

Effect of diallyl disulfide and garlic oil on different human astrocytoma cell lines

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Abstract. Gliomas are a group of malignant brain tumors. Despite significant efforts to optimize treatment options for patients with high-grade glioma, the prognosis of the overwhelming majority of patients remain poor. This bleak prognosis despite treatment of the glioma, is partly due to the tendency of therapeutics to diffusely penetrate into the neighboring brain tissues, but also due to the innate resistance of these tumors to chemotherapy and radiation. Garlic contains water-soluble and oil-soluble sulfur compounds. The oil-soluble compounds, including diallyl sulfide, diallyl disulfide (DADS), diallyl trisulfide and ajoene, are more effective potential anti-cancer treatments than the water-soluble compounds. There are several studies examining the effects of oil-soluble compounds on various types of cancer cells, although, to the best of our knowledge, there are no studies examining the effects of these compounds on glioma cells. The aim of the present study was to investigate the potential anti-glioma properties of DAD and garlic oil on proliferation and induction of apoptosis in four different types of glioma cell lines representative of different grades of the disease. The results showed that garlic oil exhibits favorable anti-cancer potential towards gliomas of various degrees of differentiation.

Introduction

Gliomas are a group of malignant brain tumors, and despite the significant efforts that have been made to develop and optimize novel therapeutics for treatment of patients with high-grade glioma, the prognosis of these patients remains poor (1). Surgery is often incomplete due to the inaccessibility of the tumor location. Additionally, glioma cells are often resistant to chemotherapy and radiation (2). Thus, novel treatment

strategies are required. The use of compounds of natural origin may serve as an alternative method of treatment. The health-promoting and healing properties of garlic have been used for thousands of years. Garlic contains several water- and oil-soluble organosulfur compounds which have conditioning properties, such as antimicrobial, antithrombotic, antiarthritic and antitumor properties (3). Oil-soluble compounds which include: Diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and ajoene are more effective than the water-soluble compounds when used to prevent or treat cancer. DADS and DATS are the primary components of garlic oil, which has a yellowish hue, and is insoluble in water (1).

A number of studies have focused on the evaluation of the impact of DADS on the human body in health and disease. These studies have shown that DADS exhibits protective properties and at the same time target cancer cells (4,5). *In vivo* studies have shown that DADS effectively inhibits carcinogens by modulating the action of cytochrome P450-dependent monooxygenases, as well as by affecting the activation of phase II enzymes which are responsible for detoxifying xenobiotics (6). These include glutathione reductase and glutathione transferase (7).

The anti-proliferative properties of DADS are related to its ability to decrease the proportion of cells in the G1 and G2/M phases (8). Furthermore, several studies have suggested that DADS may induce apoptosis in several types of cancer cells, and can work as an inhibitor of histone deacetylase (3,4,9). Previous studies have suggested that the above mentioned abilities reduce cell proliferation (10), angiogenesis, as well as invasion and metastasis in cancer cells (9). A number of studies have demonstrated the chemopreventative properties of different forms of products and compounds derived from garlic, including fresh and aged garlic extract, as well as garlic oil (11). The anti-cancer properties of garlic oil is attributed to the presence of organic sulfur compounds in garlic. The modes of organic sulfur action include its effect on drug metabolizing enzymes, antioxidant properties and tumor growth inhibition (12). Therefore, the consumption of garlic may assist in cancer prevention, while the use of specific compounds derived from garlic may increase the therapeutic potential in regard to cancer prevention or even treat existing cancers. The effectiveness of these compounds has been demonstrated in several studies on various cancer cell lines including breast, colon and prostate cancer cells (13); however,

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its effects have not been studied on human glioblastoma cells, to the best of our knowledge. Taking into consideration the above, the aim of the present study was to determine whether oil-soluble organosulfur compounds derived from garlic exhibited anti-cancer activity against human glioma cells. The effect of DADS and garlic oil (which is a combination of several oil-soluble organosulfur compounds) were assessed on cell viability and apoptosis induction. The effects were assessed on four different glioma cell lines of different grades.

Materials and methods

Cell culture. In the present study, four human astrocytoma cell lines of differing grades were used: CCF-STTG1 (grade IV, astrocytoma), SW1783 (grade III, astrocytoma), SW1088 (astrocytoma) and CHLA-03-AA (anaplastic astrocytoma). All cell lines were purchased from ATCC[®]. SW1783 and SW1088 cells were grown as a monolayer in ATCC-formulated Leibovitz's L-15 medium (cat. no. 30-2008; ATCC) supplemented with 10% FBS and 50 $\mu\text{g/ml}$ streptomycin (Sigma-Aldrich; Merck KGaA). These two cell lines were grown at 37°C in 100% air. For CCF-STTG1 and CHLA-03-AA cells, ATCC-formulated RPMI-1640 medium (catalog no. 30-2001; ATCC) supplemented with 10% FBS and 50 $\mu\text{g/ml}$ streptomycin was used. These cells were grown at 37°C with 5% CO₂. Prior to each experiment, the cells were detached using 0.25% trypsin with 0.02% EDTA (Sigma-Aldrich; Merck KGaA).

Chemicals. Garlic oil and DADS was purchased from Sigma-Aldrich; Merck KGaA (cat. no. 8000-78-0 and SMB00378, respectively). A range of concentrations of both chemicals were used in the present study (0.015-150 $\mu\text{g/ml}$). Both garlic oil and DADS were diluted in the appropriate culture medium for each cell line.

MTT assay and determination of the IC₅₀. The viability of cells was determined using an MTT assay (Sigma-Aldrich; Merck KGaA) after 24 h of incubation with different concentrations of garlic oil and DADS (0,015-150 $\mu\text{g/ml}$). To assess the effect of garlic oil, concentrations of 15 and 150 $\mu\text{g/ml}$ were used, the results obtained were not included in the determination of cell viability and IC₅₀, as the absorbance obtained in those samples was clearly overvalued due to the presence of the separate oil layer in the suspension.

The MTT assay was used for evaluation of mitochondrial metabolic function according to the manufacturer's protocol (Sigma-Aldrich; Merck KGaA). A total of 1x10⁴ cells/well were seeded into 96-well microculture plates. The absorbance was determined using an Enspire Multiplate reader spectrophotometer at 570 nm (Perkin Elmer, Inc.). Mitochondrial metabolic function was expressed as a percentage of the viable treated cells to the untreated control cells. Based on the viability values obtained in the MTT assay, the IC₅₀ values for DADS and garlic oil were calculated using GraphPad Prism version 7.03 (GraphPad Software, Inc.).

Evaluation of apoptosis and necrosis. Annexin V conjugated to fluorescein isothiocyanate (FITC) fluorochrome was used for flow cytometry analysis of cells which were undergoing apoptosis. Staining with Annexin V/FITC was performed at

Table I. IC₅₀ values of DADS and garlic oil in glioma cell lines.

Cell lines	IC ₅₀ , $\mu\text{g/ml}$	
	DADS	Garlic oil
CCF STTG1	5.8	4.05
SW1783	Non-toxic	1.11
SW1088	Non-toxic	0.15
CHLA-03-AA	2.64	0.072

DADS, diallyl disulfide.

room temperature for 15 min. The externalization of phosphatidylserine occurs during the early stages of apoptosis, and Annexin V/FITC staining can identify apoptosis at an earlier stage than assays based on nuclear changes, such as DNA fragmentation (13). Necrotic changes were identified using propidium iodide (PI), fluorescence of which is enhanced 20-30-fold upon binding to nucleic acids (13). The analysis was performed using a commercial FITC Annexin V kit (BioLegend, Inc.; cat. no. 640914). For evaluation of apoptosis and necrosis, 0.15, 0.75 and 1.5 $\mu\text{g/ml}$ DADS or garlic oil were used. Analysis was performed using CyFlow Cube 6 cytometer (Sysmex Europe, GmbH), where FL-1 was used for Annexin-V-FITC and FL-2 detector was used for necrotic cells (PI stained) measurements. A representative example of the gating strategy used in each experiment is shown in Fig. 1. In the quadrant gates, Q1, Q2, Q3 and Q4 fields correspond respectively to necrotic, late-apoptotic, alive cells and early apoptotic cells.

Statistical analysis. Data were analyzed using GraphPad Prism version 7.03. A two-way ANOVA followed by a post-hoc Tukey's test was used to compare the data were $\alpha=0.05$. All samples were analyzed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability analysis showed that the highest assessed DADS concentrations (15 and 150 $\mu\text{g/ml}$) were cytotoxic for CCF-STTG1 and CHLA-03-AA cells (Fig. 2). IC₅₀ for CCF-STTG1 and CHLA-03-AA cells were 5.8 and 2.64 $\mu\text{g/ml}$, respectively (Table I). For SW1783 and SW1088 cells, DADS was not cytotoxic and even the highest tested concentration-150 $\mu\text{g/ml}$, only slightly reduced cell viability to 88.7% for the SW1783 cell line and to 77.7% for the SW1088 cells (Fig. 2). The viability assay for garlic oil was performed using a concentration range of 0.015 to 1.5 $\mu\text{g/ml}$ due to the presence of a separate oil layer at the higher tested concentrations. In contrast to DADS, 1.5 $\mu\text{g/ml}$ garlic oil significantly reduced cell viability in all tested cell lines to 30.9% in CCF-STTG1, 45.9% in CHLA-03-AA, 31.1% in SW1783 and 40.6% in SW1088 cells (Fig. 3). The IC₅₀ values were 4.05 $\mu\text{g/ml}$ for CCF-STTG1, 0.072 $\mu\text{g/ml}$ for CHLA-03-AA, 1.11 $\mu\text{g/ml}$ for SW1783 and 0.15 $\mu\text{g/ml}$ in SW1088 (Table I).

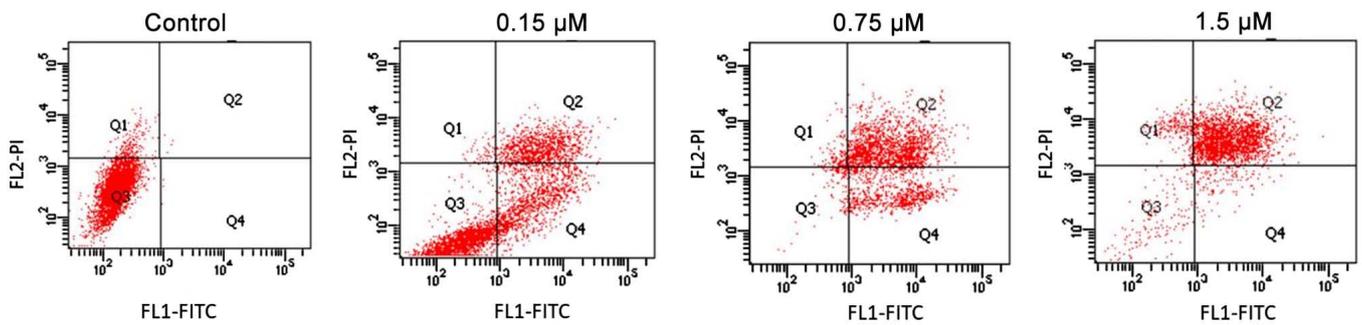


Figure 1. Gating strategy used for flow cytometry analysis. Representative examples of fluorescence activated cell sorting dot plots of CCF-STTG1 cells treated with increasing concentrations of garlic oil are presented, and the gating strategy used is also shown. In the quadrant gates, the Q1, Q2, Q3 and Q4 fields correspond respectively to necrotic, late-apoptotic, alive cells and early apoptotic cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.

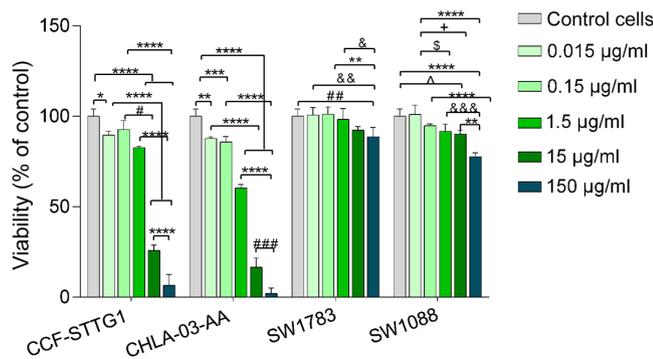


Figure 2. Viability of CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells after 24 h of treatment with increasing concentrations of DADS. Viability is expressed as a percentage of the control cells (untreated cells). Data are presented as the mean \pm standard deviation of 3 repeats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$; * $P < 0.05$, * $P < 0.05$, * $P < 0.05$. DADS, diallyl disulfide.

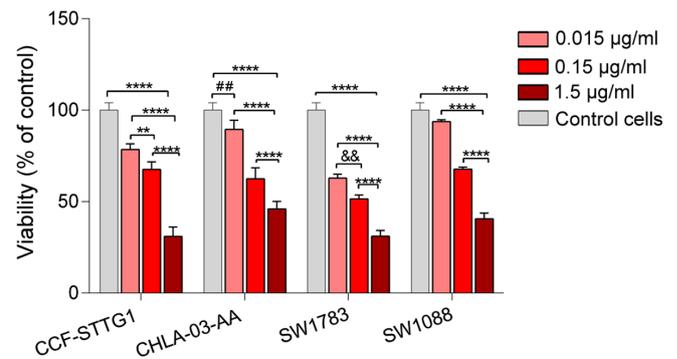


Figure 3. Viability of CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells after 24 h of treatment with increasing concentrations of garlic oil. Viability is expressed as a percentage of the control cells (untreated cells). Data are presented as the mean \pm standard deviation of 3 repeats. ** $P < 0.01$, **** $P < 0.0001$, ## $P < 0.01$; && $P < 0.01$.

The results of the flow cytometry analysis were consistent with the viability test. There was a significantly higher percentage of live cells observed in the DADS-treated samples for SW1783 and SW1088 cell lines (Fig. 4). For samples treated with 1.5 $\mu\text{g/ml}$ garlic oil, the percentages of live cells were 3, 10, 15 and 36% for CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells, respectively (Fig. 5). Flow cytometric analyses were performed to determine the proportion of dead cells using an Annexin V/PI double staining kit. Annexin V signal detection provides a very sensitive method for detecting cellular apoptosis, whereas PI is used to detect necrotic or late apoptotic cells, characterized by the loss of integrity of the plasma and nuclear membranes (13). Data generated by flow cytometry was plotted in 2D dot plots, in which PI was represented vs. Annexin V-FITC. These plots were divided into four regions corresponding to: i) Viable cells which were negative to both probes; ii) apoptotic cells which were PI negative and Annexin-V positive; iii) late apoptotic cells which were PI and Annexin-V positive; and iv) necrotic cells which were PI positive and Annexin-V negative. For each condition tested, 10,000 cells were analyzed, and the percentage of alive, early apoptotic, late apoptotic and necrotic cells was expressed as a percentage of the total number of cells assessed. Flow cytometry analysis showed that DADS induced both, apoptotic

and necrotic cell death at a similar level in the four tested glioma cell lines (Fig. 4). Using 1.5 $\mu\text{g/ml}$, DADS induced apoptosis in 28% of CCF-STTG1 cells and necrosis in 30% of these cells. For SW1783 cells, 15% of the cells were apoptotic and 15% of the cells were necrotic. For SW1088 cell, 13% of the cells were apoptotic and 11% were necrotic. In CHLA-03-AA cells, 43% of the cells were apoptotic cells and 19% of the cells were necrotic. Garlic oil notably induced apoptotic death in all the tested cell lines at a low concentration. A dose of 0.75 $\mu\text{g/ml}$ resulted in 78, 43, 54 and 34% proportion of apoptotic cells in the CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells, respectively. At a concentration of 1.5 $\mu\text{g/ml}$ garlic oil, 78, 74, 59 and 57% of the CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells were apoptotic, respectively (Fig. 5).

Discussion

The prominent effects of garlic on cell-cycle arrest, apoptosis and differentiation has been the subject of several studies (14-16). At present, there have been no attempts to analyze the effects of the compounds derived from garlic on glioma of varying degrees of differentiation, to the best of our knowledge. Hong *et al* (17) examined the effect of DAS and DADS on H460 (p53-wild type) and H1299 (p53-null) non-small cell lung cancer cells. Both DAS and DADS induced

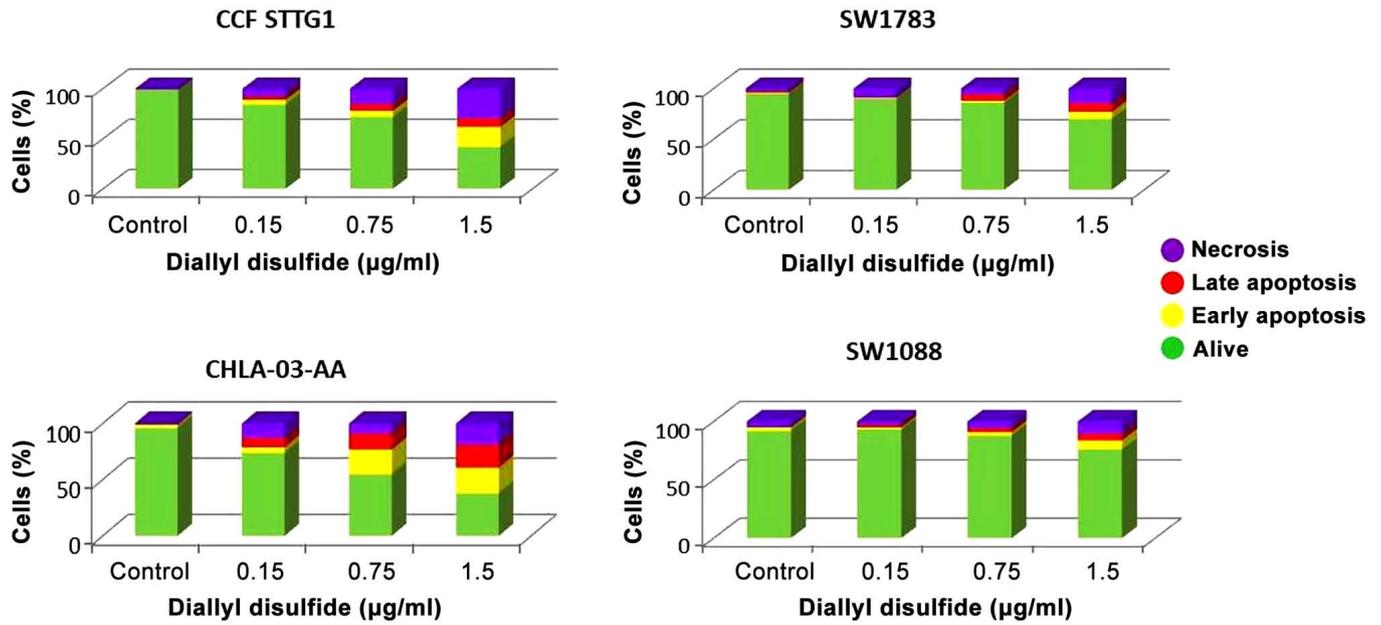


Figure 4. Flow cytometry analysis evaluating the type of induced cell death in CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells after incubation with increasing concentrations of diallyl disulfide. Control cells were incubated in cell culture medium without any treatment.

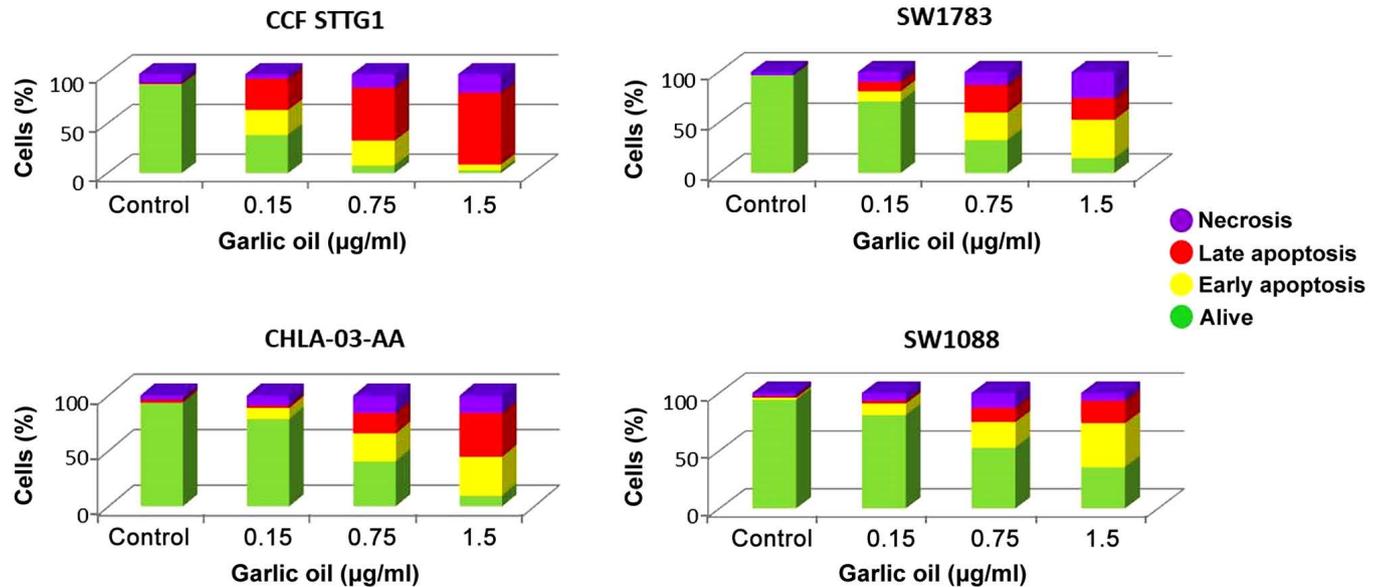


Figure 5. Flow cytometry analysis evaluating the type of induced cell death in CCF-STTG1, CHLA-03-AA, SW1783 and SW1088 cells after incubation with increasing concentrations of garlic oil. Control cells were incubated in cell culture medium without any treatment.

apoptosis in the non-small cell lung cancer cells, which was correlated with a marked increase in the protein expression levels of p53 and Bax, as well as a decrease in Bcl-2 protein expression (17). Nakagawa *et al* (18) examined the inhibitory effect of DADS on human estrogen receptor-positive and -negative breast cancer cell lines. In both these breast cancer cell lines, DADS resulted in a significant increase in Bax and caspase-3 protein expression, resulting in apoptotic cell death. In addition, DADS in the estrogen receptor-negative cells acted synergistically with eicosapentaenoic acid, which is a tumor cell suppressor. Similarly, *in vivo*, DADS resulted in a reduction of tumor weight compared to the DADS-untreated mice (18).

The toxic effects of DADS and garlic oil were assessed on four glioma cells in the present study. The results showed that DADS did not result in cytotoxicity at any of the assessed concentrations in SW1783 and SW1088 cells. In the CCF-STTG1 and CHLA-03-AA cells, there was a significant reduction in cell viability observed after treatment with 15 and 150 µg/ml. The lack of toxicity at lower doses of DADS and the cytotoxic effects at higher doses of DADS were consistent with the results of Koh *et al* (19). In a previous study performed on PC-12 cells (cells derived from a transplantable rat pheochromocytoma), it was shown that the lowest tested concentration of DADS, 20 µM, exhibited neuroprotective

effects by inducing an increase in Akt kinase activity, triggering the PI3K/Akt signaling pathway and decreasing the activity of proapoptotic factors (cytochrome *c*, caspase 3 and PARP protein) (20). Lower concentrations of DADS exhibited cytoprotective potential, whereas 50 μM of DADS resulted in an increase in the levels of free radicals and lipid peroxidation of PC-12 cell membranes. Furthermore, 100 μM DADS inhibited the PI3K/Akt pathway and increased proapoptotic activity (21). The high degree of invasiveness of gliomas may be due to aberrant signal transduction of pathways downstream of PI3K and Akt/PKB kinases, which are responsible for the regulation of cell proliferation, differentiation and survival (22-24). Mutations of genes encoding the above enzymes are commonly observed in gliomas, and lead to an increase in their activity, resulting in increased resistance to chemotherapy (25). The absence of a cytotoxic effect on SW1783 and SW1088 cells following incubation with DADS may be due to the enhanced resistance exhibited by these cell lines caused by mutations in the PI3K-Akt/PKB-mTOR signaling pathway gene (26). It is likely that for the CHLA-03-AA and CCF-STTG1 cells, higher doses of DADS induced cytotoxicity by decreasing Akt/PKB factor activity, a mechanism that was observed by Koh *et al* (19). Apoptosis inhibition via the Akt/PKB signaling pathway is very complex and can occur at different levels of the signal transduction pathways initiating programmed cell death. PI3K/AKT kinase phosphorylates the pro-apoptotic BAD protein and promotes the binding of BAD to the cytoplasmic 14-3-3 protein, resulting in inhibition of apoptosis (22). Das *et al* (27) demonstrated that the garlic compounds DAS, DADS and DATS are effective in inducing apoptosis in human glioblastoma T98G and U87MG cells. It was suggested that the compounds present in garlic activate multiple pathways which result in induction of apoptosis in these cells by increasing the production of reactive oxygen species (ROS). They have shown that ROS induces apoptosis via the phosphorylation of p38 MAPK and activation of the redox-sensitive JNK1 pathway. Production of ROS in these cells also increased endoplasmic reticulum stress, and mitochondrial release of cytochrome C and Smac into the cytosol, which also result in apoptosis (27). Liu *et al* (28) showed that different variants of the glutathione S-transferase (GST) genes may affect the pathogenesis of glioma, and thus may have an impact on the variable responses to the various types of therapeutic compounds in gliomas with distinct variants of these genes. The aforementioned differences in GST gene variants may underlie the disparities observed in DADS-induced cytotoxicity in the present study. However, multi-center controlled prospective studies are required to verify this assumption.

Garlic oil contains several active substances that possess a high therapeutic potential. These include diallyl, allyl methyl and dimethyl mono- to hexa-sulfides (29). Furthermore, the biological effects of garlic can be attributed to all of the characteristic organosulfur compounds that it contains (30). Thus, it is hypothesized that the substances contained in garlic oil may amplify the induction of apoptotic cell death by activating different pathways to DADS, which induced apoptosis only in two tested cell lines, while garlic oil caused activation of apoptotic death pathways in all four tested cell lines. Additionally, only 0.75 $\mu\text{g/ml}$ garlic oil was required to induce apoptosis. The anti-cancer potential of garlic oil

remains poorly understood. The majority of the studies in this field of study have been performed on blood cancer cell lines (31-33). Seki *et al* (31) examined the effects of garlic oil on HL-60 cells (a human promyelocytic leukemia cell line). They found that garlic oil markedly reduced cell proliferation and concluded that it could induced differentiation of HL-60 cells into granulocytic cells (31). The preliminary results of the present study are promising, particularly the results obtained for garlic oil, which induced a significant cytotoxic effect in all tested astrocytoma cell lines of various degrees of differentiation. In addition, garlic oil primarily induced activation of apoptosis. It is crucially important for therapeutic applications used for the treatment of brain tumors to reduce cell death, where necrotic death is undesirable due to a resultant strong inflammatory reaction (34).

The present study has several limitations that should be addressed in subsequent studies. These include assessing the mechanism of the induced apoptotic pathway, estimation of cell-cycle phase distribution, as well as the examination of the effects of tested compounds on normal human cells. It is hypothesized that the PI3K-Akt/PKB-mTOR signaling pathway may be induced following treatment of glioma cells with DADS and garlic oil. However, it is necessary to assess the mechanisms leading to apoptosis in astrocytoma cells following treatment with active substances derived from garlic in more detail. The effects of other compounds from garlic oil and their antiproliferative properties on glioma cells should also be addressed. This can be used to identify and separate the most active substances from the mixture. Additionally, it would be useful to perform similar research with normal cells to identify the substances which exhibit selectivity against malignant cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AC and PS designed the study. AC performed the experiments and acquired the data. AC, JK and JS analyzed and interpreted the data. AC and JS wrote the first draft. AC, JS and JK contributed to writing or critical revision of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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