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*Full Length Research Paper*

# Grape seed extract mitigates garlic-induced oxidative stress in rat spleen and plasma

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**Garlic is a commonly used spice in folk medicine which could also exert adverse health effects when given at high dosage. Grape seed extract (GSE) exhibit a variety of beneficial effects even when used at high dosage. In the present study, we evaluated the toxicity of high dosage garlic treatment on spleen and plasma as well as the protective effect of GSE. Rats were intraperitoneally injected with 5 g/kg *bw* crude garlic extract daily during one month and cotreated or not with GSE (500 mg/kg *bw*). Spleen and plasma antioxidant status were evaluated. Data confirmed that high dosage garlic induced plasma and spleen toxicity and a prooxidative state characterized by increased splenic and plasmatic malondialdehyde (MDA) and decreased antioxidant enzyme activities such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Garlic also increased intracellular and plasmatic $H_2O_2$, but decreased free iron in spleen and increased it in plasma. Moreover, garlic increased ionizable calcium in spleen but decreased it in plasma. GSE either alone or in cotreatment with garlic, had just an opposite role or counteracted almost all garlic-induced deleterious effects to near control level. In conclusion, high dosage garlic induces a prooxidative state characterized by the Fenton reaction between $H_2O_2$ and free iron inducing tissue calcium repletion and GSE exerts real antioxidant properties and calcium depletion.**

**Key words:** Garlic, grape seed extract, spleen, plasma, oxidative stress, lipid peroxidation, protein carbonylation.

## INTRODUCTION

Garlic (*Allium sativum* L.) has been widely used as a flavoring agent and as a traditional medicine to treat diseases such as microbial infection, hyperlipidemia, and heart dysfunction (Abdullah et al., 1988; Agarwal, 1996). Numerous studies have also demonstrated that garlic exerted anticarcinogenic (Dorant et al., 1993; Milner, 2001), immunomodulatory (Liu et al., 1998) and antioxidant effects (Banerjee et al., 2002). However some controversies persist concerning garlic dosage or mode of administration. In prior studies, we showed that high dosage garlic administered by intraperitoneal way could even be detrimental by inducing a prooxidant effect and ultimately toxicity in red blood cells (Hamlaoui-Gasmi et al., 2011a) and liver (Hamlaoui-Gasmi et al., 2011b).

Grape seed extract (GSE) is a widely used nutritional supplement exhibiting preventive and healing effects (Suwannaphet et al., 2010). GSE contains polyphenols such as resveratrol, which is at the basis of the “French Paradox”. GSE effects are wide ranging from cardioprotective (Decordé et al., 2009) to renoprotective (Safa et al., 2010) or neuroprotective (Wang et al., 2009) owing to its antioxidant and antiinflammatory properties (Houdé et al., 2006). GSE exerts protective effects against hepatic ischemia-reperfusion injury (Sehirli et al., 2008), biliary obstruction (Dulundu et al., 2007) or

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azathioprine induced-hepatotoxicity in rats (El-Ashmawy et al., 2010). Moreover GSE also exerts antineoplasic effects by inducing cytotoxicity towards some cancer cells (Kaur et al., 2006) by cell cycle arrest and apoptosis induction (Kaur et al., 2008).

In the present paper, we characterized the toxic effect of high dosage garlic on spleen and plasma and antioxidant status as well as the protection offered by GSE treatment. Our data confirm the previously described prooxidant effect of high dosage garlic and provide new insight into the potential protective effect of GSE. The mode of action seems to involve free iron, $H_2O_2$ and ultimately calcium level disturbances. High dosage garlic induces a prooxidative state and GSE exerts potent antioxidative properties mainly by counteracting the Fenton reaction of $H_2O_2$ with free iron.

## MATERIALS AND METHODS

### Chemicals

Thiobarbituric acid (TBA); 2,6,-di-tert-butyl-4-hydroxy-toluene (BHT); trichloroacetic acid (TCA); hydrogen peroxide ($H_2O_2$); 2-methoxyphenol (gaiacol); bovine catalase and 4-(1-Hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine) and 2,4,dinitrophenyl hydrazine (DNPH) were obtained from Sigma-Aldrich Co (Germany).

### Preparation of garlic and grape seed extracts

Garlic was purchased from local market, peeled and grounded with an electric mincer. It was diluted in double distilled water at 4 g/ml instead of 2 g/ml on the basis of the weight of the starting material and centrifuged (Beckman J20, 15 min at 10,000 g and 4°C). Supernatant was aliquoted and stored at – 80°C until use.

Grape seed and skin extract (GSE) was processed from a grape cultivar (Alicante Bouschet) of *Vitis vinifera* from northern Tunisia. Polyphenolic powder mixture containing grape seed (50%) and skin (50%) was dissolved in 10% ethanol, centrifuged 15 min at 10 000 g to eliminate insoluble material and supernatant-containing soluble polyphenols was used.

### Animals and treatment

Fourty male Wistar rats (200 to 240 g) from Pasteur Institute of Tunis were used for these experiments in accordance with the local ethic committee of Tunis University and care of animals in conformity with the NIH recommendations (NIH, 1985). They were provided with food and water *ad libitum* and maintained in animal house at controlled temperature (22 ± 2°C) with a 12 h light–dark cycle. Rats were divided into four groups of ten animals each. Group I received ethanol 10% (control), group II aqueous extract of garlic (5 g/kg *bw*), group III GSE (500 mg/kg *bw*) and group IV garlic plus GSE. Animals were daily intraperitoneally injected during 30 days. Twenty-four hours after the last injection, animals were sacrificed, their spleen rapidly excised and homogenized in phosphate buffer saline pH 7.4. After centrifugation at 10,000 g for 10 min at 4°C, supernatant was used for the determination of lipoperoxidation and protein carbonylation, antioxidant enzyme activities and intracellular mediators as free iron, $H_2O_2$ and calcium. Blood was also collected and plasma processed for the same measurements.

### Plasma and spleen lipoperoxidation measurement

Lipid peroxidation was determined by MDA measurement according to the double heating method (Draper and Hadley, 1990). Briefly, aliquots from spleen homogenates and plasma were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1000 g for 5 min at 4°C. Supernatant was blended with 0.5N HCl, 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min. After cooling, absorbance of the resulting chromophore was determined at 532 nm using a BIORAD UV-visible spectrophotometer. MDA levels were determined by using an extinction coefficient for MDA-TBA complex of $1.56\ 10^5\ M^{-1}cm^{-1}$.

### Protein carbonylation

Oxidative damage to proteins was evaluated by quantifying protein carbonylation in spleen and plasma according to Levine et al. (1990). Briefly, after proteins precipitation with 20% TCA and dissolution in DNPH-containing buffer, absorbance was measured at 366 nm and results expressed as nmol carbonyl/mg protein.

### Protein determination

Total soluble proteins were determined according to the biuret method (Ohnishi and Bar,1978). Briefly, at acidic pH soluble proteins constituted with copper a colorful complex measurable at 546 nm.

### Antioxidant enzyme activity assays

All spectrophotometric analyses of liver antioxidant enzyme activities were performed with a Beckman DU 640B spectrophotometer.

### Catalase

CAT activity was assayed by measuring the initial rate of $H_2O_2$ disappearance at 240 nm (Aebi, 1984). The reaction mixture contained 33 mM $H_2O_2$ in 50 mM phosphate buffer pH 7.0 and CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}cm^{-1}$ for $H_2O_2$.

### Peroxidase

POD activity was measured at 25°C using guaiacol as hydrogen donor. The reaction mixture contained 9 mM guaiacol, 19 mM $H_2O_2$ in 50 mM phosphate buffer pH 7 and 50 µl of enzyme extract in 1 ml final volume. The reaction was initiated by the addition of $H_2O_2$ and monitored by measuring the increase in absorbance at 470 nm. Peroxidase activity was expressed in nmol of guaiacol oxidized per min with a molecular extinction coefficient of 26.2 mM-1 for calculation (Chance and Maehly, 1955).

### Superoxide dismutase

SOD activity was determined by using modified epinephrine assay (Misra and Fridovich, 1972). At alkaline pH, superoxide anion $O_2^-$ causes the autoxidation of epinephrine to adenochrome; while competing with this reaction, SOD decreased the adenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%. Enzyme extract

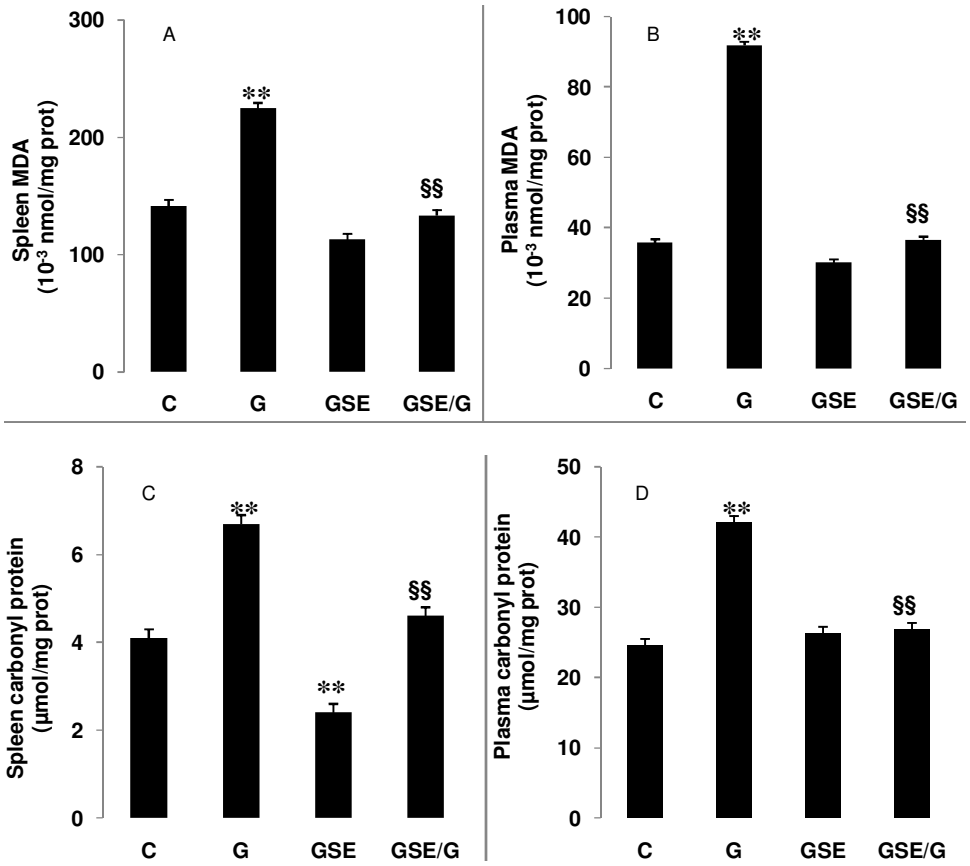


**Figure 1.** Effect of garlic and GSE on plasma and spleen oxidation. Ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) were administered to rats during one month. Splenic (Figure 1A) and plasmatic (Figure 1B) MDA and splenic (Figure 1C) and plasmatic (Figure 1D) protein carbonylation were determined. Results are expressed by mean ± SEM (n=10). ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

was added in 2 ml reaction mixture containing 10µl bovine catalase (0.4 U/µl), 20 µl epinephrine (5 mg/ml) and 62.5 mM sodium carbonate/sodium bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm. Characterization of SOD isoforms was performed using KCN (3 mM) which inhibited Cu/Zn-SOD or $H_2O_2$ (5 mM) affecting both Cu/Zn-SOD and Fe-SOD. Mn-SOD was insensitive to both inhibitors.

### Free iron determination

Spleen and plasma free iron levels were determined according to Leardi et al. (1998) using a commercially available kit from Biomaghreb, Tunisia. Briefly, at acidic pH 4.8 all $Fe^{3+}$ is released from transferrine. Ascorbic acid reduced $Fe^{3+}$ in $Fe^{2+}$ which constituted with ferrozine a colorful complex measurable at 560 nm.

### $H_2O_2$ determination

Spleen and plasma $H_2O_2$ levels were determined enzymatically according to Chance et al. (1979) using a commercially available kit from Biomaghreb, Tunisia. This colorimetric method uses the glucose oxidase. Briefly, $H_2O_2$ is oxidized by glucose oxidase into $H_2O$ and $O_2$. Changes in absorbance were measured at 505 nm.

### Calcium measurement

Ionizable calcium in spleen and plasma were determined using colorimetric method (Stern and Lewis, 1957). Briefly, at basic pH, calcium constituted with cresolphtalein a colourful complex measurable at 570 nm.

### Statistical analysis

Data were analyzed by unpaired Student's t-test or one-way analysis of variance (ANOVA) and are expressed as means ± standard error of the mean (SEM). Data are representative of ten independent experiments and assays carried out in triplicate. All statistical tests were two-tailed, and a p-value of 0.05 or less was considered significant.

## RESULTS

### Effects of garlic and GSE on spleen and plasma oxidation

Data reported on Figure 1 showed that high dosage garlic

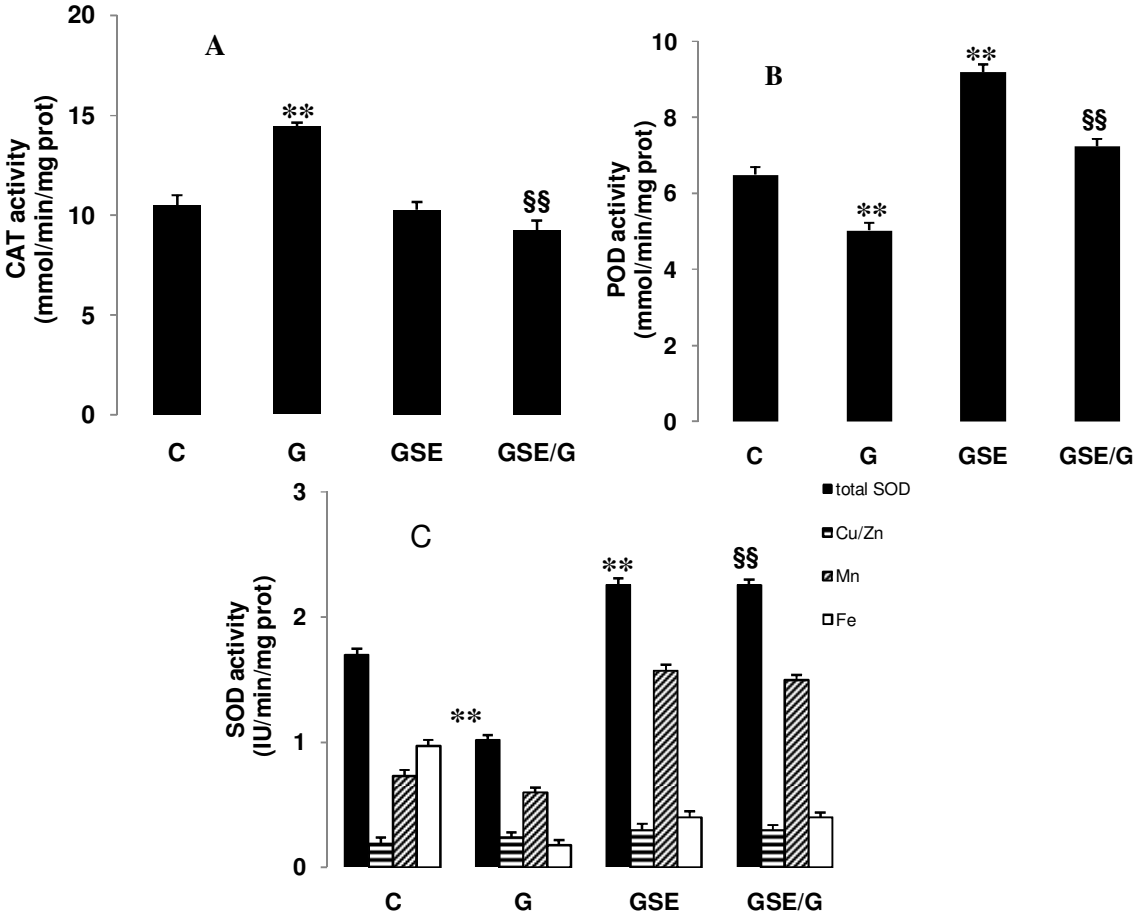


**Figure 2.** Effect of garlic and GSE on spleen antioxidant enzyme activities. Rats were daily IP injected with ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) for one month and spleen CAT (Figure 2A), POD (Figure 2B) and SOD (Figure 2C) activities determined. Results are expressed by mean ± SEM (n=10). Data are representative of 3 independent experiments. ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

increased splenic MDA and carbonylated proteins (Figures 1A and 1C) and plasmatic MDA and carbonylated proteins (Figures 1B and 1D). GSE treatment reversed garlic-induced spleen and plasma oxidations.

## Spleen antioxidant enzyme activities

We reported in Figure 2 the effect of garlic and GSE on spleen antioxidant enzyme activities. As expected from prior work, garlic increased CAT (Figure 2A) but decreased POD (Figure 2B) and SOD (Figure 2C) activities. In this latter case, garlic mainly decreased the Fe and to a lesser extent the Mn isoform. GSE alone clearly increased POD and SOD activities but has no effect on CAT. Cotreatment with GSE abrogated the garlic-induced depletion of antioxidant enzyme activities to near control level.

## Plasma antioxidant enzyme activities

We reported in Figure 3 the effect of garlic and GSE on plasma antioxidant enzyme activities. Garlic decreased CAT (Figure 3A) and POD (Figure 3B) but increased SOD (Figure 3C) activities. In this latter case, garlic mainly increased the Cu-Zn isoform. GSE alone clearly increased POD and SOD activities but decreased CAT. Cotreatment with GSE abrogated the garlic–induced depletion of antioxidant enzyme activities to near control level.

## Effects of garlic and GSE on spleen and plasma free iron levels

We further looked at spleen and plasma free iron level (Figure 4) and data showed that garlic treatment significantly decreased spleen free iron (Figure 4A),

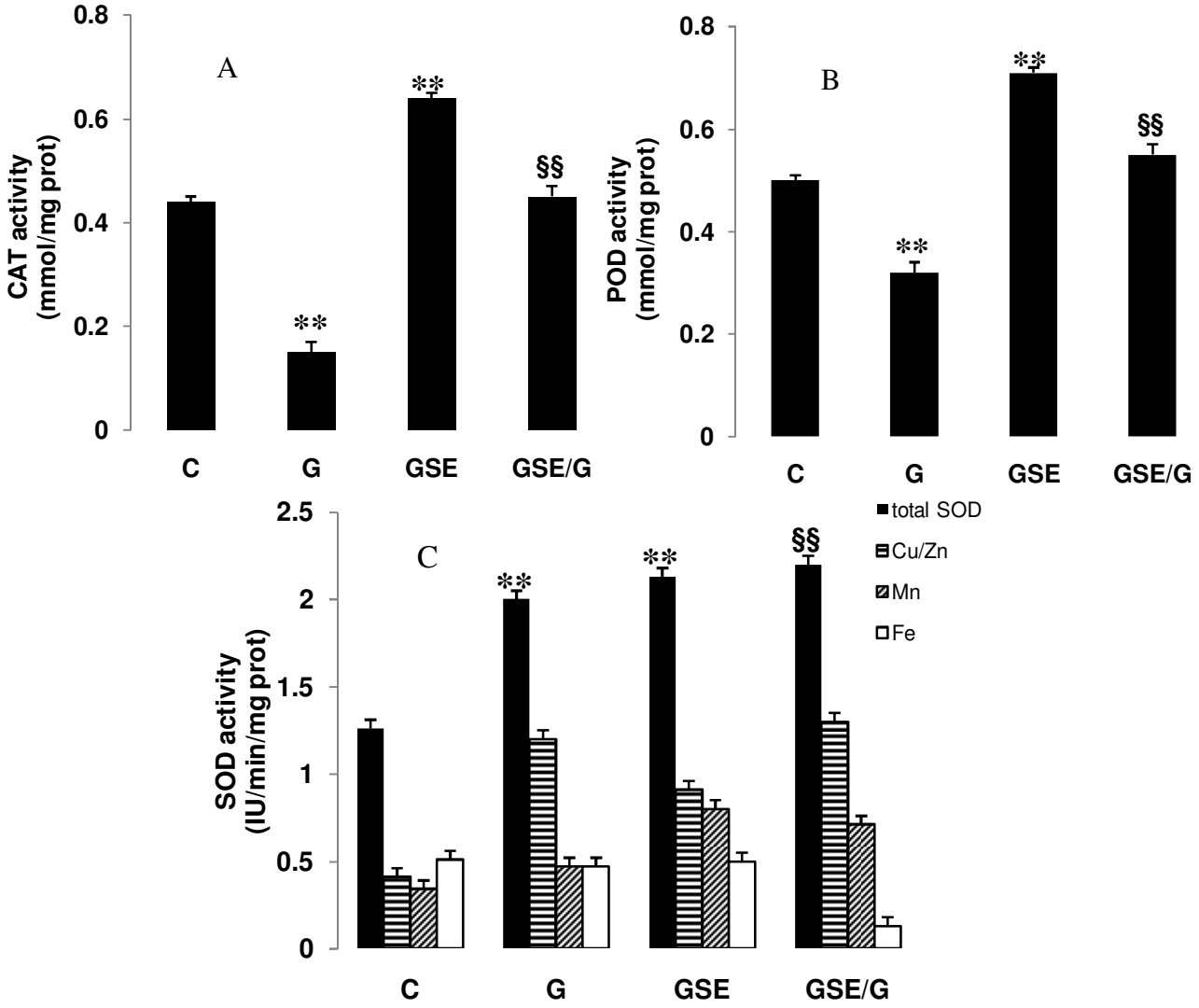


**Figure 3.** Effect of garlic and GSE on plasma antioxidant enzyme activities. Rats were daily IP injected with ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) for one month and plasma CAT (Figure 3A), POD (Figure 3B) and SOD (Figure 3C) activities determined. Results are expressed by mean ± SEM (n=10). Data are representative of 3 independent experiments. ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

whereas it increased it in plasma (Figure 4B). Co-treatment with GSE abrogated the garlic–effect of free iron to near control level.

**Effects of garlic and GSE on spleen and plasma hydrogen peroxide level.**

In the present study, the effects of garlic and GSE treatment on spleen and plasma hydrogen peroxide level were investigated and the results are presented in Figure 5. As expected garlic treatment increased $H_2O_2$ level in spleen (Figure 5A) and plasma (Figure 5B). Co-treatment with GSE abrogated the garlic–effect of $H_2O_2$ to near control level.

**Effects of garlic and GSE on spleen and plasma calcium level**

We further sought to determine the putative involvement of ionizable calcium in garlic and GSE mode of action (Figure 6). Garlic alone increased spleen calcium level (Figure 6A) but decreased it in plasma (Figure 6B). GSE alone has no effect on tissue or plasmatic calcium level however its co-treatment with garlic abrogated the garlic–induced disturbances of calcium to near control level.

## DISCUSSION

The present study revealed that IP administration of

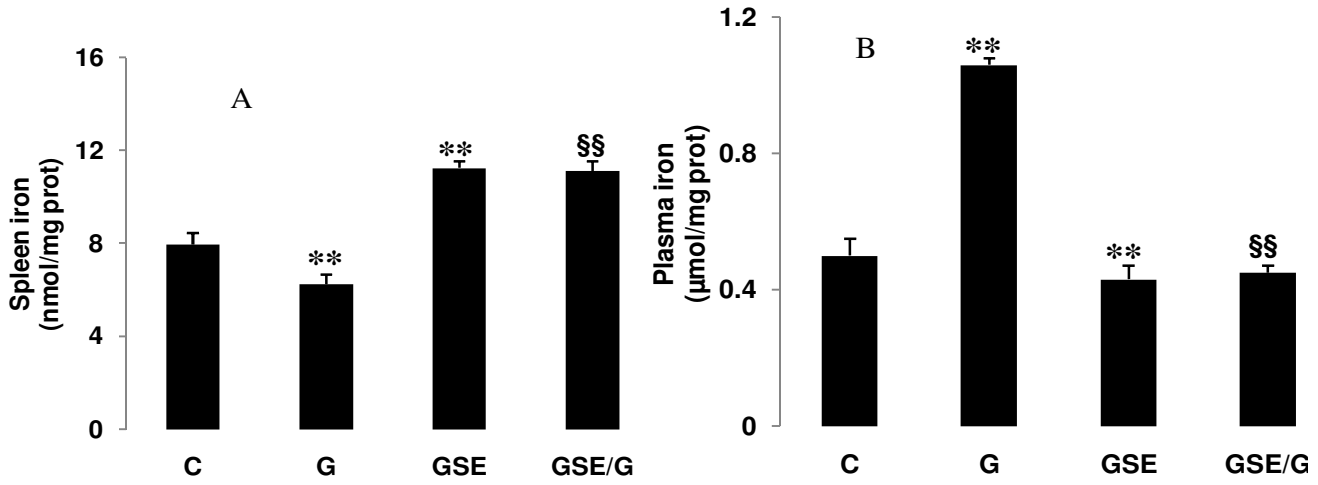


**Figure 4.** Effects of garlic and GSE on spleen and plasma free iron level. Rats were daily IP injected with ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) for one month and spleen (Figure 4A) and plasma (Figure 4B) free iron determined. Results are expressed as means ± SEM (n=10). ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

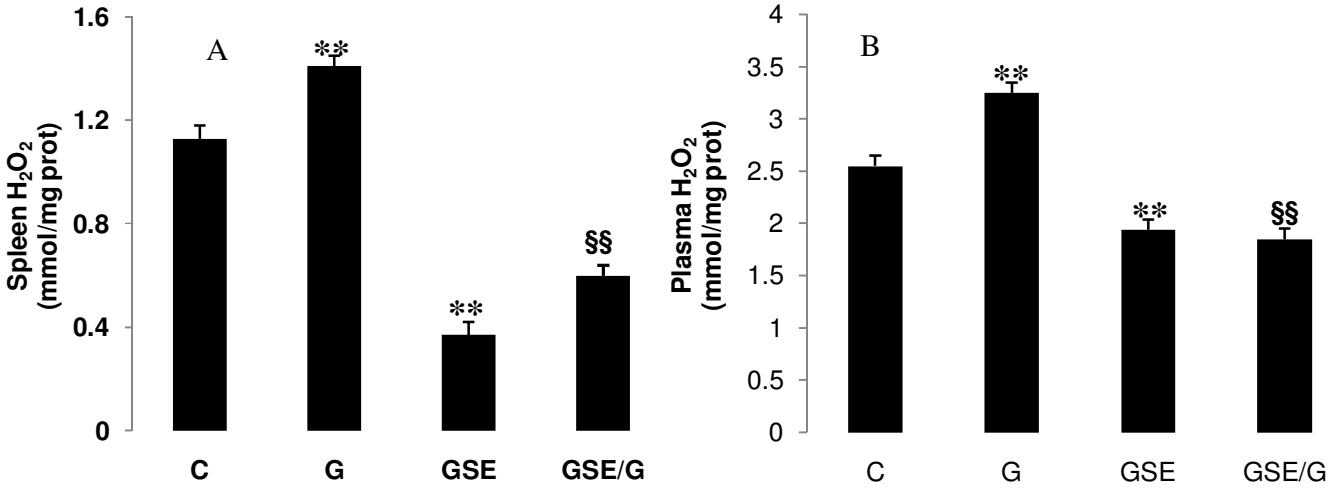


**Figure 5.** Effects of garlic and GSE on spleen and plasma $H_2O_2$ level. Rats were daily IP injected with ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) for one month and $H_2O_2$ in spleen (Figure 5A) and plasma (Figure 5B) determined. Results are expressed as means ± SEM (n=10). ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

garlic to rats during one month resulted in spleen and plasma injury and that GSE co-treatment counteracted almost all garlic deleterious effects. Garlic-induced splenotoxicity was evidenced by increased lipoperoxidation and protein carbonylation. Splenotoxicity was also reflected by depletion of antioxidant enzyme activities such as POD and SOD, a strong increase in calcium and $H_2O_2$ levels and a decrease in free iron. In the plasma compartment, garlic-induced toxicity was evidenced by increased lipoperoxidation and protein carbonylation, depletion of antioxidant enzyme activities such as CAT and POD, an increase in free iron and $H_2O_2$ levels and a decrease in calcium. Aqueous extract of garlic contains allicin, alliin, ajoene, diallylsulfide, dithiin and S-allylcysteine (Nidaullah et al., 2010). The effect of IP administration on spleen and plasma could be attributed to one of these compounds per se or in their mixture.

Our data also revealed that treatment with GSE abolished almost all parameters of garlic-induced spleen and plasma dysfunction. These protective properties of GSE may be mediated by resveratrol as recently shown for human erythrocytes (Pandey and Rizvi, 2009) or from a synergistic effect of various grape-containing polyphenols (Liu, 2004) or from the effect of flavonoids and their oligomers, the procyanidins (Bruneton, 2009).

It is well known that elevated intracellular MDA

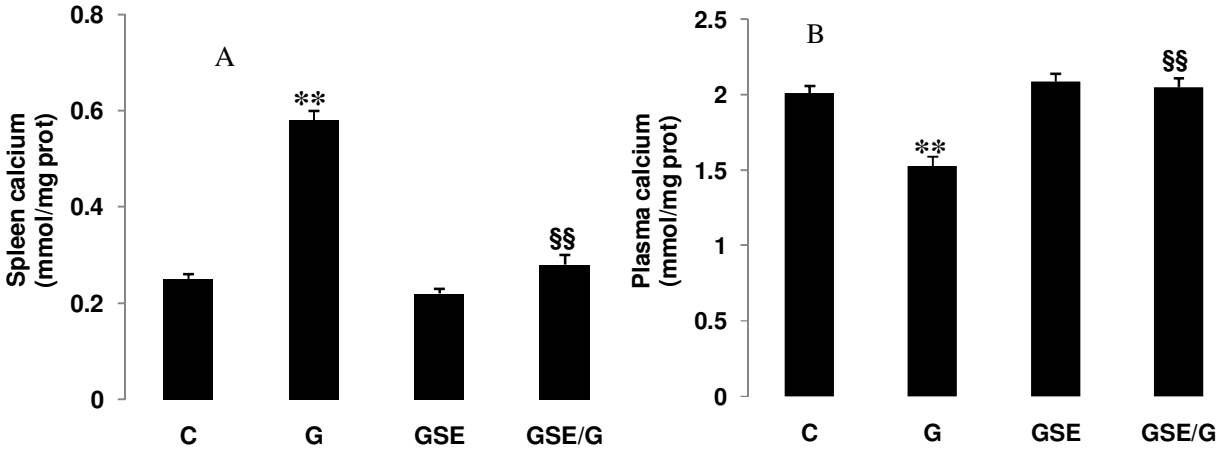


**Figure 6.** Effects of garlic and GSE on spleen and plasma calcium level. Rats were daily IP injected with ethanol 10% (C), garlic (G), grape seed extract (GSE) or GSE plus garlic (GSE/G) for one month and calcium in spleen (Figure 6A) and plasma (Figure 6B) determined. Results are expressed as means ± SEM (n=10). ** indicated $p < 0.01$ versus C. §§ indicated $p < 0.01$ versus G.

decreased the fluidity of the membrane lipid bilayer (Bryszewska et al., 1995) and that MDA is correlated to pathological conditions or stress including aging (Rizvi and Maurya, 2007). Thus, owing to its antioxidant properties (Tadolini et al., 2000), GSE reversed garlic-induced lipoperoxidation. Antioxidant activities of resveratrol, a polyphenol containing GSE were also shown to be mediated by up-regulation of antioxidant enzyme activities as reported by some of us in rat brain (Mokni et al., 2007a) or by others in the kidney (Chander et al., 2005). Resveratrol also efficiently protected the liver (Sebai et al., 2010) and the heart (Sebai et al., 2011) from oxidative stress induced by lipopolysaccharide, acetaminophen (Sener et al., 2006), cadmium (Eybl et al., 2006), alcohol (Bujanda et al., 2006), CCl4 (Rivera et al., 2008), naphthalene (Sehirli et al., 2008), pyrogallol (Upadhyay et al., 2008) and ischemia/reperfusion injury (Hassan-Khabbar et al., 2008).

To our knowledge, our report is the first one to deal with GSE protective effect on garlic-induced oxidative stress in rat spleen. Moreover, GSE was used at a dosage previously shown to be devoid of any toxicity. In fact, in the present study GSE was used at a dosage that is not harmful, near from the optimal concentration of 300 mg/kg (Hebbar et al., 2005). Furthermore, some of us previously showed that resveratrol is neuroprotective (Mokni et al., 2007a), cardioprotective (Mokni et al., 2007b) and protective against endotoxemia-induced acute phase response in rats (Sebai et al., 2009). Our data corroborates a tremendous literature dealing with antioxidant and anti-inflammatory effects of GSE that have been observed *in vitro* or *in vivo* and in various experimental settings (Bagchi et al., 2003).

Importantly, the most relevant role of GSE is its powerful ability in precluding garlic deleterious effect on spleen and plasma free iron distribution. Garlic induces spleen injury partly by acting as an iron purging agent from the spleen and GSE counteracted this effect. Iron is known to play a central role in many pathologies as cardiovascular (Alpert, 2004), neurodegenerative (Castellani et al., 2007) and hepatocellular injury (Uchiyama et al., 2008). Furthermore, as both iron deficiency and iron excess can lead to cellular dysfunction, maintaining iron homeostasis is crucial (Andrews, 1999). Iron shuttling proteins as hepcidin exerted a pivotal role in the pathogenesis of iron overload (Papanikolaou et al., 2005). High levels of hepcidin were shown to occur especially during infection and inflammation causing intracellular iron sequestration and decreased level in the plasma (Pigeon et al., 2001). It is tempting to speculate that garlic and GSE exerted opposite effects on iron shuttling proteins as hepcidin or lipocalin 2 (Devireddy et al., 2005), leading to iron homeostasis. The grape seed extracts are rich in procyanidins which have also the property to complex iron. In case of polyphenolic extracts, the balance antioxidant/prooxidant is very fine, so in certain conditions the antioxidant activity can become a prooxidant one. Also, an antioxidant effect might be observed through a prooxidant action (Azam et al., 2004). Plant polyphenols are naturally occurring antioxidants but they also exhibit prooxidant properties. Over the last several years, it has shown that various classes of plant polyphenols including flavonoids, curcuminoids and tannins are capable of catalyzing oxidative DNA cleavage particularly in the presence of transition metal ions such as copper and iron (Azam et al., 2004).

Furthermore, our data gave some new insight into garlic as well as GSE putative mode of action. Garlic which increased $H_2O_2$ but depressed free iron within the spleen likely led to increase entry of calcium, probably by acting on Ca channels, initially described in excitable

tissues as myocardium (Oudit et al., 2003). Because of the high cellular concentration of iron, splenocytes are highly susceptible to oxidative damage. A possible mechanism by which GSE exerts its beneficial effect on splenocytes is its ability to increase free iron and scavenge $H_2O_2$. Free iron is a well established catalyst of auto-oxidation and iron-mediated oxidations of cysteine residues represent a common mechanism through which $H_2O_2$ exerts its second messenger role in signal transduction pathways (Barbouti et al., 2007). Moreover $H_2O_2$ is able to induce dual roles in both survival and cell death pathways, largely depending on its concentration and also on the cell type. However, our results also raise several discrepancies.

In particular, in spleen, garlic appeared in the same time as prooxidant increasing MDA, carbonyl proteins, ionisable calcium and $H_2O_2$ and as antioxidant by its ability to decrease free iron. However, garlic which exerted strong prooxidant effect also attenuated POD and SOD (especially the Fe isoform) but unexpectedly increased CAT activity. This apparent discrepancy is reminiscent of the paradoxal prooxidant effect of catalase (Heck et al., 2003) which should be interpreted in the light of oxidative stress-induced ROS activation of non receptor tyrosine kinases associated with CAT phosphorylation and activity stimulation (Borchi et al., 2010). These later found increased CAT and GPx activities due solely to increase tyrosine phosphorylation. In our case, high dosage garlic could provoke up-phosphorylation of CAT and under-phosphorylates POD and SOD activities contrary to GSE which has exactly the opposite effects.

In the plasma compartment, garlic exerted strong prooxidant effect, which attenuated CAT and POD activities, but unexpectedly increased SOD especially the Cu/Zn isoform. First, this isoform could correspond to the secreted form of the enzyme (Marklund, 1982). Second this apparent discrepancy could be interpreted in the light of deleterious increase in ROS production exerted by Cu-Zn/SOD (Goldsteins et al., 2008) especially in such a free iron overload setting (Hamlaoui-Gasmi et al., 2011c). Anyway, one should also keep in mind that a positive influence of garlic on plasma SOD activity was insufficient to overcome the elevation of ROS (and consequently MDA) linked to down-regulation of both CAT and POD activities.

In conclusion we showed that high dosage garlic induced spleen and plasma toxicity by its prooxidative effect and that GSE is protective partly by its antioxidant way.

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