^{a}	0.347 ± 0.245 ^{a}
Acacetin	0.091 ± 0.001 ^{a}	0.308 ± 0.036 ^{b}	**0.863 ± 0.001 ^{c}**	0.209 ± 0.103 ^{ab}
Apigenin	**0.429 ± 0.001 ^{a}**	0.298 ± 0.154 ^{a}	0.307 ± 0.173 ^{a}	0.238 ± 0.069 ^{a}
Caffeic acid	nd	0.052 ± 0.037 ^{a}	nd	0.052 ± 0.037 ^{a}
Catechin	**3.892 ± 0.016 ^{a}**	3.189 ± 1.554 ^{ab}	0.553 ± 0.006 ^{b}	nd
Chrogenic acid	nd	0.067 ± 0.047 ^{a}	nd	0.067 ± 0.047 ^{a}
Epicatechin	1.460 ± 0.013 ^{a}	1.171 ± 0.828 ^{a}	**1.775 ± 0.011 ^{a}**	1.171 ± 0.828 ^{a}
Ferulic acid	**0.055 ± 0.001 ^{a}**	nd	nd	nd
Gallic acid	nd	nd	nd	3.236 ± 2.288 ^{a}
Hesperidin	0.100 ± 0.000 ^{a}	nd	**0.065 ± 0.045 ^{a}**	nd
Hispidulin	0.418 ± 0.007 ^{a}	0.393 ± 0.005 ^{a}	**0.426 ± 0.001 ^{a}**	0.282 ± 0.161 ^{a}
Isoharmentin	**0.200 ± 0.004 ^{a}**	0.012 ± 0.016 ^{b}	nd	0.129 ± 0.074 ^{ab}
Kaempferol	0.233 ± 0.001 ^{a}	0.249 ± 0.024 ^{a}	0.245 ± 0.003 ^{a}	**0.329 ± 0.089 ^{a}**
Luteolin	0.260 ± 0.001 ^{a}	0.259 ± 0.083 ^{a}	0.348 ± 0.007 ^{a}	**0.550 ± 0.494 ^{a}**
Myricetin	0.615 ± 0.001 ^{a}	0.569 ± 0.402 ^{a}	**1.796 ± 0.086 ^{b}**	0.569 ± 0.402 ^{a}
Naringenin	0.678 ± 0.002 ^{a}	1.349 ± 0.849 ^{a}	**2.590 ± 0.005 ^{a}**	1.275 ± 0.901 ^{a}
p-Coumaric acid	0.045 ± 0.000 ^{a}	nd	nd	nd
Quercetin	nd	nd	nd	nd
Rosmarinic acid	nd	nd	nd	nd
Rutin	0.176 ± 0.007 ^{a}	nd	nd	**0.207 ± 0.293 ^{a}**
Sinapic acid	**0.085 ± 0.000 ^{a}**	nd	0.069 ± 0.001 ^{b}	nd
Syrigynic acid	nd	0.064 ± 0.045 ^{a}	0.064 ± 0.001 ^{a}	0.064 ± 0.045 ^{a}
Vanillic acid	**0.059 ± 0.000 ^{a}**	0.047 ± 0.033 ^{a}	nd	0.047 ± 0.033 ^{a}

3. Discussion

It is well documented that high levels of ROS often cause toxicity or initiate apoptosis [23,24]. Therefore, in the first part of this study, we examined the influence of extracts from the Harnaś, Ornak, Violeta, and Morado garlic cultivars on ROS production in the SCC-15, CACO-2, and BJ cell lines. In our experiments, the Ornak cultivar extract most strongly enhanced ROS production across all studied cell lines. Conversely, the Violeta cultivar induced the smallest increase in ROS production compared with the other tested garlic cultivars. It should also be noted that all tested garlic extracts minimally increased ROS production in the normal BJ cell line compared with the cancerous SCC-15 and CACO-2 cell lines. Previous studies have shown that aged garlic extract increased ROS production in human neuroblastoma SH-SY5Y cells, but when co-treated with 6-hydroxydopamine (6-OHDA), ROS production was reduced [13]. However, it is important to note that while SH-SY5Y is a neuroblastoma cell line, it retains many normal cellular mechanisms and is

often considered a model for normal cells [25]. Elevated ROS levels in cancer cells have been shown to initiate cell cycle arrest [26]. Moreover, increased ROS levels contribute to destabilisation of the cancer cell genome, leading to DNA damage and ultimately cell death via necrosis or apoptosis [27]. Ethanolic garlic extracts have been reported to induce ROS-dependent cell death in human triple-negative breast cancer cell lines (MCF-7, MDA-MB-231) without harming normal Vero cells [28]. These extracts were shown to initiate cell cycle arrest and activate apoptosis via caspase-3 involvement [28]. Similarly, Özkan et al. described that small extracellular vesicles isolated from garlic induced ROS-dependent cytotoxicity and apoptosis in human kidney carcinoma (A498) and lung carcinoma (A549) cell lines, while toxicity and apoptosis were significantly lower in normal human dermal fibroblast cell lines [29]. Our previous study demonstrated that extracts from fresh garlic cloves of the Harnaś and Morado cultivars induced ROS-dependent cytotoxicity in SCC-15 cells after 6, 24, and 48 h of exposure, without significant activation of caspase-3 [14]. Our present study also suggests that the tested garlic extracts may contribute to cancer cell death by a ROS-dependent mechanism but activate caspase-3. Differences were likely due to the distinct nature of the extracts used in the previous study.

Due to the presence of ROS in cancer cells often resulting in toxicity, the next stage of the research involved determining cytotoxicity and the impact on cell metabolic status. LDH release from cells into the culture medium was used as an indicator of cytotoxicity. As a cytosolic enzyme, LDH's presence in the culture medium effectively indicates cell membrane damage or cell death [30]. Conversely, resazurin is a water-soluble dye widely used in in vitro cell tests. This method detects the metabolic activity of cells and can indirectly suggest cell numbers, although this requires further confirmation. Moreover, resazurin is considered a more sensitive alternative to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [31]. In our experiments, the Harnaś, Ornak, and Violeta cultivars increased LDH release only at the highest extract concentration (1.000 mg/mL) in the normal BJ cell line. The extract from the Morado cultivar increased LDH release at concentrations of 0.250, 0.500, and 1.000 mg/mL, suggesting that this extract has the strongest toxicity in normal cells. In the SCC-15 cell line, only the Violeta cultivar did not cause LDH release into the culture medium. Interestingly, in the CACO-2 cell line, the Ornak cultivar decreased LDH release across a wide range of concentrations. According to the literature, a decrease in LDH release can occur in vitro with highly toxic compounds that cause rapid cell death, resulting in proteolytic degradation of LDH in the culture medium [32]. Aged garlic extract has been shown to reduce LDH release in normal bovine pulmonary artery endothelial cells, murine macrophage cells (J774), and human umbilical vein endothelial cells when initiated by various stimuli [33,34]. However, at very high concentrations, the extract alone can cause LDH release in normal cells. Conversely, in cancerous cells, garlic extract increases LDH release across a wide range of concentrations. This finding was confirmed in our previous study, which showed increased LDH release in the SCC-15 cell line at concentrations of 0.250 to 1.000 mg/mL, and in another study that showed a similar effect in the MCF-7 and MDA-MB-231 cell lines at concentrations of 0.312 to 1.000 mg/mL [14,35]. Moreover, Isbilen and Volkan reported that at the highest extract concentration, LDH release significantly decreased to levels comparable with the control [35]. Therefore, we can assume that the present data align with the current state of knowledge. In our experiments, the strongest decrease in cell metabolism, as measured by the resazurin reduction test, was observed in BJ and SCC-15 cell lines following treatment with the Ornak and Morado cultivars. In the CACO-2 cell line, the Ornak cultivar increased cell metabolism across a wide range of concentrations, as measured by the resazurin reduction test. Among all the studied cell lines, the Violeta cultivar had the least effect on cell metabolism. Our previous study demonstrated that the Harnaś and Morado cultivars, at

concentrations ranging from 0.250 to 1.000 mg/mL, decreased cell numbers in SCC-15 cells, as measured by the Neutral Red assay [14]. Similarly, Siegers et al. [36] reported that garlic extracts inhibited cancer cell proliferation, as measured by the same test, at a concentration of 0.330 mg/mL in the human hepatoma cell line (HepG2) and 0.480 mg/mL in the CACO-2 cell line. A decrease in cell viability, measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, was also observed in the A498 and A549 cell lines after treatment with small extracellular vesicles derived from garlic at concentrations ranging from 5 to 50 µg/mL [29]. Additionally, decreases in cell viability and cell numbers were reported in MCF-7 and MDA-MB-231 cells at concentrations ranging from 0.625 to 1.000 mg/mL [35].

In addition to toxicity, apoptosis is another type of cell death, with caspase-3 activity serving as its main marker. Our research shows that the studied garlic extracts had a limited effect on caspase-3 activity. In the BJ cell line, the Morado cultivar increased caspase-3 activity at all studied concentrations (0.062 to 1.000 mg/mL), while the Violeta cultivar slightly decreased caspase-3 activity at the two highest concentrations. In the CACO-2 cell line, almost no changes in caspase-3 activity were observed. In the SCC-15 cell line, no changes in caspase-3 activity were observed with the Harnaś or Morado extracts, which is consistent with our previous studies [14]. Interestingly, the Ornak cultivar increased caspase-3 activity at all studied concentrations. To date, studies conducted by other teams have been consistent in showing that garlic extracts initiate ROS-dependent cell death, although the specific type of cell death (necrosis or apoptosis) depends on factors such as extract concentration, type of extract, or garlic cultivar [29,33,34]. Previous research has shown that aged black garlic extract induces apoptosis in the human gastric cancer cell line (SGC-7901) at a concentration of 100 mg/mL [37]. Similarly, Özkan et al. reported that garlic-derived small extracellular vesicles at a concentration of 50 µg/mL increased *Caspase-3*, *Caspase-9*, and *P53* mRNA levels in A498 and A549 cell lines, confirming ROS-dependent apoptosis [29]. Apoptosis, measured by caspase-3 and caspase-9 activity, has also been confirmed in MCF-7 and MDA-MB-231 cell lines at concentrations ranging from 0.156 to 1.000 mg/mL [28,35].

It is well established that garlic is rich in various polyphenols, which contribute to its numerous health-promoting properties [38]. In addition, some studies have attempted to correlate the polyphenol content with antiproliferative properties [39]. However, it should be noted that these results are inconclusive because the authors tested entirely different garlic varieties without conducting such detailed chromatographic analyses. Our previous study linked the strong cytotoxic properties of the Morado cultivar to its suspected high polyphenol content. Several polyphenols have been described as PPARγ agonists. Particularly, (+)-catechin [40], or kaempferol, are well-known full and partial PPARγ agonists, respectively [41]. Interestingly, the current study revealed that the Morado cultivar did not contain catechins. However, some studies have shown that aged garlic extract initiates various cellular processes through PPARγ pathways [20–22]. Therefore, based on previously described findings, we selected PPARγ and enzymes related to the PPARγ pathway, such as LC3A, SOD1, and CAT, for further study. Our data showed that in the normal BJ cell line, only the Morado cultivar extract increased both *PPARγ* mRNA and PPARγ protein expression while decreasing LC3A protein expression. Interestingly, the Violeta cultivar did not significantly affect *PPARγ* or *LC3A* mRNA expression but strongly decreased PPARγ and LC3A protein expression. Both the Harnaś and Ornak cultivars acted similarly, significantly decreasing *PPARγ* mRNA expression while increasing LC3A protein expression. In the CACO-2 cell line, extracts from all studied cultivars (Harnaś, Ornak, Violeta, and Morado) similarly decreased both PPARγ and LC3A mRNA and protein expression. Our research shows that the Morado cultivar had the strongest effect

on decreasing the expression of both studied proteins. Finally, in the SCC-15 cell line, the Harnaś and Ornak cultivars decreased *PPARγ* mRNA expression, while reductions in PPARγ protein levels were observed with the Harnaś, Violeta, and Morado cultivars. Interestingly, all the studied extracts strongly decreased LC3A protein expression.

Our findings allow us to establish certain relationships. Generally, in the studied cancerous cell lines (CACO-2 and SCC-15), the garlic cultivars decreased both PPARγ and LC3A protein expression. By contrast, in the normal BJ cells, the Harnaś and Ornak extracts did not affect PPARγ protein expression but increased LC3A protein expression. The Violeta extract acted similarly to its effects in cancerous cell lines, while the Morado extract increased PPARγ protein expression but decreased LC3A protein expression. Our chromatographic analysis revealed that the Harnaś and Ornak extracts had similar polyphenolic compositions, whereas the Violeta and Morado extracts were quite distinct. We believe this may explain the differences observed in normal cells. Moreover, the highest levels of catechin, a known PPARγ agonist, were detected in the Harnaś and Ornak cultivars. Therefore, we propose that the observed changes in protein expression and toxicity, at least in the case of the Harnaś and Ornak cultivars, are likely due to PPARγ activation. Moreover, our analysis revealed that the studied extracts differed significantly in their polyphenolic compound content. Large differences in polyphenolic levels have previously been reported in endemic Italian garlic cultivars (Schiacciato, Bianco, Torella, Salomone, and Ufita), even when grown on the same test field [12]. Similarly, substantial variations in polyphenolic content were observed in 43 Chinese garlic cultivars [42]. To date, it has been described that *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, apigenin, and vanillic acid strongly decrease *Pparγ* mRNA expression in 3T3-L1 mouse preadipocytes during differentiation [43]. By contrast, hesperidin, quercetin, resveratrol, and curcumin were shown to slightly decrease *Pparγ* mRNA expression, although the effects were not statistically significant. These findings support our hypothesis.

Our study is the first that tried to correlate the polyphenolic content of garlic with changes in PPARγ protein expression. Previously, aged black garlic extract was reported to decrease *Pparγ* mRNA expression in the adipose tissue of rats [28]. Unfortunately, most research focuses on sulfur compounds derived from garlic. For instance, alliin has been shown to ameliorate gut inflammation through PPARγ pathway activation in the RAW 264.7 mouse cell line [44]. Garlic-derived diallyl disulfide has been demonstrated to increase peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) expression in a ROS-dependent manner, inducing mitochondrial biogenesis at an early stage of treatment that precedes cell cycle arrest and apoptosis in the human neuroblastoma (SH-SY5Y) cell line [22]. The increase in PGC1α expression also suggests PPARγ activation. Therefore, we propose that both polyphenolic and organosulfur compounds contribute to PPARγ activation and the modulation of related pathways.

As mentioned above, the mechanism of action of garlic and polyphenols is accompanied by an increase in ROS production. Therefore, in the last part of our work, we examined the mRNA and protein expression of antioxidant enzymes such as SOD1 and CAT, whose expression is controlled by PPARγ. Our experiments showed that the Ornak, Violeta, and Morado cultivars acted similarly in the BJ, CACO-2, and SCC-15 cell lines. These garlic cultivars increased SOD1 protein expression. Interestingly, the pattern of *SOD1* mRNA expression did not fully align with the protein expression. Minor discrepancies between mRNA and protein levels may be caused by differences in the expression intensity of the studied proteins. It is well established that mRNA should be measured earlier than protein [45]. Moreover, high mRNA expression is often accompanied by lower protein levels and vice versa, usually as a result of feedback effects. The Harnaś cultivar appears to act differently, increasing both *SOD1* mRNA and SOD1 protein expression in BJ cells.

In the CACO-2 cell line, an increase in *SOD1* mRNA expression was observed, but this was accompanied by a decrease in SOD1 protein expression. Interestingly, in the SCC-15 cell line, no significant changes were observed at either the mRNA or protein level. For CAT protein expression, the Harnaś, Ornak, Violeta, and Morado cultivars either increased or had no effect on CAT protein levels in BJ and SCC-15 cell lines. A notable observation was that the strong decrease in *CAT* mRNA expression caused by the Ornak cultivar was accompanied by an increase in CAT protein expression. An opposite phenomenon was observed in the CACO-2 cell line, where the Harnaś and Violeta cultivars increased *CAT* mRNA expression but slightly decreased CAT protein expression. To date, it has been reported that in rats, after 2 weeks on a diet containing 2% garlic, a decrease in *Cat* mRNA and CAT protein expression was observed in the kidney and liver [18]. This diet did not significantly affect SOD activity in kidney or liver tissues [18]. Similarly, a decrease in CAT activity was observed in rat liver after 7 days of treatment with fresh garlic [46]. However, the response strongly depends on the type of tissue. In rats fed fresh garlic homogenate for 30 days, cardioprotective effects were observed [47]. Garlic prevented oxidative stress and mitigated decreases in CAT and SOD activity in the rat heart induced by the cytostatic agent doxorubicin. Similar protective effects of aged garlic extract were observed in the rat liver, where aged garlic extract prevented ethephon-induced decreases in CAT and SOD activity [48]. Discrepancies between studies are likely due to differences in the garlic dose, cultivar, type of cells, and exposure duration. In normal human osteoarthritic chondrocytes, allicin, sulforaphane, and lycopene derived from garlic increased protein expression of SOD1 and CAT [49]. In human breast cancer (MCF-7) cell lines inoculated in mice, black garlic extracts were shown to increase SOD activity in the serum of the mice [37]. Additionally, 120 µM diallyl disulfide has been reported to increase *SOD1* mRNA expression in human colon cancer HT-29 cells, which, according to the authors, results in cell cycle arrest and apoptosis [50]. Therefore, we can conclude that our findings are consistent with the current state of knowledge on the impact of garlic extracts on SOD and CAT expression and activity.

4. Materials and Methods

4.1. Reagents

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), and phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) were purchased from Corning (Manassas, VA, USA). Real-time polymerase chain reaction (RT-PCR) TaqMan probes corresponding to a specific nucleotide sequence encoding—CAT (Hs00156308_m1), SOD1 (Hs00533490_m1), LC3A (Hs01076567_g1), PPARγ (Hs00234592_m1), and ACTB (Hs01060665_g1)—and a High-Capacity cDNA Reverse Transcription Kit were obtained from Life Technologies (Paisley, UK). Trypsin, penicillin, streptomycin, radioimmunoprecipitation assay buffer (RIPA), staurosporine, and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The substrate for measuring caspase-3 activity (235400) was purchased from Merck Millipore (Darmstadt, Germany). The 6-, 12-, and 96-well culture plates were purchased from TPP Techno Plastic Products AG (Trasadingen, Switzerland). The kit for determining lactate dehydrogenase (LDH) release (11644793001) was obtained from Roche Applied Science (Munich, Germany). Basic laboratory reagents, including acids, bases, salts, alcohols, and simple chemical compounds, were purchased from POCH S.A. (Gliwice, Poland). Foetal bovine serum (FBS), a Universal RNA Purification Kit (E3598-02), and quantitative PCR (qPCR) master mix (Fast Probe qPCR Master Mix) were obtained from EURx (Gdańsk, Poland). Enzyme-linked immunosorbent assay (ELISA) kits for PPARγ (H1361),

SOD1 (H1113), CAT (H0643), and LC3A were purchased from Elabscience Biotechnology (Wuhan, China).

4.2. Preparation of Extracts

The Harnaś, Ornak, Violeta, and Morado garlic cultivars were kindly donated by Krzysztof Markiewicz from the Markie-Pol company (Dąbrówka Wielka, Poland). Two varieties, Harnaś and Ornak, were grown in Poland in the Świętokrzyskie Voivodeship, while Morado and Violeta were cultivated on the Iberian Peninsula in Spain, in the commune of Las Pedroñeras. The garlic was lyophilised, powdered, and stored at −30 °C. Aqueous extracts (in PBS) were prepared from the lyophilised powder according to a previously described procedure [51]. Briefly, 10 g of lyophilised garlic powder was mixed with 100 mL of PBS and blended in an ice bath. The extracts were then incubated for 30 min at 4 °C and centrifuged for 10 min at 3900× *g*. The supernatants were collected and sterilised by passing them through a 0.22-µm filter (Merck Millipore). The extracts were stored in aliquots at −20 °C.

4.3. Cell Culture and Experiments

Human squamous cell carcinoma (SCC-15 (CRL-1623)), colon adenocarcinoma (CACO-2 (HTB-37)), and normal skin fibroblast (BJ (CRL-2522)) cell lines were obtained from the American Type Culture Collection (distributor: LGC Standards, Łomianki, Poland). The SCC-15 cell line was maintained in DMEM/F12, while the BJ and CACO-2 cell lines were maintained in MEM. The media used in the experiments were phenol red-free and contained 2.5 mM L-glutamine. Additionally, the media were supplemented with 10% FBS, and for SCC-15 cells, 400 ng/mL hydrocortisone was also included. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells were seeded into 96-well culture plates (Costar, St. Louis, MO, USA) at a density of 6×10^3 per well (for the 24 h treatment) and initially cultured for 24 h prior to the experiment. Subsequently, the medium was replaced with fresh medium containing increasing concentrations of extracts from garlic cultivars (*Allium sativum* L.)—Harnaś, Ornak, Violeta, and Morado—at 0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL for 24 h. Afterwards, analyses were performed according to the protocols described below.

4.4. ROS Measurement

In our study, the fluorogenic dye H_2DCFDA was used to detect intracellular ROS, following a previously described protocol [14]. Briefly, 5 µM H_2DCFDA was applied in serum-free medium for 45 min before treatment with the extracts. After 24 h of incubation with the garlic cultivar extracts (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL), fluorescence was measured using a microplate readerFilterMax F5 Multi Mode (Molecular Devices, Corp., Sunnyvale, CA, USA). Fluorescence was measured with excitation at 485 nm and emission at 535 nm.

4.5. LDH Release

The level of LDH release was measured on a 96-well plate after 24 h of incubation with garlic cultivar extracts (0.062, 0.125, 0.250, 0.500, and 1.000 mg/mL). The toxicity of the extracts was assessed following a previously described protocol [52]. Briefly, after exposing the cells to the extracts, the culture medium was transferred to a new 96-well plate. The plate containing the cells was frozen at −80 °C for subsequent caspase-3 activity analysis. A reaction solution was added to the medium on the new plate and incubated for 30 min at room temperature in the dark. The colorimetric product was then measured. Absorbance measurements were taken at 490 nm.

4.6. Resazurin Reduction Assay

The resazurin measurement was conducted according to a previously described protocol [53]. Briefly, the cells were incubated with increasing concentrations of garlic cultivar extracts for 6, 24, or 48 h. The medium was then replaced with fresh medium containing 10% resazurin and 1% FBS. After 60 min of incubation, fluorescence was measured. Fluorescence was measured with excitation at 530 nm and emission at 590 nm.

4.7. Caspase-3 Activity Assay

The caspase-3 activity was measured according to a previously described protocol [54] with minor modifications. Briefly, after the LDH release experiments, the 24 h medium was removed and the cells were frozen at −80 °C until measurement. After thawing, the cells were lysed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT) at 10 °C for 10 min. The lysates were then incubated with the caspase-3 substrate Ac-DEVD-pNA in the same buffer at 37 °C. After 30 min, absorbance was measured. Absorbance measurements were taken at 405 nm.

4.8. Gene Expression Analysis by RT-PCR

Cells (BJ, CACO-2, or SCC-15) were treated with 0.250 mg/mL of extracts from the Harnaś, Ornak, Violeta, and Morado garlic cultivars for 6 h. Total RNA was then extracted using the Universal RNA Purification Kit, following the manufacturer's protocol. RNA quality and quantity were assessed using a Nanodrop device. The reverse transcription reaction was performed according to the manufacturer's instructions (ThermoFisher, Waltham, MA, USA) in a final volume of 20 µL, using 800 ng of RNA as the cDNA template. RT-PCR was conducted using the Fast Probe qPCR Master Mix (2x) kit (EURx, Gdańsk, Poland) with TaqMan probes specific for the genes PPARγ, SOD1, CAT, LC3A, and ACTB in a total volume of 20 µL, including 1 µL of cDNA. The standard qPCR procedure was as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 55 °C. ACTB was used as the reference gene, and the RefFinder (http://blooge.cn/RefFinder/, access date: 14 April 2023) web-based tool was employed to evaluate reference gene expression.

4.9. ELISA

The levels of PPARγ, SOD1, CAT, and LC3A proteins were determined after 24 h of treatment with 0.250 mg/mL of extracts from the Harnaś, Ornak, Violeta, and Morado garlic cultivars via ELISA. The expression of the studied proteins was determined in cell culture lysates collected in RIPA. ELISA was performed according to the manufacturer's instructions. Briefly, a 96-well plate was pre-coated with monoclonal antibodies specific to PPARγ, SOD1, CAT, and LC3A proteins. Standards and collected cell extracts were incubated for 90 min at 37 °C in the pre-coated 96-well plate. After removing the liquid, 100 µL of biotinylated detection antibodies was added and incubated for 60 min. The plate was washed three times to remove unbound substances, and horseradish peroxidase-conjugated avidin was added. Following additional washing, in the final step, 90 µL of substrate solution was added to the wells and incubated for 15 min. Then, 50 µL of stop solution was added, and the absorbance was measured. The absorbance value was proportional to the levels of the studied proteins. Protein levels were measured and standardised using a Nanodrop device. Absorbance measurements were taken at 450 nm.

4.10. Chromatographic Determination of Biologically Active Compounds

Analysis of polyphenolic compounds in garlic extracts was performed using a Prominence-i LC 2030C D3 Plus system (Shimadzu, Kyoto, Japan) with a diode array detector and a Luna Omega 5-µm Polar C18, 100 Å, 250 × 10 mm Phenomenex column

(Torrance, CA, USA). The mobile phase flow rate was 1.2 mL/min, and the sample injection volume was 20 µL. The mobile phase consisted of two mixtures: mixture A (distilled water acidified with 0.1% formic acid) and mixture B (methanol acidified with 0.1% formic acid). Each analysis lasted 60 min and followed these gradient conditions: 20% to 40% B over 10 min, 40% B for 10 min, 40% to 50% B over 10 min, 50% to 60% B over 5 min, 60% B for 5 min, 60% to 70% B over 5 min, 70% to 90% B over 5 min, 90% B for 5 min, 90% to 20% B (initial condition) over 1 min, and 20% B for 4 min. The process was carried out at 40 °C. Polyphenolic compounds were identified at the following wavelengths: 254 nm (myricetin, quercetin, luteolin, isoharmentil), 256 nm (rutin), 260 nm (vanillalic acid), 264 nm (kaempferol) 267 nm (apigenin, acacetin) 271 nm (gallic acid), 274 nm (syringic acid), 278 nm (catechin, epicatechin), 283 nm (naringenin), 284 nm (hesperidin), 310 nm (*p*-coumaric acid), 323 nm (caffeic acid, ferulic acid, sinapinic acid), 326 nm (chlorogenic acid), and 329 nm (rosmarinic acid). Representative chromatograms have been added as a Supplementary File. A calibration curve of standards was prepared to quantify the tested compounds. Standard solutions were prepared in 70% methanol acidified with 0.1% formic acid (POCh, Katowice, Poland) at a concentration of 100 µg/mL. Stock standard solutions were diluted to obtain final concentrations in the range of 0.5 to 4.0 µg/mL. All solutions were filtered through a membrane filter (PTFE 25 mm, 0.22 µm; ChemLand, Starogard, Poland) [55]. Selected polyphenolic compounds were interpreted using LabSolution ver. 5.93 software (Shimadzu Corporation, Kyoto, Japan).

4.11. Statistical Analysis

The data were normalised to vehicle-treated controls (% of control). Results are presented as mean ± standard deviation (SD) from three independent experiments, with each treatment repeated at least three times. Statistical analysis was performed using GraphPad Prism 8 software with a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test. Statistically significant differences between individual values were denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In the case of chromatographical one-way analysis of variance (ANOVA) with Dunnett's intergroup post hoc test, the same letters (a, b, or c) indicate groups that do not differ statistically significantly at $p < 0.05$ in the analysis.

5. Conclusions

Our study demonstrated that high ROS production was correlated with the strong toxicity of the studied garlic extracts. The conducted experiments showed that all the studied extracts produced a lesser increase in ROS in normal BJ fibroblasts and were less toxic to these cells. The expression patterns of PPARγ, LC3A, SOD1, and CAT, as well as the chromatographic findings, suggest different mechanisms of action for the studied garlic cultivars (Figure 9). The current study showed the health-promoting and anticancer properties of garlic. The highest levels of catechin, a known PPARγ agonist, were detected in the Harnaś and Ornak cultivars, correlating with changes in PPARγ and LC3A protein expression. Unfortunately, we could not definitively identify which polyphenol, or how it is involved, contributes to PPARγ activation. Therefore, further studies are required to elucidate the role of PPARγ in the mechanism of action of garlic extracts.

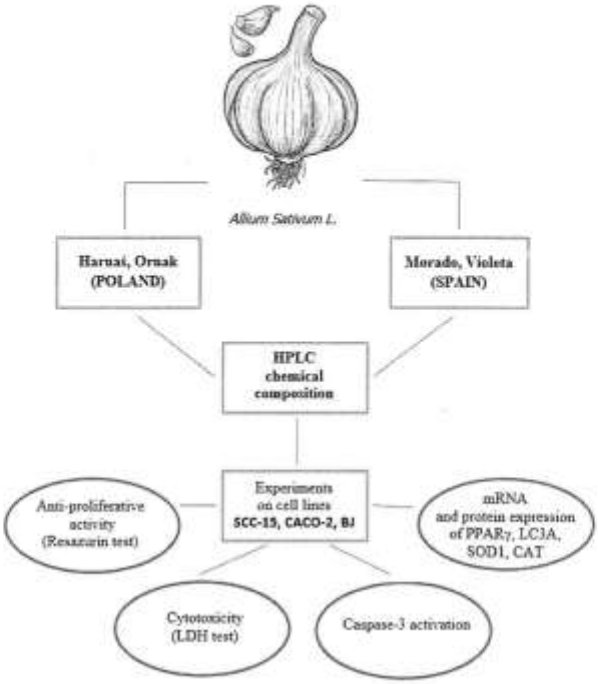

Figure 9. Proposed mechanism of action of the tested garlic extracts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms26010387/s1.

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Supplementary data

Title: Comparison of the cytotoxic mechanisms of different garlic (Allium sativum L.) cultivars with the crucial involvement of peroxisome proliferator-activated receptor gamma.

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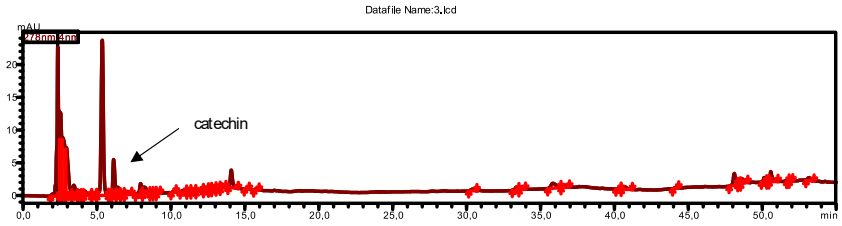
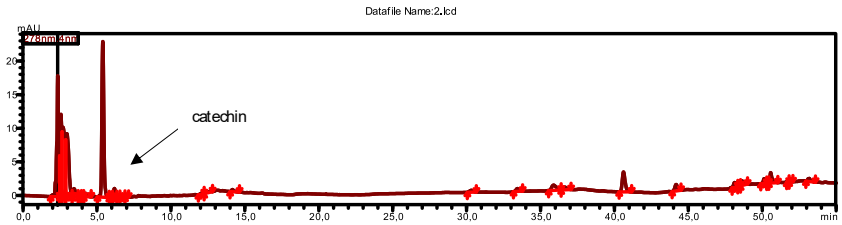
Example chromatograms from the analysis.

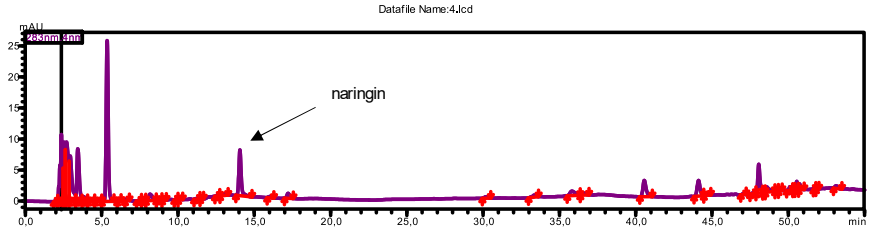
Garlic Harnaś cultivar, catechin – 6,126 – retention time



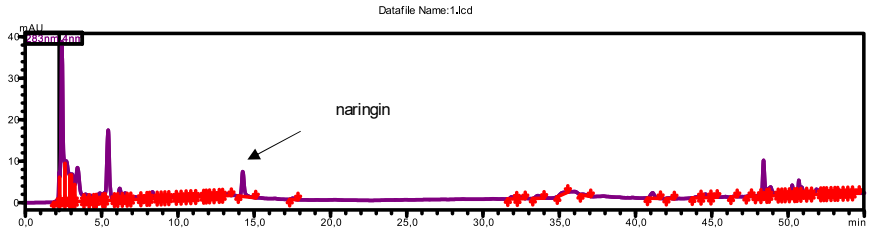
Garlic Ornak cultivar, catechin – 6,176 – retention time



Garlic Violeta cultivar, naringin – 14,058 – retention time



Garlic Morado cultivar, naringin – 14,254 – retention time



Publikacja 3

Journal of Traditional and Complementary Medicine

Current state of knowledge on the anticancer properties of polyphenolic compounds from garlic (Allium sativum L.)

--Manuscript Draft--

Manuscript Number:	EJTCM-D-25-00174R1
Article Type:	Review Article
Keywords:	Garlic; polyphenols; anticancer; PPARs; ROS
Corresponding Author:	Urszula Binduga Wyzsza Szkola Informatyki i Zarzadzania z siedziba w Rzeszowie POLAND
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	Konrad A. Szychowski, Ph.D., D.Sc., Prof.
Abstract:	Garlic (Allium sativum L.) belongs to the Allium genus and is one of the main bulbous plants consumed fresh, powdered, or cooked. Numerous studies have shown that garlic exhibits antihyperlipidaemic, antioxidant, anti-inflammatory, cardiovascular disease preventive, antihypertensive, antibacterial, antiviral, antifungal, antiparasitic, antidiabetic, anticarcinogenic, hepatoprotective, immunomodulatory, and hypoglycaemic effects. Moreover, studies on polyphenols detected in garlic reveal strong anticancer properties in various cell lines. The aim of this review is to summarise the current state of knowledge regarding the anticancer properties and shared molecular mechanisms of action of garlic-derived polyphenolic compounds. Our analysis demonstrates that the polyphenol content in garlic is highly variable and depends on numerous factors, including the part of the plant, processing methods, place of cultivation, and other conditions. Additionally, garlic is rich in anticancer polyphenols, the properties of which have been demonstrated in in vitro studies. The anticancer mechanism of action varies depending on the type of polyphenol. Several polyphenols from garlic activate peroxisome proliferator-activated receptors, which appear to contribute to at least part of garlic's anticancer activity. The primary mechanism of garlic's anticancer properties relies on reactive oxygen species-dependent toxicity and/or apoptosis, and Nrf2 is also implicated in the mechanism of action of garlic polyphenols. Our review provides evidence that under in vitro conditions, polyphenols present in garlic may exhibit anticancer properties. Garlic is not only a valuable culinary ingredient but also a natural medicine. Regular consumption in moderate amounts may offer numerous health benefits.
Opposed Reviewers:	
Response to Reviewers:	Reviewer Reviewer comment: Revision Suggestions for EJTCM-D-25-174 Thank you for submitting your manuscript to the Journal of Traditional and Complementary Medicine. Your review on the anticancer activity and molecular mechanisms of garlic-derived polyphenols addresses a highly relevant topic and offers promising value. However, after an initial evaluation, we believe that the manuscript could benefit from further development to clearly distinguish its contributions, particularly in comparison to existing literature (e.g., Molecules 2021, 26, 5028). Below are specific suggestions to enhance your manuscript: 1. Strengthen the Comparison with Existing Literature While your manuscript focuses specifically on garlic polyphenols, which is more targeted than the broader review of black garlic bioactives in the Molecules article, we suggest explicitly positioning your work as a complementary and more focused follow-up. Please consider adding a paragraph in the introduction or discussion to summarize the key contributions and limitations of prior reviews, and clarify how your study offers novel insights, particularly in mechanistic analysis. Response to the Reviewer We sincerely thank Reviewer for the time and effort devoted to reviewing our

manuscript. According to suggestion additional information has been added to introduction. "Although the literature includes broad overviews of garlic's health benefits, few focus specifically on the polyphenol-related anticancer mechanisms. For instance, Ahmed and Wang (2021) reviewed the bioactive compounds and health benefits of black garlic24. While they discussed its anticancer activity, they mostly catalogued general effects—such as antioxidant or anti-inflammatory actions—without exploring the molecular mechanisms of individual polyphenols. Similarly, Farhat et al. (2021) described various garlic preparations and their anticancer benefits, focusing primarily on organosulphur compounds and general antioxidant activity 25. A recent review by Talib et al. (2024) also concentrated on garlic's anticancer properties, mainly in the context of organosulphur constituents like allicin and ajoene. They mentioned flavonoids and phenolics only briefly26.In contrast, the present manuscript offers a novel contribution by focusing on garlic-derived polyphenols (e.g., quercetin, caffeic acid, ferulic acid) and their specific anticancer mechanisms. We highlight how these polyphenols modulate key molecular targets—such as the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) survival pathway, the nuclear factor erythroid 2–related factor 2 (Nrf2) oxidative stress response, and transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ)—to induce apoptosis, inhibit tumour proliferation, and suppress metastasis. This mechanistic depth and focused analysis of polyphenolic compounds distinguish our work from prior garlic reviews. By doing so, we aim to fill a critical gap in understanding how garlic's polyphenols exert anticancer effects at the molecular level."

Response to the Reviewer
2. Deepen Analysis of Polyphenol Classes and Mechanisms
Consider categorizing the major polyphenolic compounds (e.g., quercetin, caffeic acid, ferulic acid, etc.) and linking them to their respective anticancer pathways and molecular targets. A table format would improve clarity and readability. Strengthen the scientific foundation by citing more specific in vitro or in vivo studies that support the proposed effects. Mechanisms involving ROS, Nrf2, and PPAR activation should be visually supported with illustrative pathway diagrams showing how each compound influences these cancer-related processes.
Reviewer comment:
According to Reviewer suggestion "Table 4. Major Garlic-Derived Polyphenols and Anticancer Mechanisms" has been added. Moreover, a new figure illustrating the proposed mechanism of action of garlic polyphenols has been added to the manuscript.
PolyphenolKey Molecular Targets/PathwaysAnticancer PathwaysEvidenceKey Supporting Studies
QuercetinPI3K/Akt, NF-κB, Nrf2, JAK2/STAT3apoptosis, autophagy, cell cycle arrest, anti-angiogenesis, anti-metastasisIn vitro, In vivo135,206

Caffeic AcidNrf2/ARE, PKCδ, MAPKNrf2-mediated antioxidation, apoptosis, angiogenesis inhibitionIn vitro, In vivo207,208

Ferulic AcidCyclins/CDKs, autophagycell cycle arrest, autophagy, chemoresistance reversalIn vitro, In vivo208,209

p-Coumaric AcidGRP78/UPR, Bax/Bcl-2, NF-κBER-stress apoptosis, mitochondrial apoptosis, inflammation inhibitionIn vitro, In vivo210,211

KaempferolPI3K/Akt, MMP-2/9, p53Cell survival inhibition, apoptosis, anti-metastasis, anti-angiogenesisIn vitro, In vivo212,213

Gallic AcidJAK2/STAT3, EGFR/MAPK, ROSApoptosis, chemotherapy potentiation, ROS induction, anti-metastasisIn vitro, In vivo214,215

Response to the Reviewer
3. Improve Visual Presentation and Structure

	Rework the anticancer mechanism diagram to better highlight specific molecular pathways modulated by polyphenols, such as PI3K/Akt, MAPK, or Keap1/Nrf2, rather than showing general antioxidant activity. Including a summary table of garlic polyphenols and their biological targets/effects would enhance the practical value of the review. Reviewer comment: According to Reviewer suggestion more esthetic Figures has been added to discussion. Moreover entire new chapter "5. Detailed mechanisms of action of selected garlic-derived polyphenols" with 6 subchapters concerning detailed mechanism of action of Quercetin, Caffeic Acid, Ferulic Acid, p-Coumaric Acid, Kaempferol and Gallic Acid has been added. According to Reviewer suggestion "Table 4. Major Garlic-Derived Polyphenols and Anticancer Mechanisms" has been added. Detailed legends for figures are in the manuscript. Figure 1. Impact of garlic polyphenols on oxidative homeostasis. Figure 2. Proposed mechanism of action for garlic polyphenols. Figure 3. Mechanisms of action of the crucial polyphenols. Detailed legends for figures are in the manuscript. Reviewer comment: 4. Technical and Language Refinements Some sections of the manuscript contain long or redundant sentences. We recommend a round of language polishing to improve clarity, coherence, and conciseness. Ensure that figure legends and references follow the eJTCM style guide and formatting requirements. Summary Your review addresses a timely and important topic. With revisions focusing on comparative positioning, detailed mechanistic insights, and enhanced visual clarity, we believe the manuscript can make a significant contribution to the literature on garlic polyphenols and cancer prevention. We look forward to receiving your revised manuscript and wish you continued success in your research. Response to the Reviewer In accordance with the Reviewer's suggestions, we subjected the manuscript to linguistic proofreading with an emphasis on shortening sentences and simplifying the text.

Cover Letter

Editors-in-chief,

Rzeszów, 28.01.2025

Dear Editor,

We wish to submit a new manuscript entitled: "*Current state of knowledge on the anticancer properties of polyphenolic compounds from garlic (Allium sativum L.)*" (Urszula E. Binduga, Konrad A. Szychowski) for consideration by the "Journal of Traditional and Complementary Medicine". We confirm that this work is original and has not been published elsewhere. We have read and have abided by the statement of ethical standards for manuscripts submitted to Journal of Traditional and Complementary Medicine. All authors have approved the final article. My study does not involve human subjects. I have not submitted my manuscript to a preprint server before submitting it to Journal of Traditional and Complementary Medicine.

This review manuscript provides evidence that under *in vitro* conditions, polyphenols present in garlic may exhibit anticancer properties. Garlic is not only a valuable culinary ingredient but also a natural medicine. Regular consumption in moderate amounts may offer numerous health benefits..

Thank you for your consideration of this manuscript.

Sincerely Yours,

Urszula E. Binduga, MSc
Department of Civilization Diseases and Regenerative Medicine,
Medical College,
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Sucharskiego 2, 35-225 Rzeszow, Poland,
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Reviewer

Reviewer comment:

Revision Suggestions for EJTCM-D-25-174

Thank you for submitting your manuscript to the Journal of Traditional and Complementary Medicine. Your review on the anticancer activity and molecular mechanisms of garlic-derived polyphenols addresses a highly relevant topic and offers promising value. However, after an initial evaluation, we believe that the manuscript could benefit from further development to clearly distinguish its contributions, particularly in comparison to existing literature (e.g., Molecules 2021, 26, 5028). Below are specific suggestions to enhance your manuscript:

1. Strengthen the Comparison with Existing Literature

While your manuscript focuses specifically on garlic polyphenols, which is more targeted than the broader review of black garlic bioactives in the Molecules article, we suggest explicitly positioning your work as a complementary and more focused follow-up. Please consider adding a paragraph in the introduction or discussion to summarize the key contributions and limitations of prior reviews, and clarify how your study offers novel insights, particularly in mechanistic analysis.

Response to the Reviewer

We sincerely thank Reviewer for the time and effort devoted to reviewing our manuscript. According to suggestion additional information has been added to introduction. "Although the literature includes broad overviews of garlic's health benefits, few focus specifically on the polyphenol-related anticancer mechanisms. For instance, Ahmed and Wang (2021) reviewed the bioactive compounds and health benefits of black garlic[24]. While they discussed its anticancer activity, they mostly catalogued general effects—such as antioxidant or anti-inflammatory actions—without exploring the molecular mechanisms of individual polyphenols. Similarly, Farhat et al. (2021) described various garlic preparations and their anticancer benefits, focusing primarily on organosulphur compounds and general antioxidant activity [25]. A recent review by Talib et al. (2024) also concentrated on garlic's anticancer properties, mainly in the context of organosulphur constituents like allicin and ajoene. They mentioned flavonoids and phenolics only briefly[26].In contrast, the present manuscript offers a novel contribution by focusing on garlic-derived polyphenols (e.g., quercetin, caffeic acid, ferulic acid) and their specific anticancer mechanisms. We highlight how these polyphenols modulate key molecular targets—such as the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) survival pathway, the nuclear factor erythroid 2–related factor 2 (Nrf2) oxidative stress response, and transcription factors like nuclear factor kappa-light-chain-enhancer of

activated B cells (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ)—to induce apoptosis, inhibit tumour proliferation, and suppress metastasis. This mechanistic depth and focused analysis of polyphenolic compounds distinguish our work from prior garlic reviews. By doing so, we aim to fill a critical gap in understanding how garlic's polyphenols exert anticancer effects at the molecular level."

Response to the Reviewer

2. Deepen Analysis of Polyphenol Classes and Mechanisms

Consider categorizing the major polyphenolic compounds (e.g., quercetin, caffeic acid, ferulic acid, etc.) and linking them to their respective anticancer pathways and molecular targets. A table format would improve clarity and readability. Strengthen the scientific foundation by citing more specific in vitro or in vivo studies that support the proposed effects. Mechanisms involving ROS, Nrf2, and PPAR activation should be visually supported with illustrative pathway diagrams showing how each compound influences these cancer-related processes.

Reviewer comment:

According to Reviewer suggestion "Table 4. Major Garlic-Derived Polyphenols and Anticancer Mechanisms" has been added. Moreover, a new figure illustrating the proposed mechanism of action of garlic polyphenols has been added to the manuscript.

Polyphenol	***Key Molecular Targets/Pathways***	***Anticancer Pathways***	***Evidence***	***Key Supporting Studies***
Quercetin	PI3K/Akt, NF-κB, Nrf2, JAK2/STAT3	apoptosis, autophagy, cell cycle arrest, anti-angiogenesis, anti-metastasis	In vitro, In vivo	135,206
Caffeic Acid	Nrf2/ARE, PKCδ, MAPK	Nrf2-mediated antioxidation, apoptosis, angiogenesis inhibition	In vitro, In vivo	207,208
Ferulic Acid	Cyclins/CDKs, autophagy	cell cycle arrest, autophagy, chemoresistance reversal	In vitro, In vivo	208,209
p-Coumaric Acid	GRP78/UPR, Bax/Bcl-2, NF-κB	ER-stress apoptosis, mitochondrial apoptosis, inflammation inhibition	In vitro, In vivo	210,211

Kaempferol	PI3K/Akt, MMP-2/9, p53	Cell survival inhibition, apoptosis, anti-metastasis, anti-angiogenesis	In vitro, In vivo	212,213
Gallic Acid	JAK2/STAT3, EGFR/MAPK, ROS	Apoptosis, chemotherapy potentiation, ROS induction, anti-metastasis	In vitro, In vivo	214,215

Response to the Reviewer

3. Improve Visual Presentation and Structure

Rework the anticancer mechanism diagram to better highlight specific molecular pathways modulated by polyphenols, such as PI3K/Akt, MAPK, or Keap1/Nrf2, rather than showing general antioxidant activity. Including a summary table of garlic polyphenols and their biological targets/effects would enhance the practical value of the review.

Reviewer comment:

According to Reviewer suggestion more esthetic Figures has been added to discussion. Moreover entire new chapter "5. Detailed mechanisms of action of selected garlic-derived polyphenols" with 6 subchapters concerning detailed mechanism of action of Quercetin, Caffeic Acid, Ferulic Acid, p-Coumaric Acid, Kaempferol and Gallic Acid has been added. According to Reviewer suggestion "Table 4. Major Garlic-Derived Polyphenols and Anticancer Mechanisms" has been added. Detailed legends for figures are in the manuscript.

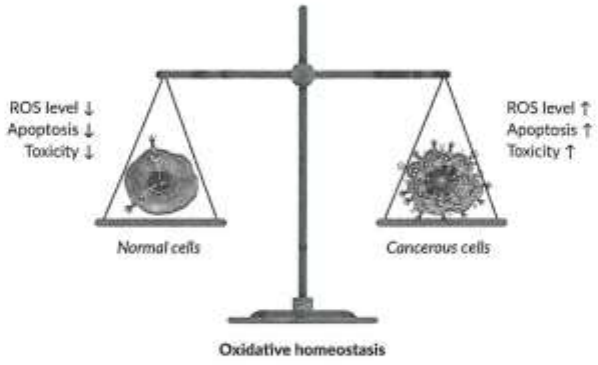


Figure 1. Impact of garlic polyphenols on oxidative homeostasis.

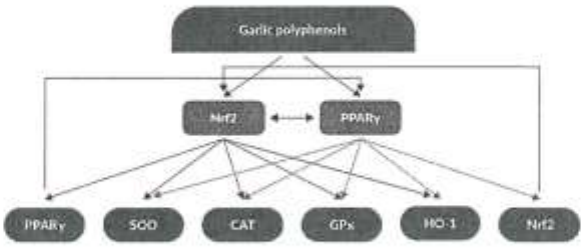


Figure 2. Proposed mechanism of action for garlic polyphenols.

Figure 3. Mechanisms of action of the crucial polyphenols.

Detailed legends for figures are in the manuscript.

Reviewer comment:

4. Technical and Language Refinements

Some sections of the manuscript contain long or redundant sentences. We recommend a round of language polishing to improve clarity, coherence, and conciseness. Ensure that figure legends and references follow the eJTCM style guide and formatting requirements.

Summary

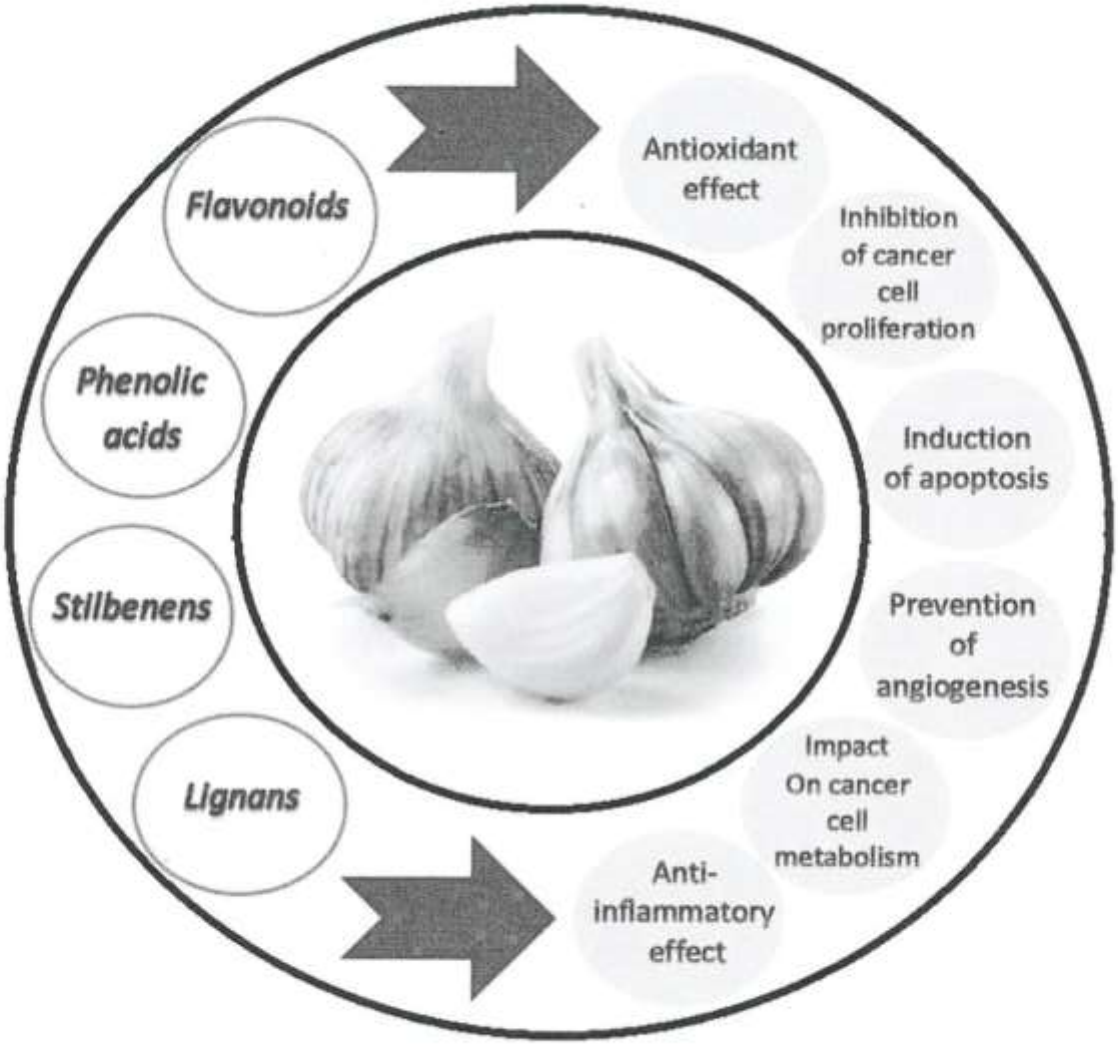
Your review addresses a timely and important topic. With revisions focusing on comparative positioning, detailed mechanistic insights, and enhanced visual clarity, we believe the manuscript can make a significant contribution to the literature on garlic polyphenols and cancer prevention. We look forward to receiving your revised manuscript and wish you continued success in your research.

Response to the Reviewer

In accordance with the Reviewer's suggestions, we subjected the manuscript to linguistic proofreading with an emphasis on shortening sentences and simplifying the text.

Graphical Abstract

Click here to access/download;Graphical Abstract;Abstract graph.jpg



Title: Current state of knowledge on the anticancer properties of polyphenolic compounds from garlic (*Allium sativum* L.)

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Keywords: garlic; polyphenols; anticancer; PPARs; ROS; Nrf2

Highlights

- Numerous polyphenols from garlic exhibit anticancer properties.
- The anticancer mechanism of action varies depending on the type of polyphenol.
- PPARs appear to play a role in the anticancer activity of several compounds.
- The major mechanism of garlic polyphenols' anticancer properties depends on ROS.
- Nrf2 is involved in the mechanism of action of garlic polyphenols.

Abstract

Garlic (*Allium sativum* L.) belongs to the *Allium* genus and is one of the main bulbous plants consumed fresh, powdered, or cooked. Numerous studies have shown that garlic exhibits antihyperlipidaemic, antioxidant, anti-inflammatory, cardiovascular disease preventive, antihypertensive, antibacterial, antiviral, antifungal, antiparasitic, antidiabetic, anticarcinogenic, hepatoprotective, immunomodulatory, and hypoglycaemic effects. Moreover, studies on polyphenols detected in garlic reveal strong anticancer properties in various cell lines. The aim of this review is to summarise the current state of knowledge regarding the anticancer properties and shared molecular mechanisms of action of garlic-derived polyphenolic compounds. Our analysis demonstrates that the polyphenol content in garlic is highly variable and depends on numerous factors, including the part of the plant, processing methods, place of cultivation, and other conditions. Additionally, garlic is rich in anticancer polyphenols, the properties of which have been demonstrated in *in vitro* studies. The anticancer mechanism of action varies depending on the type of polyphenol. Several polyphenols from garlic activate peroxisome proliferator-activated receptors, which appear to contribute to at least part of garlic's anticancer activity. The primary mechanism of garlic's anticancer properties relies on reactive oxygen species-dependent toxicity and/or apoptosis, and Nrf2 is also implicated in the mechanism of action of garlic polyphenols. Our review provides evidence that under *in vitro* conditions, polyphenols present in garlic may exhibit anticancer properties. Garlic is not only a valuable culinary ingredient but also a natural medicine. Regular consumption in moderate amounts may offer numerous health benefits.

1. Introduction

Garlic (*Allium sativum* L.) belongs to the *Allium* genus and is one of the primary bulbous plants consumed fresh, powdered, or cooked. It is also traditionally used as a medicinal plant worldwide[1]. Fresh garlic cloves contain approximately 63% water, 28% carbohydrates, 7% protein, 0.2% fat, 0.8% fibre, and a significant amount of sulphur compounds. These compounds contribute to the characteristic pungent taste and odour of garlic [2]. To date, it is widely accepted that garlic is rich in various biologically active substances[3]. Its diverse chemical composition provides a wide range of biological effects. Numerous studies indicate that garlic extracts or the consumption of raw cloves exhibit antihyperlipidaemic[4], antioxidant[5], anti-inflammatory[6], cardiovascular[7], antihypertensive[8], antibacterial[9], antiviral[10], antifungal[11], antiparasitic[12], antidiabetic[13], anticarcinogenic[14], hepatoprotective[15], immunomodulatory[16], and hypoglycaemic[17] effects.

Garlic's biologically active compounds can be broadly classified into two groups: sulphur-containing and sulphur-free compounds[18]. The first group includes alliin, γ-glutamylcysteine, and their derivatives such as allicin, diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DATS), ajoene, S-allylcysteine (SAC), and S-allylmercaptocysteine (SAMC)[18]. Garlic contains the highest concentration of sulphur compounds among all *Allium* species. Therefore, this class of compounds is widely considered responsible for its health-promoting properties[19]. However, the second group—sulphur-free compounds—also includes important bioactives, particularly polyphenols[20]. Despite being often underestimated, these compounds exhibit health-promoting properties comparable to, or even stronger than, those of sulphur-containing molecules[21]. Moreover, some polyphenol groups are present in higher amounts in aged garlic extract or black garlic (BG) compared to raw garlic[20,22].

In 2022, an estimated 20 million new cancer cases and 9.7 million cancer-related deaths were reported worldwide[23]. As a result, the search for new therapeutic options and, more importantly, effective cancer prevention strategies is a major focus of the scientific community. Garlic, as a functional food, holds promise in this context. However, current data on the role and significance of garlic-derived polyphenolic compounds in cancer prevention remain fragmented and scattered. The aim of this manuscript is to summarise the current state of knowledge regarding the anticancer properties and molecular mechanisms of action of garlic-derived polyphenols.

Although the literature includes broad overviews of garlic's health benefits, few focus specifically on the polyphenol-related anticancer mechanisms. For instance, Ahmed and

Wang (2021) reviewed the bioactive compounds and health benefits of black garlic[24]. While they discussed its anticancer activity, they mostly catalogued general effects—such as antioxidant or anti-inflammatory actions—without exploring the molecular mechanisms of individual polyphenols. Similarly, Farhat et al. (2021) described various garlic preparations and their anticancer benefits, focusing primarily on organosulphur compounds and general antioxidant activity [25]. A recent review by Talib et al. (2024) also concentrated on garlic's anticancer properties, mainly in the context of organosulphur constituents like allicin and ajoene. They mentioned flavonoids and phenolics only briefly[26]. In contrast, the present manuscript offers a novel contribution by focusing on garlic-derived polyphenols (e.g., quercetin, caffeic acid, ferulic acid) and their specific anticancer mechanisms. This manuscript highlight how these polyphenols modulate key molecular targets—such as the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) survival pathway, the nuclear factor erythroid 2–related factor 2 (Nrf2) oxidative stress response, and transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ)—to induce apoptosis, inhibit tumour proliferation, and suppress metastasis. This mechanistic depth and focused analysis of polyphenolic compounds distinguish our work from prior garlic reviews. By doing so, we aim to fill a critical gap in understanding how garlic's polyphenols exert anticancer effects at the molecular level.

2. Types of polyphenolic compounds in garlic

Polyphenols are naturally occurring compounds primarily found in fruits, vegetables, and various plant-derived materials. To date, more than 8,000 polyphenolic compounds have been identified in plants[27]. Typically, plants synthesise polyphenols in response to stress. These compounds help protect young plants from pests, ultraviolet radiation, and pathogens[28]. Polyphenols also influence key sensory and stability-related properties of food, including taste, colour, oxidative stability, and aroma. The polyphenol content in garlic is modulated by numerous factors. In addition to environmental stressors, these include the garlic variety, soil type, country of origin, sunlight exposure, rainfall, degree of plant maturity, harvest time, processing methods, and storage conditions[1,22,29–31].

Polyphenols can be classified into several groups based on the number of phenol rings and the structural linkages between them[20,32]. These groups include flavonoids (e.g., quercetin, catechins found in green tea) [33], phenolic acids (e.g., chlorogenic acid from coffee) [34], stilbenes (e.g., resveratrol from red wine) [35], and lignans (e.g., from linseed) [36].

Lignans are diphenol derivatives containing a dibenzylbutane skeleton. They exhibit characteristics similar to those of phytoestrogens[37]. It has been suggested that lignans may confer several health benefits, including antioxidant, antitumor, oestrogenic and anti-oestrogenic effects, as well as cardiovascular protection[38]. In the Mediterranean diet, lignan sources include garlic, onion, leafy and non-leafy vegetables, cereals, and seasonal fruits such as citrus. Each of these contributes differently—ranging from 11% to 70%—to the total polyphenol intake[39].

Stilbenes are composed of a benzoin ring structure with 15 carbon atoms and a phenylpropane unit. Their biological activity is determined by diverse structural modifications, which also overlap with those seen in flavonoids[40,41]. Although present in food only in small quantities, the best-known stilbene is resveratrol, found in red wine. Notably, studies on garlic from Nigeria have also identified resveratrol as the dominant stilbene in this variety[42].

Phenolic acids are generally classified into two types: benzoic acid derivatives and cinnamic acid derivatives. These compounds feature a benzoic acid ring with carboxyl and hydroxyl groups. Among them, hydroxycinnamic acids are more prevalent and include p-coumaric acid, caffeic acid, sinapic acid, and ferulic acid. In contrast, hydroxybenzoic acids include gallic acid, vanillic acid, p-hydroxybenzoic acid, and syringic acid[43]. Phenolic acids demonstrate strong antioxidant activity through multiple mechanisms. These include reducing activity (via electron or hydrogen donation), free radical scavenging, and interruption of radical chain reactions. Additionally, they act as oxidase inhibitors and metal ion chelators[44]. The antioxidant nature of phenolic acids significantly contributes to their recognized health-promoting effects.

Flavonoids are the largest and most extensively studied group of polyphenols. They are further classified into subgroups such as flavonols, flavones, catechins, proanthocyanidins, anthocyanidins, and isoflavonoids, as summarised in Table 1.

Types of flavonoids	Example compounds	Example sources	References
FLAVONOLS	Quercetin, kaempferol, fisetin	Raw/black garlic, red onion, fresh capers	[45,46]
FLAVONES	Apigenin, acacetin, luteolin	Raw/black garlic, parsley, rosemary	[20,29,47]

CATECHINS	Epicatechin, epigallocatechin, epicatechin gallate	Raw garlic, broad beans, green tea	[48,49]
ISOFLAVONES	Daidzein, genistein, glycitein	Raw garlic, soya, broccoli	[50,51]
FLAVANONES	Naringenin, hesperetin, eriodictyol	Raw garlic, orange, lemon	[50,52]
FLAVANOLS	Epigallocatechin gallate, epicatechin, theflavin-3,3'-digallate	Raw/black garlic, green tea, cocoa	[1,53]
CHALCONES	Lonchocarpin, cardamonin, licochalcones	Tomato, *Ginkgo biloba* leaves, liquorice roots	[54,55]

Table 1. Classification and sources of flavonoids

Many flavonoids and related compounds are known for their strong antioxidant properties[56]. They have been widely studied as potential bioactive ingredients for incorporation into functional foods. The primary activity of polyphenols is their ability to scavenge free radicals. This includes their capacity to chelate reactive metals and stabilise radicals[57,58]. However, under certain conditions, polyphenols may undergo oxidation. Factors contributing to peroxidation include alkaline pH, oxygen exposure, and high concentrations of specific metals[57,58]. The compound's structure plays a key role in its radical scavenging activity, with critical features including the number of hydroxyl (-OH) groups and the degree of methylation[58].

Plants from the Allium genus, including garlic, are recognised for containing significant amounts of polyphenols[59]. Our analyses revealed that raw garlic bulbs contain the highest levels of β-resorcylic acid, allyl isothiocyanate, pyrogallol, p-hydroxybenzoic acid, and syringic acid (Table 2). Unfortunately, these compounds have not yet been studied in BG. A literature review shows that apigenin, kaempferol, and naringenin have not been detected in BG. Moreover, compounds such as caffeic acid, catechin, chlorogenic acid, epicatechin, gallic acid, and quercitrin are present in BG but in lower quantities than in raw garlic. BG is

produced by aging whole garlic bulbs or separated cloves under controlled humidity (80%–90%) and temperatures ranging from 60°C to 90°C, typically for 15 to 90 days[60]. In recent years, BG products have become increasingly popular in the Korean market as health-oriented foods, largely due to growing public awareness of garlic's health benefits[20]. However, many health-promoting compounds may degrade during the aging process. For example, allyl isothiocyanate is unstable and gradually decomposes in water at room temperature and 37°C, forming garlic-like odorous compounds[61]. It undergoes multiple chemical reactions, including hydrolysis, oxidation, thermal degradation, and interactions with proteins[62]. This instability may account for the absence or reduced concentration of certain substances in BG compared to raw garlic. On the other hand, the concentrations of epigallocatechin gallate, m-coumaric acid, morin, o-coumaric acid, p-coumaric acid, quercetin, resveratrol, and vanillic acid are higher in BG. This increase is likely due to the technological processing. Previous studies have shown that the BG production process increases total polyphenol and flavonoid content, while selectively enhancing certain compound classes[63]. A similar trend has been observed in other foods. For instance, during the aging of tangerine peel, flavonol levels tend to decrease, while isoflavones and chalcones increase. Dihydroflavones and flavanones decrease during the same process[64]. These findings support the hypothesis that technological processing significantly influences the polyphenol profile in BG.

Literature data also indicate that dried garlic contains lower levels of several polyphenols, such as catechin, kaempferol, protocatechuic acid, and rutin, compared to raw garlic. The exception is quercetin, which is found in higher concentrations in dried garlic. As discussed above, thermosensitive polyphenols can degrade during garlic processing. However, total polyphenol content generally increases during drying[65]. In onions, it has also been reported that quercetin levels rise in dried onion skins[66]. Thus, our findings are consistent with the current understanding of how technological processes affect polyphenol composition.

A comparison between garlic leaves and cloves showed that leaves contain significantly higher levels of apigenin, catechin, chlorogenic acid, ferulic acid, hesperidin, luteolin, naringin, p-coumaric acid, p-hydroxybenzoic acid, quercitrin, rutin, sinapic acid, and vanillic acid. Therefore, garlic leaves may serve as a valuable source of bioactive compounds, particularly during early spring. Studies have also shown that garlic leaves contain more vitamin C, a higher total polyphenol content, and exhibit stronger antioxidant activity than cloves[29]. Although garlic leaves are less commonly consumed in Europe and North America, they are widely used in Southeast Asia and China, especially in cuisines that emphasise fresh, seasonal ingredients[20,67].

Our analysis also revealed significant variability in polyphenol content among garlic samples. For example, catechin ranged from not detected (N.D.) to 95.03 mg/kg, ellagic acid from N.D. to 103.50 mg/kg, hyperoside from 0.37 to 89.24 mg/kg, myricetin from 30.80 to 139.50 mg/kg, p-hydroxybenzoic acid from N.D. to 218.97 mg/kg, and rutin from 64.90 to 687.80 mg/kg. This variability likely reflects genetic differences among garlic cultivars. Additional contributing factors include soil composition, fertilisation practices, harvest maturity, postharvest storage, handling procedures, and climatic conditions[68,69].

Polyphenol compounds	*Range of detection (mg/kg)*	*Type of garlic*	*References*
Acacetin	25.20	Raw garlic	48
Allyl isothiocyanate	124.90–340.45	Raw garlic	70,71
Apigenin	3.24–23.20	Raw garlic	30,71,72
	N.D.	Black garlic	20
	17.90–38.80	Raw garlic leaves	30
β-Resorcylic acid	313.50–452.10	Raw garlic	73
Benzoic acid	N.D.–39.22	Raw garlic	1,48
Caffeic acid	0.06–81.97	Raw garlic	1,20,30,48,71,72
	12.21–25.58	Black garlic	1,20
	14.80–36.70	Raw garlic leaves	30
Catechin	N.D.–95.03	Raw garlic	1,20,30,31,48
	17.51	Black garlic	20
	5780.60	Raw garlic leaves	30
	1.12	Dried garlic	74
Catechol	9.53	Raw garlic	48
Chlorogenic acid	N.D.–93.94	Raw garlic	1,20,30,48,70–72
	13.40	Black garlic	20

	133.30–1068.70	Raw garlic leaves	[30]
Cinnamic acid	0.60	Raw garlic	[48]
Daidzein	N.D.–0.10	Raw garlic	[50,51]
Ellagic acid	14.29	Raw garlic	[48]
Epicatechin	N.D.–103.50	Raw garlic	[1,20,30,45,48,70–72]
	38.32	Black garlic	[20]
	42.60	Raw garlic leaves	[30]
Epigallocatechin	0.06	Raw garlic	[1]
Epigallocatechin gallate	0.55–1.03	Raw garlic	[1,20]
	19.52–23.12	Black garlic	[20]
Ferulic acid	0.06–39.66	Raw garlic	[20,30,48,70–72]
	31.00–138.50	Raw garlic leaves	[30]
Gallic acid	0.97–96.48	Raw garlic	[1,20,31,48,70–72]
	2.50–45.53	Black garlic	[1,20]
	7.74	Dried garlic	[74]
Genistein	0.10–0.20	Raw garlic	[50,51]
Hesperidin	N.D.–8.30	Raw garlic	[30,48]
	58.80–472.80	Raw garlic leaves	[30]
Hydroxytyrosol	0.16	Raw garlic	[48]
Hyperoside	0.37–89.24	Raw garlic	[70–72]
Isoferulic acid	2.78	Raw garlic	[48]
Isoorientin	0.91	Dried garlic	[74]
Isovanillic acid	3.02	Dried garlic	[74]
Kaempferol	0.07–23.90	Raw garlic	[20,30,48]

	N.D.	Black garlic	20
	7.90–26.60	Raw garlic leaves	30
	1.13	Dried garlic	74
Luteolin	0.15–22.92	Raw garlic	30,71,72
	9.30–46.70	Raw garlic leaves	30
m-Coumaric acid	4.84	Raw garlic	20,72
	13.99	Black garlic	20
Morin	1.06–1.33	Raw garlic	20
	6.19–7.74	Black garlic	20
Myricetin	30.80–139.50	Raw garlic	30
	81.40–105.30	Raw garlic leaves	30
Naringenin	N.D.–56.71	Raw garlic	20,71,72
	N.D.	Black garlic	20
Naringin	22.30–70.40	Raw garlic	30
	73.60–221.70	Raw garlic leaves	30
o-Coumaric acid	0.66	Raw garlic	20,72
	14.44	Black garlic	20
Orientin	0.95	Dried garlic	31
p-Coumaric acid	0.55–22.80	Raw garlic	20,30,48,71,72
	32.73	Black garlic	20
	29.50–106.0	Raw garlic leaves	30
p-Hydroxybenzoic acid	N.D.–218.97	Raw garlic	30,71,72
	125.50–914.80	Raw garlic leaves	30
Protocatechuic acid	4.22	Raw garlic	48

	2.23	Dried garlic	31
Pyrogallol	426.26	Raw garlic	48
Quercetin	N.D.–0.13	Raw garlic	20,48
	7.31	Black garlic	20
	0.56	Dried garlic	74
Quercitrin	0.14–14.52	Raw garlic	20,30,48,71
	9.89	Black garlic	20
	6.00–62.80	Raw garlic leaves	30
Resveratrol	0.58–0.61	Raw garlic	20,48
	6.42	Black garlic	20
Rosmarinic acid	0.31	Raw garlic	48
Rutin	N.D.–43.43	Raw garlic	30,48,71,72
	64.90–687.80	Raw garlic leaves	30
	0.10	Dried garlic	74
Salicylic acid	1.77	Raw garlic	48
Sinapic acid	1.00–5.40	Raw garlic	30
	139.60–233.90	Raw garlic leaves	30
Syringic acid	1.77–200.02	Raw garlic	31,48
Vanillic acid	N.D.–3.20	Raw garlic	1,20,30,48,72
	6.41	Black garlic	20
	22.60–64.80	Raw garlic leaves	30
Vitexin	1.98	Dried garlic	74

Table 2. Summary of polyphenols detected in different garlic parts or processed forms.

The table summarises the minimum and maximum amounts of polyphenols detected in garlic. In cases where only one reference study measured the content of a specific polyphenol, a single value is provided in the table. All units, such as parts per million (ppm), nmol/g, µg/g, and mg/100g, have been converted to mg/kg. N.D., not detected.

3. Garlic polyphenol anticancer properties

Cancer remains one of the most pressing health challenges in modern society. Consequently, the vast majority of polyphenols have been investigated for their anticancer potential. Most studies have assessed these effects in cell culture models (Table 3). However, experimental studies involving animals or human participants remain scarce. According to the 2022 World Health Organization (WHO) report, the three most prevalent cancer types worldwide are lung, breast, and colorectal cancers[23]. It is therefore not surprising that most in vitro studies have focused on cell lines derived from these cancer types. Current evidence indicates that all polyphenols identified in garlic exhibit anticancer activity in vitro (Table 3). The table summarises representative publications describing the in vitro anticancer effects of garlic-derived polyphenols.

Type of polyphenol	*Name of cell line*	*Observed effect*	*Reference*
Acacetin	MCF-7, 143B, MG63, SJSA, HOS	cell viability↓, apoptosis↑, DNA fragmentation↑, caspase-3, -8, -9↑	75,76
Allyl isothiocyanate	GBM8401, MCF-7	cell viability↓, apoptosis↑, DNA fragmentation↑, caspase-3, -8, -9↑	77,78
Benzoic acid	HeLa, HUH7, CACO-2, MG63, HT-29, A673, SW48, PC3, HCT-116, HCT-15	cell viability↓, apoptosis↑, ROS↑	79,80
Caffeic acid	ME-180, HeLa, HCT-116	apoptosis↑, DNA damage↑, ROS↑	81,82
Catechin	HCT-15, HCT-116, HepG2, A549	cell viability↓, apoptosis↑, DNA damage↑, cell cycle arrest↑	83,84

Catechol	MCF-7, MDA-MB-231, KP2, H460	DNA damage↑, apoptosis↑, cell cycle arrest↑, colony formation↓	85,86
Chlorogenic acid	A549, HT-29	cell viability↓, cell proliferation↓, apoptosis↑, ROS↑	87,88
Cinnamic acid	HT-144, CACO-2	cell viability↓, cell cycle arrest↑, apoptosis↑, caspase-9↑, cell number↓, DNA synthesis↓	89,90
Coumaric acid isomers	HCT-15, HT-29, U-138MG	cell viability↓, apoptosis↑, DNA fragmentation↑, colony formation↓, ROS↑	91,92
Curcumin	SKBR3, BT474, MBA-MB-231, MCF-7	cell cycle arrest↑, apoptosis↑, DNA fragmentation↑	93,94
Ellagic acid	U87MG, U118, MCF-7, MDA-MB-231	cell viability↓, cell proliferation↓, DNA damage↑, apoptosis↑, cell cycle arrest↑, colony formation↓	95,96
Epicatechin	MCF-7, MDA-MB-231, U937, MKN-4	cell viability↓, cell proliferation↓, apoptosis↑, cell cycle arrest↑, ROS↑, DNA fragmentation↑	97,98
Epigallocatechin gallate	H1299, A549, HepG2	cell viability↓, cell proliferation↓, apoptosis↑, colony formation↓, cell migration↓	99,100
Ferulic acid	DU-145, MCF-7, PANC-1, MDA-MB-231	cell viability↓, cell cycle arrest↑, cell proliferation↓, apoptosis↑	101,102
Gallic acid	A549, HepG2, SMMC-7721	cell proliferation↓, apoptosis↑, DNA fragmentation↑, ROS↑, caspase-3 and -9 expression↑	103,104

Hesperidin	HN6, HN15, DU145, PNT1A	cell proliferation↓, cell migration↓, cell viability↓, colony formation↓, ROS↑, LDH release↑, apoptosis↑	105,106
Hispidulin	NCI-H460, A549, A2058	cell proliferation↓, apoptosis↑, tumour volume↓, cell migration↓, cell viability↓, caspase-3 expression↑	107,108
Hydroxytyrosol	LS180, Jurkat, HL60, Raw264.7	cell viability↓, apoptosis↑, cell cycle arrest↑	109,110
Hyperoside	A431, A432, HS-4, MCF-7, 4T1	cell viability↓, apoptosis↑, cell number↓, cell migration↓, caspase-3 and -9 expression↑	111,112
Isoferulic acid	Raji, K562, Jurkat	apoptosis↑, cell cycle arrest↑	113
Isoorientin	HT-29, A549, NCI-H23, NCI-H460	cell viability↓, apoptosis↑, caspase-3 and -8 mRNA expression and activities↑, ROS↑, cycle arrest↑	114,115
Kaempferol	A375, MDA-MB-231, BT474	cell viability↓, apoptosis↑, cycle arrest↑, colony formation↓, caspase-3 and -9 expression↑	116,117
Luteolin	MDA-MB-231, MKN45, BGC823	cell viability↓, apoptosis↑, cycle arrest↑, colony formation↓, caspase-3 and -9 expression↑	118,119
Morin	SK-BR-3, SW480	cell viability↓, apoptosis↑, caspase-3 and -7 expression↑, cycle arrest↑, colony formation↓	120,121

Myricetin	A549, HT-29	cell viability↓, apoptosis↑, cell migration↓	122,123
Naringenin	MDA-MB-231, SKBR3, B16F10, SK-MEL-28	cell viability↓, apoptosis↑, cycle arrest↑, cell migration↓	124,125
Naringin	MDA-MB-231, WiDr	cell viability↓, caspase-3 expression↑	126
Orientin	T24, HT-29	cell viability↓, apoptosis↑, cycle arrest↑	127,128
p-Hydroxybenzoic acid	K562, PC-3, LNCaP, MDA-MB-231, MCF-7, MCF-10A	cell viability↓, apoptosis↑, cycle arrest↑, cell migration↓	129,130
Protocatechuic acid	A549, HepG2	cell viability↓	131,132
Pyrogallol	HT-29, ASPC-1, A2780, DU145, PC3	cell viability↓, apoptosis↑, cycle arrest↑	133,134
Quercetin	DU145, PC3, HepG2, Hep3B, HCT-116	cell viability↓, colony formation↓, ROS↑	135,136
Quercitrin	A549, NCI-H358, DLD-1	cell viability↓, apoptosis↑, caspase-3 activity↑	137,138
Resveratrol	HK-2, L02, 4T1, HepG2, PC-3	cell viability↓, toxicity↑, apoptosis↑, cycle arrest↑	139,140
Rosmarinic acid	A549, MDA-MB-231, U251, U343	cell viability↓, apoptosis↑, cell migration↓, DNA fragmentation↑	141,142
Rutin	SAOS2, 786-O	cell viability↓, apoptosis↑	143,144
Salicylic acid	B16F10, J774, MCF-7, MCF10A, HCT-116	cell viability↓, apoptosis↑, colony formation↓	145,146
Sinapic acid	HT-29, PC-3, LNCaP	cell viability↓, apoptosis↑, caspase-3 expression↑	147,148

Syringic acid	Primary gastric cancer, DU-145	cell viability↓, apoptosis↑, ROS↑	149,150
Vanillic acid	A549, MCF-7	cell viability↓, apoptosis↑, ROS↑	151,152
Vitexin	HCT-116, A549	cell viability↓, toxicity↑, apoptosis↑, caspase-3 expression↑	153,154

Table 3. Selected anticancer effects of polyphenols detected in garlic on cancerous cell lines *in vitro*. ↑, increase; ↓, decrease.

Our analysis showed that polyphenols detected in garlic were primarily studied on similar cell lines derived from the digestive system (e.g., CACO-2, HT-29, HCT-15, HCT-116, SW480), breast cancers (e.g., MCF-7, MDA-MB-231, 4T1, BT474, MCF10A, SK-BR-3), and lung cancers (e.g., A549, H1299). These cancer types correspond to the most prevalent cancers identified by the WHO (summarised in Table 3). However, other cancer types have also been studied in the context of polyphenols derived from plants, including garlic. The most commonly investigated cancers beyond these include those of the nervous system (e.g., U118, U87MG, SH-SY5Y, U251, U343), liver (e.g., HepG2, Hep3B), and prostate (e.g., DU145, PC3, LNCaP). In the cell lines mentioned, similar mechanisms of action for the studied polyphenols have been observed. These include inhibition of cell proliferation, a decrease in cell viability, and, in most cases, initiation of the apoptosis process[79,80,89,90,124,125] (summarised in Table 3). In the vast majority of studied cell lines, the described polyphenols selectively induce cell death in cancerous cells without causing significant damage to normal cells. Moreover, research consistently shows that apoptosis is mediated by an increased level of reactive oxygen species (ROS) [79,80,87,88,103,104].

The anticancer effect of individual polyphenols has also been studied in animal models. Researchers demonstrated that, in a rat oesophageal in vivo model, ellagic acid (0.4 and 4 g/kg) significantly decreased the average number of tumours induced by N-nitroso-N-methylbenzylamine (NBMA). Additionally, ellagic acid inhibited both preneoplastic and neoplastic lesions in this bioassay[155]. It was shown that ellagic acid reduces the formation of NBMA metabolites as well as their binding to DNA in cultured rat oesophageal explants[156]. However, the potential use of ellagic acid as a cancer chemopreventive agent is limited by its poor bioavailability when administered orally and its low solubility in water[157,158]. This

limitation was overcome by administering ellagic acid as a subdermal implant, which was evaluated in oestrogen-induced rat mammary tumours. Notably, a much smaller dose of ellagic acid was required via subdermal administration to achieve a similar reduction in tumour burden, compared with dietary intake[159]. Mechanistically, in terms of its cancer chemopreventive potential, ellagic acid modulates various cell regulatory proteins. This includes the downregulation of p-STAT3, p-Akt, and p-ERK1/2, which leads to cell apoptosis or inhibition of proliferation[157,160].

In another study, resveratrol inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture. It also suppressed tumourigenesis in a mouse skin cancer model[161]. Additionally, a separate study demonstrated the antioxidant activity of resveratrol in preventing tumour initiation. This was achieved by inhibiting angiogenesis through interactions with vascular endothelial growth factor (VEGF) and metalloproteases[46]. In vivo experiments further showed that resveratrol inhibits tumour growth in athymic mice by suppressing angiogenesis and increasing apoptosis in LNCaP xenograft cells[162]. This compound also prevents the progression and growth of prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) models by inducing apoptosis and reducing androgen receptor expression in tumour cells[163]. Animal studies have also confirmed curcumin's ability to inhibit tumour growth and angiogenesis in athymic mice implanted with PC3 xenografts. These effects are mediated by reduced proliferation and enhanced apoptosis[164,165].

To date, animal and human studies on the anticancer effects of garlic have primarily focused on sulphur compounds. However, the relationship between organic sulphur compounds and phenolic compounds, as well as their combined biological activity, remains poorly understood. Researchers emphasise the synergistic therapeutic effects of these compound classes[166]. Li et al. reported that in C57BL/6 mice inoculated with highly aggressive mouse lymphoma cells, injection of raw garlic extract eliminated cancer cells without signs of adverse effects. In contrast, all animals in the control group succumbed to the disease[167]. Other studies have shown that supplementation with raw garlic powder reduced the incidence of mammary cancer in rats and prevented mammary carcinogenesis induced by N-methyl-N-nitrosourea[168,169]. Unfortunately, most available studies focus on sulphur compounds. These have been shown to mildly reduce tumour incidence and severity in models of N-nitroso compound-induced carcinogenesis[170]. This reductionist approach has led to numerous studies evaluating individual garlic constituents for their anticancer properties. In

most animal studies, oral administration of garlic or its isolated compounds resulted in weak direct anticancer effects at best[171]. These and other findings suggest that garlic's anticancer properties may be more effective upon direct exposure to cancer cells, rather than after absorption through the gastrointestinal epithelium. Moreover, it cannot be excluded that garlic polyphenols exert their strongest effects as a mixture, where compound interactions enhance bioactivity.

Most human studies on garlic's anticancer effects have been retrospective surveys, aimed at identifying potential associations between cooked garlic consumption and cancer incidence or progression[172–174]. Some studies have involved dietary interventions in which participants consumed garlic. However, direct evidence supporting garlic's anticancer potential in humans remains weak[175,176]. Studies specifically addressing polyphenol effects in humans are particularly rare. In clinical trials on healthy volunteers, Goldberg et al. investigated the absorption kinetics of resveratrol, quercetin, and catechin after ingestion[177]. Their results showed that peak plasma concentrations occurred at 30 minutes post-consumption. However, levels returned to baseline within 4 hours[177]. Further research by Walle et al. and Goldberg et al. confirmed the low bioavailability of these polyphenols in their active forms[177,178]. Therefore, to exert sustained biological effects, polyphenols would require continuous dietary intake. Currently, it is believed that regular garlic consumption, as part of a balanced diet, may support cancer prevention and therapy[14]. However, it is important to emphasise that garlic provides supportive effects only and does not replace conventional cancer treatments. The relationship between polyphenol intake and cancer risk has been regularly assessed and meta-analysed over the past 10–15 years. For example, a meta-analysis of prospective studies found that isoflavone consumption was associated with a 19% reduction in gastric cancer risk[179]. Despite decades of research, the anticancer properties of garlic still lack conclusive evidence, particularly regarding curative effects against aggressive cancers in animal models or humans.

4. The common mechanism of action of polyphenols found in garlic

The high polyphenol content in garlic is commonly associated with strong antioxidant and health-promoting properties. This is largely attributed to their ability to scavenge endogenous reactive oxygen species (ROS). Numerous studies on garlic extracts have shown that high polyphenol levels protect normal cells from ROS-dependent cell death[180,181]. For instance, aged garlic extract was reported to reduce ROS-dependent cell death in SH-SY5Y cells—a model of human neuroblasts—by activating Nrf2[180]. Similarly, reduced

oxidative stress via Nrf2 activation has been observed in rats fed raw garlic homogenate[182]. It is well-documented that polyphenols such as chlorogenic acid, resveratrol, quercetin, ferulic acid, and rosmarinic acid activate Nrf2[183]. However, depending on their type and concentration, some polyphenols may also inhibit Nrf2 activity[184]. Nrf2 binds to the antioxidant response element (ARE) and plays a key role in regulating the expression of various detoxifying and antioxidant genes, including heme oxygenase-1 (HO-1). It also controls enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [185]. Increased expression of these enzymes protects normal cells from oxidative stress and ROS-induced apoptosis. In contrast, in cancer cells, high levels of polyphenols often increase toxicity[186]. Plant-derived polyphenols have been reported to exert pro-oxidant effects through interactions with Nrf2, which may contribute to their anticancer and apoptosis-inducing properties[187]. Many of the studies summarized in Table 3 describe the anticancer activity of garlic-derived polyphenols by comparing their effects on cancerous and normal cells. These studies generally show that cancer cells exhibit higher basal ROS levels. An additional increase in ROS due to the oxidative action of polyphenols or their interaction with Nrf2 may drive ROS-dependent cell death[188]. This elevated ROS level in cancer cells reflects an imbalance between oxidants and antioxidants. Treatment with polyphenols can further modulate molecular pathways, amplifying their anticancer effects. In normal cells, polyphenols usually cause only a modest increase in ROS production. However, this is accompanied by upregulation or activation of antioxidant enzymes, ultimately providing long-term protection (Figure 1).

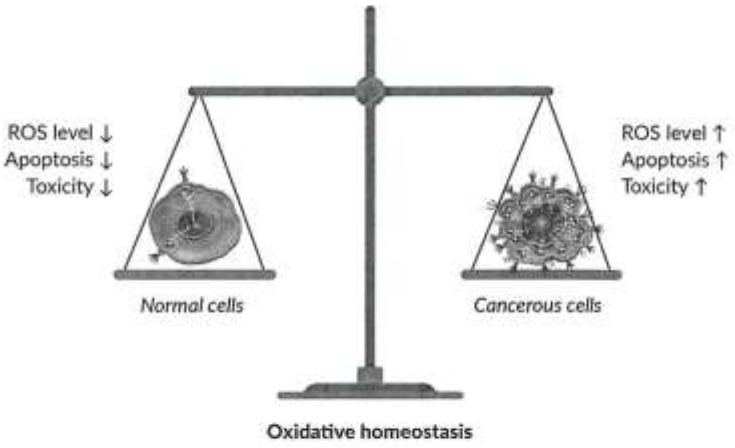

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Figure 1. Impact of garlic polyphenols on oxidative homeostasis.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. They comprise three subtypes: PPARα, PPARβ/δ, and PPARγ[189]. PPARs regulate energy metabolism in various tissues, including the liver, adipose tissue, and muscle[190]. All PPAR isoforms modulate lipid accumulation, cell differentiation, and apoptosis in cancer cells[191,192]. Additionally, PPARs can act as receptors for environmental factors, including dietary polyphenols[71]. Several phenolic acids, such as gentisic, p-hydroxybenzoic, chlorogenic, caffeic, p-anisic, ferulic, and gallic acid, as well as flavonoids like hesperidin, naringin, and epicatechin, have been shown to increase PPARα mRNA expression[193]. In contrast, compounds such as epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, catechin, and gallocatechin increase PPARγ mRNA expression, while exhibiting little or no effect on PPARα[194]. Moreover, these flavonoids also function as partial PPARγ agonists[195]. To date, several polyphenols listed in Table 2—including luteolin, quercetin, p-coumaric acid, kaempferol, apigenin, ferulic acid, and ellagic acid—have been described to directly interact with PPARγ and influence the differentiation of mouse preadipocytes in the 3T3-L1 cell line[71]. Interestingly, some flavonoids such as quercetin, kaempferol, and resveratrol act as agonists of all PPAR subtypes and affect both their expression and activity[196–199]. All PPARs influence ROS homeostasis and exert antioxidant effects by regulating the transcription of endogenous antioxidant genes. Additionally, they coordinate intracellular signaling pathways that reduce ROS production, either directly or indirectly[200]. Numerous studies have shown that ligands of PPARα, PPARβ/δ, and PPARγ enhance the expression and activity of catalase (CAT) [200]. Furthermore, other enzymes involved in ROS homeostasis—including SOD1, SOD2, GPx, and HO-1—are also regulated by PPAR ligands[200,201]. Garlic-derived polyphenols have been shown to increase the expression and/or activity of antioxidant enzymes. In a previous study, our team demonstrated that the Spanish garlic cultivars Morado and Castano exhibited the highest total polyphenol content among the analyzed garlic varieties. This high polyphenol content was correlated with increased toxicity in human skin fibroblast (BJ) cells[31]. Further research revealed that the Morado cultivar induced ROS-dependent cell death in the human squamous carcinoma (SCC-15) cell line[181]. In our most recent study, we showed that Morado also activated both mRNA and protein expression of PPARγ, along with upregulation of SOD1 and CAT[202].

A functional interaction between the Nrf2 and PPARγ signaling pathways has also been reported[203]. An antioxidant response element (ARE) has been identified within the *PPARγ* promoter, while peroxisome proliferator response elements (PPREs) have been detected in the *Nrf2* promoter region[204]. The presence of these response elements suggests a positive feedback loop between the Nrf2 and PPARγ pathways. This crosstalk may facilitate mutual upregulation of both transcription factors and their downstream antioxidant targets[203,205]. Based on current evidence, we propose that the interplay between Nrf2 and PPARγ contributes to the anticancer activity of garlic-derived polyphenols (Figure 2). While many of these compounds share common mechanisms of action, individual polyphenols may also exert distinct, compound-specific effects, as described in the following chapter.

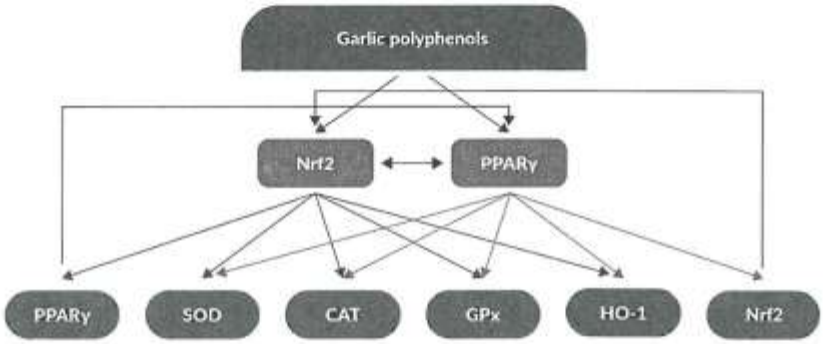

Figure 2. Proposed mechanism of action for garlic polyphenols.

Garlic polyphenols may interact with both Nrf2 and PPARγ receptors, regulating detoxification, cell apoptosis, differentiation, and oxidative stress homeostasis. Both Nrf2 and PPARγ influence antioxidant enzymes such as SOD, CAT, GPX, and HO-1. Furthermore, an ARE has been identified in the PPARγ promoter, and PPREs have been found in the Nrf2 promoter region, resulting in cross-talk between these receptors. Abbreviations: CAT - catalase; GPx - glutathione peroxidase; HO-1 - heme oxygenase-1; Nrf2 - nuclear factor erythroid 2-related factor 2; PPARγ - peroxisome proliferator-activated receptor gamma; SOD - superoxide dismutase.

5. Detailed mechanisms of action of selected garlic-derived polyphenols

Garlic-derived polyphenols exert common anticancer effects by engaging a network of cellular pathways that regulate oxidative stress, inflammation, proliferation, and cell death. However, each polyphenol also possesses distinct molecular targets and potencies. Below, we discuss the key mechanisms and cancer-related pathways modulated by six prominent garlic

polyphenols – quercetin, kaempferol, caffeic acid, ferulic acid, p-coumaric acid, and gallic acid – highlighting both their shared pathways and unique features.

To highlight these differences and similarities, Table 4 summarises the major garlic-derived polyphenols along with their proposed anticancer mechanisms. Importantly, the overall anticancer activity of garlic is likely mediated by the synergistic interaction of its multiple polyphenolic constituents. Therefore, the extent and nature of its biological effects may vary depending on factors such as garlic cultivar, cultivation conditions, and post-harvest processing methods. The main mechanisms of action of the following polyphenols have been compiled and presented in Figure 3.

Polyphenol	***Key Molecular Targets/Pathways***	***Anticancer Pathways***	***Evidence***	***Key Supporting Studies***
Quercetin	PI3K/Akt, NF-κB, Nrf2, JAK2/STAT3	apoptosis, autophagy, cell cycle arrest, anti-angiogenesis, anti-metastasis	In vitro, In vivo	135,206
Caffeic Acid	Nrf2/ARE, PKCδ, MAPK	Nrf2-mediated antioxidation, apoptosis, angiogenesis inhibition	In vitro, In vivo	207,208
Ferulic Acid	Cyclins/CDKs, autophagy	cell cycle arrest, autophagy, chemoresistance reversal	In vitro, In vivo	208,209
p-Coumaric Acid	GRP78/UPR, Bax/Bcl-2, NF-κB	ER-stress apoptosis, mitochondrial apoptosis, inflammation inhibition	In vitro, In vivo	210,211
Kaempferol	PI3K/Akt, MMP-2/9, p53	Cell survival inhibition, apoptosis, anti-metastasis, anti-angiogenesis	In vitro, In vivo	212,213
Gallic Acid	JAK2/STAT3, EGFR/MAPK, ROS	Apoptosis, chemotherapy potentiation, ROS induction, anti-metastasis	In vitro, In vivo	214,215

Table 4. Major Garlic-Derived Polyphenols and Anticancer Mechanisms

All compounds are present in garlic (*Allium sativum*) or its preparations (including aged/black garlic) and have demonstrated anticancer bioactivity in preclinical studies. Molecular targets refer to the primary proteins or pathways modulated. Anticancer pathways indicate the resultant cellular processes (e.g., apoptosis, cell cycle arrest, anti-invasion) affected by the compound. Abbreviations: ARE – Antioxidant Response Element; Bax/Bcl-2 – Bcl-2-associated X protein / B-cell lymphoma 2; CDKs – Cyclin-Dependent Kinases; EGFR – Epidermal Growth Factor Receptor; GRP78/UPR – Glucose-Regulated Protein 78/Unfolded Protein Response; JAK2/STAT3 – Janus kinase 2 / Signal transducer and activator of transcription 3; MAPK – Mitogen-Activated Protein Kinase; MMP-2/9 – Matrix Metalloproteinases 2 and 9; NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells; Nrf2 – Nuclear factor erythroid 2–related factor 2; p53 – Tumor Protein p53; PI3K/Akt – Phosphoinositide 3-kinase / Protein kinase B; PKCδ – Protein Kinase C delta isoform; ROS – Reactive Oxygen Species

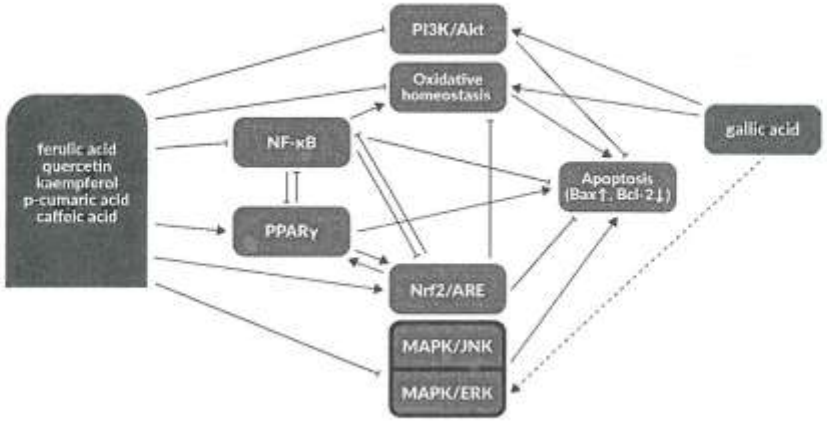

Figure 3. Mechanisms of action of the crucial polyphenols.

The dashed line indicates a potential effect, red lines indicate stimulation, and dark lines indicate inhibition. Abbreviations: ARE – Antioxidant Response Element; Bax/Bcl-2 – Bcl-2-associated X protein / B-cell lymphoma 2; ERK – extracellular signal-regulated kinase; JNK – c-Jun N-terminal kinase; MAPK – Mitogen-Activated Protein Kinase; NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells; Nrf2 – Nuclear factor erythroid 2–related factor 2; PI3K/Akt – Phosphoinositide 3-kinase / Protein kinase B; PPARγ – peroxisome proliferator-activated receptor gamma

5.1. Quercetin

Quercetin is a flavonol that robustly modulates multiple signaling pathways to inhibit tumor growth. A wealth of studies indicates quercetin can suppress cancer cell proliferation and induce apoptosis by interfering with the PI3K/Akt pathway and downstream transcription factors like NF-κB and STAT3 [216,217]. Through inhibition of NF-κB, quercetin downregulates the expression of anti-apoptotic and pro-inflammatory genes, thereby sensitizing cells to cell death signals [218]. Concurrently, quercetin activates the Nrf2/ARE pathway, leading to increased expression of cytoprotective antioxidant enzymes such as HO-1 and SOD [219]. This dual action – dampening NF-κB while boosting Nrf2 – skews cells away from a pro-survival, pro-oxidant state and towards an environment hostile to cancer growth [220]. Mechanistic studies in lung, colon, prostate, and other tumor models have confirmed quercetin's capacity to induce cell cycle arrest (often at G0/G1 or G2/M) and trigger intrinsic apoptosis via modulation of Bcl-2 family proteins [221]. For instance, treatment of colon carcinoma cells with quercetin elevates the Bax/Bcl-2 ratio, promoting mitochondrial cytochrome c release and caspase-3 activation [222]. In prostate cancer and leukemia cells, quercetin was shown to reduce cell viability and NF-κB activity, leading to increased apoptotic markers [135]. In vivo studies further underscore quercetin's anticancer efficacy. Rubio et al. (2018) demonstrated that 25 μM quercetin attenuated NF-κB signaling in leukemia cells, and in mice bearing Ehrlich ascites carcinomas, quercetin (200 mg/kg) selectively induced tumor cell apoptosis via caspase-3 while sparing normal tissues [223]. Taken together, quercetin's anticancer activity stems from its multi-targeted modulation of survival pathways (PI3K/Akt, NF-κB), stress responses (Nrf2/ARE), and apoptotic regulators (Bax/Bcl-2, caspases), yielding a potent anti-proliferative and pro-apoptotic effect across diverse cancer types.

5.2. Kaempferol

Kaempferol, another major flavonol in garlic, shares several anticancer mechanisms with quercetin but also exhibits distinct effects on specific pathways. Like quercetin, kaempferol inhibits the PI3K/Akt kinase cascade, leading to downstream suppression of NF-κB and other pro-survival signals [224]. Kaempferol treatment consistently increases expression of the tumor suppressor the tensin homolog deleted on the chromosome 10 (PTEN) in various models, which further antagonizes PI3K/Akt signaling and facilitates apoptotic onset [225]. Through NF-κB inhibition, kaempferol attenuates the production of inflammatory mediators (tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2)) that can fuel tumor progression [226]. In parallel, kaempferol can activate Nrf2 and upregulate ARE-driven genes, contributing to its antioxidant and cytoprotective profile [227]. A notable

feature of kaempferol is its impact on the mitogen-activated protein kinase (MAPK) pathways: it tends to inhibit mitogenic ERK signaling while it may activate stress-related MAPKs (JNK/p38) in a context-dependent manner [228]. In human gastric carcinoma cells, for example, kaempferol inactivated ERK and upregulated a pro-apoptotic microRNA (miR-181a), resulting in reduced proliferation [229]. In lung cancer models (A549 cells), kaempferol was found to inhibit Akt phosphorylation and enhance MEK/ERK activity as a necessary step for full caspase-7 activation – an intriguing crosstalk wherein a transient MAPK activation contributed to apoptosis induction [230]. Kaempferol also exerts anti-metastatic effects by targeting extracellular proteases and motility pathways. It downregulates matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) expression, enzymes critical for tumor invasion, and can interfere with transforming growth factor beta (TGF-β)/Smad signaling to prevent epithelial–mesenchymal transition (EMT) [231]. In vivo, kaempferol has demonstrated the ability to reduce metastasis and tumor growth. In a B16 melanoma mouse model, kaempferol significantly decreased pulmonary metastatic nodules, correlating with reduced MAPK/PKC signaling and MMP-9 activity in the tumors [232]. Moreover, kaempferol can synergize with chemotherapeutics; co-treatment of colon cancer xenografts with kaempferol and fluorouracil (5-FU) enhanced tumor regression via augmented Akt inhibition and apoptosis [233]. In summary, kaempferol broadly converges on the same anti-inflammatory and pro-apoptotic pathways as quercetin (notably NF-κB and Akt suppression), but also distinctly impacts cell migration and cell cycle regulators (e.g. CDKs and p53-dependent pathways), underscoring its multi-faceted anticancer profile.

5.3. Caffeic Acid

Caffeic acid is a hydroxycinnamic acid that contributes to garlic's antioxidant and anticancer properties. A primary mode of action of caffeic acid is the mitigation of oxidative stress: it is a well-known ROS scavenger and also an inducer of the Nrf2/ARE pathway [234]. By stabilizing Nrf2, caffeic acid increases cellular glutathione and detoxifying enzymes, which protect normal cells from carcinogenic insults and oxidative DNA damage. In parallel, caffeic acid inhibits major pro-tumorigenic signaling pathways. It has been shown to block the activation of NF-κB, resulting in lowered expression of NF-κB target genes such as vascular endothelial growth factor (VEGF) (a key angiogenic factor) and MMP-9 (a metastasis-promoting protease) in hepatocellular carcinoma models [235]. This corresponds with a reduction in tumor angiogenesis and invasiveness. Additionally, caffeic acid can interfere with the MAPK cascade; for example, in skin cancer cells, it significantly reduced

phosphorylation of ERK1/2, JNK, and p38 MAPK, thereby disrupting mitogenic and stress signaling needed for tumor growth [236]. Through inhibition of both NF-κB and MAPKs, caffeic acid exerts an anti-inflammatory effect that is linked to decreased production of IL-6, IL-1β, and COX-2 in the tumor microenvironment [237]. On the PI3K/Akt axis, caffeic acid was found to decrease phosphorylation of Akt and its downstream effector mTOR, contributing to growth arrest and autophagic cell death in certain cancer cell lines [238]. At higher concentrations, caffeic acid directly induces mitochondrial dysfunction – it can depolarize the mitochondrial membrane (loss of $\Delta\Psi m$) and elevate intracellular ROS within cancer cells, leading to apoptosis via the intrinsic pathway [239]. Indeed, treatment of hepatoma cells with caffeic acid triggered cytochrome c release and caspase-9/-3 activation, consistent with mitochondrial apoptotic induction [240]. Caffeic acid has shown chemopreventive efficacy *in vivo* as well. Low-dose caffeic acid (and its derivative caffeic acid phenethyl ester) caused complete regression of implanted hepatomas in mice, an effect attributed to a "dual mechanism" of NF-κB inhibition and pro-oxidant DNA damage in tumor cells [241]. Animal models of skin cancer and colon cancer have similarly noted that dietary caffeic acid can reduce tumor incidence and multiplicity, accompanied by lower inflammatory markers and increased antioxidant enzyme levels in tissues [242]. Collectively, caffeic acid's anticancer mechanisms center on reducing oxidative and inflammatory stimuli (*via* Nrf2 activation and NF-κB inhibition) and directly promoting apoptotic pathways, making it an effective agent in both cancer prevention and therapy.

5.4. Ferulic Acid

Ferulic acid, another abundant hydroxycinnamic acid in garlic, is distinguished by its potent antioxidant action and ability to modulate cell cycle regulators. Ferulic acid strongly activates the Nrf2/ARE pathway; in fact, it can upregulate Nrf2 and its target genes to a degree comparable to classic chemopreventive agents, thereby fortifying cells' antioxidant defenses [243]. It also engages PPARγ, a nuclear receptor involved in cellular metabolism and differentiation: ferulic acid treatment has been shown to increase PPARγ expression and activity in certain models, an effect linked to anti-inflammatory outcomes via repression of NF-κB [244]. Through PPARγ activation, ferulic acid may promote a more differentiated, less proliferative state in tumor cells and enhance lipid metabolic reprogramming unfavorable to rapid growth [245]. Like other polyphenols, ferulic acid inhibits the NF-κB pathway; for instance, in LPS-stimulated macrophages, ferulic acid prevented IκBα degradation and nuclear p65 translocation, thereby blocking NF-κB–mediated transcription of IL-6 and TNF-α

[246]. In cancer cells, this NF-κB inhibition translates to lower expression of survival proteins (e.g., Bcl-xL, survivin) and reduced chronic inflammation that can drive tumor progression. Ferulic acid also targets the PI3K/Akt pathway. In cervical carcinoma cells, ferulic acid was observed to markedly reduce phosphorylated Akt levels and downstream kinase signals, coinciding with G0/G1 cell cycle arrest [209]. The cell cycle blockade by ferulic acid is attributed to downregulation of cyclins (Cyclin D1, E) and CDKs alongside upregulation of CDK inhibitors like p21[Cip1209,247]. Such changes halt the transition from G1 to S phase, giving ferulic acid a strong antiproliferative effect. On triggering apoptosis, ferulic acid utilizes both intrinsic and extrinsic pathways. It can increase pro-apoptotic Bax and decrease anti-apoptotic Bcl-2, tilting the balance toward mitochondrial apoptosis [209]. In HeLa and CaSki cervical cancer cells, ferulic acid induced DNA fragmentation and PARP-1 cleavage, hallmarks of apoptosis, and these effects were accompanied by activation of caspase-3[248,249]. Interestingly, ferulic acid may also interfere with growth factor signaling; a study by Yang et al. (2015) reported that ferulic acid suppressed fibroblast growth factor-1 (FGF1) and its receptor FGFR1 in melanoma cells, leading to downstream inhibition of the PI3K/Akt pathway and inhibited tumor cell migration [250]. In vivo, ferulic acid displays chemopreventive and therapeutic properties. Dietary ferulic acid supplementation in a rat hepatocarcinogenesis model significantly upregulated hepatic Nrf2 and p53 levels while downregulating phosphorylated Akt and NF-κB, correlating with reduced tumor burden [251] Ferulic acid has also been shown to protect normal tissues from chemotherapy-induced damage (e.g., nephrotoxicity) by activating Nrf2/HO-1 and PPARγ in vivo, all the while sensitizing tumor cells to the chemotherapy *via* NF-κB suppression [252]. In summary, ferulic acid's anticancer mechanism is multi-pronged: it restrains cell proliferation through cell cycle arrest, promotes apoptosis by modulating Bcl-2 family proteins and caspases, and attenuates inflammation and oxidative stress *via* NF-κB inhibition and Nrf2/PPARγ activation. These properties make it a compelling adjunct in cancer prevention and in enhancing the efficacy of conventional therapies.

5.5. p-Coumaric Acid

p-Coumaric acid is a phenolic acid that, although less studied than ferulic or caffeic acid, has shown notable anti-cancer effects, particularly in colorectal cancer models. Mechanistically, p-coumaric acid powerfully activates the Nrf2 pathway, which is thought to underlie its chemopreventive activity in the colon [253]. In a short-term carcinogen model, Sharma et al. (2019) found that p-coumaric acid supplementation induced nuclear

translocation of Nrf2 in colon mucosal cells, elevating phase II enzyme expression and significantly reducing early precancerous lesions [253]. This Nrf2-driven antioxidant response protects cells from DNA damage and also indirectly reduces NF-κB activity (as Nrf2 and NF-κB can negatively regulate each other). Indeed, an anti-inflammatory role of p-coumaric acid is well documented: it inhibits NF-κB activation, resulting in decreased levels of pro-inflammatory cytokines and mediators (IL-6, IL-1β, COX-2) in vitro [254]. By impairing NF-κB, p-coumaric acid also diminishes expression of cyclin D1 and other proliferation drivers, contributing to cell cycle arrest in cancer cells [255]. A distinct feature of p-coumaric acid is its ability to induce endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in tumor cells. Treatment of colon cancer cells with p-coumaric acid was shown to downregulate the chaperone GRP78 and activate PERK-eIF2α signaling, leading to C/EBP homologous protein (CHOP)-mediated apoptosis [210]. This links p-coumaric acid to the disruption of proteostasis in cancer cells, a stress that can selectively kill malignant cells laden with misfolded proteins. On the mitochondrial apoptosis front, p-coumaric acid increases the Bax/Bcl-2 ratio and elevates Cyt-c release, effectively engaging the intrinsic apoptotic pathway [211]. Caspase-9 and -3 activation has been observed following p-coumaric acid treatment in breast and colon cancer cell lines, confirming execution of apoptosis [211]. Additionally, p-coumaric acid can inhibit growth factor signaling such as IGF-1R/Akt, and it modestly suppresses MAPK/ERK activity, thereby blocking proliferative and survival cues [256,257]. In vivo studies reinforce these findings: p-coumaric acid administration in rats attenuated dimethylhydrazine-induced colon carcinogenesis, with treated animals showing lower NF-κB and higher Nrf2 activity in colonic tissues compared to controls [253]. The treated rats also exhibited reduced inflammatory cell infiltration and a decline in dysplastic aberrant crypt foci, indicating a chemo-preventive effect via modulation of the Nrf2–NF-κB axis. Beyond the colon, p-coumaric acid has demonstrated anti-neoplastic effects in models of skin and liver cancer, again correlating with enhanced antioxidant enzyme levels and reduced oxidative damage [258]. In summary, p-coumaric acid combats cancer through antioxidant activation (Nrf2), inflammation inhibition (NF-κB), and pro-apoptotic stress induction (ER stress and mitochondrial pathways). Its capacity to simultaneously trigger protective responses in normal cells and lethal stress in cancer cells exemplifies the nuanced chemopreventive potential of dietary phenolics.

5.6. Gallic Acid

Gallic acid is a benzoic acid-type polyphenol with a well-established reputation for antioxidant activity; paradoxically, in cancer cells gallic acid often acts as a pro-oxidant and potent inducer of apoptosis. This dual nature is key to gallic acid's mechanism. In normal physiological settings, gallic acid can chelate metal ions and scavenge radicals [259]. However, within the high-ROS environment of tumor cells, gallic acid undergoes oxidative conversion to generate semiquinone radicals and hydrogen peroxide, effectively raising intracellular ROS to cytotoxic levels [260]. This surge in ROS causes oxidative DNA damage and dysfunction of mitochondria in cancer cells, overwhelming their redox defenses. Consistently, gallic acid has been shown to collapse the mitochondrial membrane potential in a dose-dependent manner and increase lipid peroxidation in cancer cell membranes [261]. The result is activation of the intrinsic apoptotic pathway: gallic acid-treated cancer cells exhibit Bax upregulation, Bcl-2 downregulation, cytochrome c release, and activation of caspase-9 and -3 [262]. In bladder carcinoma T24 cells, for example, gallic acid caused a significant drop in phosphorylated Akt and IκBα, accompanied by increased Bax/Bcl-2 expression, leading to apoptotic cell death [263]. That study linked gallic acid's effects to simultaneous suppression of the PI3K/Akt/NF-κB pathway, suggesting that gallic acid not only induces direct oxidative injury but also blocks key survival signals. Indeed, gallic acid interferes with multiple cell signaling pathways relevant to cancer progression. It can inhibit constitutive STAT3 and NF-κB activity, reducing transcription of genes involved in proliferation (cyclins, c-Myc), inflammation (COX-2, IL-6), and angiogenesis (VEGF) [264]. Additionally, gallic acid targets receptor tyrosine kinase pathways: in triple-negative breast cancer cells, gallic acid downregulated EGFR expression and attenuated downstream MAPK/ERK signaling, contributing to growth arrest and apoptosis [264,265]. Another facet of gallic acid's action is the induction of autophagy – some studies report that gallic acid triggers a cytotoxic form of autophagy in cancer cells through AMPK activation and mTOR inhibition, which can synergistically enhance apoptosis [266]. Gallic acid also exhibits anti-metastatic properties. It has been noted to inhibit migration and invasion in melanoma and breast cancer cells by suppressing focal adhesion kinase (FAK) signaling and matrix metalloproteinase expression [267]. In vivo, gallic acid is effective at both reducing tumor growth and potentiating the effects of chemotherapy. For instance, in an A549 lung cancer xenograft model, intraperitoneal gallic acid over 4 weeks significantly reduced tumor volume and when combined with cisplatin led to greater tumor regression than chemotherapy alone [268]. Treated tumor tissues showed increased cleaved caspase-3 and decreased Ki-67 proliferation indices, in line with enhanced apoptosis and cell-cycle arrest. Importantly, gallic acid's pro-oxidant killing of tumor cells does not translate into systemic

toxicity when used at moderate doses, owing to normal cells' higher reserve of antioxidants and lower baseline ROS – a selectivity that is highly advantageous in cancer therapy [269]. In summary, gallic acid combats cancer through a combination of ROS-mediated cytotoxicity and inhibition of survival pathways like Akt, NF-κB, and EGFR/MAPK. This leads to robust apoptosis and can also curb invasion and sensitize tumors to other treatments, marking gallic acid as a uniquely potent polyphenol in the context of cancer.

6. Conclusions and perspectives

Garlic is rich in anticancer polyphenols, as demonstrated in *in vitro* studies. The anticancer mechanism of action varies depending on the type of polyphenol. Several polyphenols found in garlic activate PPARs, which appear to be responsible for at least part of garlic's anticancer activity. A major mechanism underlying garlic's anticancer properties is ROS-dependent toxicity and/or apoptosis. Additionally, Nrf2 is also involved in the mechanism of action of garlic polyphenols. Our review highlights that garlic polyphenols may play a role in cancer prevention and support conventional cancer treatments. Unfortunately, for many polyphenols, the precise mechanisms of action remain unknown. According to the current state of knowledge, the available studies have been conducted primarily on cell lines. In our opinion, more research on animal models, followed by clinical trials, is essential. The anticancer effects of garlic polyphenols arise from their broad impact on molecular processes related to cancer formation and progression. Regular consumption of garlic as part of a healthy diet may enhance the body's protection against cancer, particularly due to its antioxidant, anti-inflammatory, and cancer cell-signalling properties. However, for comprehensive protection, garlic should be included as part of a balanced lifestyle that incorporates other healthy habits.

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Conflict of interests

The authors declare no conflict of interests.

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Conflict of Interest

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

4. Podsumowanie i wnioski

4.1. Wprowadzenie i uzasadnienie wyboru tematu

Produkty naturalne oraz medycyna tradycyjna odgrywają istotną rolę we współczesnym podejściu do zdrowia. Wiele osób dostrzega, że nowoczesna medycyna nie zawsze stanowi jedyne skuteczne rozwiązanie dla obecnych dolegliwości. W związku z tym coraz większą popularnością cieszą się naturalne metody leczenia, takie jak fitoterapia, ponieważ rośliny lecznicze są bogatym źródłem substancji farmakologicznie czynnych (Kumar i in., 2021; Prakash i in., 2021a, 2021b).

Czosnek zwyczajny (*Allium sativum* L.) należy do rodziny roślin amarylkowatych (*Amaryllidaceae*) i jest jedną z najstarszych roślin użytkowych znanych człowiekowi od tysięcy lat (Rivlin, 2001). Roślina ta pochodzi z Azji Środkowej i od dawna stanowi ważną uprawę w regionie Morza Śródziemnego, a także popularną przyprawę w takich częściach świata jak Afryka i Europa (Damián i in., 2022). Obecnie uprawia się go w większości krajów strefy umiarkowanej, a jego znaczenie wykracza daleko poza zastosowanie kulinarne – czosnek zaliczany jest do tzw. żywności funkcjonalnej ze względu na szerokie spektrum właściwości prozdrowotnych (Verma i in., 2023). Według danych Organizacji Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (FAO, ang. *Food and Agriculture Organization of the United Nations*), w 2022 roku światowa produkcja czosnku wyniosła około 29,15 miliona ton. Największym producentem były Chiny, które odpowiadały za około 73% globalnej produkcji, co przekłada się na 21,34 miliona ton. Pozostałymi znaczącymi producentami czosnku w 2022 roku były: Indie – 3,21 miliona ton, Bangladesz – 526,8 tysiąca ton, Egipt – 396,5 tysiąca ton, Hiszpania – 281,9 tysiąca ton. Te pięć krajów łącznie odpowiadało za ponad 92% światowej produkcji czosnku. Natomiast Polska, z 25 200 ton produkcji rocznie jest oceniana na 34 miejscu wśród producentów tej rośliny (Food, 2022).

Od czasów starożytnych czosnek ceniony jest ze względu na swój aromat i smak. Pierwsze wzmianki o czosnku można znaleźć w egipskim papirusie, Codex Ebers, pochodzącym z 1550 r. p.n.e., zawierającym setki przepisów wskazujących tą roślinę na przykład jako lek na na bóle głowy i ukąszenia owadów oraz jako środek przeciwbólowy (Sarpaki, 2021). Dowodzi to stosowania czosnku nie tylko jako produktu spożywczego, ale w dużej mierze jako rośliny leczniczej. W starożytnej Grecji był on tradycyjnie stosowany do leczenia wielu różnych zaburzeń, w tym trądu, biegunki, zaparć, astmy, gorączki i infekcji (Petrovska i Cekovska, 2010). Obecnie wiadomo, że świeże ząbki czosnku

zawierają około 60% wody, 32% węglowodanów i 6,45% białka (Moreno-Ortega i in., 2020). Zawartość kalorii w czosnku jest wysoka i wynosi 146 kcal/100 g świeżej masy produktu. W główkach można zidentyfikować takie pierwiastki jak potas (400 mg), fosfor (153 mg), magnez (25 mg), sód (17 mg) czy wapń (41 mg/100 g świeżej masy). W czosnku występują również selen i german, a ich ilość zależy od zawartości minerałów obecnych w glebie (Oosthuizen i in., 2018; Tavares i in., 2021). Ponadto, ząbki czosnku są bogatym źródłem witaminy C (ok. 31 mg/100 g świeżej masy) i dostarczają niewielkich ilości witamin z grupy B, szczególnie witaminy B1 (Ciuba i in., 2016). Powszechnie uznaje się, że czosnek jest również bogaty w złożone substancje biologicznie czynne. W dotychczasowych badaniach zidentyfikowano ponad 200 substancji chemicznych o różnorodnych właściwościach (Fallah-Rostami i in., 2013). Jego zróżnicowany skład chemiczny zapewnia szeroki zakres efektów biologicznych. Współczesne badania naukowe wykazały, że ekstrakty z czosnku lub spożycie surowych ząbków wykazują działanie przeciwhiperlipidemiczne (Nickavar, 2022), przeciwutleniające (Recinella i in., 2022), przeciwzapalne (Shao i in., 2022), wspomagające układ sercowo-naczyniowy (Imaizumi i in., 2023), obniżające ciśnienie krwi (Xiang i in., 2024), przeciwbakteryjne (B. Adjei-Mensah i in., 2023), przeciwwirusowe (Benjamin Adjei-Mensah i in., 2023), przeciwgrzybicze (Agustantina i Soekartono, 2021), przeciwpasożytnicze (Qaemifar i in., 2023), przeciwdziałające cukrzycy (Gil i Rabiej, 2024), przeciwnowotworowe (Stępień i in., 2024), ochronne dla wątroby(Almatroodi i in., 2020), immunomodulujące (Song i in., 2023) oraz obniżające poziom glukozy we krwi (Song i in., 2023). Co pokazuje znaczący potencjał tej rośliny.

Biologicznie aktywne związki czosnku można podzielić na dwie główne grupy: substancje zawierające siarkę i substancje nie zawierajcie siarki (El-Saadony i in., 2024). Pierwsza grupa obejmuje aliin, γ-glutamylocysteinę oraz ich pochodne, takie jak allicyna, disulfid diallilowy, trisulfid diallilowy, ajoen, S-allylcystein oraz S-allylmerkaptocystein (El-Saadony i in., 2024). Należy jednak zaznaczyć, że np. allicyna nie występuje w czosnku, dopóki nie zostanie on uszkodzony (zmiażdżony, pocięty, pogryziony, odwodniony, sproszkowany lub wystawiony na działanie wody) co aktywuje enzym alliinazę, który metabolizuje alliinę do allicyny (El-Saadony i in., 2024). Allicyna natychmiast rozkłada się na pozostałe w/w pochodne. Ponieważ czosnek zawiera najwyższe stężenie związków siarkowych spośród wszystkich gatunków z rodzaju *Allium sp.*, ta grupa związków jest najczęściej badana i uznawana za odpowiedzialną za jego właściwości prozdrowotne (Aviello i in., 2009; El-Saadony i in., 2024). Druga grupa substancji obecnych w czosnku to

substancje wolne od siarki z pośród których najważniejsze to polifenole (Kim i in., 2013). Związki polifenolowe są często niedocenianą grupą którą również charakteryzują właściwości prozdrowotne porównywalne a często nawet silniejsze niż te przypisywane związkom siarkowym (Lanzotti, 2006). Ponadto należy zaznaczyć, że związki polifenolowe w czosnku są generalnie stabilniejsze niż związki siarkowe**,** szczególnie pod względem reaktywności chemicznej i odporności na czynniki środowiskowe (np. temperatura, tlen)(Fujisawa i in., 2008; Pedisić i in., 2018). Co ciekawe, odpowiednia obróbka tej rośliny (np. fermentacja w czosnku czarnym) może przyczyniać się nawet do wzrostu zawartości tak niedocenianych polifenoli (Ma i in., 2021). Dzięki wysokiej zawartości polifenoli i naturalnych przeciwutleniaczy (np. wspomniana wcześniej witamina C), czosnek może bezpośrednio i pośrednio zwiększać ekspresję enzymów antyoksydacyjnych, chroniąc prawidłowe komórki przed stresem oksydacyjnym (Askari i in., 2021).

Reaktywne formy tlenu (ROS, ang. *reactive oxygen species*) to zazwyczaj naturalne produkty uboczne metabolizmu komórkowego (Apel i Hirt, 2004). Mogą również powstawać w wyniku procesów patologicznych czy działania substancji chemicznych oraz czynników fizycznych. Mimo niezbędnej roli ROS w fizjologii komórki, ich nadmiar – szczególnie ze źródeł zewnętrznych, prowadzi do zaburzeń homeostazy, uszkodzeń DNA, białek i lipidów, a w konsekwencji do apoptozy (Wang i in., 2023). Wykazano, że ekstrakty z czosnku oraz jego składniki aktywne wykazują zdolność do neutralizacji ROS, zwiększania poziomu glutationu i stymulowania ekspresji enzymów antyoksydacyjnych w zdrowych komórkach (Kohda i in., 2013). Co ciekawe, podczas gdy niewielka stymulacja ROS jest korzystna dla komórek prawidłowych, komórki nowotworowe, ze względu na ich wysoki metabolizm i zaburzenia mitochondrialne, są szczególnie wrażliwe na dodatkowy stres oksydacyjny (Aboelella i in., 2021). Udokumentowano, że ekstrakty z czosnku mogą wywoływać apoptozę w liniach komórkowych raka wątroby (HepG2), jelita grubego (CACO-2), prostaty (PC-3) i piersi (MCF-7), poprzez aktywację kaspazy-3 – enzymu kluczowego dla szlaku apoptozy (Bagul i in., 2015). Równocześnie, czosnek może indukować śmierć komórek niezależnie od kaspazy, np. za pośrednictwem allicyny czy kwasu galusowego (De Martino i in., 2016). Jednym z interesujących kierunków badań jest rola receptora aktywowanego przez proliferatory peroksysomów gamma (PPARγ, ang. *peroxisome proliferator-activated receptor gamma*), który reguluje różnicowanie komórek, metabolizm, autofagię oraz produkcję enzymów antyoksydacyjnych (Elrod i Sun, 2008; Rupérez i Anguita-Ruiz, 2018). Badania wykazały, że polifenole obecne w czosnku zwyczajnym, takie jak kwercetyna, kwas kawowy i kwas ferulowy, mogą aktywować

receptor PPARγ, modulując jego aktywność transkrypcyjną (Shin i in., 2009). Na przykład, kwercetyna wiąże się z domeną wiążącą ligand PPARγ, indukując ekspresję genów związanych z różnicowaniem komórkowym i metabolizmem lipidów (Sun i in., 2015). Dzięki temu czosnek może wywierać działanie przeciwzapalne i metaboliczne poprzez mechanizmy zależne od PPARγ.

Badania dotyczące czosnku prowadzone przez zespół badawczy w którym uczestniczyłam od 2016 roku wykazały, że różne odmiany czosnku charakteryzują się znaczącymi różnicami w zawartości związków polifenolowych, co odpowiadało toksyczności ekstraktów (Szychowski i in., 2018). Zainspirowało to mnie do sformułowania pierwszych hipotez rozprawy doktorskiej (publikacja nr 1). Co więcej, szczegółowy przegląd literatury, w którym brałam udział, wykazał, że mechanizm działania czosnku może znacząco zależeć od formy jego przetworzenia, a także wpływać na zawartość polifenoli. Temat ten stał się przedmiotem moich zainteresowań naukowych. (Bar i in., 2022). Było to inspiracją do sformułowania koncepcji publikacji 2 i 3.

Do badań wybrałam trzy linie komórkowe, z czego dwie stanowiły linie nowotworowe: gruczolakorak okrężnicy (CACO-2), oraz płaskonabłonkowy rak języka (SCC-15). Nowotwory języka i jelita grubego należą do często diagnozowanych i trudnych w leczeniu typów raka (Marcellinaro i in., 2023). Nowe metody profilaktyki i leczenia są więc pilnie poszukiwane, a ponieważ są to nowotwory występujące w tkankach mających kontakt z pokarmem, czosnek mógłby stanowić wartościową substancję żywieniową wspomagającą terapię. Co więcej, związki roślinne są zazwyczaj mniej toksyczne niż tradycyjne leki przeciwnowotworowe (Reuter, 1995) i mogą być stosowane jako uzupełnienie terapii lub w profilaktyce. W celu oceny toksyczności związków aktywnych zawartych w czosnku na komórki prawidłowe, do badań włączono linię fibroblastów (BJ).
Dlatego, głównymi celami rozprawy doktorskiej było:

- Określenie potencjału antynowotworowego surowych ekstraktów czosnkowych w warunkach *in vitro* – publikacja 1
- Określenie potencjału antynowotworowego liofilizowanych ekstraktów czosnkowych w warunkach *in vitro* – publikacja 2
- Próba powiązania zawartości flawonoidów z prozdrowotnymi właściwościami ekstraktów – publikacja 3

4.2. Omówienie publikacji stanowiących cykl

Publikacja 1: K. A. Szychowski, **U. E. Binduga**, K. Rybczyńska-Tkaczyk, M. L. Leja, J. Gmiński. Cytotoxic effects of two extracts from garlic (*Allium sativum* L.) cultivars on the human squamous carcinoma cell line SCC-15. Saudi Journal of Biological Sciences 2018, 25 (8): 1703-1712

Celami pierwszej publikacji było:

1) Określenie, czy ROS jest zaangażowany w mechanizm antynowotworowego działania surowych ekstraktów z odmian uprawnych czosnku Harnaś i Morado.
2) Określenie toksyczności oraz działania proapoptotycznego badanych ekstraktów.

W przedstawionej pracy badawczej oceniono cytotoksyczność wodnych ekstraktów dwóch odmian czosnku zwyczajnego – polskiej odmiany Harnaś oraz hiszpańskiej odmiany Morado w komórkach ludzkiej linii raka płaskonabłonkowego języka SCC-15. Głównym celem badania było określenie udziału ROS oraz procesu apoptozy w mechanizmie działania cytotoksycznego wspomnianych ekstraktów czosnkowych. Badania przeprowadzono z wykorzystaniem testów biochemicznych, takich jak: oznaczenie uwolnienia dehydrogenazy mleczanowej (LDH, ang. *lactate dehydrogenase*) do pożywki hodowlanej, test wychwytu czerwieni obojętnej (ang. *Neutral Red Uptake Assay*), oznaczenie aktywności kaspazy-3, testy z wykorzystaniem wskaźników fluorescencyjnych żywotności komórek (kalceina-AM), detekcja ROS przy użyciu sondy H_2DCFDA (ang. *2',7'-dichlorodihydrofluorescein diacetate*) oraz barwienie jąder komórkowych i ciałek apoptotycznych barwnikiem Hoechst 33342. Procedura ekstrakcji wodnej była prowadzona według wcześniej opisanej metodyki (Lemar i in., 2002), która została skrótowo przedstawiona w załączonej publikacji (publikacja 1).

Przeprowadzone badania wykazały, że oba ekstrakty czosnkowe indukują znaczący wzrost poziomu ROS w komórkach SCC-15 już po 6 godzinach inkubacji, przy czym efekt ten był bardziej intensywny i długotrwały w przypadku odmiany Harnaś. Po 24 i 48 godzinach aktywność prooksydacyjna odmiany Morado ulegała osłabieniu, natomiast Harnaś nadal indukował silną produkcję ROS. Jest to zgodne z wcześniejszymi doniesieniami wskazującymi, że komórki nowotworowe charakteryzują się wyższym poziomem endogennego stresu oksydacyjnego, co czyni je szczególnie podatnymi na dalszy wzrost ROS wywołany przez czynniki egzogenne (Liou i in., 2010; Pelicano i in., 2004).

Cytotoksyczność ekstraktów potwierdzono za pomocą dwóch wspomnianych testów: LDH oraz Neutral Red. Zarówno odmiana Harnaś, jak i Morado wywoływały zależne od dawki i czasu uszkodzenie błon komórkowych oraz spadek żywotności komórek. Co ciekawe, po 48 godzinach, nawet niższe stężenia ekstraktów wykazywały wyraźne działanie cytotoksyczne, co może wskazywać na narastające działanie stresu oksydacyjnego i akumulację uszkodzeń komórkowych. Obserwowane różnice pomiędzy wynikami LDH i Neutral Red potwierdzają wcześniejsze sugestie, że różne testy cytotoksyczności mogą różnić się czułością i czasem odpowiedzi na bodźce toksyczne (Riss i Moravec, 2004).

Wbrew przewidywaniom, ekstrakty czosnkowe nie aktywowały znacząco kaspazy-3 – głównego efektora szlaku apoptozy – nawet po 48 godzinach hodowli. Wyjątkiem był nieznaczny wzrost aktywności kaspazy-3 po zastosowaniu niskiego stężenia ekstraktu Morado. Co więcej, najwyższe stężenia obu ekstraktów obniżały aktywność tego enzymu. Sugeruje to, że dominującym mechanizmem śmierci komórkowej może być nekroza lub mniej prawdopodobnie alternatywne formy śmierci komórek, takie jak ferroptoza czy kaspazo-niezależna apoptoza. Podobne obserwacje poczynili Park i in. (2005) oraz De Martino i in. (2016), którzy opisali indukcję śmierci komórkowej przez czosnek i jego składniki (np. allicynę) w sposób niezależny od kaspaz (De Martino i in., 2016; Park i in., 2005).

Kluczowym aspektem opisywanej publikacji była rola ROS w cytotoksyczności. Użycie związku narzędziowego w postaci N-acetylo-L-cysteiny (NAC), będącej skutecznym zmiataczem wolnych rodników, prowadził do wyraźnego zahamowania produkcji ROS oraz ochrony komórek przed działaniem ekstraktów czosnkowych potwierdzone testami LDH i Neutral Red. Wyniki te jednoznacznie potwierdzają, że cytotoksyczność była zależna od stresu oksydacyjnego. Podobny mechanizm działania opisano w pracy Yang i in. (2009), gdzie diallilodisiarczek (składnik czosnku) indukował śmierć komórek nowotworowych poprzez ROS i aktywację szlaku stresu retikulum endoplazmatycznego (Yang i in., 2009).

Uzyskane w opisywanej publikacji wyniki wpisują się w szerszy kontekst danych literaturowych, według których czosnek i jego składniki wywierają efekt przeciwnowotworowy poprzez indukcję stresu oksydacyjnego, uszkodzeń DNA oraz zakłócenia w integralności błon komórkowych (Amagase, 2006; Bagul i in., 2015). Obserwowane różnice pomiędzy odmianami czosnku – silniejszy, długotrwały efekt odmiany Harnaś w porównaniu do Morado – mogą wynikać z odmiennych profili fitochemicznych, w tym zawartości związków siarkowych oraz polifenoli, co wcześniej

wykazano w badaniach porównujących odmiany czosnku z różnych regionów geograficznych (Beato i in., 2011; Matysiak i in., 2015; Szychowski i in., 2018). Wykonane w niniejszej pracy próby wizualizacji ciałek apoptotycznych (Hoechst 33342) oraz metabolizmu komórek (Kalceina AM) wykazały spadek liczby komórek w grupach poddanych działaniu ekstraktów czosnku, bez istotnego wpływu na spadek metabolizmu oraz formowanie ciałek apoptotycznych. Dodatek NAC odwracał efekt co powiedziało zaangażowanie ROS w antyproliferacyjne działanie badanych ekstraktów.

Podsumowując, wyniki potwierdzają istotną rolę stresu oksydacyjnego w mechanizmach działania czosnku na komórki nowotworowe SCC-15. Brak aktywacji kaspazy-3 sugeruje, że indukowana śmierć komórkowa nie jest klasyczną apoptozą, lecz raczej formą ROS-zależnej śmierci alternatywnej np. nekrozą w skutek silnego odziaływania ROS. Nie można również wykluczyć działania antyproliferacyjnego. Obie odmiany czosnku – Harnaś i Morado – wykazują istotny potencjał przeciwnowotworowy w warunkach *in vitro*, przy czym odmiana polska Harnaś może mieć silniejsze i bardziej długofalowe działanie. Ponieważ badania wykonywane były na świeżo pozyskanych ekstraktach czosnkowych, przechowywanych w zamrożeniu do czasu dodania do hodowli komórkowej, najprawdopodobniej w tej publikacji obserwowane efekty były skutkiem działania związków siarkowych zawartych w czosnku.

Głównymi wnioskami z pracy jest to że:

- polska odmiana czosnku Harnaś wykazywała większy potencjał stymulacji produkcji ROS w komórkach SCC-15.
- obydwie badane odmiany czosnku Harnaś i Morado stymulowały cytotoksyczność zależną od ROS w komórkach linii SCC-15.
- najwyższe stężenie badanych ekstraktów nie powodowało aktywacji kaspazy-3 charakterystycznej dla apoptozy.

Publikacja 2: U. E. Binduga, A. Kopeć, J. Skoczylas, K. A. Szychowski. Comparison of the Cytotoxic Mechanisms of Different Garlic (*Allium sativum* L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. International Journal of Molecular Sciences, 2025, 26 (1): 387

Celami drugiej publikacji było:

1) Określenie toksyczności oraz działania proapoptotycznego badanych ekstraktów uzyskanych z liofilizatów
2) Próba powiązania zawartości polifenoli z działaniem antynowotworowym danego ekstraktu
3) Określenie zmian ekspresji receptora PPARγ po ekspozycji na badane ekstrakty

W drugiej z omawianych prac dokonano porównawczej analizy czterech odmian czosnku, w tym dwóch pochodzących z Polski (Harnaś, Ornak) oraz dwóch z Hiszpani (Violeta i Morado), pod kątem ich właściwości przeciwnowotworowych. Badania przeprowadzono na dwóch liniach komórek nowotworowych: SCC-15 i CACO-2, a także na prawidłowych fibroblastach linii BJ. Metody stosowane w tej pracy były podobne do stosowanych we wcześniej omówionej publikacji. Dodatkowo wykonano analizę chromatograficzną badanych ekstraktów, a także ich wpływ na ekspresję genów i białek w badanych komórkach. Eksperymenty prowadzone były w dwóch przedziałach czasowych: 6 godzin – dla badania ekspresji genów oraz 24 godziny w przypadku badania ekspresji białek i testów metabolicznych. W drugiej z prac ekstrakty wodne powstały z proszku powstałego po zmieleniu zliofilizowanych plastrów pokrojonego czosnku. Liofilizaty zostały rozdrobione za pomocą młynka laboratoryjnego Pulverisette 14 z sitem 0,5 mm. Obecnie wiadomo, że związki siarkowe takie jak np. allicyna są szczególnie niestabilne i w ciągu kilkunastu godzin ulegają rozkładowi w roztworach wodnych. Należy również zaznaczyć, że w produktach komercyjnych czosnek najczęściej dostępny jest w formie sproszkowanej po liofilizacji lub suszeniu. Obecnie wiadomo, że zarówno suszenie jak i liofilizacja inaktywuje enzym allinazę który przekształca alline do allicyny (Amagase, 2006). Ponadto, suszenie oprócz degradacji związków siarkowych niszczy również pozostałe w produkcie polifenole (Lanzotti, 2006). Tak więc, aby badać substancje polifenolowe w uzyskanym ekstrakcie i zredukować efekt działania związków siarkowych w opisywanej publikacji wybrano ekstrakty wodne przygotowane na bazie liofilizatów.

Głównym celem badań zawartych w tej publikacji było zbadanie cytotoksyczności ekstraktów oraz zidentyfikowanie mechanizmów molekularnych odpowiedzialnych za ich działanie, ze szczególnym uwzględnieniem roli receptora PPARγ.

Wszystkie badane ekstrakty wykazywały zdolność do generowania ROS, przy czym efekt ten był wyraźnie silniejszy w liniach komórek nowotworowych niż w fibroblastach. Największy wzrost poziomu ROS odnotowano dla odmiany Harnaś w komórkach CACO-2 – ponad 15-krotny względem kontroli. Tak silna indukcja ROS w komórkach

nowotworowych jest zgodna z doniesieniami literaturowymi wskazującymi, że stres oksydacyjny może być wykorzystywany terapeutycznie do selektywnego uszkadzania komórek nowotworowych (Trachootham i in., 2009). Zdolność czosnku do generowania ROS przypisuje się głównie obecności związków siarkowych (np. allicyna, ajoen) ale również niedocenianych związków fenolowych (np. katechina) (Amagase, 2006).

Cytotoksyczność badanych ekstraktów została oceniona za pomocą testu LDH oraz testu z redukcji rezazuryny (komercyjnie znany pod nazwą ang. *Alamar Blue,* bazuje na aktywności mitochondrialnej). Ekstrakt Morado istotnie zwiększał poziom LDH w komórkach SCC-15 i fibroblastach BJ, wskazując na potencjalną toksyczność również wobec komórek prawidłowych. Z kolei ekstrakty Harnaś i Ornak wywoływały mniejszy efekt w komórkach BJ, co może sugerować większą selektywność względem komórek nowotworowych – cechę pożądaną w kontekście terapii przeciwnowotworowych (Lee i in., 2011). W teście redukcji resazuryny obserwowano znaczne spadki aktywności metabolicznej po zastosowaniu ekstraktów z odmian Ornak i Morado, co potwierdzało ich silne działanie cytotoksyczne. Co interesujące, w linii komórkowej CACO-2 po ekspozycji na ekstrakt z czosnku Ornak obserwowano wzrost aktywności metabolicznej, co mogło być związane z mechanizmem obronnym komórki i aktywnością enzymów z rodziny cytochromów P450.

Interesujące wyniki uzyskano w analizie aktywności kaspazy-3 – kluczowego enzymu odpowiedzialnego za wykonawczy etap apoptozy. Ekstrakt Ornak istotnie zwiększał aktywność kaspazy-3 we wszystkich zastosowanych stężeniach w komórkach SCC-15. Podobnie, ekstrakt z czosnku Morado zwiększał aktywność kaspazy-3 we wszystkich zastosowanych stężeniach w komórkach BJ. Sugeruje to aktywację procesu apoptozy we wskazanych przypadkach. Pozostałe ekstrakty, zwłaszcza Harnaś i Violeta, nie wpływały istotnie na aktywację kaspazy-3, co sugeruje udział innych mechanizmów, np. śmierci komórek poprzez autofagię lub nekrozę. Doniesienia literaturowe potwierdzają, że ekstrakty roślinne mogą różnicować mechanizmy śmierci w zależności od zawartości związków aktywnych i typu komórek (Jasamai i in., 2016).

W analizie ekspresji genów i białek szczególną uwagę poświęcono PPARγ – receptorowi jądrowemu regulującemu szlaki związane z proliferacją, apoptozą i metabolizmem lipidów. Stwierdzono, że odmiany Harnaś i Ornak wyraźnie obniżały poziomy mRNA i białka PPARγ w komórkach nowotworowych, natomiast Morado zwiększał ekspresję tego receptora w fibroblastach. Rola PPARγ w nowotworach jest złożona – z jednej strony może promować różnicowanie i zahamowanie proliferacji,

z drugiej strony jego aktywacja może sprzyjać przeżyciu niektórych typów komórek nowotworowych (Kota i in., 2005). Zmniejszenie poziomu PPARγ w SCC-15 po zastosowaniu Harnaś i Ornak może sugerować jego aktywacje i być elementem po aktywacyjnej degradacji receptora. Ponadto wykazano, że wszystkie odmiany czosnku obniżają poziom białka LC3A – markera autofagii – w komórkach nowotworowych, co może oznaczać zahamowanie tego procesu i skierowanie komórek na szlak apoptozy. Równocześnie zaobserwowano wzrost poziomu enzymów antyoksydacyjnych dysmutazy ponadtlenkowej (SOD1, ang. *superoxide dismutase 1*) i katalzay (CAT, ang. *catalase*)), szczególnie w fibroblastach BJ, co można interpretować jako odpowiedź ochronną komórek prawidłowych na stres oksydacyjny. Wyjątkowy pod tym względem był jednak ekstrakt z odmiany Ornak. Zjawisko wzrostu ekspresji enzymów antyoksydacyjnych w odpowiedzi na ROS zostało już wcześniej opisane w pracy Kim'a i współpracowników (2010), gdzie komórki nowotworowe były bardziej podatne na indukowany stresem ROS, podczas gdy komórki prawidłowe aktywowały mechanizmy kompensacyjne (Kim i in., 2010).

Jak wspomniano, w prezentowanej publikacji badano również profil polifenolowy ekstraktów, analizowany metodą HPLC (ang. *high-performance liquid chromatography*). Badanie wykazało obecność wielu bioaktywnych związków o znanym potencjale przeciwnowotworowym. Ekstrakty Harnaś i Ornak zawierały najwięcej katechiny – związku będącego agonistą PPARγ – co może tłumaczyć ich zdolność do modulacji szlaków sygnałowych zależnych od tego receptora (Pham Ngoc i in., 2019). Odmiana Violeta wyróżniała się obecnością acacetyny, epikatechiny i naryngeniny, a Morado był bogaty w kemferol i rutynę – flawonoidy o udowodnionym działaniu cytotoksycznym i proapoptotycznym w różnych typach nowotworów (Brusselmans i in., 2005).

Oprócz badań znajdujących się w opisywanej publikacji wykonano dodatkowo analizę składu podstawowego (sucha masa, węglowodany [wyliczone z wzoru], białko [metodą Kjeldahla], tłuszcze [metodą Soxhleta] oraz popiół) według dobrze opisanej i szeroko stosowanej metodologii i norm (AOAC, 2011) (Tabela 1). Ponadto, wykonano analizę zawartości całkowitych polifenoli metodą z wykorzystaniem odczynnikiem Folina-Ciocalteu oraz właściwości antyoksydacyjnych metodą wykożystująca $ABTS^{\bullet+}$ (ang. *2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid*) (Re i in., 1999) (Tabela 1). Przeprowadzona dodatkowa analiza wykazała, że odmiana Ornak wyróżniała się spośród pozostałych najwyższą zawartością białka (24,20 g/100g), polifenoli (778,88 mg/100g) i najsilniejszymi właściwościami antyoksydacyjnymi (88,69 μmol Troloxu/1g). Wyniki te mogą dodatkowo tłumaczyć nieznaczne odrębne działanie tego ekstraktu na poziomie

ekspresji genów (*PPARγ, LC3A, SOD1* i *CAT*). Ekstrakt z czosnku Ornak działał również odmiennie na omawiane parametry takie jak aktywność kaspazy-3 oraz redukcję resazuryny. Nie można wykluczyć, że było to właśnie rezultatem odmiennego składu. Co ciekawe najwyższą zawartość polifenoli miała odmiana Morado (848,84 mg/100g) przy najniższej aktywności antyoksydacyjnej (42,28 µmol Troloxu/1g). Ta obserwacja sugeruje, że polifenole nie są głównym składnikiem odpowiedzialnym za właściwości antyoksydacyjne czosnku. Zgodnie z literaturą oprócz polifenoli za silne właściwości antyoksydacyjne czosnku odpowiedzialne są selen, witamina C oraz liczne związki siarkowe które nie były badane w niniejszej rozprawie. W świetle powyższego nie można wykluczyć wpływu w/w substancji na otrzymane wyniki.

	Harnaś	***Ornak***	***Violeta***	***Morado***
Sucha Masa *g/100g**	37,20 ± 0,05^{a}	40,63 ± 1,83^{b}	37,10 ± 0,82^{a}	36,80 ± 0,05^{a}
Węglowodany Ogółem *g/100g***	77,55 ± 0,35^{c}	70,20 ± 0,12^{a}	84,13 ± 0,23^{d}	72,27 ± 0,01^{b}
Białka *g/100g***	19,00 ± 0,08^{b}	24,20 ± 0,04^{d}	16,50 ± 0,47^{a}	22,90± 0,20^{c}
Tłuszcze *g/100g***	1,18 ± 0,03^{a}	1,77 ± 0,17^{b}	1,32 ± 0,20^{a}	1,51 ± 0,03^{ab}
Popiół Ogółem *g/100g***	2,25 ± 0,22^{b}	3,78 ± 0,06^{a}	3,79 ± 0,09^{a}	3,27 ± 0,15^{c}
Polifenole Ogółem *mg/100g***	608,68 ± 27,93^{b}	778,88 ± 16,92^{c}	411,14 ± 11,31^{a}	848,84 ± 29,07^{d}
Aktywność Antyoksydacyjna *µmol Troloxu/1g*	79,45 ± 2,96^{a}	88,69 ± 1,08^{c}	79,60 ± 1,52^{b}	42,28 ± 1,06^{a}

Tabela 1. Skład podstawowy, ogólna zawartość polifenoli oraz aktywność antyoksydacyjna badanych ekstraktów. W przypadku chromatograficznej jednokierunkowej analizy wariancji (ANOVA) z zastosowaniem testu post-hoc Dunnetta, te same litery (a, b, c lub d) oznaczają grupy, które nie różnią się statystycznie istotnie przy $p < 0,05$ w analizie. Wyniki przedstawiają średnią z trzech powtórzeń ± odchyleniem standardowym. * - g/100g świeżej masy; ** - g/100g suchej masy.

Podsumowując, ekstrakty czosnkowe wykazują zróżnicowane działanie cytotoksyczne zależne od odmiany i typu komórek. Największy potencjał przeciwnowotworowy wykazały odmiany Harnaś i Ornak, charakteryzujące się wysoką aktywnością prooksydacyjną, modulacją ekspresji PPARγ oraz selektywnością względem komórek nowotworowych. Morado, choć silnie cytotoksyczny, wykazywał niższą selektywność, natomiast Violeta okazała się najłagodniejsza. Wyniki te potwierdzają, że wybór konkretnej odmiany czosnku ma istotne znaczenie dla jego potencjału terapeutycznego, a dalsze badania molekularne powinny skupić się na synergii składników aktywnych i ich wpływie na szlaki komórkowe regulowane przez PPARγ.

Głównymi wnioskami z pracy jest to że:

- wysoka produkcja ROS (w najwyższym stężeniu) ekstraktów była powiązana z silną toksycznością badanych ekstraktów czosnku
- wszystkie badane ekstrakty powodowały mniejszy wzrost ROS w prawidłowych fibroblastach BJ i były mniej toksyczne dla tych komórek
- wzory ekspresji PPARγ, LC3A, SOD1 i CAT sugerują odmienne mechanizmy działania badanych odmian czosnku
- najwyższe poziomy katechiny, znanego agonisty PPARγ, wykryto u odmian Harnaś i Ornak – odpowiada to spadkowi ekspresji mRNA *PPARγ*
- odmiana Ornak, posiadająca najsilniejsze właściwości antyoksydacyjne, najsilniej wpływała na ekspresje mRNA *PPARγ* i *LC3A* we wszystkich badanych liniach komórkowych

Publikacja 3: U. E. Binduga, K. A. Szychowski, Current state of knowledge on the anticancer properties of polyphenolic compounds from garlic (*Allium sativum* L.) – **publikacja w recenzji**

Celami trzeciej publikacji było:

1) Analiza dostępnej literatury na temat zawartości polifenoli w ekstraktach pochodzących z różnych form czosnku
2) Podsumowanie dostępnych danych literaturowych na temat antynowotworowego działania czosnkowych polifenoli w warunkach *in vitro*

W niniejszej pracy przeglądowej dokonano obszernej analizy aktualnego stanu wiedzy na temat przeciwnowotworowych właściwości związków polifenolowych pochodzących z czosnku zwyczajnego. Praca skupia się na podkreśleniu, że zawartość poszczególnych polifenoli zależeć może od formy czosnku (np. świeży, fermentowany, suszony) a także jego elementów (np. ząbki, liście), co znaleźć może odzwierciedlenie w molekularnym mechanizmie działania ekstraktu. Głównym celem opracowania było ukazanie, że oprócz dobrze poznanych związków siarkowych (np. allicyny), to właśnie polifenole stanowią istotny komponent o potencjale przeciwnowotworowym, często pomijanym w literaturze.

W pracy sklasyfikowano polifenole występujące w czosnku do czterech głównych grup: flawonoidy (np. kwercetyna, katechina, naryngenina), kwasy fenolowe (np. kwas galusowy, ferulowy, kawowy), stilbeny (np. resweratrol) oraz lignany (np. matairezynol). Zawartość i skład jakościowy tych związków zależy od wielu czynników, w tym odmiany czosnku, etapu dojrzałości, sposobu obróbki (czosnek surowy, suszony, czarny), jak również od warunków środowiskowych. Podkreślono, że liście czosnku zawierają znacznie więcej polifenoli niż ząbki, co czyni je obiecującym surowcem farmaceutycznym, mimo ich ograniczonego zastosowania kulinarnego w Europie.

W kontekście mechanizmów działania przeciwnowotworowego autorzy przedstawiają dowody na to, że polifenole z czosnku działają wielotorowo, m.in. poprzez:

- indukcję procesu apoptozy (głównie przez wzrost ROS, aktywację kaspaz, fragmentację DNA),
- zatrzymanie cyklu komórkowego (głównie w fazach G1 i G2/M),
- hamowanie angiogenezy i migracji komórek nowotworowych (m.in. przez wpływ na MMP-2/9 (ang. *matrix metalloproteinase-2 and -9*), VEGF (ang. *vascular endothelial growth factor*)),
- modulację szlaków PI3K/Akt (ang. *phosphoinositide 3-kinase / protein kinase B (Akt)*), JAK2/STAT3 (ang. *janus kinase 2 / signal transducer and activator of*

transcription 3), NF-κB (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*) oraz Nrf2 (ang. *nuclear factor erythroid 2–related factor 2*),

- aktywację lub represję receptora PPARγ, w zależności od typu polifenolu i modelu komórkowego.

W porównaniu do dotychczasowych opracowań literaturowych praca wyróżnia się szczegółowym podejściem mechanistycznym – każdy z kluczowych polifenoli (m.in. kwercetyna, kwas kawowy, ferulowy, p-kumarowy, galusowy, kemferol) został opisany pod kątem molekularnych celów działania. Na przykład kwercetyna aktywuje Nrf2 i hamuje szlak PI3K/Akt, a p-kumarowy kwas prowadzi do stresu ER i aktywacji osi Bax/Bcl-2. Tego typu podejście rzadko występuje w poprzednich przeglądach, które skupiały się głównie na ogólnych właściwościach przeciwutleniających czosnku lub dominowały w nich związki siarkowe.

W publikacji zwrócono również uwagę na problem ograniczonej biodostępności niektórych polifenoli, takich jak resweratrol czy kwas elagowy, co stanowi istotną barierę w ich zastosowaniu w terapii nowotworów u ludzi. Mimo to, dane z badań *in vitro* oraz wybranych modeli zwierzęcych potwierdzają silne działanie antyproliferacyjne i proapoptotyczne wielu związków. W pracy sugeruje się, że synergizm działania polifenoli oraz ich kombinacje z innymi klasami bioaktywnych substancji (np. związkami siarkowymi) mogą wzmocnić ich efektywność biologiczną – co jest zgodne z koncepcją "całościowego efektu rośliny" w fitoterapii.

W porównaniu z dotychczasowymi przeglądami (Ahmed i Wang, 2021; Talib i in., 2024), niniejsza praca stanowi uzupełnienie wiedzy – oferując pogłębioną analizę mechanizmów działania konkretnych związków polifenolowych obecnych w czosnku. W publikacji nie tylko następuje katalogowanie efektów biologicznych polifenoli, ale także prezentowana są szczegółowe szlaki sygnałowe, co wyróżnia tę pracę na tle innych.

Podsumowując, przegląd literatury przedstawia czosnek jako bogate źródło bioaktywnych polifenoli o potencjale przeciwnowotworowym. Ich działanie zależy od struktury chemicznej, biodostępności oraz zdolności do modulowania kluczowych szlaków sygnałowych w komórkach nowotworowych. Praca również wskazuje na potrzebę dalszych badań *in vivo* i klinicznych, a także opracowania preparatów czosnkowych standaryzowanych pod kątem zawartości polifenoli, co może zwiększyć ich skuteczność i bezpieczeństwo w terapii wspomagającej nowotwory.

Głównymi wnioskami z pracy jest to że:

- Dokładny mechanizm działania niektórych polifenoli czosnku jest nadal nieznany, a dotychczasowe badania przeprowadzono głównie na liniach komórkowych.
- Potrzebne są dalsze badania na zwierzętach i próby kliniczne.
- Czosnek może wspierać ochronę przed rakiem dzięki właściwościom antyoksydacyjnym, przeciwzapalnym oraz oddziaływaniu na sygnalizację komórek rakowych.
- Aby zapewnić pełną ochronę, czosnek powinien być stosowany w ramach zrównoważonego stylu życia, który obejmuje również inne zdrowe nawyki.

4.3. Syntetyczne podsumowanie

W badaniach *in vitro* wykazano, że ekstrakty wodne z różnych odmian czosnku (*Allium sativum* L.), zarówno w postaci świeżej, jak i liofilizowanej, wykazują silne działanie cytotoksyczne względem komórek nowotworowych (SCC-15 i CACO-2), co wiąże się z indukcją ROS. Najwyższy potencjał przeciwnowotworowy zaobserwowano dla odmian Harnaś i Ornak, które silnie generowały ROS, modulowały ekspresję receptora PPARγ i cechowały się selektywnością względem komórek nowotworowych. Jednocześnie nie wszystkie ekstrakty aktywowały klasyczny szlak apoptozy – sugerując udział alternatywnych mechanizmów śmierci komórkowej. Wyniki badań przeglądowych potwierdziły istotny, choć niedoceniany udział polifenoli czosnku w jego działaniu przeciwnowotworowym, podkreślając konieczność standaryza cji ekstraktów i dalszych badań *in vivo*. Całokształt wyników wskazuje, że odpowiednio przygotowane ekstrakty czosnkowe, szczególnie z odmian bogatych w katechinę, mogą stanowić obiecujący komponent terapii wspomagającej nowotwory.

4.4. Ograniczenia rozprawy

1. Brak badań *in vivo* lub klinicznych

Choć badania *in vitro* dostarczają cennych informacji na temat mechanizmów molekularnych działania ekstraktów czosnkowych, ich wyniki nie zawsze w pełni odzwierciedlają złożoność organizmu żywego. W celu potwierdzenia skuteczności oraz bezpieczeństwa ekstraktów w warunkach fizjologicznych, niezbędne są dalsze badania *in vivo* oraz próby kliniczne.

2. Ograniczona liczba badanych linii komórkowych

Analizy przeprowadzono na trzech liniach komórkowych, co stanowi standardowy zakres badań *in vitro*. Niemniej jednak, rozszerzenie modelu o dodatkowe typy nowotworów lub inne linie komórek prawidłowych mogłoby ułatwić określenie selektywności działania ekstraktów wobec różnych typów tkanek.

3. Różnorodność fitochemiczna odmian czosnku

Choć praca zawiera szczegółowe porównanie kilku odmian czosnku, ich skład chemiczny może ulegać zmianom w zależności od warunków uprawy, miejsca pochodzenia i sezonowości. Dlatego konieczna byłyby badania standaryzujące warunki uprawy i przetwarzania materiału roślinnego.

4. Brak standaryzacji zawartości składników aktywnych w ekstraktach

Ekstrakty wodne z czosnków świeżych i z liofilizatów stosowane w badaniach różniły się zawartością związków aktywnych (szczególnie polifenoli i związków siarki), jednak nie wszystkie zostały w pełni zidentyfikowane ilościowo. W przyszłości należałoby rozważyć przygotowanie ekstraktów standaryzowanych pod względem zawartości wybranych związków bioaktywnych.

5. Potencjalna degradacja związków siarkowych

Ekstrakty uzyskiwano zarówno ze świeżych, jak i liofilizowanych ząbków czosnku. W przypadku tych drugich, proces liofilizacji mógł wpłynąć na zawartość nietrwałych związków siarkowych (np. allicyny), co wpływało na obserwowane efekty biologiczne szczególnie widoczne w przypadku linii SCC-15 odmiany Harnaś i Morado. Przyszłe badania powinny porównać ekstrakty uzyskane różnymi metodami, przy zachowaniu pełnej stabilności związków siarkowych.

6. Złożoność działania PPARγ i jego różnorodna rola w nowotworach

Choć wyniki sugerują udział receptora PPARγ w odpowiedzi komórek nowotworowych na ekstrakty czosnkowe, rola tego receptora może być kontekstowo zależna od typu nowotworu i mikrośrodowiska komórkowego. Dalsze badania powinny obejmować analizę jego aktywności w szerszym zakresie modeli nowotworowych oraz w obecności selektywnych agonistów/antagonistów.

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6. Streszczenie w języku polskim

Zdecydowana większość badań obecnych w literaturze naukowej przypisuje prozdrowotne właściwości czosnku zwyczajnego (*Allium sativum* L.) obecności w tej roślinie substancji siarkowych. Należy jednak zaznaczyć, że związki z tej grupy są niestabilne i szybko ulegają degradacji szczególnie w trakcie obróbki i przetwarzania czosnku. Związki polifenolowe stanowią niedocenianą grupę substancji dużo dłużej stabilnych w przetworzonych formach czosnku. Celem niniejszej rozprawy doktorskiej była ocena przeciwnowotworowych właściwości ekstraktów z różnych odmian czosnku zwyczajnego (*Allium sativum* L.) w warunkach *in vitro* oraz określenie mechanizmów molekularnych odpowiedzialnych za ich działanie cytotoksyczne, ze szczególnym uwzględnieniem roli reaktywnych form tlenu (ROS), receptora jądrowego (PPARγ) i związków polifenolowych. W dwóch publikacjach oryginalnych przeprowadzono szczegółowe badania *in vitro* z użyciem komórek nowotworowych (gruczolakorak okrężnicy (CACO-2), oraz płaskonabłonkowy rak języka (SCC-15)) oraz prawidłowych fibroblastów (BJ), wykorzystując testy biochemiczne, analizy ekspresji genów i białek, a także chromatografię HPLC. Pierwsza z prac koncentrowała się na badaniu świeżych ekstraktów z czosnku na linii SCC-15. Druga publikacja obejmowała linie SCC-15, CACO-2 i BJ w których badano ekstrakty uzyskane z liofilizatów pochodzących z czosnków Harnaś, Ornak, Morado i Violeta. W przeprowadzonych badaniach wykazano, że ekstrakty z odmian Harnaś i Ornak charakteryzują się największą aktywnością prooksydacyjną, selektywnym działaniem wobec komórek nowotworowych oraz istotną modulacją ekspresji PPARγ i markerów autofagii (LC3A). Równocześnie w przeglądzie literaturowym przedstawiono aktualny stan wiedzy o właściwościach przeciwnowotworowych czosnkowych polifenoli, klasyfikując je i analizując ich wpływ na kluczowe szlaki sygnałowe w komórkach nowotworowych. Wyniki rozprawy wskazują na potencjał terapeutyczny czosnku jako źródła bioaktywnych związków, których skuteczność zależy od odmiany, formy przetworzenia oraz składu chemicznego. Wnioski te podkreślają konieczność dalszych badań *in vivo* i klinicznych oraz opracowania standaryzowanych preparatów czosnkowych o ukierunkowanym działaniu biologicznym.

7. Streszczenie w języku angielskim

The vast majority of studies in the scientific literature attribute the health-promoting properties of garlic (*Allium sativum* L.) to the presence of sulfur-containing compounds. However, it should be noted that these compounds are unstable and degrade rapidly, especially during processing and preparation of garlic. In contrast, polyphenolic compounds represent an underappreciated group of substances that are significantly more stable in processed forms of garlic. The aim of this doctoral dissertation was to evaluate the anticancer properties of extracts derived from various garlic (*Allium sativum* L.) cultivars under in vitro conditions and to determine the molecular mechanisms underlying their cytotoxic activity, with particular emphasis on the role of reactive oxygen species (ROS), the nuclear receptor PPARγ, and polyphenolic compounds. Two original research publications present detailed *in vitro* studies using cancer cell lines—colorectal adenocarcinoma (CACO-2) and oral squamous cell carcinoma (SCC-15)—as well as normal human fibroblasts (BJ). The methodologies included biochemical assays, gene and protein expression analyses, and high-performance liquid chromatography (HPLC). The first study focused on the effects of fresh garlic extracts on SCC-15 cells. The second study investigated extracts obtained from lyophilized cloves of four garlic cultivars—Harnaś, Ornak, Morado, and Violeta—tested on SCC-15, CACO-2, and BJ cells. The results demonstrated that extracts from Harnaś and Ornak cultivars exhibited the highest pro-oxidative activity, selective cytotoxicity toward cancer cells, and significant modulation of PPARγ expression and autophagy marker LC3A. Concurrently, the literature review included in the dissertation presented the current state of knowledge regarding the anticancer potential of garlic-derived polyphenols, classifying these compounds and analyzing their impact on key intracellular signaling pathways in cancer cells. Overall, the findings highlight the therapeutic potential of garlic as a source of bioactive compounds, whose efficacy depends on cultivar type, processing method, and chemical composition. These conclusions emphasize the need for further in vivo and clinical studies, as well as the development of standardized garlic preparations with targeted biological activity.

8. Opinia komisji bioetycznej

W niniejszej rozprawie eksperymenty były wykonywane na ludzkich liniach komórkowych takich jak: fibroblasty (BJ), nowotwór płaskonabłonkowy języka (SCC-15) oraz gruczolakoraka okrężnicy człowieka (CACO-2). Zdonie z obowiązującym ustawodawstwem, badania prowadzone na liniach komórkowych nie są uznawane za eksperymenty medyczne, ponieważ nie są przeprowadzane na materiale biologicznym pobranym od osób dla celów naukowych. W związku z tym, zgoda Komisji Bioetycznej nie jest wymagana.

Do prowadzenia badań nie wykorzystywano zwierząt. Zgodnie z Ustawą z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych, doświadczenia na zwierzętach wymagają zgody odpowiedniej komisji etycznej. Jednakże, prace *in vitro* na liniach komórkowych nie obejmują użycia żywych zwierząt, a jedynie ich pochodne komórkowe. W związku z tym, takie badania nie podlegają obowiązkowi uzyskania zgody Komisji ds. Doświadczeń na Zwierzętach.

Aby zapewnić najwyższy standard badań były one prowadzone na materiale pochodzącym od renomowanego dostawcy ATCC (ang. *American Type Culture Collection*) zgodnie z GLP (ang. *good laboratory practice*).

9. Oświadczenia współautorów publikacji

Rzeszów, dnia 25.10.2024r.

Imię i nazwisko współautora pracy: Konrad Szychowski

Adres do korespondencji: Kielnarowa 386A
36-020 Tyczyn k.Rzeszowa
Nr telefonu: 17 866 12 93
E-mail: kszychowski@wsiz.edu.pl

Rada Naukowa Dyscypliny Nauki Medyczne
Wyższej Szkoły Informatyki i Zarządzania
z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Szychowski K.A., Binduga U.E., Rybczyńska-Tkaczyk K., Leja M., Gmiński J., *Cytotoxic effects of two extracts from garlic (Allium sativum L.) cultivars on the human squamous carcinoma cell line SCC-15.* Saudi J Biol Sci. 2018 Dec;25(8):1703-1712. doi: 10.1016/j.sjbs.2016.10.005 mój udział wynosił 20% i polegał na stworzeniu koncepcji pracy, wykonanie wstępnych eksperymentów, zdjęć fluorescencyjnych, nadzorze merytorycznym oraz wykonaniu analiz statystycznych.

.....
Podpis

Rzeszów, dnia 25.10.2024r.

Imię i nazwisko współautora pracy: Urszula Binduga

Adres do korespondencji: Kielnarowa 386A

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Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Szychowski K.A., Binduga U.E., Rybczyńska-Tkaczyk K., Leja M., Gmiński J., *Cytotoxic effects of two extracts from garlic (Allium sativum L.) cultivars on the human squamous carcinoma cell line SCC-15.* Saudi J Biol Sci. 2018 Dec;25(8):1703-1712. doi: 10.1016/j.sjbs.2016.10.005 mój udział wynosił 60% i polegał na wykonywaniu analiz kolorymetrycznych (uwolnienie LDH, pomiar wychwytu czerwieni obojętnej, aktywność kaspazy-3), fluorescencyjnych (produkcja ROS) oraz przygotowaniu i poprawie manuskryptu.

Urszula Binduga

.....

Podpis

Rzeszów, dnia 25.10.2024r.

Imię i nazwisko współautora pracy: Kamila Rybczyńska - Tkaczyk
Adres do korespondencji: Leszczyńskiego 7
20 – 069 Lublin
Nr telefonu: 81 524 81 04
E-mail: kamila.rybczynska-tkaczyk@up.lublin.pl

Rada Naukowa Dyscypliny Nauki Medyczne
Wyższej Szkoły Informatyki i Zarządzania
z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Szychowski K.A., Binduga U.E., Rybczyńska-Tkaczyk K., Leja M.L., Gmiński J., *Cytotoxic effects of two extracts from garlic (Allium sativum L.) cultivars on the human squamous carcinoma cell line SCC-15*. Saudi J Biol Sci. 2018 Dec;25(8):1703-1712. doi: 10.1016/j.sjbs.2016.10.005 mój udział wynosił 10% i polegał na pomocy w przygotowaniu ekstraktów.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

Kamila Rybczyńska-Tkaczyk

Podpis

Rzeszów, dnia 25.10.2024r.

Imię i nazwisko współautora pracy: Marcin Ludwik Leja

Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Szychowski K.A., Binduga U.E., Rybczyńska-Tkaczyk K., Leja M.L., Gmiński J., *Cytotoxic effects of two extracts from garlic (Allium sativum L.) cultivars on the human squamous carcinoma cell line SCC-15.* Saudi J Biol Sci. 2018 Dec;25(8):1703-1712. doi: 10.1016/j.sjbs.2016.10.005 mój udział wynosił 5% i polegał na pomocy w hodowli i namnażaniu komórek.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

Marcin Leja

.....

Podpis

Rzeszów, dnia 15.01.2025

OŚWIADCZENIE

My – niżej podpisani – zgodnie oświadczamy, że z przyczyn niezależnych od nas nie jest możliwe uzyskanie oświadczenia o współautorstwie w publikacji: Szychowski K.A., Binduga U.E., Rybczyńska-Tkaczyk K., Leja M., Gmiński J., *Cytotoxic effects of two extracts from garlic (Allium sativum L.) cultivars on the human squamous carcinoma cell line SCC-15*. Saudi J Biol Sci. 2018 Dec;25(8):1703-1712. doi: 10.1016/j.sjbs.2016.10.005 od p. Jana Gmińskiego.

Wkład współautora w powstanie ww. pracy polegał na nadzorze merytorycznym i wynosił 5%.

prof. dr hab. n. med. Konrad Szychowski
Kierownik Katedry Biotechnologii i Biologii Komórki
Promotor

Kandydat

Rzeszów, dnia 15.01.2025

Imię i nazwisko współautora pracy: Urszula Binduga

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Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Kopeć, A., Skoczylas, J., Szychowski, K.A., *Comparison of the Cytotoxic Mechanisms of Different Garlic (Allium sativum L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. Int. J. Mol. Sci.* 2025, *26*, 387. doi: 10.3390/ijms26010387 mój udział wynosił 85% i polegał na wykonywaniu analiz kolorymetrycznych (uwolnienie LDH, aktywność kaspazy-3), fluorescencyjnych (produkcja ROS, redukcja resazuryny), wykonaniu reakcji qRT-PCR oraz wykonanie testów ELISA.

Ponadto oświadczam, że jestem autorem korespondencyjnym wskazanej pracy.

Urszula Binduga

.....

Podpis

Kraków, dnia 09.01.2025r.

Imię i nazwisko współautora pracy: Aneta Kopeć
Adres do korespondencji: ul. Balicka 122
30-149 Kraków
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Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Kopeć, A., Skoczylas, J., Szychowski, K.A., *Comparison of the Cytotoxic Mechanisms of Different Garlic (Allium sativum L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. Int. J. Mol. Sci.* 2025, *26*, 387. doi: 10.3390/ijms26010387 mój udział wynosił 5% i polegał na nadzorze merytorycznym prowadzonych prac i pomocy w przygotowaniu tekstu manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

Signed by / Podpisano przez:
Aneta Maria Kopeć
Date / Data: 2025-01-13 11:43

.....

Podpis

2-Kopeć.pdf

Rzeszów, dnia 07.01.2025r.

Imię i nazwisko współautora pracy: Joanna Skoczylas

Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Kopeć, A., Skoczylas, J., Szychowski, K.A., *Comparison of the Cytotoxic Mechanisms of Different Garlic (Allium sativum L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. Int. J. Mol. Sci.* 2025, *26*, 387. doi: 10.3390/ijms26010387 mój udział wynosił 5% i polegał na wykonaniu oznaczeń wybranych związków polifenolowych metodą HPLC.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

Joanna Skoczylas

.....

Podpis

Rzeszów, dnia 18.01.2025

Imię i nazwisko współautora pracy: Konrad Szychowski

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Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Kopeć, A., Skoczylas, J., Szychowski, K.A., *Comparison of the Cytotoxic Mechanisms of Different Garlic (Allium sativum L.) Cultivars with the Crucial Involvement of Peroxisome Proliferator-Activated Receptor Gamma. Int. J. Mol. Sci.* 2025, *26*, 387. doi: 10.3390/ijms26010387 mój udział wynosił 5% i polegał na opiece merytorycznej i nadzorze nad całością pracy.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

.....

Podpis

Rzeszów, dnia 15.01.2025

Imię i nazwisko współautora pracy: Urszula Binduga

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Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Szychowski, K.A., *Current state of knowledge of anticancer properties of polyphenolic compounds from garlic (Allium sativum L.)* - **praca w recenzji,** mój udział wynosił 90% i polegał na zebraniu literatury, krytycznym przeglądzie literatury, przygotowaniu tabel oraz koncepcji schematów.

Ponadto oświadczam, że jestem autorem korespondencyjnym wskazanej pracy.

Urszula Binduga

.....

Podpis

Rzeszów, dnia 18.01.2025

Imię i nazwisko współautora pracy: Konrad Szychowski

Adres do korespondencji: Kielnarowa 386A

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E-mail: kszychowski@wsiz.edu.pl

Rada Naukowa Dyscypliny Nauki Medyczne

Wyższej Szkoły Informatyki i Zarządzania

z siedzibą w Rzeszowie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Binduga U.E., Szychowski, K.A., *Current state of knowledge of anticancer properties of polyphenolic compounds from garlic (Allium sativum L.)* – **praca w recenzji,** mój udział wynosił 10% i polegał na opiece merytorycznej, współpracy w przygotowaniu schematów i nadzorze nad całością pracy.

Jednocześnie wyrażam zgodę na wykorzystanie w/w publikacji w postępowaniu doktorskim Pani mgr Urszuli Bindugi.

.....

Podpis

10. Całościowy dorobek kandydata

10.1. Edukacja i przerwy w pracy naukowej

Edukacja:

- 2022-2023 – Wyższa Szkoła Informatyki i Zarządzania z siedzibą w Rzeszowie, Studia podyplomowe: Dietetyka i planowanie żywienia
- 2010- 2011 Wyższa Szkoła Ekonomii i Innowacji w Lublinie, Podyplomowe studia kwalifikacyjne w zakresie przygotowania pedagogicznego
- 2006- 2007 Uniwersytet Rzeszowski, Studia podyplomowe w zakresie zarządzania zasobami ludzkimi
- 2004 - 2006 Wyższa Szkoła Informatyki i Zarządzania w Rzeszowie, studia magisterskie kierunek turystyka i rekreacja, specjalność: turystyka międzynarodowa
- 2001- 2004 Wyższa Szkoła Informatyki i Zarządzania w Rzeszowie, studia licencjackie, kierunek turystyka i rekreacja, specjalności: zarządzanie w turystyce, hotelarstwo i gastronomia

Przerwy w pracy naukowej:

- 18.07.2020 - 16.07.2021 - Urlop macierzyński i rodzicielski
- 10.02.2020 -17.07.2020 - zwolnienie chorobowe
- 02.02.2018 - 31.01.2019 - Urlop macierzyński i rodzicielski
- 13.07.2017 - 01.02.2018 - zwolnienie chorobowe

10.2. Publikacje niewchodzące w skład cyklu

Do roku 2010 pod nazwiskiem rodowym **Rogala**.

1. Bar M, **Binduga UE**, Szychowski KA. Methods of Isolation of Active Substances from Garlic (*Allium sativum* L.) and Its Impact on the Composition and Biological Properties of Garlic Extracts. Antioxidants (Basel). 2022; 11:1345. DOI: 10.3390/antiox11071345. (^{2022}IF:7,0; pkt MNiSW: 100) *Czasopismo z oficjalnej listy Ministerstwa z dyscypliny Nauki medyczne*
2. Jakubczyk A, Karaś M, Stanikowski P, Rutkowska B, Dziedzic M, Zielińska E, Szychowski KA, **Binduga UE**, Rybczyńska-Tkaczyk K, Baraniak B. Characterisation of Biologically Active Hydrolysates and Peptide Fractions of Vacuum Packaging String Bean (*Phaseolus vulgaris* L.). Foods. 2020; 9:842. DOI:

10.3390/foods9070842. ([2020]IF:4,35; pkt MNiSW: 100) *Czasopismo z oficjalnej listy Ministerstwa z dyscypliny Nauki o zdrowiu*

3. Szychowski KA, Rybczyńska-Tkaczyk K., Gaweł-Bęben K., Świeca M., Karaś, M., Jakubczyk, A., Matysiak, M., **Binduga U.**, Gmiński J.: Characterization of active compounds of different garlic (*Allium sativum* L.) cultivars, Pol. J. Food Nutr. Sci., 2018; 68:73-81 DOI: 10.1515/pjfns-2017-0005 ([2018]IF: 1,514; pkt MNiSW: 15) *Czasopismo z oficjalnej listy Ministerstwa z dyscypliny Nauki o zdrowiu*
4. Szychowski KA, Leja M.L., Kaminskyy D.V., Kryshchyshyn A.P., **Binduga U.E.**, Pinyazhko O.R., Lesyk R.B., Tobiasz J., Gmiński J.: Anticancer properties of 4-thiazolidinone derivatives depend on peroxisome proliferator-activated receptor gamma (PPARγ), European Journal of Medicinal Chemistry, 2017; 14:162-168 DOI: 10.1016/j.ejmech.2017.09.071. ([2017]IF: 4,816; pkt MNiSW: 40) *Czasopismo z oficjalnej listy Ministerstwa z dyscypliny Nauki medyczne*
5. Szychowski KA, Leja M.L., Kaminskyy D.V., **Binduga U.E.**, Pinyazhko O.R., Lesyk R.B., Gmiński J.: Study of novel anticancer 4-thiazolidinone derivatives. Chemico-Biological Interactions, 2017; 262:46-56 DOI: 10.1016/j.cbi.2016.12.008. ([2017]IF: 3,296; pkt MNiSW: 30) *Czasopismo z oficjalnej listy Ministerstwa z dyscypliny Nauki o zdrowiu*
6. Jodłowska-Jędrych B., Jędrych M., Ruta E. **Binduga U.E.** Stawecka-Wiatrowska A., Wpływ stanów emocjonalnych na relacje z rodziną u osób z nieswoistymi zapaleniami jelit, Pol J Public Health 2012; 122 (1) : 7-12; (pkt MNiSW: 7)
7. Krupa J., **Binduga U**., Skiba U., Koncepcja programów nauczania na kierunkach Turystyka i rekreacja oraz Zdrowie publiczne w kontekście potrzeb rynku pracy Polski Południowo Wschodniej, Instytut Gospodarki, Wyższa Szkoła Informatyki i Zarządzania, Rzeszów 2011. (pkt MNiSW: 12)
8. Jędrych M., Polz-Dacewicz, **Rogala U**., Głowniak-Józefczak E., The assessment of patients' satisfaction with nursing care in hospitals taking into consideration the patients' gender. (Ocena satysfakcji pacjentów z opieki pielęgniarskiej w szpitalach wśród kobiet i mężczyzn.). W: Environmental factors shaping wellness in sickness and disability: monografia. Pod red. Józefa Bergiera, Lublin 2010, NeuroCentrum, s. 105-122. (pkt MNiSW: 7)
9. Pietryka-Michałowska E., Jędrych M., **Rogala U**.,Effects of selected dietary factors and physical activity on the prevalence of overweight and obesity in the town population (Ocena wpływu wybranych czynników żywieniowych i aktywności

fizycznej na występowanie nadwagi i otyłości w populacji miejskiej). Chapter XXII [w:]Public health and research; red.Leszek Wdowiak, Wacław Kruk, Monika Binkowska-Bury, wyd. NeuroCentrum, Lublin 2009, s. 279-290; (pkt MNiSW: 7)

10. Jędrych M., Polz-Dacewicz M., **Rogala U**., Pietryka-Michałowska E., Assesment of patient's satisfaction with nursing care provided in primary health care depending on its accessibility (Ocena staysfakcji pacjentów z opieki pielęgniarskiej w podstawowej opiece zdrowotnej w zależności od dostępu do opieki). Chapter XI [w:]Public health and research; red.Leszek Wdowiak, Wacław Kruk, Monika Binkowska-Bury, wyd. NeuroCentrum, Lublin 2009, s. 147-163; (pkt MNiSW: 7)
11. Jędrych M., **Rogala U.,** The awareness of some chosen lifestyle factors on the health condition of contemporary young people on the example of secondary school students (Świadomość wpływu wybranych czynników stylu zycia na stan zdrowia współczesnej młodzieży na przykładzie uczniów szkół ponadgimnazjalnych). Chapter X [w:]Wellness and success; red. Józef Bergier, wyd. NeuroCentrum, Lublin 2009, s. 139-152; (pkt MNiSW: 7)
12. Jędrych M., **Rogala U**., Psychosociological and pharmacological motives of using addictive substances among the school teenagers (Psychologiczne oraz farmakologiczne motywy stosowania środków uzależniających wśród młodzieży szkolnej). Chapter [w:]Environment and human health; red. Andrzej Borzęcki wyd. WSCHÓD Agencja Usługowa, Lublin 2009, s. 203-213 (pkt MNiSW: 7)
13. Jędrych M., Polz-Dacewicz M., Płotka A., **Rogala U**., Nowaczewska A., The assessment of patients' satisfaction with nursing care in basic medical care depending on age (Ocena satysfakcji pacjentów z opieki pielęgniarskiej w podstawowej opiece zdrowotnej w zależności od wieku). Chapter IX [w:] Wellness in different phases of life; red. Grażyna Olchowik, wyd. NeuroCentrum, Lublin 2008, s. 79-87; (pkt MNiSW: 7)

10.3. Konferencje naukowe

1. Szychowski KA, Leja M.L., **Binduga U.E.**, Tobiasz J., Gmiński J.: Impact of elastin-derived peptides (EDPs) on matrix metalloproteinases -2, -9 (MMPs-2, -9) mRNA expression in mouse astrocytes *in vitro*. Neurochemical Conference 2017, Advances in molecular and epigenetic mechanisms in neurodegeneration and neuroinflammation: novel therapeutic approaches, Warszawa, 19-20 października 2017

2. Tobiasz J., Szychowski KA, Leja M.L., Yelnytska V., **Binduga U.E.**, Gmiński J.: Antyproliferacyjne działanie ekstraktu z grzyba Inonotus obliquus. Badania i Rozwój Młodych Naukowców w Polsce 2017, Lublin, 12 maja 2017
3. Szychowski KA, Wnuk A, **Binduga U**, Kajta M, Wójtowicz A.K.: Impact of triclosan on expression of NMDA receptor subunits in mouse neocortical neurons. The 13th International Symposium "Molecular basis of pathology and therapy in neurological disorders" and The 4th International Conference "Stem cells: therapeutic outlook for nervous system disorders", Warsaw, November 17 – 18, 2016

10.4. Udział w zewnętrznych projektach naukowych

1. Wykonawca w projekcie badawczym: „Program przebudowy rolnictwa w kierunku zmian strukturalnych i zasad zrównoważonego rozwoju" finansowany jest ze środków budżetu państwa, przyznanych przez Ministra Nauki i Szkolnictwa Wyższego w ramach Programu „Nauka dla Społeczeństwa II". Numer umowy: UMOWA Nr NdS-II/SN/0159/2023/01 o realizację projektu nr rej. NdS-II/SN/0159/2023/01, czas realizacji: 21.12.2023 - 21.12.2025
2. Wykonawca w projekcie badawczym pt. „Pozyskanie substancji bioaktywnych z Inonotus obliquus w formie możliwej do zastosowania we wzbogacaniu żywności". Źródło finansowania Podkarpackie Centrum Innowacji, numer 27/WSIZ/1/DG/PCI/2021, czas realizacji 02.2022 – 07.2022 (kwota 252 173,30 zł).

10.5. Zgłoszenia patentowe

1. W dniu 19.07.2022, złożono zgłoszenie patentowe nr P.441769, pt. „Sposób ekstrakcji substancji aktywnych z Błyskoporka podkorowego". Autorzy głoszenia patentowego Konrad Szychowski, Tadeusz Pomianek, Bartosz Skóra, Urszula Binduga. Zgłoszenie patentowe zostało przygotowane na podstawie uzyskanego projektu badawczego.

10.6. Działalność popularyzująca naukę

1. Prelegentka TEDxRzeszów Countdown, Wystąpienie: „Czy zioło może dużo?"
2. Prelegentka Konferencji w Podkarpackim Ośrodku Doradztwa Rolniczego w Boguchwale:
    a) Konferencja w ramach realizowanej operacji pn. „Żywność funkcjonalna i jej bezcenny wpływ na organizm" ze środków pochodzących z Unii

Europejskiej Schematu II Pomocy Technicznej, Krajowej sieci Obszarów wiejskich, Planu Rozwoju Obszarów wiejskich na lata 2014-2020 , wystąpienie: „Żywność funkcjonalna i jej bezcenny wpływ na organizm'' – 29.02.2024

b) Konferencja „Ograniczenie strat i marnotrawstwa w krótkim łańcuchu dostaw – wyzwaniem najbliższych lat", wystąpienie: „Nie wyrzucaj wykorzystaj – niekonwencjonalne wykorzystanie odpadów żywnościowych w dietetyce i kosmetologii" – 08.03.2023

c) Konferencja „Racjonalne odżywianie profilaktyką zdrowotną", wystąpienie: „Znaczenie postów krótkotrwałych w profilaktyce chorób". – 11.10.2022

3. Pomysłodawca i koordynatorka akcji profilaktyczno-zdrowotnej dla pracowników WSIiZ pn. „Tydzień Zdrowia WSIiZ".
4. Audycje w radiu Rzeszów w cyklu „Bliżej natury" „Science" oraz „Szlachetne zdrowie" wywiady:
   a) Wpływ diety na odporność.13.01.2025
   b) Zioła przeciwzapalne. Jak je stosować? 28.11.2023
   c) Właściwości słodzików – 23.03.2023
   d) Mikro i makro składniki w diecie – 09.11.2022
5. Wywiady w telewizji TVP3 Rzeszów, wywiad: „Cudze chwalicie swego nie znacie – o właściwościach zdrowotnych jabłek" – 02.10.2023
6. Prelekcje w szkołach ponadpodstawowych – prowadząc dla uczniów zajęcia praktyczne w ramach projektu „Ciekawa lekcja" z tematyki zasad zdrowego odżywiania oraz profilaktyki zdrowotnej. Od 2022 roku do chwili obecnej lekcje odbyły się w ponad 50 szkołach , zrealizowano – 217 godzin.
7. Prelekcje w Akademii 50+ prowadząc wykłady oraz warsztaty z tematów m.in.: Świadome kształtowanie diety – niebezpieczne dodatki do żywności, A gdyby tak cofnąć zegar biologiczny – o sile antyoksydantów w diecie, Analiza składu masy ciała, Produkty wspierające odporność organizmu, Tabletka cud - fakty i mity na temat suplementów diety, Racjonalne odżywianie - nowa piramida żywieniowa.
8. Współautorka artykułów popularnonaukowych na Zielonym Blogu WSIiZ:
   a) „Kondycja gleb a zdrowie człowieka": 23.01.2025 https://zielonyblog.wsiz.edu.pl/kondycja-gleb-a-zdrowie-czlowieka/
   b) „Mikroplastik powoli zabija nas od środka!": 01.08.2024 https://zielonyblog.wsiz.edu.pl/mikroplastik-powoli-zabija-nas-od-srodka/

Rzeszów, dnia 6.06.2025 r.

Urszula Binduga
imię i nazwisko kandydata

OŚWIADCZENIE

Oświadczam, że złożona przeze mnie rozprawa doktorska:

„Mechanizm działania ekstraktów z czosnku zwyczajnego (*Allium sativum* L.) ze szczególnym uwzględnieniem roli substancji polifenolowych i receptora aktywowanego proliferatorami peroksysomów gamma (PPARγ) w warunkach *in vitro* została napisana przeze mnie samodzielnie".

Oświadczam również, że przedstawiona rozprawa doktorska nie była wcześniej przedmiotem procedur związanych z uzyskaniem stopnia naukowego.

Jednocześnie wyrażam zgodę na jej publiczne udostępnianie.

Urszula Binduga
podpis