ISSN : 0974 - 7532 Volume 5 Issue 3

Trade Science Inc.

Research & Reviews in

BioSciences

Regular Paper

RRBS, 5(3), 2011 [139-145]

Allium sativum prevents the induced-hepatotoxicity, abnormal blood hemolysis and viscosity in rats

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Received: 6th September, 2011 ; Accepted: 6th October, 2011

ABSTRACT

The present study was conducted to investigate the chemopreventive effects of garlic extract on carbon tetrachloride (CCl_4)-induced hepatotoxicity in male albino rats, were studied for its protective effects against hepatotoxic, abnormal blood hemolysis and viscosity induced using carbon tetrachloride (CCl_4) in rats. Male Wistar rats were administered CCl_4 by intraperitoneal injection for 7 weeks and received a normal diet or normal diet with extracted garlic for the same period. Extracted garlic significantly reduced the liver damage, including fibrosis and fatty infiltration, vaculation, and sinusoid dilated in a dose dependent manner. Moreover, extracted garlic significantly decreased the elevation in plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin ($P < 0.01$). It also restored the decrease in blood hemolysis ($P < 0.05$) and viscosity ($P < 0.01$) during CCl_4 treatment. These results suggested that extracted garlic may protect the liver against CCl4-induced fibrosis, abnormal blood hemolysis and viscosity. This protective effect appears due to garlic antioxidant properties.

KEYWORDS

Garlic;
CCL_4;
Hepatotoxicity;
Blood hemolysis;
Viscosity.

INTRODUCTION

Garlic, *Allium sativum,* is a member of the *lily* family that has been cultivated by humans as a food plant for over 10,000 years. Ancient Egyptian records mentioned that use of garlic as a remedy for a variety of diseases[5]. Recently, it has been found that the sulfur-containing compounds of garlic have anti-mutagenesis and anti-carcinogenesis effects. *In vivo* studies show that garlic and its associated sulfur components suppress the incidence of tumors in rodent models[19, 30]. Epidemiological findings also demonstrated an inverse relationship between garlic consumption and the incidence of the stomach, colorectal and prostate cancer[14]. Epidemiological studies have also suggested that garlic may have protective effects against cardiovascular diseases[6]. Garlic as an herbal remedy reduces a multitude of risk factors, which play decisive roles in the genesis and progression of cardiovascular abnormalities. These effects include a decrease in LDL- and total cholesterol, an increase in HDL-cholesterol, a reduction of serum triglyceride and fibrinogen concentration,

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lowering of arterial blood pressure and promotion of organ perfusion, an enhancement in fibrinolysis, inhibition of platelet aggregation, and diminution of plasma viscosity[28]. Furthermore, garlic (2.0%) in the diet, produced the hypotriglyceridemic effect, appeared to beneficially correct the altered osmotic fragility of erythrocytes in CCl_4 action[16].

Liver injury commonly observed with exposure to viruses, alcohol, drugs and environmental toxins results in liver fibrosis and eventually liver cirrhosis. Many hepatotoxins require metabolic activation, particularly by the liver cytochrome (CY) P_{450} enzymes to form reactive, toxic metabolites, which in turn cause liver injury in experimental animals and humans[12, 27].

Toxic liver injury induced by carbon tetrachloride (CCl_4) is the well characterized system of the xenobiotic induced hepatotoxicity and is a commonly used model for the screening the hepatotoxicity and hepatoprotective activity of drugs[7]. CCl_4 is converted to trichloromethyl radical ($CCl^{\bullet}_3$) by cytochrome P_{450} (CYP), especially CYP 2E1. $CCl^{\bullet}_3$ can react with oxygen to form another reactive oxygen species (ROS), trichloromethylperoxy radical ($CCl_3OO^{\bullet}$), which triggers lipid peroxidation[18]. Parenchymal and nonparenchymal cells, especially activated Kupffer cells, mediate the hepatic inflammation process by producing tumor necrosis factor-α (TNF-α) and other cytotoxic cytokine. A previous study showed that the production of these inflammatory factors is associated with the nuclear factor-κ B (NF-κ B) pathway and increased activating protein 1 (AP-1) expression in liver after CCl_4 treatment[15, 33].

Blood viscosity mediates the relationship between blood pressure gradients and blood flow. At a fixed driving pressure, blood flow is indirectly proportional to the viscosity of blood. Blood is a shear thinning non-Newtonian fluid, meaning its viscosity decreases as the shear rate increases. The shear rate is determined by the velocity of blood flow and by the size of the blood vessel. High shear rates are typically present in large arteries with high blood flow velocity, whereas low shear rates are typically present in the microcirculation where blood flow velocity is low. Thus, under normal physiologic conditions, the viscosity of blood within the circulation at any instant or location varies depending on the shear rate within the particular vessel. Viscosity is defined as the factor of proportionality relating shear stress and shear rate for the fluid[29].

The rheologic properties of blood depend on hematocrit and plasma constituents[3]. As the cellular fraction of blood increases, so does viscosity. Plasma expanders, such as hydroxyethyl starch (HES) and albumin, have their own distinct rheologic properties that are different from those of blood. A plasma expander used under clinical conditions will alter blood viscosity as a consequence of both hemodilution and its inherent physical properties[8].

The osmotic fragility test is employed to help diagnose different types of anemia, in which the physical properties of the red blood cell are altered. The main factor affecting the osmotic fragility test is the shape of the red blood cell, which, in turn is independent on the volume, surface area, and function state of red blood cell membrane.

If red blood cells are placed in an isotonic solution, 0.85% sodium chloride, water will leave the red cell. If red blood cells are placed in a 0.25% solution of sodium chloride, water enters the red blood cell; the cell swells up, and eventually hemolyzes or ruptures. A spherocyte, which is almost round, swell up in 0.25% sodium chloride and ruptures much more quickly than a normal red cell than the cells that have a large surface area per volume, such as target cells or sickle cells. The fragility of the red blood cell is said to be increase when the rate of hemolysis is increased. An increased osmotic fragility is found in hemolytic anemia. Decreased osmotic fragility occurs in liver disorder and polycythemia. In osmotic fragility test, whole blood is added to varying concentrations of buffered sodium chloride solution and allowed to incubate at room temperature. The amount of hemolysis is then determined by reading supernatants on a spectrophotometer[2].

The consumption of fruits and vegetables[22] containing antioxidants has been found to offer protection against these diseases. Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay the oxidation process[25].

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THE MATERIALS AND METHODS

Preparation of garlic extract

Extracted garlic was prepared by homogenizing the required amount of peeled garlic in an appropriate volume of distilled water to prepare a concentration of 20 mg/ml[1]. The homogenate was centrifuged at 3000 x g for 10 minutes to remove particulate matter and the supernatant fraction was used for the experiment. Extracted g is composed of different beneficial molecules, especially antioxidants and disulfide.

EXPERIMENTAL ANIMALS

This study was carried out in the Departments of Physics and Zoology, Mansoura and fayoum Universities respectively. A total of 40 adult albino rats of the Wistar strain weighing 150 - 200 g were used in this study. They were purchased from the animal house of the Department of Urology and Nephrology Centre, Mansoura University. Following an acclimation period of 2 weeks, the rats were kept in plastic cages at room temperature of 25±4°C and <30% relative humidity with a 12 h light/dark cycle. They had access to standard laboratory diet and drinking water *ad libitum.*

Rats were divided into four main groups (n= 10) as follow: 1) the

control rats were treated with corn oil and fed a normal diet.; 2) Garlic extract-treated group, received standard diet and ip injected with extracted garlic (200 mg/kg b.w.) daily for a period of 7 weeks; 3) CCl_4-treated group, received standard diet supplemented and were treated intraperitoneally with CCl_4 (8% CCl_4 in corn oil; 1 ml/ kg body weight twice a week; Mon and Thu) for 7 weeks to produce slowly reversible cirrhosis, as described by[13]; and 4) CCl_4 then treated with garlic extract-treated group, received standard diet and were supplemented orally with similar doses of CCl_4 and extracted garlicas group 3 for the similar period of 7 weeks.

BIOPHYSICAL ASSAYS

Osmotic fragility (OF) for rat RBCs

Normal RBCs hemolysis was determined by measurement of hemoglobin released from the cells relative to the total cellular hemoglobin content. Ten microliter of whole fresh blood was incubating in 5 ml normal saline for 30 min. The samples were centrifuged at 3,000 rpm for 10 min, and the absorbance of the supernatant was measured calorimetrically at 540 nm[9]. The percentage of hemolysis was taken against complete blood hemolysis:

$$\%H = \frac{A_{sample}}{A_{100\%lysis}} \times 100$$

Where A_{sample} and $A_{100\%\ lysis}$ are the absorbance of the hemoglobin released from RBCs in normal saline and after complete hemolysis, respectively.

The process of osmosis was studied by mixing small volumes of blood with buffered saline solutions, (pH 7.4) of different tonicity, in the proportion of 1–100. The test was carried out at room temperature. The osmotic lysis of RBCs is detected by the release of hemoglobin into the extracellular fluid. The amount of hemoglobin appearing in media was determined calorimetrically according to the method reported by Dacie and Lewis[23]. From these data, a fragility curve can be drawn by plotting percentage of hemolysis versus NaCl concentration.

Rheological properties

In order to determine the rheological properties of a blood with anomalous flow properties, it is necessary to use an instrument in which all the samples under test are exposed to a uniform shear stress and shear rate, and in which each of these effects is separately determinable. The rheometer employed in this study is the Brookfield DV-III Programmable Rheometer. It is a cone-plate viscometer that measures fluid parameters of shear stress and viscosity at given shear rates. The applied shear rate was 40–500 s^{-1}, and the measurements were carried out at temperature 37^0C. The data was collected from the rheometer by the software program ''Rheocalc for Windows''. The apparent viscosity of the blood which is non-Newtonian fluid is not constant but rather depends on the magnitude of the shear stress or shear rate, and can be calculated as the ratio of shear rate to shear stress. It decreases as the shear rate increases (Flow curve). The analysis of the flow curve was performed by applying the Bingham

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plastic model:

$F=F_0+nD$

Where F is the shear stress (dyne/cm^2) and D is the shear rate (s^{-1}), and the yield stress (F_0), and viscosity(n) can be calculated.

Determination of serum biochemical parameters

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin levels were determined to assess liver function by using a Modular Autoanalyzer (Roche Diagnostics, GmbH, D-68298, Mannheim, Germany).

Histological analysis

The liver was sectioned and used for histological analysis. The tissue was fixed by immersion in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5-μm sections and stained with hematoxylin-eosin, followed by blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated. The histological changes were evaluated in non-consecutive, randomly chosen × 200 histological fields.

Statistical analysis

Data were presented as mean ± standard deviation (SD). The difference between groups was assessed using F-test (one way analysis of variance ANOVA) by SPSS program, Version 14.

THE RESULTS

Serum biochemical parameters

Serum AST, ALT, ALP and total bilirubin levels were determined to assess liver function. Rats with CCl_4-induced fibrosis showed serum levels of ALT and ALP higher than those of normal rats. Treatment with extracted garlic reported these values within the normal range. The statistically significant difference in total bilirubin concentration was found in the different groups (TABLE.1).

Histopathological observation

The normal architecture of liver was completely lost in rats treated with CCl_4 with the appearance of vacuolated hepatocytes and degenerated nuclei. Vacuolization, fatty changes and necrosis of hepatocytes were severe. CCl_4 poisoning led to the excessive formation of deposition of connective tissue and development of scars and such liver sections were shown. The histology of the liver sections of normal control animals showed normal hepatic cells with preserved cytoplasm, prominent nucleus and well brought out the central vein. The liver sections of CCl_4 intoxicated rats showed fatty changes, necrosis, infiltration of lymphocytes and Kupffer cells. CCl_4- induced group was more severe than other groups. The histopathological architecture of liver sections of rats treated with extracted garlicshowed the more or less normal lobular features with a mild degree of necrosis, dilated liver sinusoids and fat vacuoles almost comparable to the normal control group (Figure 1).

TABLE 1 : the biochemical assays of liver functions in different animal groups

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	T. Bilirubin (mg/dl)
Control group	48.67±2.11	67.36±3.44	88.02±4.20	1.09±0.07
Garlic group (200 mg/kg b.w.)	47.85±2.78	60.66±2.67	92.34±3.32	1.00±0.05
CCl_4 group (1 ml/ kg b.w.)	215.23±4.25[a]	199.33±3.39[a]	190.98±3.54[a]	3.07±0.03[a]
CCl_4 & garlic group	50.78±4.99[b]	65.23±2.50[b]	90.18±3.75[b]	1.15±0.06[b]

Values are expressed as Mean±S.E.M. [a]Activities of AST, ALT, ALP and TBil are increased significantly in Group III (CCl_4 group) than Group I (normal control) and GroupII ($P < 0.01$). [b]Activities of AST, ALT, ALP and TBil are inhibited significantly in Group IV than Group III (CCl_4 group) ($P < 0.01$).

Biophysical assays results

Osmotic fragility (OF) curve for rat RBCs

Changes in the typical hemolytic curve for rat RBCs exposed to CCL_4 and other animal groups are shown in Figure 2. The curves were shifted right in a parallel manner with increases in the group of the carbon tetrachloride, where the significant increase (P<0.05) in hemolysis was present in the CCL_4 treated group (68.20±32.94) when compared with the control group

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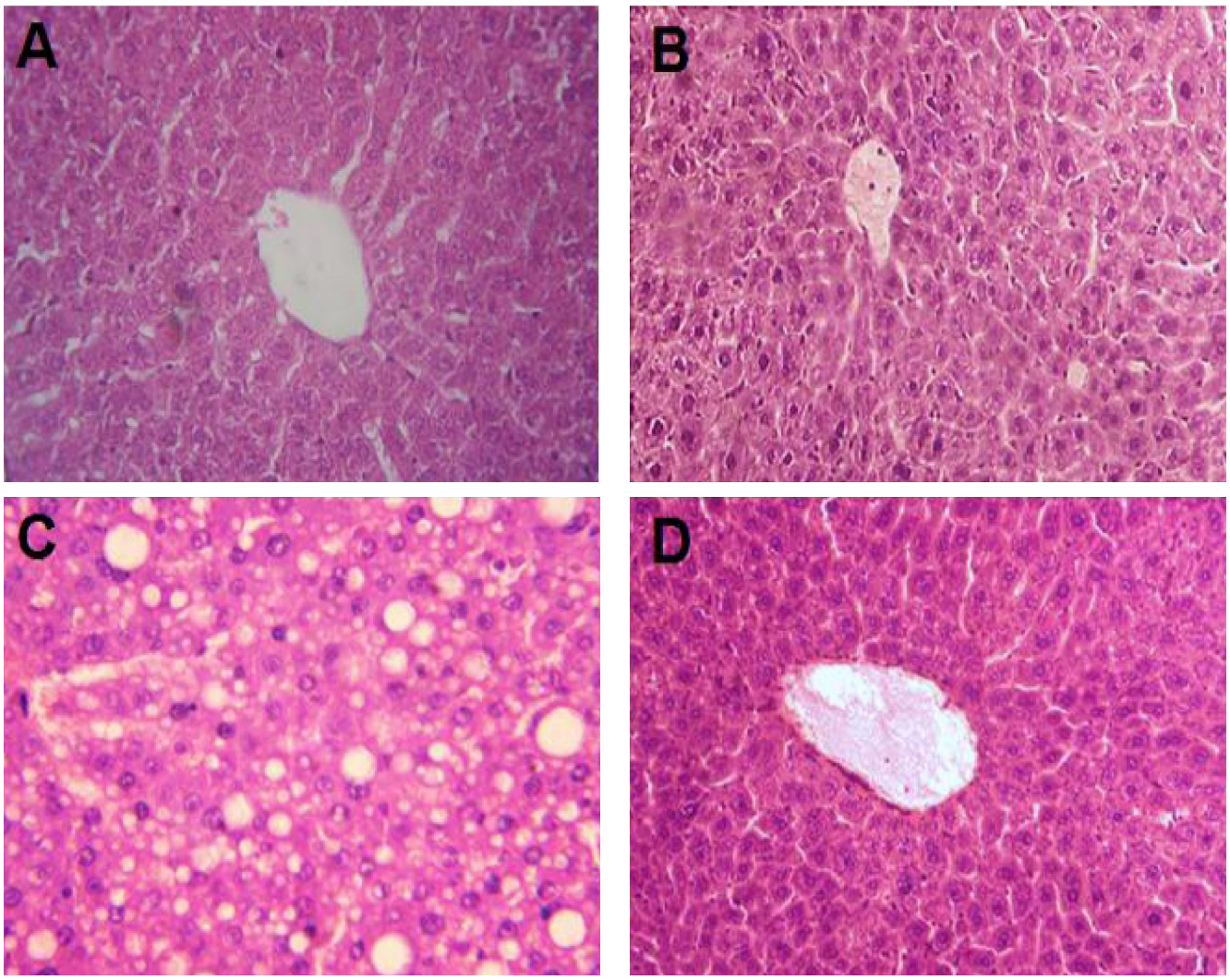

Figure 1 : Histopathology of liver showing the normal architecture and cells with granulated cytoplasm and small uniform nuclei of control and garlic liver treatment respectively (A and B, HE X 250). CCl_4-induced hepatotoxicity rats show loss of architecture, fibrosis and fatty infiltration (C, HE X 250) CCl_4-induced hepatotoxicity rats and treated with garlic showing minimal pleomorphism, vaculation, less disarrangement and sinusoid dilated (D, HE X 250).

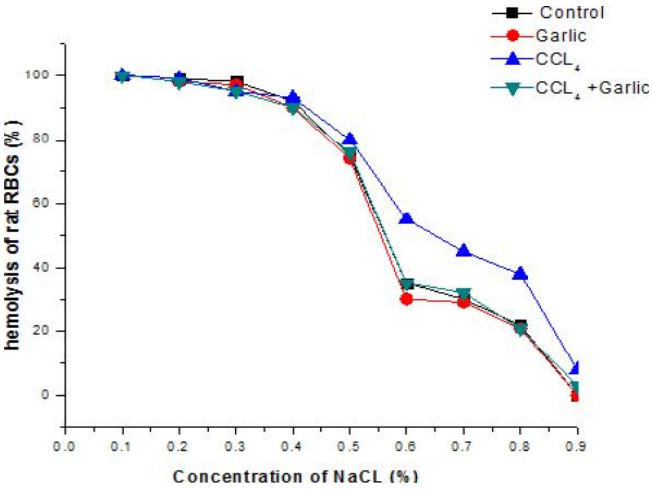

Figure 2 : The RBCs hemolysis in different treated animal groups

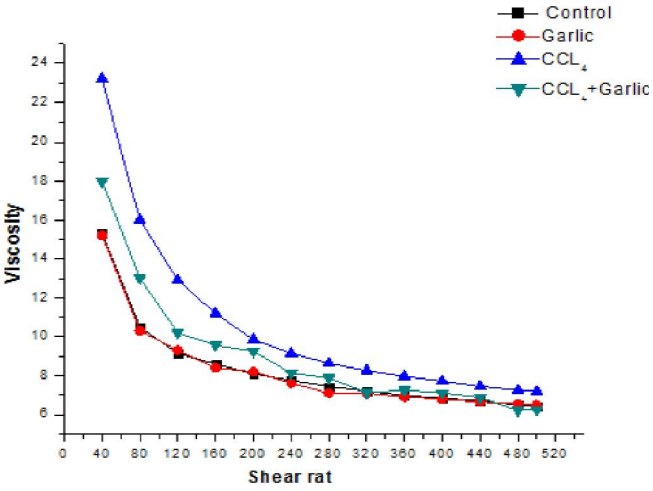

Figure 3 : The blood viscosity in different treated animal groups

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(61.30±39.32).

Extracted garlic at the dose of 200 mg/kg b.w. can be significantly reduced the sheer rates of whole blood viscosity ($P<0.01$) as shown in Figure 3, where the significant increase in viscosity was present in the CCL_4 treated group (10.52±4.50) when compared with the control group (8.27±2.41).

DISCUSSION

In the present study, the capability of extracted garlic to protect against CCl_4-induced hepatotoxicity, and oxidative stress was investigated. The hepatotoxicity induced by CCl_4 is due to its activation by cytochrome P450 to form a trichloromethyl radical, $CCl_3^{\cdot}$ The trichloromethyl radical leads to liver damage by alkylating cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids to produce lipid peroxides[31]. Hepatocellular necrosis leads to elevations of serum AST and ALT activities and an increased incidence and severity of histopathological hepatic lesions in rats. The present study revealed a significant increase in the activities of AST and ALT on exposure to CCl_4 indicating considerable hepatocellular injury. This elevation could potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage by CCl_4. Serum AST, ALT and ALP are biomarkers in the diagnosis of hepatic damage because they are released into the circulation after cellular damage[20]. Administration of garlic or significantly reduced the activity of liver enzymes in CCl_4- induced rats. Due to its ability to reduce free radical-induced oxidative damage in the liver[11], extracted garlic has been shown to decrease liver enzymes in serum and prevent liver damage of rats with liver fibrosis[21].

The protective effect of extracted garlic was also reflected in the histological studies. CCl_4 treatment increased the incidence and severity of histopathological hepatic lesions in rats. The acute hepatotoxic effects induced by CCl4 administration were confirmed histopathologically, revealing extensive hepatocellular degeneration and necrosis, fatty changes, inflammatory cell infiltration, congestion, and sinusoidal dilatation. The obtained results are in accordance with those of the previous reports[10]. In contrast, our histological results showed that treatment with extracted garlic effectively protected rats against CCl_4-induced hepatic toxicity. Treatment with extracted garlic prevented the necrosis and the other histopathological changes induced by CCl_4 treatment.

Under normal physiological conditions, low concentrations of lipid peroxidation products are always seen in tissues and cells. In pathological conditions, more peroxidation products are formed during cell damage. The damage to RBC membranes by CCL_4, is evidenced by the increased amount of lipid peroxidation products, the increased membrane fluidity, and the reduced activities of membrane-bound enzymes. Oxidative stress is normally challenged by cellular antioxidant defenses including reduced glutathione, GPx, and CAT[32]. The results of the experiment demonstrated that the rate of percent hemolysis was significantly lower in extracted garlic group in which CCL_4 is used as inducer, indicating the potential free radical scavenging effect of extracted garlic. The biochemical mechanisms involved in the hepatotoxic effect of CCL_4 are well documented. It is now well known that the formation of reactive trichloromethyl radicals from the CCL_4 metabolism is a crucial factor in the pathogengesis of CCL_4 in hepatotoxicity. Besides to the normal functions of the liver, is to excrete the breakdown product of hemoglobin (namely bilirubin) into the bile. As CCl_4 affects the integrity of liver cells, by the same manner it affects on the structure and function of the erythrocyte membrane, and increase the erythrocyte fragility, this in turn lead to erythrocyte hemolysis and hemoglobin breakdown[17].

In the present study, a higher blood viscosity was found in the (CCL_4) exposed animals compared to the control animals at different shear rates. Erythrocyte deformability is one of the parameters having an effect on blood viscosity. CCL_4 is known to impair membrane structure and function. The effects on the red blood cell membrane in particular have been intensely analyzed because red blood cells (RBC) have a high affinity for CCL_4, contain a majority of the CCL_4 found in the blood stream, and are more vulnerable to oxidative damage than many other cells[24]. CCL_4 was reported to increase the osmotic and mechanic susceptibility of RBC with a concomitant decreased deformability and a shortened life span. Finally, CCL_4 increases the blood viscosity

by production of ROS and resulting destruction of the red blood cell membrane and its function.

From these observations, it can be concluded that the extracted garlic may suppress the formation of free radicals induced hepatotoxicity, abnormal blood hemolysis and viscosity in rats by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants.

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