

Hepato-protective effect of *Allium sativum* against immobilization stress in rats

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Abstract: The purpose of the current study was to examine immobilization stress-induced antioxidant defense changes and estimation of the antioxidant potential of pre and post stress treatment of aqueous garlic extract in rat's liver. For this purpose, male Albino Wistar rats were treated with aqueous garlic extract both pre and after 6 h of immobilization stress. Pro-oxidant status of rat liver was evaluated by determining the levels of reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), glucose, uric acid and the activities of super oxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). In response to 6 h of immobilization stress a significant rise in the level of above mentioned liver enzymes were recorded. However, SOD, CAT and GST enzymatic activities showed a sharp decline. The extract treatment before and after stress, almost reverted the activities of studied biochemical parameters towards their control values. Current study highlighted the antioxidant potential of garlic extracts. Based on our study, we recommend the use of garlic extract as nutritional supplement for combating oxidative stress induced damage.

Keywords: Stress, garlic, antioxidant enzymes, AST, ALT, ALP, MDA.

INTRODUCTION

Liver is a vital organ of the body, involved in a vast array of biochemical and metabolic functions, including detoxification of xenobiotic substances and excretion of drug metabolites (Ademiluyi *et al.*, 2013). Oxidative stress has been demonstrated to cause a significant extent of liver injury through numerous oxidant-induced mechanisms (Omurtag *et al.*, 2005). Additionally, certain predisposed conditions such as alcohol consumption, obesity, fatty-liver disease and diabetes are also known to further aggravate liver damage during stress (Paradies *et al.*, 2014). The liver injury may manifest as hepatic fibrosis, a dynamic and highly integrated cellular response to chronic liver injury and cirrhosis, a disperse fibrosis with nodular rebirth of hepatocytes which is accompanied by altered prothrombin, albumin, cholesterol synthesis and insulin resistance (Sadiq and Baseer, 1991). The mild degree of liver necrosis that is usually present in response to cirrhosis may be responsible for an increase in the levels of serum enzymes viz. aspartate aminotransferase (AST) [serum glutamic oxaloacetic transaminase (SGOT)], alanine aminotransferase (ALT) [serum glutamic pyruvic transaminase (SGPT)] and lactate dehydrogenase-5 (LDH) (Popper *et al.*, 1981).

Immobilization stress is a useful and reproducible experimental animal model for studying oxidative stress (Zafir and Banu, 2009; Jia *et al.*, 2007). Formation of oxidative stress induced molecules, increased lipid per oxidation (LPO) and decreased activities of free radical scavenging enzymes may facilitate tissue and cellular events responsible for chronic liver injury (Muriel, 2009). It is widely known that vitamins A (retinol), E (tocopherol), C (ascorbic acid), glutathione (GSH) and selenium are first-line defense against oxidative stress induced damage, in biological system (Zaidi *et al.*, 2003; Zaidi and Banu, 2004; Zaidi *et al.*, 2005). Several conditions could alter GSH level via changes in the activity of γ -glutamyl cysteine synthetase, the gene expression of which has been reported to be associated with oxidative stress, activator of phase II detoxifying enzymes, antioxidants, hormones, cell proliferation and diabetes mellitus (Davis and Mehendale, 1980). Hepatic GSH level is also closely associated with nutritional status, especially cysteine content of the diet, but this does not affect the enzymes involved in its synthesis.

Garlic (*Allium sativum* L.) has been widely used as a flavoring agent in the traditional medicine (Berginc *et al.*, 2010; Shirzad *et al.*, 2011). Numerous studies have also demonstrated and validated many beneficial medicinal properties attributed to garlic preparations (Asdaq and

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Inamdar, 2010). Different types of garlic supplements viz. garlic powder (tablets), aged garlic extracts (capsules, tablets and liquid), garlic oils (capsule) are commercially available; each product reflects a different organo-sulfur compound profile (Lawson and Gardner, 2005). The aged garlic (upto 20 months old) provides better garlic preparations. During this period, the unstable and highly odorous organo-sulfur compounds get converted into more stable and odorless compounds (Amagase *et al.*, 2001).

Garlic extract contains certain phytochemicals and organo-sulfur compounds such as diallyltrisulfide (DATS), S-allyl cysteine (SAC), S-allyl cysteine sulfoxide (SACS) and flavonoids, phenolics and anthocyanins, which are believed to prevent oxidative stress induced damage. Out of these phytochemicals, fat and water soluble organo-sulfur components viz. allixin, flavonoids and selenium are noteworthy in providing antioxidant potential to the garlic extract. Several *in vitro* and *in vivo* studies have been carried out to estimate the antioxidant potential of aged garlic extract. Considerable number of studies also reported their ability to elevate the level of non-enzymatic and antioxidant enzymes through neutralization of reactive oxygen species (ROS), reactive nitrogen species (RNS) and inhibition of some pro-oxidant enzymes like cyclooxygenase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate hydrogen NADPH oxidase (Zare *et al.*, 2008; Ide and Lau, 1999). The garlic extract has also been found to prevent lipid peroxidation caused by ROS and oxidative damage to DNA and proteins (Borek, 2001). Since oxidative stress plays a major role in the progression of hepatic fibrosis and cirrhosis, we investigated the antioxidant potential of crude extract of garlic on the immobilization stress-induced oxidant/pro-oxidant status of rat hepatic tissues. Extract treatment was given to experimental rats both before and after restraint stress exposure and the observed results were interpreted in terms of modulation in hepatic and antioxidant enzyme activities, TBARS and total GSH level. We believe that the results obtained in the current study may further contribute to understanding of antioxidant property of garlic and its potential application to prevent/alleviate oxidative stress induced damage to hepatic cellular constituents.

MATERIALS AND METHODS

Reagents and chemicals

1-Chloro-2,4-dinitrobenzene (CDNB), Bovine serum albumin, and thiobarbituric acid were purchased from Sigma (St Louis, MO, USA); pyrogallol, hydrogen peroxide, 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), were purchased from E-Merk (Darmstadt, Germany). All additional chemicals used for the present study were of analytical grade and purchased from various commercial sources.

Preparation of garlic extract

One kilogram of garlic (*Allium sativum* L.) were purchased from the local market. Peeled and grinded with an electric mincer and diluted in double distilled water at 4g/mL on the basis of weight of the starting material. The diluted material was centrifuged (Beckman J20, 10,000g for 15 min at 4°C). The supernatant was aliquoted and stored at -80°C until further use.

Induction of immobilization stress and treatment procedures

Adult (10 weeks) male Albino Wistar rats weighing in the range of 180-200g were used in the study. They were housed in group cages, fed with Purina diets and tap water *ad libitum*. Experimental protocols complying with the guidelines of the animal welfare committee of the University were strictly followed to carry out the study. Prior to the commencement and throughout the experiment the rats were housed at 24±3°C room temperature and 12h light/ dark cycles (Zaidi *et al.*, 2014).

The immobilization stress was induced by restraining individual rats inside the cylindrical wire-mesh cages (approximately 8 cm diameter and 18 cm long) attached to a wooden board. Control rats were left freely walking in their respective cages.

A pilot study was performed with 50, 100, 150, 200 mg/kg of garlic extract using 3 animals per dose to find out the optimum therapeutic dose of garlic that can modulate deranged free radical metabolism (results not shown). It was observed that the extract at 100 mg/kg body weight (bw) dose has demonstrated the best preventive effect on oxidative stress induced changes in liver. Thus, the same dose was selected for evaluating the antioxidative potential of garlic extract.

Experimental protocol

To elucidate the effect of immobilization stress induced pro-oxidant changes and their attenuation by garlic extract, environment acclimatized 40 rats (male 10 weeks old, 180-200 g) were selected randomly and divided into 5 groups (N=8 per group). The groups were: Group I: A no stress no treatment group (CON-group); Group II: A group with immobilization stress without any treatments (STR-group). Animals in this group were exposed to 6 h of immobilization stress and sacrificed using pentobarbital treatment as euthanasia method, 1 h after removal of immobilization stress to account for the effect of possible natural relaxation after the stress; Group III: Normal rats treated with a single oral dose of 100mg/kg of garlic extract in order to obtain baseline data on the effect of garlic extract per se (GAR-group). These rats were sacrificed 7h after dose administration along with stress-induced post treatment animals (STR + Post-GAR-group). Group IV: A group pre-treated with a single dose of (100mg/kg bw) garlic extract 1h before they were

subjected to immobilization stress (Pre-GAR+STR-group) and Group V: A group which was treated with garlic extract after 6 h of stress (STR + Post-GAR-group). Rats were subjected to immobilization stress between 7 AM to 1 PM for 6 h by placing them in the individual wire mesh cages of appropriate size attached to a wooden board, as reported earlier (Zaidi *et al.*, 2014). They animals were deprived of food and water during the stress procedure. The rats were treated with a single dose (100mg/kg) of liquid extract via oral (*p.o*) route with the help of gavage needle. Animals were sacrificed using pentobarbital (*i.p.* 50 mg/kg body weight), 60 min after completion of the stress procedure. Non-stressed control animals (with or without garlic extract) and animals treated with garlic extract prior to immobilization stress were handled at the same time similar to stressed groups.

Preparation of homogenate

Liver tissues were quickly removed and washed with ice cold sterile physiological saline water (0.9%). A 10% homogenate was prepared in 0.1M sodium phosphate buffer (pH 7.4), centrifugation was performed on Beckman coulter centrifuge (rotor radius: 20.4 cm) at 3000 g for 15min at 4°C to remove cellular debris and the supernatant was used for further studies. (Zaidi *et al.*, 2015; Zaidi *et al.*, 2015)

Superoxide dismutase assay

The liver SOD activity was measured according to the method of Marklund (Marklund and Marklund, 1974). This procedure depends upon the autoxidation of pyrogallol (8nM) in the presence of 0.05M tris succinate buffer (pH 8.2). The inhibition of pyrogallol autoxidation by SOD was monitored at 412 nm. One unit of SOD enzyme is defined as the amount of enzyme required to inhibit the rate of pyrogallol autoxidation by 50%.

Catalase assay

Liver CAT activity was assayed according to the method of Beers and Sizer with hydrogen peroxide (30 mM) as the substrate (Beers and Sizer, 1952). One unit of CAT activity is defined as the micromoles of hydrogen peroxide consumed per minute per milligram of protein sample.

Glutathione-S-Transferase assay

The level of liver GST was estimated as per the method of Habig (Habig *et al.*, 1974) using 1-chloro-2,4-dinitrobenzene (CDNB) [1.0mM] as a substrate. In this method, enzyme activity is measured by following the increase in absorbance at 340 nm of CDNB-GSH conjugate generated in response to GST catalysis between GSH and CDNB.

Lipid peroxidation assay

Lipid peroxidation measurement was performed by employing an assay based on the stoichiometric reaction

of one molecule of malondialdehyde (MDA) with two molecules of 0.69% 2-thiobarbituric acid at pH 3.5 (Halliwell and Chirico, 1993). The pink chromogen was detected spectrophotometrically with an extinction coefficient of 156mM/cm at 532 nm.

Total GSH assay

The method of Sedlak and Lindsay was used to measure total liver GSH (Sedlak and Lindsay, 1968). The assay is based on the reduction of 0.01M 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) by sulfhydryl groups of GSH to form 2-nitro-5-mercaptobenzoic acid per moles of GSH.

ALT, AST, ALP, Glucose and Uric acid assays

The activities of above mentioned biochemical parameters were measured by the use of specific kits from Reckson Diagnostic Pvt. Ltd (Delhi, India).

Protein estimation

Total protein in the tissue homogenate was estimated as per Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

STATISTICAL ANALYSIS

Statistical analysis was performed for the control / baseline levels of the enzymes under study with respect to the treatments given to the rats. All the data are expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) test at $p=0.05$ on the data obtained by the repeated investigations. Paired *t* tests were also performed to find out the significant change in the results, followed by pair wise comparison (Tukey's honestly significant Post hoc analysis). Similar statistical treatments were also given to the data obtained for the biochemical parameters from pre and post garlic stress treatments with respect to the stress alone or non-stressed controls.

RESULTS

The present study demonstrated that 6h of immobilization stress resulted a significant decline in the activities of SOD, CAT, GST, glutathione and glucose levels along with a significant rise in thiobarbituric acid reactive substances (TBARS), AST, ALT, uric acid and ALP compared with both CON-group (untreated and non-stressed rats) and GAR group (garlic treated but non stressed) (figs. 1-3). Thus indicating that immobilization significantly induced oxidative stress in rats (STR-group). A single dose of garlic extract (100mg/kg body weight) did not result any significant change in general behavior, food intake or body weight of animals and the above mentioned biochemical parameters. However, post-treatment with garlic crude extract markedly neutralized immobilization stress induced changes in the studied antioxidant enzymes and the levels of GSH, ALP, AST,

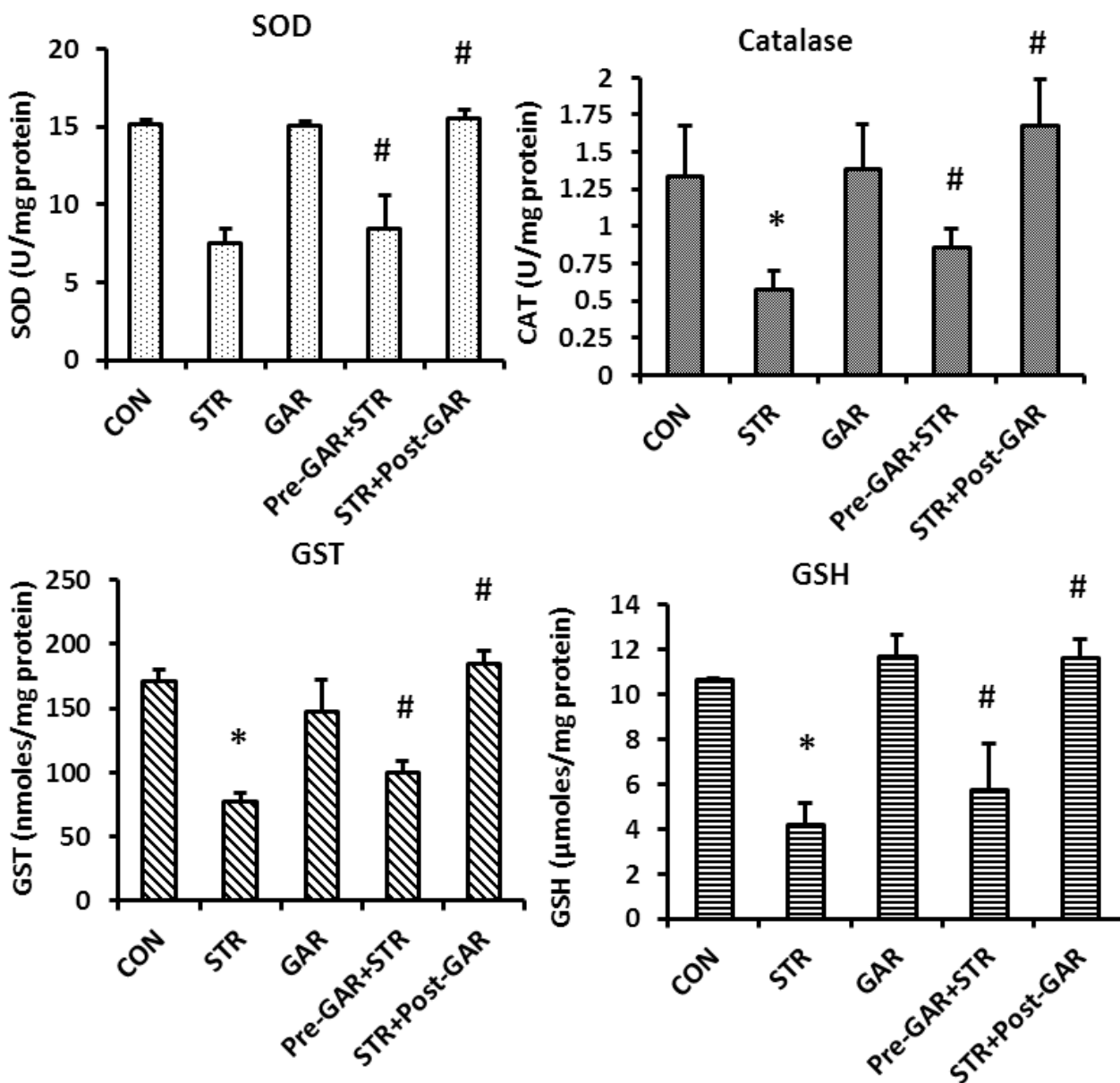


Fig. 1: Effect of crude garlic extract treatment on immobilization stress induced changes in liver (a) SOD (b) CAT (c) GST (d) GSH. Significant decrease in antioxidant enzymes activities and glutathione level were observed after immobilization stress. The pre and post stress garlic treatment revert the deranged free radical system towards their normal values with a relative dominance by later. * shows P values compared with controls, while # shows P values compared with stressed rats, where * $p < 0.05$ and # $p < 0.05$.

ALT, glucose and uric acid. Administrations of garlic aqueous extract both prior and after the immobilization stress resulted in a significant modulation in liver and antioxidant enzyme levels. However, the post stress oral administration of extract (100mg/kg body weight), was found to be more effective in restricting stress induced decline in SOD, CAT, GST and GSH and increase in levels of TBARS, AST, ALT, glucose, uric acid and ALP than pre-stress extract treatment as compared to post stress treatment alone (figs. 1-3).

DISCUSSION

It has widely been accepted that both physical and psychological stress may induce inflammatory and fibrosing changes in the liver especially under disease conditions causing a significant decline in overall health status. Therefore, we utilized an 'immobilization stress model' a convenient method for inducing both psychological (escape reaction) and physical (muscle work) stress as a result of restricted mobility and

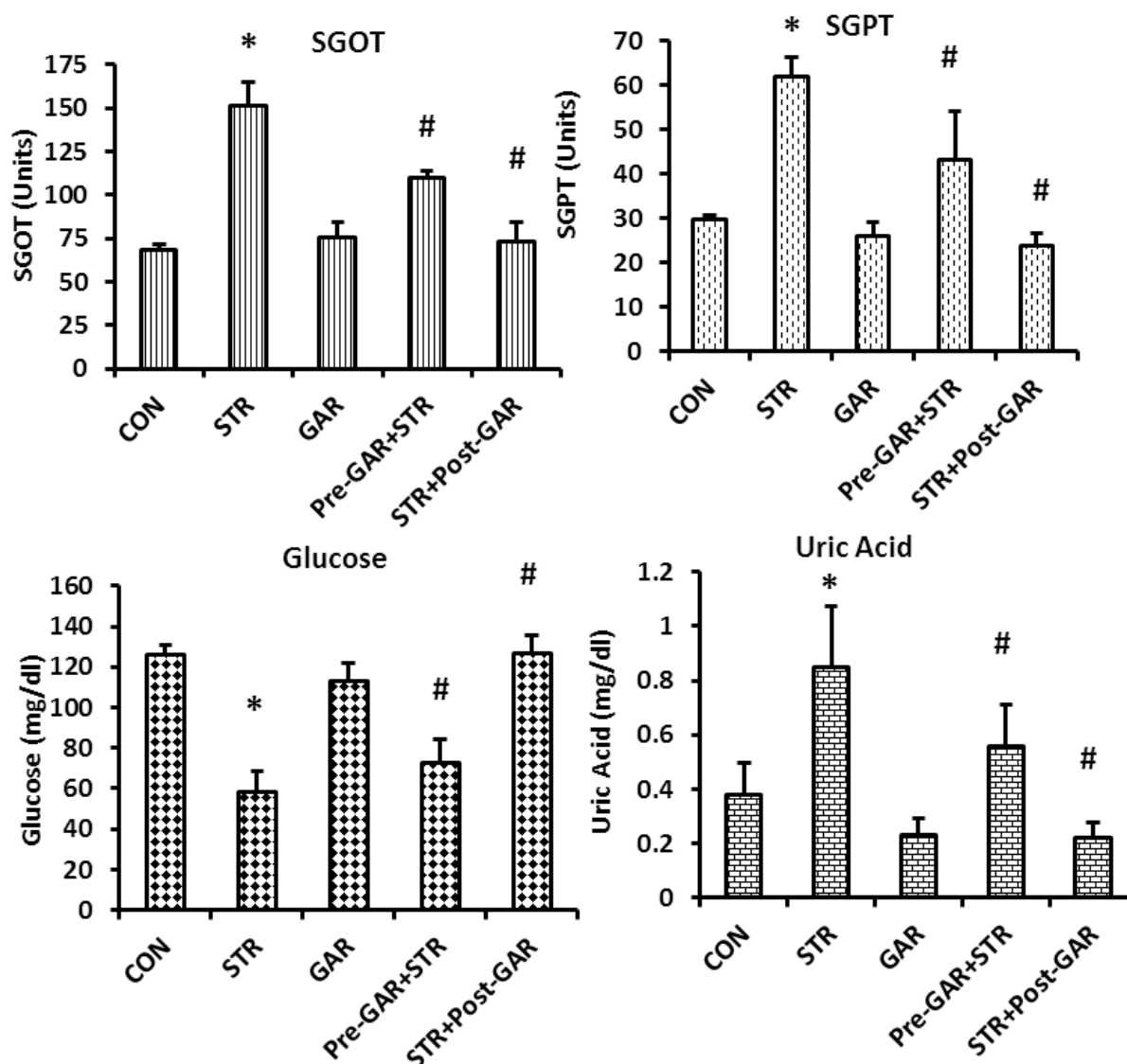


Fig. 2: Effect of crude garlic extract treatment on immobilization stress induced changes in liver (a) SGOT (b) SGPT (c) Glucose (d) Uric acid. Significant increase in liver markers enzymes and uric acid along with a significant decrease in glucose levels were observed after immobilization stress. The pre and post stress garlic treatment revert the deranged free radical system towards their normal values with a relative dominance by later. * shows P values compared with controls, while # shows P values compared with stressed rats, where * $p < 0.05$ and # $p < 0.05$.

aggression to study the effect of crude garlic extract on liver function under severe stress conditions (Liu *et al.*, 2013). The results in the present study clearly demonstrate that the immobilization stress leads to detrimental alterations in the pro-oxidant/antioxidant status. Moreover, dietary supplement with active antioxidants such as aged garlic extract are promising conventional treatment to harness the endogenous protection and defend against the oxidative stress.

In order to maintain the homeostatic stability in living organism, it is necessary to maintain critical balance between the oxidative and antioxidant defense. It is well known that SOD, GST and CAT play an important role in

scavenging oxy-radicals and their products. In our study, 6 h of immobilization stress resulted in the generation of oxidative stress in rat liver as shown by the decrease in levels of GSH and antioxidant enzyme levels and enhanced level of TBARS (fig. 1 and 2). The generated ROS could directly attack biomolecules, with consequent increase in membrane lipid per oxidation (LPO), which is believed to be the major factor involved in the mechanism of liver cell injury (Videla *et al.*, 2003; Jaeschke, 2011; Jaeschke *et al.*, 2002). The enhanced hepatic LPO process has been demonstrated to be associated with the depletion of endogenous GSH (Davis and Mehendale, 1980; Stohs and Bagchi, 1995). Additionally, it has also been reported that the combined action of GSH and SOD forms an

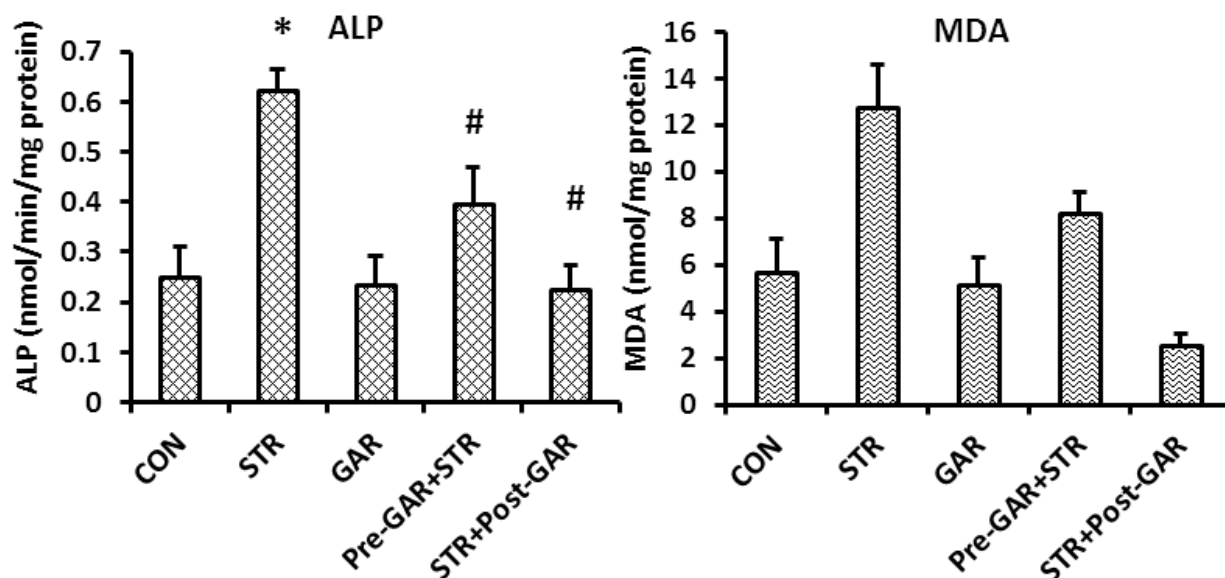


Fig. 3: Effect of crude garlic extract treatment on immobilization stress induced changes in liver (a) ALP (b) MDA. Significant increase in ALP and MDA level were observed after immobilization stress. The pre and post stress garlic treatment revert the deranged free radical system towards their normal values with a relative dominance by later. * shows P values compared with controls, while # shows P values compared with stressed rats, where * $p < 0.05$ and # $p < 0.05$.

integral component of cellular anti- high levels of catecholamines and glucagon, which is a key protective factor against oxidant damage. (Zaidi *et al.*, 2005) Thus, the depletion of GSH observed in our study might be the result of decreased activities of free radical scavenging enzymes viz. GST, SOD and CAT. The GSH depletion in immobilization stress rats may be correlated with increased susceptibility of the plasma membrane to peroxide attack, as reflected by rise in TBARS level. Moreover, both short-term (1 hour) and long-term (24 hours), immobilization stress have also been reported to cause an increase in TBARS level in brain, liver and heart tissues, accompanied by decrease in GSH levels (Valko *et al.*, 2005; Seçkin *et al.*, 1997). The oral administration of crude garlic extract significantly increased the hepatic activities of SOD, CAT and GST and the levels of glucose and GSH while the level of LPO, AST, ALT, ALP and uric acid was found to be decreased (figs. 1-3). Garlic extract was found to attenuate oxidative stress generated by immobilization stress, which was evident by the reversal of deranged antioxidant enzymatic activities and liver function enzymes including glucose and uric acid towards their control values. This is possibly due to the presence of organosulfur compounds in the garlic like allicin, alliin, SAC and SAMC which are well known potent free radical scavengers (Mellon *et al.*, 2000; Imai *et al.*, 1994). These antioxidant compounds present in garlic may perform a dual function. They can up regulate the antioxidant enzymatic activities and GSH level during stress to combat free radicals and down regulate LPO at the same time (Zaidi and Banu, 2004). While other researchers

have also reported similar benefits of garlic extract in stressed cardiac muscles (Banerjee *et al.*, 2002), the results obtained in the current study highlight liver protective effects of garlic treatment under conditions of immobilization stress.

The altered activity of AST and ALT are sensitive indicators of acute hepatic functional impairment and increase in ALP level is an indicator of hepatobiliary disease (Tabrez and Ahmad, 2009). The decrease in these surrogate biomarkers in the plasma indicates degenerative changes, metabolic alterations and hypo function of heart and liver, which are adversely affected by immobilization stress. Our findings further support the notion that the intra-gastric garlic extract treatment (both pre- and post-stress) reverses the hypo function of heart and liver (Naik *et al.*, 2011).

The level of glucose in liver was found to be significantly decreased in response to immobilization stress (fig. 2C). This could be due to the enhanced catecholamine level in response to immobilization stress. The catecholamines secretion evokes an initial repression of insulin secretion, followed by rebound hyper secretion of pancreatic hormone, subsequently leading to hypoglycemia, which ultimately decrease liver glucose level observed during immobilization stress (Middleton *et al.*, 2000). Six hours of stress resulted decrease in glucose level in liver which was found to be reverted towards their control values by both pre- and post-stress garlic extract treatment (fig. 2C). Uric acid is suggested as non-enzymatic antioxidant, but its increased production in response to immobilization

stress can cause increase in free radicals generation via activation of xanthine oxidase enzymes system (Zaidi et al., 2015; Radak et al., 2008). The elevated levels of uric acid can be detrimental under depleted GSH concentration during immobilization stress. The treatment with garlic extract resulted in a significant decrease in the uric acid level both in pre- and post-stress extract treatment animal groups. However, immobilization stressed groups demonstrated marked changes in uric acid level as compared to respective controls. The increase in uric acid concentration in immobilization stress could be due to body's natural response to combat ROS produced due to decreased antioxidant enzyme activities, increased xanthine oxidase activity and/or due to high levels of catecholamines (Marchitti et al., 2007).

CONCLUSION

Immobilization stress was found to induce oxidative stress through decrease in the activities of SOD, CAT, GST and levels of GSH and glucose, along with increase in uric acid, AST, ALT, ALP and LPO levels. The pre and post-stress oral administration of aqueous garlic extract was effective in protecting immobilization stress induced oxidative changes. The extract treatment alone did not showed any effect, but the post stress extract treatments was found to be more effective than pre-stress extract treatments in preventing/restoring the stress induced decline in SOD, CAT, GST, GSH and glucose levels and increase in uric acid AST, ALT, ALP and LPO levels. Based on our study, we recommend the use of garlic extract as nutritional supplement for combating oxidative stress induced damage.

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