

# Effect of raw garlic juice and diallyl disulfide on cell viability and reactive oxygen species production in ethanol-treated HepG2 cells

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**Abstract:** Several reports indicate that garlic compounds inhibit ethanol metabolism in the liver and also attenuate ethanol-induced lipid peroxidation and enhance the activity of antioxidative enzymes. The aim of this study is to evaluate whether raw garlic juice and diallyl disulfide (DADS) could modulate the viability of ethanol-treated HepG2 cells as a model of hepatocytes. The results of our experiments strongly indicate that raw garlic juice and DADS at low, non-toxic concentrations inhibit ethanol-induced HepG2 cell death. To assess the possible mechanisms of cytoprotection, we examined the influence of garlic juice and DADS on ethanol-induced reactive oxygen species (ROS) production in HepG2 cells. The results revealed that the decrease in ethanol-induced cell death was caused, at least partially, by inhibition of superoxide and hydrogen peroxide production in ethanol-treated HepG2 cells, mainly via the CYP2E1 system. Moreover, garlic juice and DADS were superoxide anion scavengers. These data indicate that garlic juice and DADS may be useful as agents for preventing ethanol-induced hepatotoxicity.

**Key words:** garlic juice, diallyl disulfide (DADS), cell viability, ethanol, reactive oxygen species

## INTRODUCTION

Alcohol consumption by humans is increasing steadily worldwide and has become one of the most serious health hazards. Several harmful effects of alcohol are known, but among the organs, the liver is most susceptible to the toxic effect of ethanol.

The main pathway for ethanol oxidation involves hepatic alcohol dehydrogenase (ADH), a cytosolic enzyme that catalyses the transformation of ethanol into acetaldehyde, which in turn is converted to acetate through acetaldehyde dehydrogenase (ALDH). Both enzyme reactions generate an excess of reducing equivalents in the liver, primarily in the form of NADH [1]. Another adaptive system of ethanol oxidation is called the microsomal ethanol-oxidizing system (MEOS). This system involves an ethanol-inducible isoform of cytochrome P450 known as CYP2E1. Ethanol-induced liver metabolic disturbances are especially due to oxidative stress generated via the ADH pathway of the liver and changes in the redox state leading to NADH generation. On the other hand, the induction of CYP2E1 and formation of reactive oxygen species (ROS) appear to be mechanism by which ethanol is hepatotoxic [2, 3].

Garlic (*Allium sativum*, Linn) and compounds derived from it have been found to have many therapeutic effects. There are several reports on the effect of garlic extracts on lipid peroxidation and antioxidant enzymes [4, 5, 6], and also on hepatic ethanol metabolism in mice [7]. It has been reported that fresh garlic extract inhibits MEOS as well as ALDH activity in the liver of ethanol-treated mice (2A). Garlic oil

has a gastroprotective activity against ethanol-induced ulcers by reducing lipid peroxidation and normalizing the activity of antioxidant enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) [8].

Allicin (diallyl thiosulfinate) is believed to be one of the major bioactive compounds of garlic. Allicin is formed by alliinase cleavage of naturally occurring alliin upon crushing or mincing of garlic, and is the progenitor of a number of other bioactive products [9].

Garlic oil obtained by steam distillation contains more than 20 organosulfur compounds, among which diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are three major compounds. Garlic oil and its compounds have been reported to benefit the hepatic oxidation and detoxication systems [10, 11] in rat primary hepatocytes.

HepG2, human hepatoma cells, are very often used as a model for the study of mechanisms involved in ethanol-induced liver injury [12, 13]. Moreover, HepG2 cells have been shown to be a useful model for investigating the capacity of potential chemoprotectants of natural origin to enhance the antioxidative mechanisms of cells [14]. Therefore, we used HepG2 cells to evaluate whether raw garlic juice, containing mainly allicin and other water-soluble compounds, and diallyl disulfide, as an oil-soluble garlic compound, could modulate the viability of ethanol-treated HepG2 cells. To assess the possible mechanisms involved in hepatoprotection, we examined the influence of garlic juice and DADS on ethanol-induced ROS production in HepG2 cells.

## MATERIALS AND METHOD

**Materials.** Raw garlic juice was prepared from skinned raw garlic cloves crushed in a blender for 1 min together with an equal weight of distilled water (1g of garlic/ml of water).

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The mixture was then allowed to stand for 30 min at 25°C. After filtration through cheesecloth, raw garlic juice samples were obtained, filtrated through 0.22 µm filters, distributed into Eppendorf tubes and stored at -80°C before use. Diallyl disulfide (ICN Biomedicals, Inc. Irvine, CA, USA) was diluted in DMSO as a 1 mM stock solution.

The human hepatoma cell line HepG2 (American Type Culture Collection, Manassas, VA, USA) was cultured in Eagle's Medium (MEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 1% nonessential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin, pH 7.4. Cells were seeded in tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Treatment of HepG2 cells with alcohol and garlic compounds.** HepG2 cells were grown on 96-well plastic plates (Falcon, Bedford, MA, USA),  $4 \times 10^4$  cells/well, and treated or not treated with different garlic juice or DADS concentrations and different ethanol concentrations. The toxicity of ethanol and garlic compounds was determined by the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. For further experiments, three ethanol concentrations (5, 10 and 50 mM) and two DADS concentrations, 50 µM and 100 µM, were chosen. Also, two concentrations of garlic juice were used – 1 mg or 0.1 mg of wet garlic weight per ml of culture medium. The cells treated with ethanol alone (ethanol control) or with ethanol and garlic compounds were incubated for 24 h in a humidified atmosphere at 37°C, 5% CO<sub>2</sub>. After incubation, cell culture viability was estimated by the MTT assay. MTT was metabolized by viable, metabolically active cells to purple formazan crystals, which were solubilized overnight in SDS buffer, and the product quantified spectrophotometrically by measuring absorbance at 570 nm in a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA, USA).

**Measurement of superoxide anion (O<sub>2</sub><sup>-</sup>) production by cytochrome C reduction assay [15].** HepG2 cells were grown in 96-well plastic plates ( $4 \times 10^4$  cells/well) with or without garlic compounds. After 24 h incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, cultures were washed twice with HBSS and an assay for superoxide anion was performed. Briefly, HBSS (207.5 µl), 12.5 µl of cytochrome C solution in HBSS (final concentration 75 µM), 5 µl of either SOD solution (final concentration 60 U/ml) or 5 µl of distilled water, and 25 µl ethanol solution in HBSS (final concentration 5, 10 or 50 mM) were added into each well on a 96-well plate. There were also control wells, where cells were incubated without ethanol. The microplate was incubated at 37°C for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of O<sub>2</sub><sup>-</sup> based on the extinction coefficient of cytochrome C:  $\Delta E_{550} = 21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . The results were expressed as nanomoles of O<sub>2</sub><sup>-</sup> per  $1 \times 10^6$  cells per 60 min. (All chemicals purchased from Sigma, St. Louis, MO, USA).

**Measurement of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production [16].** HepG2 cells were grown on 96-well plastic plates ( $4 \times 10^4$  cells/well) with or without garlic compounds. After 24 h incubation at 37°C in a humidified atmosphere of 5%

CO<sub>2</sub>, cultures were washed twice with Hanks' Balanced Salt Solution (HBSS) and the medium was replaced by HBSS (100 µl/well) with an addition of ethanol (final concentration 5, 10 or 50 mM) for 60 min at 37°C. There were also control wells where cells were incubated without ethanol. Next, the wells were washed twice with HBSS and a measurement of intracellular hydrogen peroxide was performed. The assay is based on horseradish-dependent peroxidation (HRPO) of phenol red by H<sub>2</sub>O<sub>2</sub> leading to the formation of a compound that exhibits absorbance at 600 nm. Briefly, the cells were covered with 100 µl/well of the assay solution, prepared on the day of the experiment, and consisted of HBSS, phenol red (Sigma, final concentration 0.56 mM), and HRPO (Serva, Heidelberg, Germany, final concentration 20 U/ml), to which 10 µl/well of 1N NaOH was added. After 3 min of incubation, the plate was read at 600 nm in the microplate reader. The results were expressed as nanomoles of H<sub>2</sub>O<sub>2</sub> per  $1 \times 10^6$  cells per 60 min based on the phenol red extinction coefficient ( $\Delta E_{600} = 19.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ ).

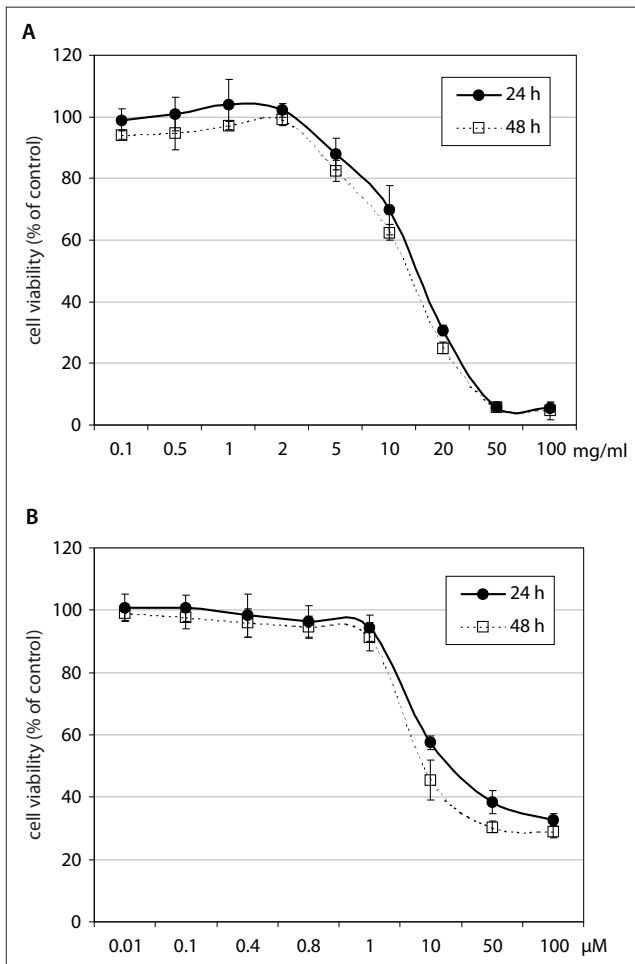
**Influence of garlic juice and DADS on superoxide anion (O<sub>2</sub><sup>-</sup>) generation by ethanol-induced cells in the presence of an alcohol dehydrogenase (ALD) inhibitor.** Before the experiments, HepG2 cells were incubated for 72 h with 2 mM pyrazole (an ALD inhibitor). The cells were then continuously grown on 96-well plastic plates ( $4 \times 10^4$  cells/well) with or without an addition of the inhibitor and also in the absence or presence of 50 µM DADS or 1mg/ml of garlic juice. After 24 h incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, the cultures were washed twice with HBSS and assayed for superoxide anion, as described above.

**Measurement of garlic juice and DADS ability to scavenge superoxide anion (O<sub>2</sub><sup>-</sup>) production.** HepG2 cells were grown on 96-well plastic plates ( $4 \times 10^4$  cells/well) for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cultures were then washed with HBSS, and into each well was added: HBSS (182.5 µl), 12.5 µl of cytochrome C solution in HBSS (final concentration 75 µM), 5 µl of either SOD solution (final concentration 60 U/ml) or 5 µl of distilled water, and 25 µl ethanol solution in HBSS (final concentration 5, 10 or 50 mM). After 30 min of incubation at 37°C, 25 µl of 10 µM (in HBSS) garlic compounds were added (final concentration 50 µM or 100 µM of DADS and 1 mg/ml or 0.1 mg/ml of garlic juice). There were also control wells with either ethanol or garlic compounds and also control wells without treatment (cell control). After another 30 min of incubation at 37°C, the plates were transferred to the microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of O<sub>2</sub><sup>-</sup> based on the extinction coefficient of cytochrome C:  $\Delta E_{550} = 21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . The results were expressed as nanomoles of O<sub>2</sub><sup>-</sup> per  $1 \times 10^6$  cells per 60 min.

**Statistical analysis.** Experiments were conducted at least in triplicate. Values are expressed as mean ± SD. The significance of differences was determined with the use of an analysis of variance (Statistica computer package). The following statistical tests were used: a two-way ANOVA test with *post-hoc* Tukey's test and Wilcoxon paired test for comparison inside groups. P values ≤ 0.05 were considered significant.

**RESULTS**

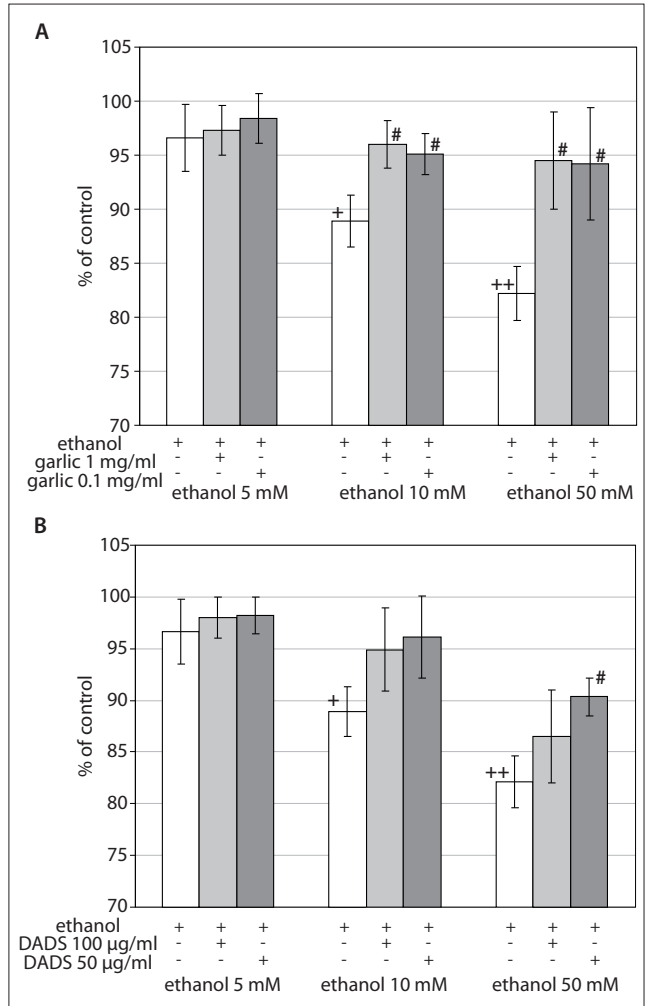
As can be seen from Fig. 1, garlic juice and DADS inhibited the viability of HepG2 cells depending on the concentration used. For further experiments, nontoxic concentrations of garlic juice (1 mg/ml and 0.1 mg/ml) and DADS (50 µM and 100 µM) were chosen.



**Figure 1** Influence of different concentrations of garlic juice (A) and DADS (B) on HepG2 viability. After 24 h or 48 h of incubation, cell viability was estimated by MTT method and expressed as a percent of viable cells in comparison to not treated controls.

A significantly higher cell viability was observed in cells pretreated with garlic compounds before ethanol treatment in comparison to cells treated with ethanol alone. This hepatoprotection was especially pronounced when high, 10 mM and 50 mM ethanol concentrations were used. Ethanol significantly decreased cell viability, while garlic juice increased cell viability nearly to the control, non-treated cell level. DADS was a little less active, but its 50 µM concentration significantly increased cell viability (Fig. 2).

When HepG2 cells were pretreated for 24 h with garlic juice or DADS and after that induced for superoxide anion production by treatment for 1 h with different ethanol concentrations, a significant decrease in O<sub>2</sub><sup>-</sup> level was observed (Fig. 3). Also the level of hydrogen peroxide was diminished in HepG2 cells treated with DADS or garlic juice (Fig. 4). The lower concentrations of DADS and garlic juice, 50 µM and 0.1 mg/ml respectively were more effective in decreasing ROS production



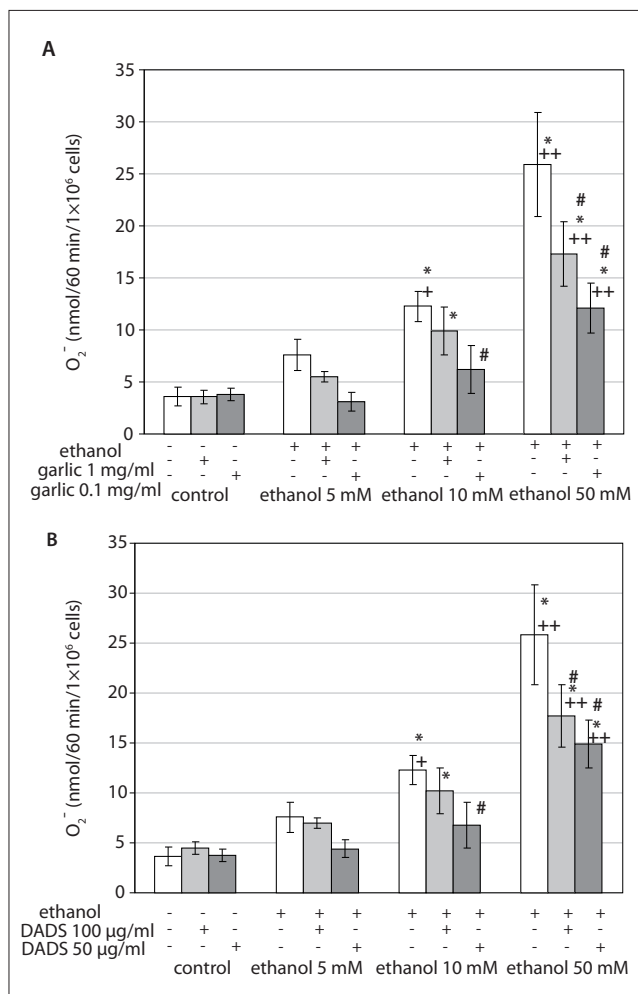
**Figure 2** Influence of different concentrations of garlic juice (A) and DADS (B) on HepG2 viability (estimated by MTT method) after treatment with 5 mM, 10 mM or 50 mM ethanol. Results expressed graphically as a percentage of the viability of control (not treated) cells. Values are means ± SD of results from five experiments.

+ Significantly different in comparison to cells treated with 5 mM ethanol, p<0.01.  
 ++ Significantly different in comparison to cells treated with 10 mM ethanol, p<0.001.  
 # Significantly different from ethanol control in a respective group (indicates garlic influence), p<0.01. Garlic significantly changed the effect of ethanol, p<0.003 (two-way ANOVA).

than the higher concentrations. The results strongly suggest that the decrease in ROS production was connected with the hepatoprotective activity of both compounds examined against ethanol cytotoxicity.

To assess the mechanisms of antioxidative activity of garlic compounds, we blocked the ethanol oxidation pathway via alcohol dehydrogenase by incubation of HepG2 cells for 3 days with 2mM pyrazole. The cells treated with pyrazole were viable (data not shown) and still produced superoxide anion after ethanol induction, probably via the MEOS system (Fig. 5). In such experimental conditions, both garlic juice and DADS used together with pyrazole still inhibited ethanol-induced superoxide anion production. These results indicated that garlic juice and DADS inhibited ROS production generated by the MEOS system.

We also used garlic juice and DADS as superoxide anion scavengers. HepG2 cells were induced with different concentrations of ethanol for ROS generation, and after 30 min garlic juice or DADS were added. In this model,



**Figure 3** Influence of garlic juice (A) and DADS (B) supplementation on superoxide anion (nmol/60 min/ $1 \times 10^6$  cells) release from HepG2 cells treated with different concentrations of ethanol. Results expressed as mean  $\pm$  SD of four independent experiments.

\* Significantly different from respective control (cells not treated or treated only with garlic or DADS),  $p \leq 0.0001$ .

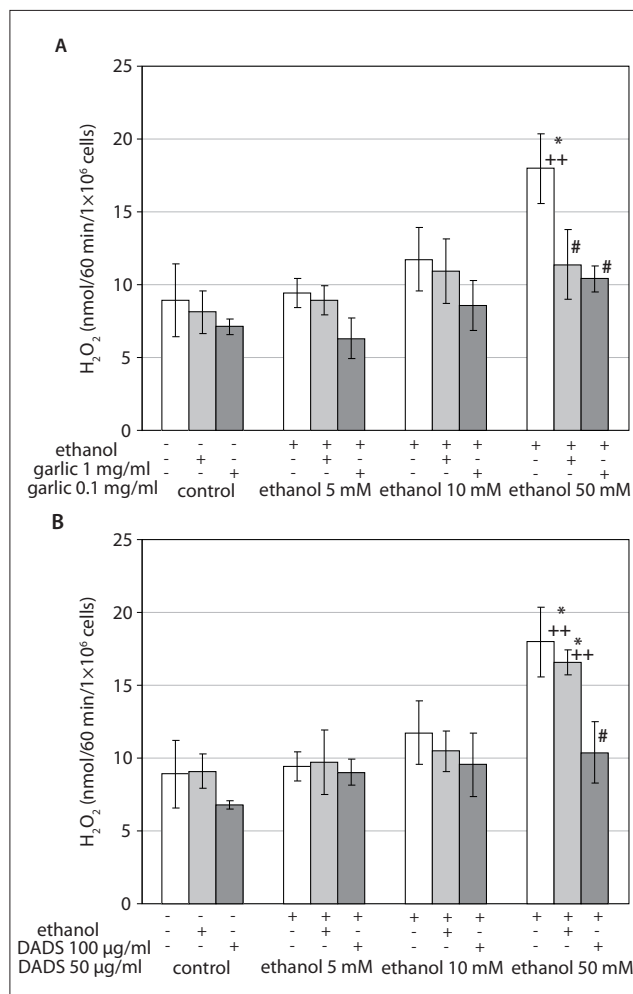
+ Significantly different in comparison to cells treated with 5 mM ethanol,  $p \leq 0.001$ .

++ Significantly different in comparison to cells treated with 10 mM ethanol,  $p \leq 0.001$ . # Significantly different from ethanol control in a respective group,  $p \leq 0.0001$ . Garlic and DADS significantly changed the effect of ethanol,  $p \leq 0.0001$  (two-way ANOVA).

higher garlic juice and DADS concentrations, namely 100  $\mu$ M DADS and 1 mg/ml garlic juice, were more effective in scavenging superoxide anion than the lower concentrations (Fig. 6).

## DISCUSSION

Several studies have demonstrated that an aqueous extract of garlic in which alliin (diallylthiosulfinate), S-allylcysteine sulfoxide (SACS) and also S-allylcysteine (SAC) are the main compounds, can significantly improve the oxidant-antioxidant status in the liver and protect hepatocytes against lipid peroxidation in different *in vivo* models [17-19]. Mice fed with fresh garlic had significantly diminished concentrations of acetaldehyde and acetate in serum after ethanol administration. Moreover, MEOS activity was significantly lower, as was aldehyde dehydrogenase (ALDH)



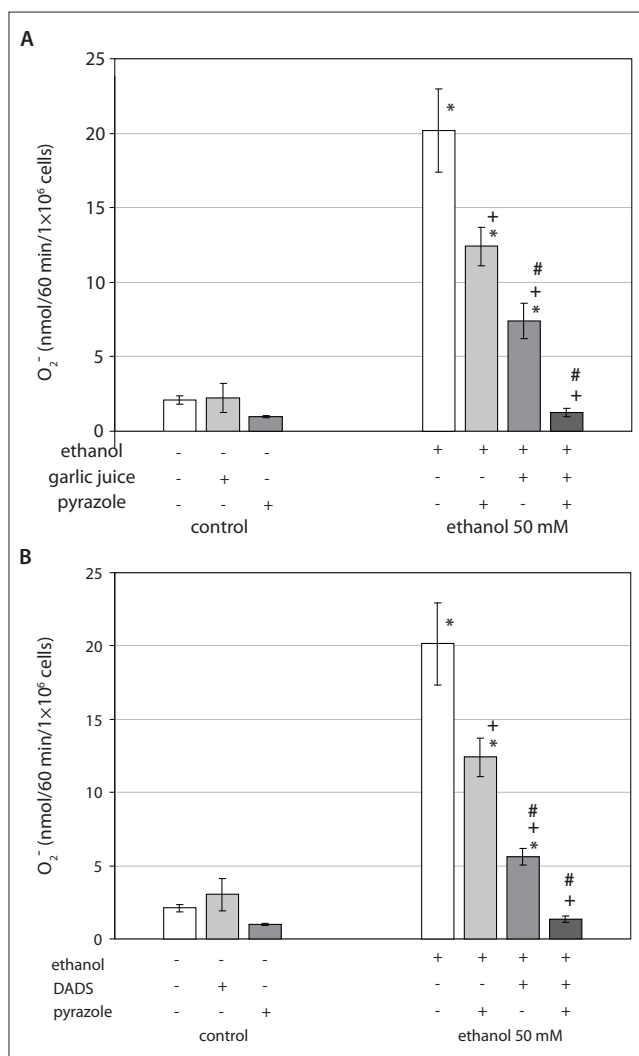
**Figure 4** Influence of garlic juice (A) and DADS (B) supplementation on hydrogen peroxide (nmol/60 min/ $1 \times 10^6$  cells) release from HepG2 cells treated with different concentrations of ethanol. Results expressed as mean  $\pm$  SD of four independent experiments.

\* Significantly different from respective control (cells not treated or treated with garlic or DADS alone),  $p \leq 0.001$ .

++ Significantly different in comparison to cells treated with 10 mM ethanol,  $p \leq 0.001$ . # Significantly different from ethanol control in a respective group,  $p \leq 0.0001$ . Garlic ( $p \leq 0.001$ ) and DADS ( $p \leq 0.0006$ ) significantly changed the effect of ethanol (two-way ANOVA).

activity. Alcohol dehydrogenase (ADH) activity, however, was not changed [7]. It is well known that the toxicity of ethanol is connected with acetaldehyde generation via the ADH system as well as via MEOS. However, in ethanol-induced hepatotoxicity, the generation of ROS and lipid peroxidation are also very important. In our *in vitro* experiments, we detected that both garlic juice and DADS significantly inhibited superoxide anion as well as hydrogen peroxide production. Therefore, it can be expected that the protection of HepG2 cells against ethanol-induced cytotoxicity observed in our study, at least in part, was connected with a decrease in ROS generation. Moreover, this protection was not caused by ethanol metabolism via ADH, but probably by MEOS (CYP2E1 inducible P450 isoenzyme), because when ADH was blocked by pyrazole, garlic juice and DADS still inhibited ethanol-induced superoxide anion production.

Water garlic extract and S-allylcysteine have already been described as suppressing the production of hydroxyl radicals in activated rat macrophages [20], and a chloroform extract

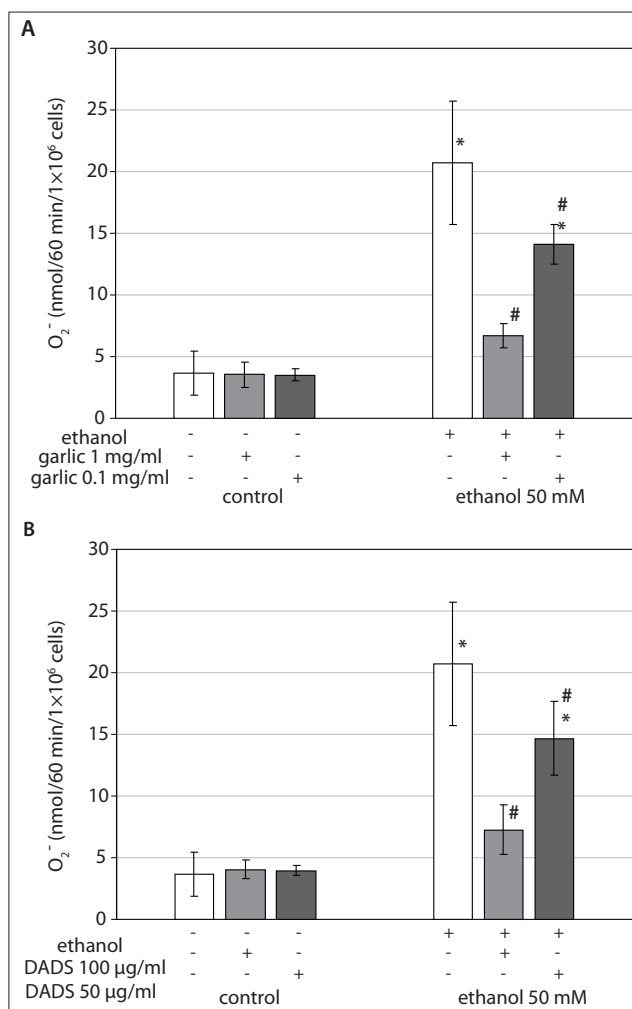


**Figure 5** Influence of 0.1 mg/ml of garlic juice (A) and 50 μM DADS (B) on superoxide anion (nmol/60 min/ $1 \times 10^6$  cells) release from HepG2 cells treated with 50 mM ethanol in the presence of 2 mM pyrazole as inhibitor of alcohol dehydrogenase.

\* Significantly different from respective control (cells not treated or treated only with garlic juice, DADS or pyrazole),  $p \leq 0.0001$ .  
 + Significantly different from cells treated with ethanol alone,  $p \leq 0.001$ .  
 # Significantly different from cells treated with pyrazole and 50 mM ethanol,  $p \leq 0.01$  (Wilcoxon paired test).

of garlic was active in attenuating free radical generation from neutrophils [21]. Moreover, garlic products decreased oxidative stress and preserved antioxidant enzymes activity in gentamycin-induced and cadmium-induced renal damage in rats [22, 23], as well as oxidative organ damage connected with thermal skin injury in mice [6]. S-allylcysteine sulfoxide (SACS) isolated from fresh garlic was found to significantly decrease lipid peroxidation in alcohol-fed rats [24]. Moreover, allicin alone can protect mice against concanavalin-A induced hepatic injury [25]. Since garlic extract and compounds present in fresh garlic extract inhibited lipid peroxidation in all these *in vivo* systems, it seems likely that endogenous production of ROS can be inhibited by water-soluble fresh garlic compounds independently of the inducer used.

However, in several papers, aged garlic extract (AGE) obtained by extracting garlic cloves for several months with a water-ethanol mixture, was reported to have higher hepatoprotective and antioxidative properties than raw garlic



**Figure 6** Garlic juice (A) and DADS (B) as scavengers of superoxide anion (nmol/60 min/ $1 \times 10^6$  cells) release from HepG2 cells treated with 50 mM ethanol.

\* Significantly different from respective control (cells not treated or treated with garlic juice or DADS alone),  $p \leq 0.0001$ .  
 # Significantly different from cells treated with 50 mM ethanol alone,  $p \leq 0.0001$ . Garlic ( $p \leq 0.00008$ ) and DADS ( $p \leq 0.0001$ ) significantly changed the effect of ethanol (two-way ANOVA).

juice [26-29]. It is known that AGE contains SAC and several other compounds, such as allylmercaptocysteine (SAMC). Lipid soluble compounds in AGE include diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS). Garlic oil containing DAS, DADS, and DATS has been described to protect mice against ethanol-induced gastric ulcers by decreasing lipid peroxidation and normalizing antioxidative enzyme activity of GPx, catalase and SOD [8]. AGE was also shown to protect against oxidative damage by inhibiting lipid peroxidation in liver cells exposed to phenobarbital, a sedative, and bromobenzene-3,4-oxide, an environmental toxic agent. Other studies have shown that AGE protects against liver toxicity by benzo(a)pyrene and aflatoxin, two potent free radical-producing carcinogens [30, 31].

It has been demonstrated that 0.5 mmol/l DADS might enhance detoxification and antioxidation capabilities of rat primary hepatocytes by increasing the intracellular level of GHS and the activity of GPx as well as glutathione reductase (GRd) and glutathione transferase (GST) [32]. In our study, such a high concentration of DADS was toxic to HepG2 cells. These differences in cell cytotoxicity are connected with the

kind of cells used for experiments: it has been detected that DADS is several times more toxic to hepatoma cells than to normal hepatocytes. In studies concerning the anti-tumour effect of DADS, the authors detected that 100 µmol/l DADS induced a significant level of apoptosis in HepG2 cells after 24 h of incubation [32, 33]. If this is so, the lower hepatoprotective activity of 100 µM DADS in comparison to 50 µM observed in our study could be caused by apoptosis of some cells in the culture.

In our study, both garlic juice and DADS exhibited an ability to scavenge superoxide anion generated in HepG2 cells treated with ethanol. There are discrepancies in the literature concerning the scavenging activity of different garlic preparations. In some papers [26], only AGE, but not other garlic extracts, had antioxidant properties when the inhibition of low level chemiluminescence in a liver microsomal fraction initiated by t-butyl hydroperoxide was examined. Also, raw garlic juice had prooxidant properties in contrast to AGE, which exhibited antioxidant activity [34]. However, in a recently used *in vitro* method, in which the garlic extracts were examined as inhibitors of methyl linolate oxidation in acetonitrile solution, it was detected that garlic extract, especially allicin, which is a major component of this extract, were active antioxidants. Moreover, allicin added to solutions generating peroxy radicals was capable of scavenging a peroxy radical [35].

It should be stressed that DADS is preferentially metabolised to allicin by CYP2E1 in the human liver [36]; therefore the inhibition of ROS generation in ethanol-treated HepG2 cells by DADS can be partially caused by the antioxidant properties of allicin.

To summarise, raw garlic juice and DADS at low non-toxic doses decreased ethanol-induced HepG2 cell death, at least partially, by inhibiting superoxide and hydrogen peroxide generation by metabolizing ethanol HepG2 cells, mainly via ethanol-inducible MEOS. Moreover, raw garlic juice and DADS were superoxide anion scavengers. These data suggest that garlic juice and DADS at low doses may be useful agents for the prevention of alcohol-induced hepatotoxicity, and their effect might be due to their antioxidant activity.

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