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Effects of water garlic extracts on cell cycle and viability of HepG2 hepatoma cells

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Abstract

Garlic extracts, either aqueous or oily, are commonly employed to prepare garlic derivative supplements used as nutraceuticals for the treatment of different pathologies. In this study, we investigated the effects of water garlic extracts from two different areas of Italy well known for garlic production, Latina (GEL) and Sulmona (GES), on cell cycle and death of HepG2 hepatoma cells. The effects of the treatments with GEL and GES were also compared with the oil-soluble sulfur compound of garlic, diallyl disulfide (DADS). GEL and GES induced a p53/p21-dependent cell cycle arrest in G2/M phase and apoptosis, although to a different extent, whereas DADS, under the experimental conditions used, was not detrimental to HepG2 cells. GEL and GES committed HepG2 cells to apoptosis by the activation of c-Jun-NH_2 terminal kinase (JNK)/c-Jun phosphorylative cascade without a detectable increase in the flux of reactive oxygen species. Moreover, differentiation of HepG2 cells induced by retinoic acid determined resistance to GEL and GES treatments without the activation of JNK signaling pathway.

Overall, the results obtained indicate that water-soluble garlic extracts are more inhibitory of the growth of transformed hepatoma cells than the oil-soluble isolated compound DADS, and that their antiproliferative properties are different depending on the area of origin of the starting material.

Keywords: Garlic; Diallyl disulfide; JNK; p53; Apoptosis

1. Introduction

The health benefits of garlic (*Allium sativum*) are derived from a wide variety of components and from the different ways it is administered. The complex chemistry of garlic makes it plausible that changes in processing may yield quite different preparations. For example, some unstable thiosulfinate compounds disappear during processing and are quickly transformed into different organosulfur components [1–3]. A variety of biological activities have been reported for garlic including effects on tumorigenesis, atherosclerosis, microbial growth and blood sugar modulation [4–7]. The antitumorigenic effects of garlic have been related to induction of apoptosis in tumor cells [8–12].

It is possible that such a variety of effects partly depends on the processing of garlic extract preparations. In particular, many adverse reactions are associated with an excess of oil-soluble organosulfur components: for example, the lipid-lowering effects of some oil-soluble sulfur compounds observed in vitro are associated with cytotoxicity, as revealed by the increased extracellular level of lactate dehydrogenase and by the presence of acetone in the breath after their oral consumption by humans [13,14]. On the other hand, water-soluble garlic extracts, although equally effective in decreasing cholesterol level, are not cytotoxic [15–18]. Moreover, the oil-soluble organosulfur compounds of garlic, such as allicin, ajoene and their intermediate products, are not found in urine or blood even after consumption of a large amount of garlic because alliinase, the enzyme that transforms alliin in allicin, is inactivated by low pH in the stomach [19]. However, in the case of enteric-coated garlic powders, which are able to bypass inactivation by gastric juice, the intestinal fluids inhibit production of allicin from the garlic powder by 40% and the intestinal epithelial cells decompose allicin [20]. Therefore, allicin is not a bioavailable stable compound and cannot reach target

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organs via circulation [21]. It has also been shown that an allicin-derived compound, diallyl disulfide (DADS), is metabolized and has been found as sulfate in liver [22]. After oral ingestion of 25 g of raw garlic, DADS as well as allicin have not been detected in human blood or urine from 1 to 24 h after consumption [20].

These results suggest that oil-soluble sulfur compounds cannot be reasonably considered as active principles. On the contrary, the water-soluble compounds, less odorous than the oil-soluble compounds, besides being more stable, appear to have a higher bioavailability and appear to be able to enter the blood and reach target organs. In fact, after garlic consumption, a water-soluble compound, such as *N*-acetyl-*S*-ally-cysteine, was found in human blood and urine [23]. As phytochemicals, they show antioxidant properties [24], inhibit cancer progression [25] and protect liver from toxins [26]. In a very recent study, patients with a benign prostate hyperplasia, regularly consuming aqueous garlic extract, showed a significant improvement in disease parameters [27].

In this work, we have compared the effects of garlic water extracts from bulbs cultivated in two geographic areas of Italy (Latina and Sulmona) with the oil-soluble garlic component DADS, which, in vitro, has been often demonstrated to be a potent cytostatic and cytotoxic agent. Our study shows that water garlic extracts are more effective than DADS in modulating cell cycle and inducing apoptotic death in HepG2 hepatoma cells. Moreover, we show that the bulbs produced in the Sulmona area are more effective than those from Latina in triggering cell cycle arrest and apoptosis.

2. Methods and materials

2.1. Cell cultures

Human hepatocarcinoma HepG2 cells were purchased from the European Collection of Cell Cultures (Promochem, UK), grown in glutamine-containing RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 1% sodium pyruvate, 1% non essential amino acids and 10% fetal calf serum, and incubated at 37°C in an atmosphere of 5% CO_2 in air. Cells were routinely trypsinized and plated at a density of 4×10^4 cells/cm^2.

2.2. Garlic extracts

Fresh garlic bulbs, collected the last week of July, were kindly provided by garlic producers from Sulmona and Latina areas. After immediate removal of the clove skin, the peeled cloves were placed in a Waring Blender with 1:3 w/v phosphate-buffered saline (PBS) and blended at high speed until homogeneity (approximately 60–70 s). Homogenates were filtered on a cheesecloth, and the liquid extracts were centrifuged at 50,000 rpm for 30 min. After centrifugation, supernatants were sterilized by filtration on a 0.2-μm cellulose membrane, divided in 1 ml aliquots and stored a −20°C.

2.3. Dry weight determinations

Dry weight of the garlic extracts was determined with a thermobalance (GULMI HG63, PBI International) by placing 1 ml of extracts in a preweighed aluminum weight pans and by utilizing a programmed drying method for aqueous solutions. After weighing (for at least three times until a constant weight was reached), the aluminum weight pans with dried samples were immediately placed in a glass desiccator jar containing predehydrated silica gel and let to equilibrate at room temperature for 12 h. The weight was then recorded in a Sartorius BP121S balance until a constant weight was reached.

2.4. Selenium determinations

Garlic extracts were diluted 1:2 with 65% HNO_3. After at least 1 week at room temperature, selenium concentration was measured by atomic absorption spectrometry using an AAnalist 300 Perkin-Elmer instrument, equipped with a graphite furnace with platform (HGA-800) and an AS-72 auto sampler. Nickel Nitrate was used as matrix modifier.

2.5. Cell treatments

A 50-mM solution of DADS (Sigma-Aldrich, St. Louis, MO) was prepared just before the experiments, dissolving 5.5 M DADS in dimethyl sulfoxide. Cells were treated with 50 μM DADS (which we calculated to correspond to the concentration of DADS present in 1% v/v of oil extract [23]) at 37°C in a medium supplemented with serum, because this concentration has been demonstrated to be efficient in inducing apoptosis [28] and represents a good compromise between the selective induction of apoptosis in cancer cells and the threshold of tolerance of differentiated cells toward its unspecific toxic effects. In fact, 50 μM is within the range used for in vivo study [29]. As control, an equal amount of dimethyl sulfoxide (0.1%) was added to untreated cells.

Water garlic extracts were thawed just before use and added to cells at 0.1, 0.5 and 1% v/v.

A 20-μM retinoic acid was used to induce differentiation of HepG2 [30]: before treatments with garlic extracts, HepG2 cells were preincubated with retinoic acid every 2 days over a period of 2 weeks.

2.6. Measurements of oxidative damage

Detection of intracellular reactive oxygen species (ROS) was performed by incubation of the cells with 50 μM 2′ -7′ -dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) (dissolved in dimethyl sulfoxide) for 30 min at 37°C [31] prior to the treatment with DADS and water garlic extracts. Treatment with 100 μM *tert*-butyl hydroperoxide was used as a positive control.

Carbonylated proteins were detected using the Oxyblot Kit (Intergen, Purchase, NY). Briefly, 20 μg of proteins were reacted with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25°C. Samples were resolved on 12% SDS-polyacrylamide

Table 1
Dry weight of GEL and GES

	GEL	GES
Dry weight (g/ml)	0.1002±0.0036	0.1052±0.0030

Data are expressed as mean±S.D, $n=3$.

gels and DNP-derivatized proteins were identified by immunoblotting using an anti-DNP antibody.

Levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured by a colorimetric method using the Lipid Peroxidation Assay Kit (Calbiochem-Novabiochem; La Jolla, CA) according to manufacturer instructions. Lipid peroxidation was evaluated with reference to standard curves obtained with known amounts of MDA and 4-HNE and expressed as micromole MDA+4-HNE/milligram protein.

2.7. Analysis of cell viability and apoptosis

Cell viability was evaluated in a hemocytometer chamber under a phase-contrast optical microscope by their capacity to exclude trypan blue (0.2%). Moreover, adherent apoptotic cells were detected with a fluorescence microscope directly on chamber slides by analyzing the nuclear fragmentation after staining with Hoechst 33342 (Calbiochem-Novabiochem) dye as previously described [32]. Early apoptosis was also detected with a FACScalibur instrument (Becton Dickinson, San Jose, CA) by analyzing phosphatidylserine externalization after staining with the impermeant dye annexin V-FITC (Bender MedSystems, Vienna, Austria). Alternatively, adherent (after trypsinization) and detached cells were combined, washed in PBS and stained with 50 μg/ml propidium iodide for the cytofluorimetrical evaluation of late apoptotic cells. The percentages of apoptotic cells were evaluated according to Nicoletti et al. [33] by calculating the peak area of hypodiploid nuclei. Data from 10,000 cells were collected for each data file. Cell cycle analyses were performed with WinMDI version 2.8 software.

2.8. Preparation of cell lysates and Western blot analyses

Cell pellet was resuspended in a lysis buffer containing 10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% IGEPAL CA-630 and protease inhibitors (Sigma). After 30 min of incubation in an ice-bath, cells were disrupted by 10 s of sonication. Lysates were then centrifuged at 14,000×g for 15 min at 4°C, and supernatants were removed and stored at −80°C. Twenty micrograms (for p53 and p21) or 50 μg (for the phosphoisoforms of JNK and c-Jun) of proteins were loaded on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Polyclonal anti-c-Jun (1:200), anti-JNK (1:200), anti-p21 (1:5000) or monoclonal anti-phospho-activated c-Jun and JNK isoforms (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-p53 (1:5000) and anti-β-actin (1:5000) (Sigma), were used as primary antibodies. The specific protein complex formed upon appropriate secondary antibody (Bio-Rad) treatment (1:10,000) was identified using a Fluorchem Imaging system (Alpha Innotech — Analitica De Mori, Italy) after incubation with ChemiGlow chemiluminescence substrate (Alpha Innotech).

Proteins were determined according to the method of Lowry et al. [34].

2.9. Data presentation

All experiments were carried out at least four times ($n=4$) unless otherwise indicated. Data were expressed as means ± S.D., and significance was assessed by Student's t test. Differences with P values <.05 were considered significant.

3. Results

3.1. GEL and GES inhibit cell growth in HepG2 cells

We previously demonstrated that DADS was able to induce a significant cell cycle arrest and apoptosis in neuroblastoma cells when administrated at a concentration of 50 μM [28]. In this study, we focused on the effects of water garlic extracts on HepG2 hepatoma cells. We used the same volumes of GEL or GES because, as shown in Table 1, they have the same dry weight.

Fig. 1 shows the effects of DADS, GEL or GES on the growth of HepG2 hepatoma cells. Fifty micromolar DADS slightly, but significantly, inhibited the cell growth after 24 h of treatment with a complete recovery of proliferation at 48 h, suggesting that HepG2 cells were able to rescue from

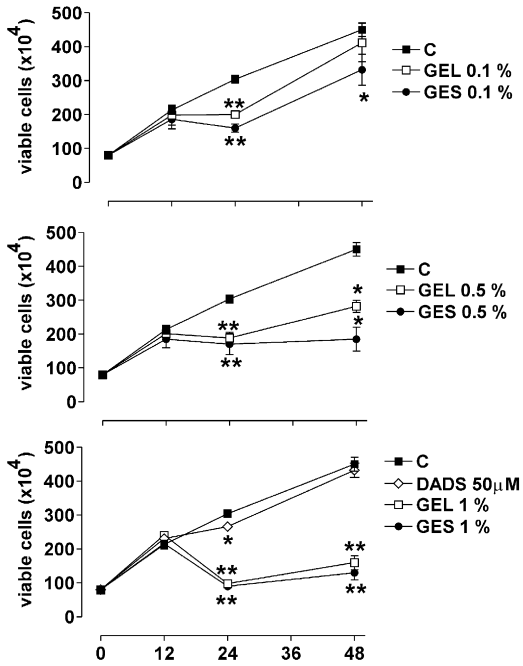
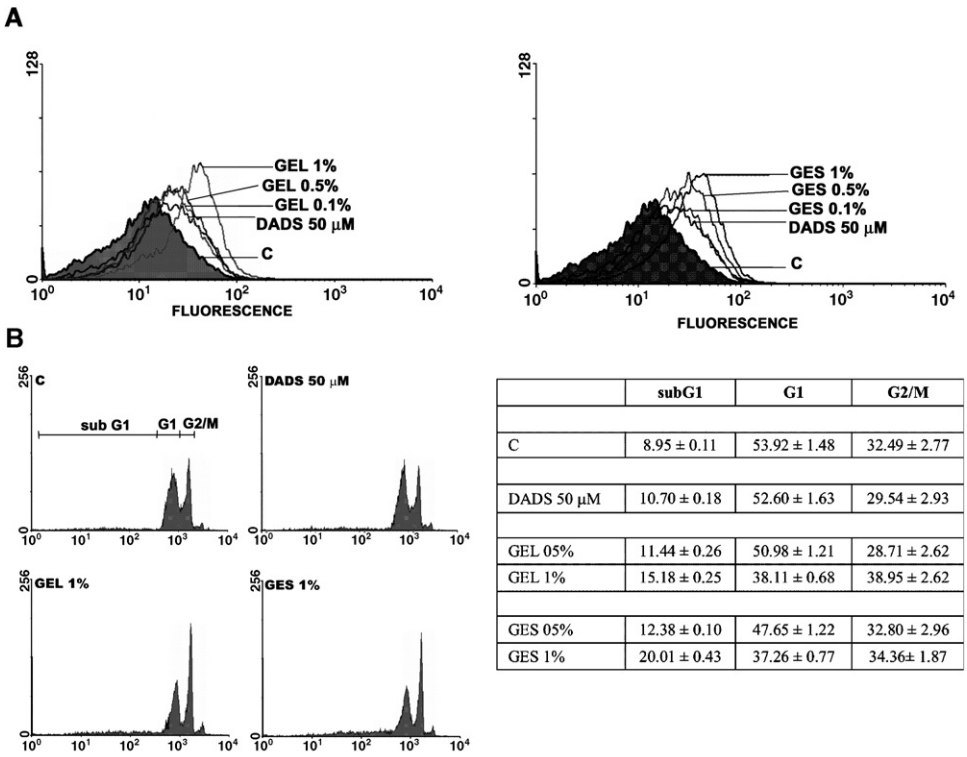


Fig. 1. Effects of DADS, GEL and GES on growth of human hepatoma cells HepG2. HepG2 cells, plated at density of $4\times10^4/cm^2$, were incubated with 50 μM DADS or 0.1%, 0.5% and 1% GEL or GES. Viable cells were evaluated by direct counts upon trypan blue staining under a phase-contrast optical microscope. Data show the only viable (trypan blue-negative) cells and are expressed as means±S.D. *P<.05, **P<.001 ($n=6$).



	subG1	G1	G2/M
C	8.95 ± 0.11	53.92 ± 1.48	32.49 ± 2.77
DADS 50 μM	10.70 ± 0.18	52.60 ± 1.63	29.54 ± 2.93
GEL 05%	11.44 ± 0.26	50.98 ± 1.21	28.71 ± 2.62
GEL 1%	15.18 ± 0.25	38.11 ± 0.68	38.95 ± 2.62
GES 05%	12.38 ± 0.10	47.65 ± 1.22	32.80 ± 2.96
GES 1%	20.01 ± 0.43	37.26 ± 0.77	34.36± 1.87

Fig. 2. GEL and GES affect cell cycle and viability of HepG2 cells. HepG2 cells plated at density of $4\times10^4/cm^2$ and treated with 50 μM DADS or 0.1%, 0.5% and 1% GEL and GES. (A) After 12 h of treatment, cells were washed twice with PBS and stained with annexin V-FITC as described under material and methods for detection of early apoptosis. Histograms reported are from a typical experiment done in triplicate out of five that gave similar results. (B) For detection of late apoptosis, cells were treated for 24 h, washed and stained with propidium iodide. Analysis of cell cycle and apoptosis was performed by a FACScalibur instrument, and percentages of staining-positive cells were calculated using WinMDI version 2.8 software. The cell cycle plots reported are from a typical experiment done in triplicate out of five that gave similar results. Tables indicating the percentages of nuclei in the G_1, G_2/M and sub-G_1 phases of cell cycle are shown at the right (sub-G_1, apoptotic cells).

DADS-mediated cytostatic effect and reenter normal cell cycle progression. On the other hand, GEL and GES showed a dose- and time-dependent effect on cell proliferation, with a significant reduction of viable cell number at 24 h but with a less pronounced recovery of cell proliferation at 48 h. GEL or GES (0.5% and 1%) induced a significant block of cell growth starting after 12 h of treatment, with GES being more effective in inducing inhibition of cell growth, as a concentration of 0.5% was already enough to completely inhibit the proliferation rate.

3.2. GEL and GES induce cell cycle arrest and apoptosis

To test whether the inhibition of cell proliferation could be the result of a massive arrest of the cell cycle or of the induction of the apoptotic process, we examined earlier apoptotic markers such as externalization of phosphatidylserine and cell cycle distribution by cytofluorimetric analysis after treatment with DADS, GEL or GES.

After 12 h of treatment, we performed cytofluorimetric analysis after staining HepG2 cells with annexin V-FITC, which is able to bind to the phosphatidylserine exposed on the surface and recognize apoptotic cells. Fig. 2A shows that GEL and GES were both more effective than 50 μM DADS in committing the cells to undergo apoptosis.

In order to evaluate whether the externalization of phosphatidylserine was followed by the execution phase of apoptosis, as monitored by internucleosomal cleavage of DNA, we performed cytofluorimetric analysis after staining with propidium iodide, a specific DNA dye. Fig. 2B shows that while the percentage of cell populations in the different phases of the cell cycle did not change upon treatment with 50 μM DADS, a significant increase of sub-G_1 (apoptotic) cells was evidenced after treatments with GEL and GES, with the latter being more efficient than the former in inducing cell death. These results were associated with an increase of cell populations in G2/M phase (Fig. 2B).

These results were confirmed by fluorescence microscopy analyses of cell nuclei stained with the DNA-specific dye Hoechst 33342. Fig. 3 shows that 12 and 24 h of treatment with 50 μM DADS resulted in the appearance of metaphasic nuclei and a slight chromatin condensation at 48 h, confirming that DADS was not able to commit cells to apoptosis. On the other hand, treatment with 1% GEL and GES affected DNA structure by inducing chromatin condensation and nuclear fragmentation already after 12 h of treatment, with a marked effect at 24 and 48 h (Fig. 3).

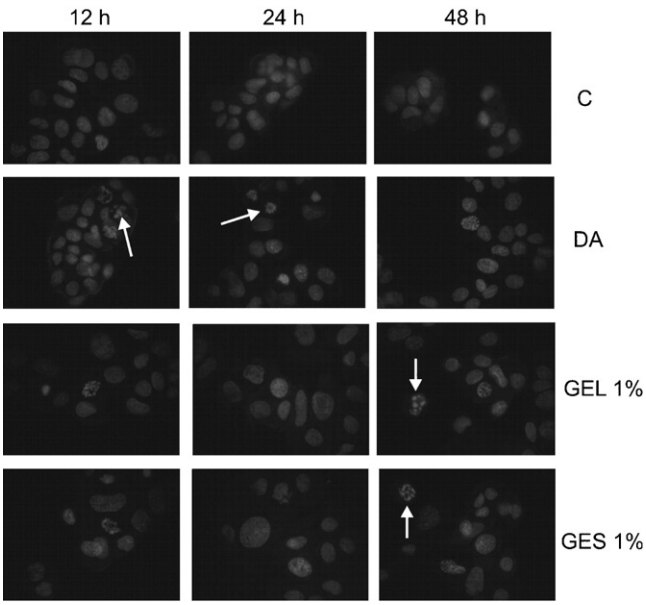


Fig. 3. GEL and GES induce apoptosis in HepG2 cells. HepG2 cells, plated at density of $4\times10^4/cm^2$, were grown on chamber-slides and treated for 12, 24 and 48 h with 50 μM DADS or 1% GEL and GES. After washing twice with PBS, cells were stained with Hoechst 33342 and visualized by fluorescence microscopy. White arrows show metaphasic nuclei (DADS) or nuclei with typical apoptotic features (GEL and GES).

3.3. GEL and GES induce p53 and p21 accumulation

The tumor suppressor gene p53 is known to be a member of the DNA damage-response pathway. It has been demonstrated that p53 protein increases at the early stages of cellular damage in response to a variety of stress-inducing agents [35], being responsible for the inhibition of cell growth and/or commitment to apoptosis [28,36]. Western blot analysis of the p53 protein, after treatments with 50 μM DADS or 1% GEL and GES (Fig. 4A), shows that DADS induced a late and slight increase of p53, while GEL and GES caused a significant rise of the protein already after 3 h of treatment in a dose-dependent fashion. In order to further dissect the role of p53 in GEL- and GES-mediated cell cycle arrest, we analyzed the expression of its downstream effector p21, which is a potent inhibitor of cell cycle kinases. In fact, also the p21 protein levels increased in a dose- and time-dependent manner (Fig. 4A), indicating that the p53/p21 system was actively induced in our system and could represent the effector of cell cycle arrest and apoptosis observed under our experimental condition. Fig. 4B shows that longer time point (48 h) analyses did not evidence changes in the p53 expression level, while a still significant accumulation of p21 could be detected only in the case of GES. These results suggest that GES affected cell cycle and viability of HepG2 cells more efficiently than GEL.

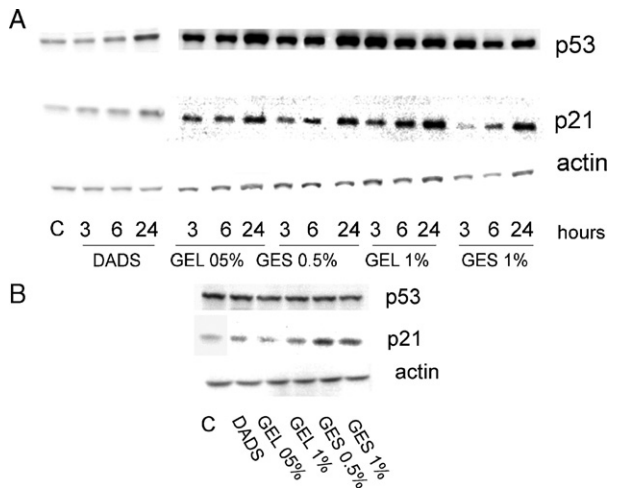


Fig. 4. GEL and GES induce cell cycle arrest by activation on p53/p21 pathway. HepG2 were treated with 50 μM DADS or 0.5% and 1% GEL and GES for 3, 6, 24 (A) and 48 h (B). Twenty micrograms of proteins of total cell lysates were loaded onto each lane for detection of p53 and p21 protein levels. β-Actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results.

3.4. GEL and GES induce the activation of JNK/Jun pathway

In a previous study, we demonstrated that DADS was able to induce a ROS-mediated activation of JNK/c-Jun pathway in neuroblastoma cells, and that this activation led to apoptosis [28]. In order to evaluate whether this pathway was operative also in HepG2 cells, we performed Western blot analyses of the active phosphorylated forms of JNK and c-Jun (p-JNK, p-Jun) after treatment with GEL and GES. Fig. 5A shows that 50 μM DADS induced a

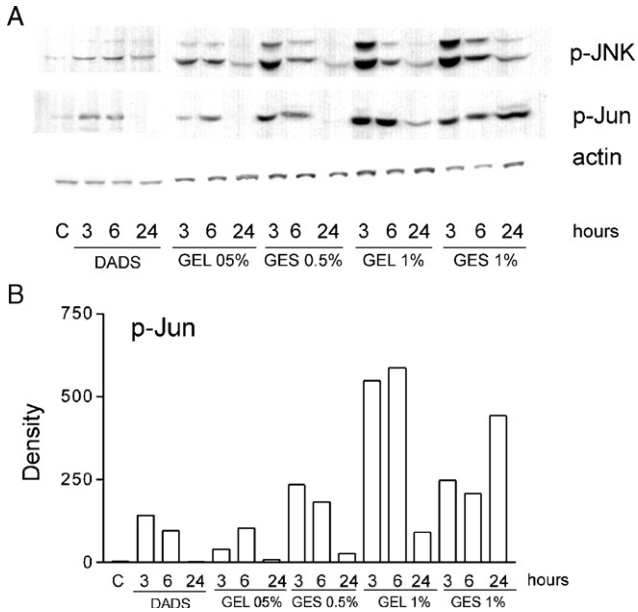


Fig. 5. GEL and GES induce a rapid activation of JNK/c-Jun signaling cascade. HepG2 were treated with 50 μM DADS or 0.5% and 1% GEL and GES for 3, 6 and 24 h. (A) Fifty micrograms of proteins of total cell lysates were loaded onto each lane for detection of the phospho-activated isoforms of JNK (p-JNK) and Jun (p-Jun). β-Actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results. (B) Densitometric analysis of p-Jun protein levels, calculated using Quantity One Software (Bio-Rad). Data are shown as arbitrary units.

slight accumulation of p-JNK and p-Jun at short time points (3 and 6 h), with the recovery of the control levels after 24 h. On the other hand, both GEL and GES were able to induce the JNK/Jun pathway in a dose-dependent manner, reaching the maximum extent as early as at 3 h. However, while during DADS and GEL treatments, the activation of this pathway was apparently terminated at 24 h, high levels of p-Jun were still maintained upon treatment with 1% GES.

To understand whether ROS were involved in the activation of the JNK/c-Jun signaling cascade, we attempted to assay their production under the treatments with DADS, GEL and GES. However, no evidence for ROS production was found under any of the treatments used (data not shown). Accordingly, we were not able to detect any significant changes either in protein or in lipid oxidation markers (data not shown).

3.5. GEL and GES contain different levels of selenium

Data so far obtained, although suggested that water garlic extracts were more effective than DADS in inducing cell cycle arrest and apoptosis in HepG2, also indicated that GES was more active of GEL in this respect.

As reported above (Table 1), GEL and GES have no significant differences in their dry weights. On the other hand, Table 2 shows that selenium concentration was significantly different in the two extracts. In particular, selenium was almost twofold higher in GES than in GEL, suggesting that it could play a role in the different cellular effects of the two extracts.

3.6. GEL and GES do not affect the viability and the expression levels of apoptotic markers in retinoic acid-differentiated cells

Therapies against tumor cell growth should not compromise cell viability of differentiated cells, or at least their efficacy should be much selective toward cancer cells. In this context, we induced differentiation of HepG2 cells by 15 days of treatment with 20 μM retinoic acid. Fig. 6A shows that, after retinoic acid incubation, the percentages of sub-G_1 cells did not increase with respect to the control values, suggesting that water garlic extracts do not affect cell viability of differentiated HepG2 cells. Accordingly, as shown in Fig. 6B, no change in p-JNK level and no phospho-activation of the downstream effector c-Jun could be evidenced after 6 h of treatment with GEL and GES of differentiated cells. It is worth to point out that also p53 and p21, although expressed at high concentrations — owing to retinoic acid-mediated induction of differentiation — were not affected by the treatments.

Table 2
Selenium concentration of GEL and GES

	GEL	GES
[Se] (nmol/ml)	18.24±0.11	27.05±0.09[a]

Garlic extract concentrations of Se, calculated by atomic absorption spectrometry. Data are expressed as mean±S.D.

[a] $P<.001$ ($n=3$).

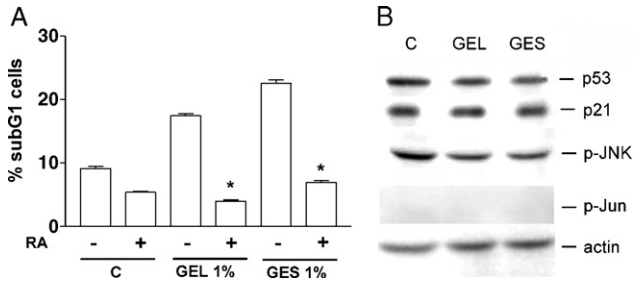


Fig. 6. GEL and GES do not induce apoptosis in differentiated HepG2 cells. HepG2 were incubated with 20 μM retinoic acid each 2 days over 2 weeks to induce differentiation. After this period, HepG2 cells were treated with 1% GEL or GES. (A) After 24 h, cells were washed and stained with propidium iodide. Analysis of cell cycle and apoptosis was performed by a FACScalibur instrument, and percentages of sub-G_1 (apoptotic) cells were calculated using WinMDI version 2.8 software. Data are expressed as means±S.D. *$P<.001$ ($n=4$). (B) After 6 h of treatment, cells were collected and lysed. Fifty micrograms of proteins of total cell lysates were loaded onto each lane for detection of the phospho-activated isoforms of JNK (p-JNK) and Jun (p-Jun), and for the p53 and p21 proteins. β-Actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results.

4. Discussion

Garlic is used worldwide as food additive, spice and medicine. As herbal supplements, garlic was found to be used more than twice as much as other supplements [37]. Recently, it has been reported that the consumption of vegetables rich in antioxidant phytochemicals and organosulfur compounds, such as garlic, could prevent tumorigenesis [4]. Consequently, a therapeutic use of purified garlic-deriving components such as DADS [28], diallyl trisulfide [38,39] and *S*-allyl mercaptocysteine [40] has been suggested in order to selectively kill tumor cells.

In this work, we have compared the effects of aqueous garlic extracts on human hepatoma HepG2 cells with those of DADS, which has been widely demonstrated to be, in vitro, an efficient antitumor agent [28,41,42]. First of all, liver is the organ where nutraceuticals are metabolized; furthermore, HepG2 cells are resistant to several anticancer therapies, thus, representing a good experimental model to compare different proapoptotic agents. In this study, we also investigated if garlic produced in different geographic areas of Italy had the same effects on tumor cells.

On the basis of the results obtained, it can be concluded that water garlic extracts are more effective than DADS in inducing cell cycle arrest and apoptosis in HepG2 cells. In fact, in our experiments, DADS did not induce a remarkable effect on cell cycle of HepG2 cells, as normal growth was still evidenced after prolonged exposures to DADS. The water garlic extracts prepared with garlic coming from Latina (GEL) and Sulmona (GES) areas, instead, induced a significant inhibition of cell growth, which was maintained for long time and resulted in apoptosis.

The apoptotic process was shown to be associated with the activation of the p53/p21 system and of the JNK/c-Jun signaling cascade. GEL and GES were able to induce a persistent activation of these pathways, suggesting that the stress conditions induced by the complex mixture present in the two water garlic extracts were not counteracted by the detoxifying cellular systems. Activation of JNK/c-Jun pathway was previously demonstrated to occur as a response of neuroblastoma cells to DADS-mediated ROS production [28]. In HepG2 cells, on the other hand, DADS is not able to strongly activate this pathway, confirming the hypothesis that the human liver cancer cells HepG2 efficiently overcome the toxic effect induced by DADS [43]. In fact, we were not able to detect either ROS increase or ROS-mediated damage of lipids and proteins upon treatment with DADS.

It was rather unexpected that neither GEL nor GES was able to give rise to ROS increase and ROS-mediated lipid damage in HepG2 cells. However, we have recently shown that in cells rich in antioxidant, such as gastric-derived AGS histotype, the resistance to DADS depends on ROS scavenging [44]. The same process can be operative in the HepG2 cells.

Since the JNK-mediated phosphorylative cascade is redox sensitive [45] and garlic extracts are rich in thiol-containing molecules, the induction of JNK/c-Jun system could reasonably depend on an alteration of the intracellular thiol redox state produced by GEL and GES without ROS production.

However, we do not yet know what components of water garlic extracts are responsible for the effect we observed on cell viability and/or if they undergo additional modifications during their metabolic processing. It has been reported [23] that water-soluble organosulfur compounds are present in human fluids long time after ingestion, and that they could undergo metabolic modification such as hydroxylation or hydrolysis. Therefore, metabolomic studies should be performed in the future to investigate which are the molecules acting as potential modulators of signaling pathways that promote cell death upon administration of water garlic extracts.

The second interesting outcome of the present work is that cytotoxic effects on cell proliferation differ in water garlic extracts depending on the areas of origin of our samples.

The only significant difference that we have so far observed between GEL and GES was in their selenium content. This difference may indicate that this oligoelement could have a role in the induction of cell death. In fact, selenium, besides being the cofactor of several enzymes involved in ROS scavenging (i.e., glutathione peroxidases) and in the protection against oxidation of sulfhydryl groups (i.e., glutaredoxin and thioredoxins), when not properly complexed, could also represent a proapoptotic compound [46]. In this context, it has been demonstrated that the efficacy of cancer prevention by selenium-enriched garlic is primarily dependent on the action of this element [47]. Of course, we are not in the condition to state that the different selenium concentration of the two extracts fully accounts for the differential cytotoxicity effects observed. Work is in progress in our laboratory in order to analyze, by mass spectrometry, whether other components contribute in determining the different effect of GEL and GES on HepG2 cells.

Induction of apoptosis by GEL and GES appears to be selective for cancer cells. In fact, water garlic extracts were not able to induce cell death in retinoic acid-differentiated HepG2 cells. Moreover, the lack of activation of the JNK/c-Jun pathway in differentiated cells shows that the induction of this system is necessary to transduce the garlic extract-mediated apoptotic signal within the cell.

In conclusion, these results suggest that the use of water garlic extract as adjuvant in cancer therapy may be beneficial, and that a proper choice of the starting material is advisable. However, further studies are necessary to establish a more rigorous protocol for their administrations.

Acknowledgments

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