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Original Article

The Protective Effect of Garlic Extract against Acetaminophen-Induced Loss of Mitochondrial Membrane Potential in Freshly Isolated Rat Hepatocytes

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Abstract

Overdose of acetaminophen causes severe hepatic necrosis in humans and experimental animals. Studies on its hepatotoxicity remain a very active area since some of current data are still uncertain. In this study, freshly isolated rat hepatocytes were used to determine the effects of garlic extract and its component, allicin on the acetaminophen-induced cell cytotoxicity and to compare with the effect of N-acetyl cysteine as a standard treatment. Garlic extract was prepared via a standard method and its allicin and allyl mercaptan contents were determined using analytical and preparative high performance liquid chromatography (HPLC). Rat hepatocytes were isolated using collagenase perfusion and mitochondrial membrane potential and cell cytotoxicity were determined using Rhodamine 123 fluorescence and trypan blue exclusion, respectively. Inclusion of garlic extract and/or N-acetyl cysteine resulted in a reduction in the loss of mitochondrial membrane potential as well as cell death which occurred after acetaminophen addition and therefore, illustrated considerable hepatoprotective effects without significant differences between two treatments. In contrast, pure allicin was not effective significantly. The hepatoprotective effects of garlic extract may be due to the compounds other than allicin such as allyl mercaptan, as allicin has been shown to transform to allyl mercaptan as a major metabolite.

Keywords: Allicin; Cytotoxicity; Garlic extract; Hepatocytres; Mitochondrial membrane potential.
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1. Introduction

Garlic (*Allium sativum*) has been used as a medicinal plant, since before the time of the Sumerian civilization (2600-2100 BC), by when it was already widely cultivated in India and China [1]. The intact bulbs of garlic contain an odorless, sulfur-containing amino acid derivative known as alliin [(+)-S-allyl-L-cysteine sulfoxide]. When garlic bulbs are crushed, alliinase rapidly lyses the cytosolic alliin to form allicin (diallyl thiosulfinate) which is the dominant sulfur compound in crushed garlic [2]. Allicin is highly odoriferous, unstable, and undergoes a number of transformations depending on pH, temperature, and solvent conditions [3]. Its half-life in tissues is too short to ensure appreciable pathogen damage [4]. Systemic levels of the metabolic products of allicin are reported to be relatively high [5] and researchers have now turned their interest to these constituents due to their considerable antimicrobial and antioxidant properties. Allicin also has been shown to transform to diallyl disulfide and allyl mercaptan in the isolated perfused rat liver, with the former being rapidly metabolized to allyl mercaptan [6]. Acetaminophen or paracetamol, as it is known in Europe (N-acetyl-para-aminophenol; APAP) is a widely used analgesic and antipyretic drug throughout the world [7]. It has been long known that an overdose of APAP can cause severe damages to the liver or even death of the experimental animals and individuals who have ingested large quantities of APAP accidentally or in an attempt to commit suicide [8]. At pharmacological doses, APAP is mainly metabolized by sulfation and glucuronidation [9]. A small proportion is metabolized through cytochrome CYP2E1 and to a lesser extent CYP1A2 and CYP3A4, which produce a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) [10, 11]. Following therapeutic doses NAPQI is efficiently detoxified by conjugation with glutathione [12]. However, in overdoses a large amount of APAP is metabolized through the P450s, leading to GSH depletion by NAPQI conjugation followed by covalent binding of NAPQI to proteins [13]. Inhibition of mitochondrial respiration has been investigated as an important mechanism in acetaminophen toxicity [14]. Although the precise mechanism of APAP hepatotoxicity is not well understood, a number of studies have suggested that NAPQI exerts a cytotoxic effect through its covalent binding to cytosolic or microsomal proteins and membrane components, inhibition of mitochondrial respiration, depletion of ATP etc. [7]. Studies on APAP hepatotoxicity remain a very active area since much is still uncertain despite continuous efforts worldwide. On the other hand, considering repugnance results about cytoprotective effects of garlic and allicin, in the following, for the first time, the protetctive effect of garlic extract, allicin, and NAC towards APAP toxicity on freshly isolated rat hepatocytes have been compared. Also, the role of MMP has been studied with regards to the acetaminophen toxicity and the protective effects of agents mentioned above.

2. Materials and methods

2.1. Chemicals and plant materials

Bovine serum albumin, collagenase A from *Clostridium histolyticum* and HEPES were obtained from Roche Diagnostics (Indianapolis, IN), acetaminophen (APAP; 4-acetamidophenol) from Sigma-Aldrich (St. Louis, MO), N-acetyl-cysteine (NAC) from Acros Pharmaceuticals, Rhodamine 123 from Fluka, β-naphto-flavon (BNF), heparin sodium salt grade 1-A, trypan blue, methanol, $MgSO_4$ and other buffer salts, were obtained from Merck (Germany). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male Sprague-Dawley rats (200-250 g) were obtained from the Laboratory of Animals Research Center of Tabriz University of Medical Sciences. The rats were housed in an air-conditioned room, under controlled temperature of 23±1 °C, relative humidity of 36±6% and 12 h light/12 h dark conditions for 1 week before starting the experiments. They were allowed to feed with standard laboratory chaw and tap water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the NIH guidelines for the care and use of laboratory animals.

2.3. Preparation of garlic extract

Fresh garlic (*Allium sativum* L.) was purchased from a retail food store (Tabriz, Iran) and identified by botanists in the herbarium of Tabriz University. On the day of experiments the garlic bulbs were peeled, weighed and ground to obtain a fine juice. It was then homogenized in either methanol or deionized water. The homogenized mixture was filtered through cheesecloth. Garlic extracts of lower concentrations were prepared by dilution of this solution with media used for cell suspensions [15].

2.4. Determination and preparation of allicin from garlic extract

In order to determine and isolate the allicin content in garlic a modified method of Vargas *et al*. [16] was applied. Briefly, aqueous extract was assayed using analytical HPLC. Separation of allicin from extract was performed using a Spherisorb ODS2 column (4.6×250mm, 5µm, Waters, Ireland) and methanol (75%) and water (phosphate buffer pH=3), as a mobile phase, with a flow rate of 1 ml/min., detecting allicin at the wavelength of 254 nm. Retention time for allicin was 5.7 min. Pure allicin was obtained on the basis of analytical HPLC from a further preparative HPLC. The achieved allicin solution was dried with freeze drier and the resulting allicin powder was used for all experiments. Allicin content of garlic bulbs was quantified with analytical HPLC mentioned above as 80 mg%.

2.5. Preparation of allicin stock solution

Allicin (32 mg) acquired from preparative HPLC was dissolved in 20 ml distilled water, so that the concentration reaches to 10 mM.

2.6. Preparation of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats by a two-step collagenase perfusion, as described previously [17, 18]. The first step involves the perfusion of a calcium-free buffer. The second step is circulation of a calcium-supplemented buffer containing collagenase. The initial perfusion facilitates desmosomal cleavage and further dispersion of liver cells. The addition of Ca^{2+} to the enzyme solution ensures adequate collagenase activity. After isolation, the cells were suspended (10^6 cells/ml) in Krebs-Henseleit buffer containing 12.5 mM HEPES and incubated under a stream of 95% O_2 and 5% CO_2 in continuously rotating round-bottomed 50 ml flasks at 37 °C. Cell viability was measured by Trypan blue exclusion method. The hepatocytes used in this study were at least 85-90% viable immediately after isolation.

2.7. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential of cells was assessed by monitoring the uptake of cationic dye Rhodamine 123 [19]. Isolated cells were extracted, and then resuspended in original media containing 1 µM Rhodamine 123. After 10 min. of incubation, the cells were centrifuged and the supernatant was measured with a Shimadzu RF-5000U spectrofluorimeter at the excitation wavelength 501 nm and the emission wavelength 530 nm. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells [20].

2.8. Experimental protocols

The animals of test group received 3 i.p. injections of BNF (80 mg/kg) during 72 h. before starting the experiments; while the animals of positive control and negative control groups received corn oil and no chemicals, respectively [21]. On the day of experiments, after induction of anesthesia using sodium pentobarbital, the liver cells were isolated via a two step model. The cells were allowed to get adapted with the incubation conditions for 20 min. before the addition of compounds to the incubation mixture. The cells were then exposed to different concentrations of allicin, garlic extract and/or NAC 30 min. before, at the same time with APAP and 30 min. after APAP addition. Aliquots of the cells were taken at different time points (0, 60, 120 and 180 min.) for determination of cell viability and MMP.

2.9. Statistical analysis

Statistical comparisons were carried out using a one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test for multiple comparisons in order to determine statistical significance ($p< 0.05$ or less) between treatments and control groups. Results represent the mean±SD of at least three independent samples.

3. Results

3.1. Protective effect of GE and/or NAC against APAP cytotoxicity

As it has been shown in Table 1, APAP (500 μM) was not toxic in normal hepatocytes (the cells without any pretreatments) under controlled conditions. In contrast, it was extremely toxic towards the cells isolated from rats pretreated with BNF. On the other hand, neither the treatments, nor the solvents or carriers, showed significant cytotoxicity in the absence of APAP even in BNF pretreated cells. This means BNF itself could not be

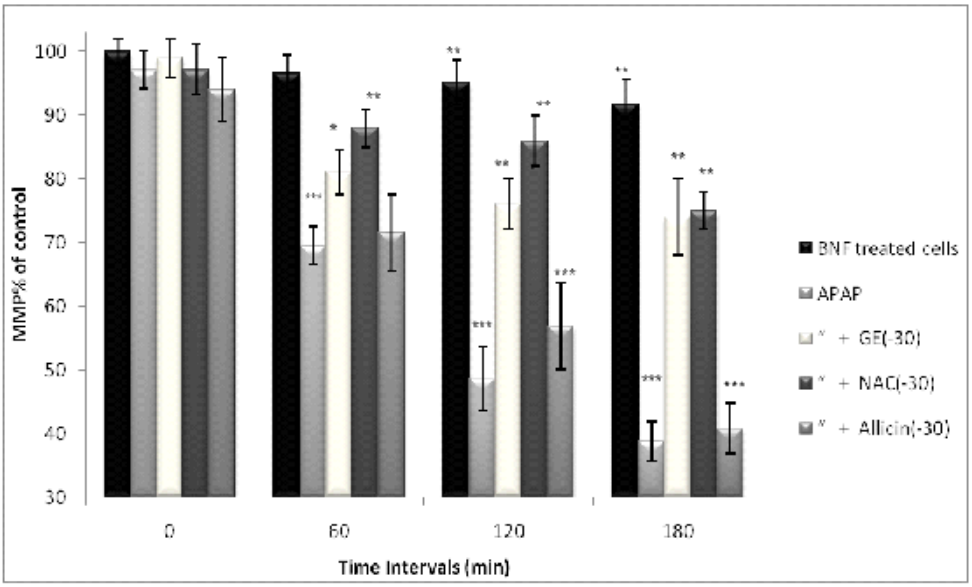

Figure 1: The effects of allicin, GE, and NAC on the APAP-induced MMP loss when added 30 min. before administration of APAP. Rhodamine was added to the aliquots of cell suspensions and the fluorescence amounts of the samples were measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. All results were shown in mean±SD and are at least from 3 independent experiments. *Shows significant difference ($p<0.05$) with APAP group. **Shows significant difference ($p<0.01$) with APAP group. ***Shows significant difference ($p<0.001$) with control group (BNF treated cells).

cytotoxic although it induces the microsomal metabolism at the doses administered. Among all control groups, only the groups receiving allicin demonstrated cytotoxicity after 180 min. There were no signs of toxicity in normal cells with treatments and chemicals even with APAP addition. Therefore, the results of normal cells have not been entered in Table 2. In order to investigate if the timing of the addition of APAP and the treatments is effective in the responses, three different protocols composed of 30 min. of time intervals were employed and the samples of control were prepared to avoid any possible experimental errors. As it can be seen in Table 2, GE, and NAC were effective against APAP hepatotoxicity in BNF treated hepatocytes, however, allicin had no protective effects. There was not a significant difference between protective effects of GE and NAC.

3.2. Protection of mitochondrial membrane potential against APAP toxicity by GE and/or NAC

Figures 1 to 3 illustrate the relative percentages of mitochondrial membrane potential (MMP) in test groups and its comparison with the control group, where the 100 percent of Rhodamine 123 trapping occurred in the intact mitochondria. In order to separate the main protocols from each other, the results have been illustrated in three figures of 30 min. before APAP addition (Figure 1), at the same time with APAP (Figure 2) and 30 min. after APAP addition (Figure 3).

Figure 1 illustrates that allicin was not only unable to maintain the mitochondrial membrane potential but also demonstrated APAP like effects; when NAC and/or GE showed significant differences with APAP treatment in MMP. On the other hand, there was no significant difference between NAC

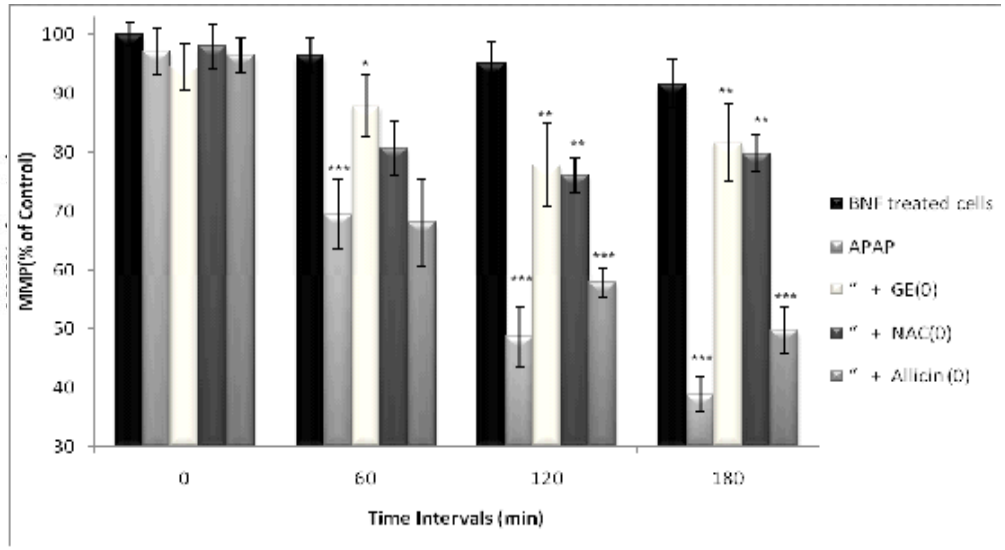

Figure 2: The effects of allicin, GE, and NAC on the APAP-induced MMP loss when added at the same time with APAP. Rhodamine was added to the aliquots of cell suspensions and the fluorescence amounts of the samples were measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. All results were shown in mean±SD and are at least from 3 independent experiments. *Shows significant difference (p<0.05) with APAP group. **Shows significant difference (p<0.01) with APAP group. ***Shows significant difference (p<0.001) with control group (BNF treated cells).

as the standard treatment and GE.

It can be deducted from Figure 2 that both NAC and/or GE were effective in preventing the loss of MMP induced by APAP, but not allicin.

In Figure 3, there is no significant protective effect by GE and/or allicin. However, NAC significantly protected MMP towards APAP toxicity. There were no significant differences between groups of GE and NAC in Figures 1 and 2, but a significant difference was observed between GE and NAC specially after 180 min. of monitoring (p<0.05) .These findings indicate the decrease in efficacy of GE when administered after APAP addition which does not happen with NAC. Meanwhile the most non protective effect was shown with allicin 30 min. after APAP addition (Figure 3).

4. Discussion

Generally the use of natural and plant medicines has been more desirable throughout the world, especially during the recent decades. Garlic juice which contains mercaptans, thiosulfinates and other sulfur containing compounds has been used in cardiovascular diseases [22] as an anti bacterial [23-25], anticancer [26, 27] and a lipid lowering agent [28]. Even today, no definitive answers have been provided for the active biochemical mechanisms responsible for the apparent beneficial effects of garlic products.

APAP has been widely used as an analgesic and antipyretic agent with very few adverse effects at therapeutic doses; but it has been reported that even borderline high APAP concentrations may induce liver toxicity [29]. The mechanism of APAP toxicity which involves NAPOI binding to hepatocytes' GSH and other proteins like membrane proteins and enzymes is well known [12, 30]. In this research, two different dimensions of APAP

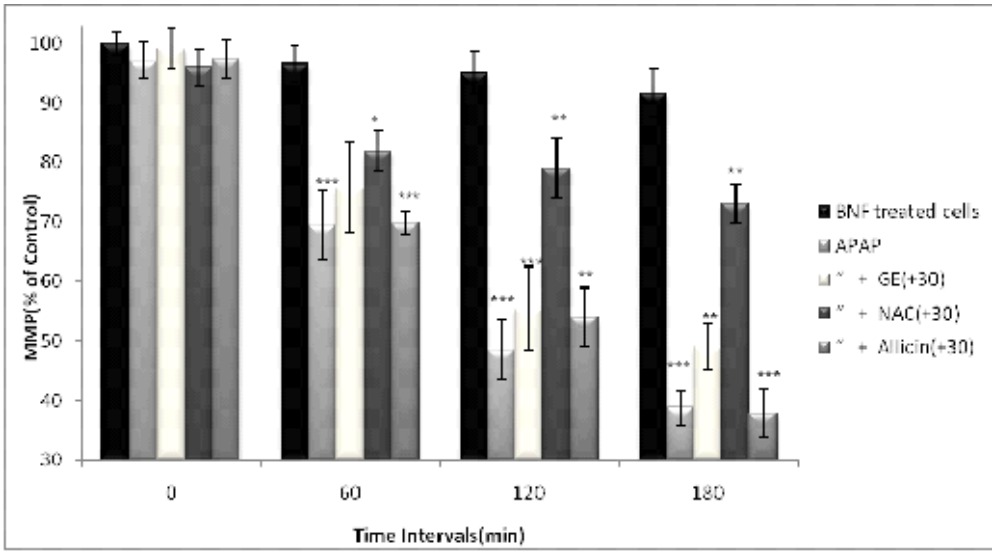

Figure 3: The effects of allicin, GE, and NAC on the APAP-induced MMP loss when added 30 min. after administration of APAP. Rhodamine was added to the aliquots of cell suspensions and the fluorescence amounts of the samples were measured with a Shimadzu RF-5000U spectrofluorimeter. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. All results were shown in mean ±SD and are at least from 3 independent experiments. *Shows significant difference (p<0.05) with APAP group. **Shows significant difference (p<0.01) with APAP group. ***Shows significant difference (p<0.001) with control group (BNF treated cells).

Table 1: The effects of pure treatments and APAP on cell viability (positive and negative control groups).

	Cytotoxicity%		
Treatments	60 min.	120 min.	180 min.
BNF pretreated cells	20±2	24±3	27±3
" + Water (200 µl)	21±3	27±3	28±4
" + Methanol (25 µl)	22±1	28±3	35±4
" + APAP (500 µM)	52±4[a]	84±6[a]	91±6[a]
" + Allicin(100 µM)	32±4	34±4	42±4[b]
" + GE (100 µl)	22±3	25±4	31±5
" + NAC (100 µM)	19±2	26±4	30±4
Normal cells (without treatment)	20±1	25±2	27±3
" + Water (200µl)	19±3	23±2	26±3
" + Methanol (25µl)	25±3	26±4	36±5
" + APAP (500 µM)	20±2	25±3	27±2
" + Allicin(100 µM)	21±4	24±2	35±6
" + GE (100 µl)	22±2	23±3	26±3
" + NAC (100 µM)	25±3	27±3	28±3

Hepatocytes were incubated in Krebs-Henseleit solution, pH 7.4 at 37 °C under the atmosphere of 95% O_2/5% CO_2.The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion method. All data were shown in mean±SD and are at least from 3 independent experiments. [a]:Significant difference (p<0.001) with control group. [b]Significant difference (p<0.05) with control group.

toxicity have been studied and the possible protective effects of garlic extract and one of its components in freshly isolated rat hepatocytes have been investigated.

According to the results obtained from trypan blue exclusion test (Tables 1 and 2), hepatic microsomal metabolism has a dominant role in the APAP toxicity, because APAP did not show any hepatic cell death in normal doses and this effect was demonstrated just after induction of CYP enzymes (CYP1A2, CYP2E1) with BNF pretreatment. It can be deducted from Table 2 that, both NAC and GE could prevent the cytotoxic effects of APAP, but allicin not only lacked this ability, but also demonstrated a significant cytotoxicity, specially in the presence of APAP.

As illustrated in Figures 1-3, both NAC and GE were capable of protecting MMP against APAP-induced membrane potential loss, which itself can push hepatocytes towards different apoptotic, necrotic and cytotoxic pathways [12]. As it can be seen from Figures 1-3, GE was as effective as NAC (our standard treatment with a known effect) in maintenance of the MMP except for the case of GE administered 30 min. after APAP. This data can support the hypothesis of other mechanisms involved in garlic protective effects such as enzyme inhibition. In addition, there has been shown some CYP2E1 enzyme inhibitory effect for diallylsulfide and allylmethylsulfide, two sulfur containing components of garlic [31], which makes it necessary to investigate the same possible role for allyl mercaptan in the future and the importance of this mechanism in the APAP hepatotoxicity.

During this research, any significant loss of membrane potential 30 min. after addition of APAP was not seen, which could support the hypothesis of NAPQI mediated MMP loss. The other novel finding of this research was the cytotoxic effect of allicin. According to other researches about possible antioxidant roles of allicin [32], many researches demonstrated bactericidal effect of allicin in the cultured media [23, 25], which introduced allicin as the cytotoxic agent of garlic components. During years it has been speculated that allicin was the major reactive component of garlic, however, recent researches detected no allicin in the blood after garlic ingestion but allyl mercaptan was detected in the blood [6, 33]; besides, some researches on anticancer effects of garlic and allicin also proved that allicin could be the

Table 2: The effects of allicin, GE and NAC on the APAP-induced hepatotoxicity

	Cytotoxicity%		
Treatments	60 min.	120 min.	180 min.
BNF pretreated cells	20±2[b]	24±3[b]	27±3[b]
" + Allicin(100 µM)	29±2[b]	38±2[b]	49±3[b]
" + GE (100 µl)	22±2[b]	25±3[b]	34±3[b]
" + NAC (100 µM)	25±4[b]	23±3[b]	33±2[b]
" + APAP (500 µM)	52±4[a]	84±6[a]	91±6[a]
" + " + Allicin (-30)	61±5[a]	65±4[a]	71±5[a]
" + " + Allicin (0)	63±4[a]	71±3[a]	89±4[a]
" + " + Allicin (+30)	55±5[a]	64±4[a]	78±5[a]
" + " + GE(-30)	32±3[b]	37±3[b]	41±4[b]
" + " + GE(0)	30±3[b]	33±4[b]	36±5[b]
" + " + GE(+30)	33±4[b]	35±3[b]	42±4[b]
" + " + NAC(-30)	26±2[b]	36±3[b]	36±3[b]
" + " + NAC(0)	25±5[b]	32±2[b]	37±4[b]
" + " + NAC(+30)	26±3[b]	35±4[b]	41±3[b]

Hepatocytes were incubated in Krebs-Henseleit solution, pH 7.4 at 37 °C under the atmosphere of 95% O_2/5% CO_2.The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion method. All data were shown in mean±SD and are at least from 3 independent experiments. [a]Significant difference (p<0.001) with control group. [b]Significant difference (p<0.05) with control group.

major cause of neoplastic cells' death induction in cultured media [34]. In this study allicin was not protective at all against APAP hepatotoxicity which supports the hypothesis of its cytotoxicity and the interference of this trait in its anticancer as well as antibacterial or antifungal effects. In addition, allicin has been shown to transform to diallyl disulfide and allyl mercaptan in the isolated perfused rat liver, with the former being rapidly metabolized to allyl mercaptan [5, 6].

According to this information, it can be deducted from the results of the present study that the allicin component of garlic is the one responsible for its toxicity, as there are other components like allyl mercaptan, diallyl disulfide and diallyl sulfide which might be the cytoprotective and antioxidant components. These data are supported by researches done with this research group on allyl mercaptan (data not published) and other studies done on dially trisulfide and DADS [35, 36].

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