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Abstract

Mitochondria are the particular target of arsenic (As) in the cell. Cell death induced by As is associated with mitochondrial membrane depolarization and release of cytochrome c from the mitochondria. DATS in a lipophillic organosulfur compound of garlic, which are known for its potent biological and pharmacological effects. Hence, this study was aimed to investigate the protective role of diallyl trisulphide against As induced oxidative stress in cardiac mitochondria. From all the groups, mitochondria were isolated from the heart tissue of rats and use for this present study. As exposed rats showed significant increases in lipid peroxidation products, mitochondrial swelling, NO concentration, H_2O_2 production, concentration of arsenic in cardiac tissue and mitochondria, and alterations in lipids profile of mitochondria. Significant decreases in mitochondrial antioxidant and Kreb's cycle enzymes, cytochrome-c-oxidase, ATP, Ca^{2+} level, oxygen consumption rate and mitochondrial membrane potential were observed in the heart mitochondria of As exposed rats. All these changes cause by As could be lessened by the pre-supplementation of diallyl trisulphide. The protective effect of DATS on the heart mitochondria was evidenced by altering all the changes induced by As and it is supported by TEM study. Since, all theses activity of DATS may be the means responsible for the defensive action of DATS against As induced mitochondrial damages in heart.

Keywords: Arsenic, Diallyl trisulphide, heart, mitochondria, oxidative stress

Introduction

Cardiac dysfunction is a major cause of morbidity and mortality worldwide due to its complex pathogenesis. Among the cells, the heart cell's cardiomyocyte is a most energy demanding cell in the body and is totally dependent on oxidative phosphorylation to supply the large amount of ATP required for beat-by-beat contraction and relaxation.[1] The energy metabolism of the heart relates essentially to the contractile mechanisms of the myofibrils and oxidative phosphorylation in the mitochondria. As mitochondria are the primary intracellular sites of oxygen consumption, they may also be primary sites of generation of reactive oxygen species (ROS).[1]

The mitochondrion is an excellent example of a subcellular organelle whose function is closely linked to maintenance of redox balance. As mitochondria are the primary intracellular sites of oxygen consumption, they may also be primary sites of generation of reactive oxygen species (ROS). Toxic or pathological conditions, such as oxidative stress, that lead to an impairment of mitochondrial function, can increase the release of ROS.[2] As the main consumers of molecular oxygen in the cardiac cells, mitochondria play a central role in molecular events leading to tissue damage occurring in the condition ischaemia.[3]

The major cause of death worldwide is mainly related to the cardiovascular diseases. Out of the known causative factors of cardiovascular diseases arsenic is thought to be one among the available factors.[4] Arsenic exposure via drinking water is associated with hypertension, peripheral vascular disease, cardiomyopathy and ischemic heart disease.[5] Long term arsenic exposure plays a key role in the pathogenesis of myocardial tissue leading to various cardiovascular complications and myocardial injury.[6] Fragmentation of DNA, reactive oxygen

species (ROS) generation, changes in cardiac ion channels and apoptosis in the myocardial tissue are the possible mechanisms of arsenic-induced cardiotoxicity.[7]

Arsenite, a trivalent form of arsenic, reacts with cellular thiols to exert its toxicity. Alternatively, arsenic compounds generate reactive oxygen species during their metabolism, in cells to cause tissue damage. Mitochondria are the major site of utilization of oxygen and many of the mitochondrial enzymes contain essential sulfhydryl groups. In addition, the inner and outer mitochondrial membranes contain unsaturated lipids. Therefore, mitochondria are more susceptible to arsenic attack as well as by the free radicals produced by it than other organelles and it is the particular target in the cell as arsenic can accumulates in it.[8]

Antioxidants play an important role in preventing free radical mediated damages by directly scavenging them. DATS is one of the most widely distributed, naturally occurring and biologically active antioxidant of garlic. It protects against lipid peroxidation most efficiently through its chain-breaking antioxidant action.[9] DATS has been found to protect the lipid-rich membrane mitochondria of arsenic treated rat liver. Although the pharmacological effects of DATS have been extensively studied, there is no direct report about the effects of DATS on As-induced cardiac damage and cardiac mitochondrial dysfunction in the current literature. In view of the potent antioxidant capacity of DATS and the role of ROS in the etiology of mitochondrial toxicity by As, the protective effects of DATS on As-induced cardiac mitochondrial injury are worthy to be studied.

Thus, we investigated the ability of DATS, to prevent arsenic-induced mitochondrial damage. Studies were carried out to evaluate the effect of DATS on cardiac mitochondrial oxidative and antioxidative markers as well as alterations on mitochondrial enzymes activities in response to short-term arsenic exposure. Moreover, the ability of DATS to attenuate the effects

of arsenic on mitochondrial function will provide insight into whether defects in mitochondrial functions are linked to the development of early stages of arsenic mediated cardiac injury.

MATERIALS AND METHODS

Reagents

Diallyl trisulfide (DATS) was purchased from Lukang Cisen Pharmaceutical Co., Ltd. (Shangdong, China). Arsenic, nitroblue tetrazolium, phenazine methosulphate, butylated hydroxy toluene, 1-chloro-2,4-dinitrobenzene, 2,4-dinitrophenylhydrazine, trisodium citrate, glutathione, potassium-α-ketoglutarate, thiamine pyrophosphate, sodium succinate, oxaloacetate and cytochrome-c were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade.

Animals

Male albino rats weighing 170–190 g were used in this study. They were maintained in an environmentally controlled animal house (temperature 24±2 °C) with a 12 h light/dark schedule and free access to deionized drinking water. The animal treatment and protocol employed were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 885/2012/CPCSEA).

Experimental Design

In the present study, arsenic was administered as Na_3AsO_4 intragastrically intubation at a dose of 5 mg/kg body weight/day for 4 weeks, which was 1/8 of the oral LD_{50} values in rats. A pilot study was conducted with three different doses of DATS (20, 40 and 80 mg/kg) to determine the dose dependent effect on As treated rats. After 4 weeks of the experiment, it was observed that DATS pre-treatment at the doses of 20, 40 and 80 mg/kg.BW significantly ($p <$

0.05) altered the biochemical changes induced in As intoxicated rats (data not shown). 80 mg/kg.BW of DATS showed significant effect when compared with the 20 and 40 mg/kg.BW. Hence, 80 mg/kg of DATS is found to be the most effective dose and we have selected this effective dose for our study.

For experiments, 24 rats were randomly selected into four group consisting six rats in each groups: group I-Normal plus control rat treated with normal saline and corn oil for 28 days, group II-treated with As (5 mg/kg.BW) in normal saline for 28 days, group III- rats were orally pre-administered with DATS (80 mg/kg.BW) 90min before the arsenic as sodium arsenate (5 mg/kg.BW) for 28 days, group IV- treated with DATS (80mg/kg.BW) for 28 days. The animals of all the groups were provided with a control diet composed of71% carbohydrate, 18% protein, 7% fat, and 4% salt mixture with free access to deionized drinking water.

Isolation of Heart Mitochondria

Heart mitochondria were isolated by the method of Takasawa.[10] The heart tissue was put into ice-cold 50 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and homogenized. The homogenates were centrifuged at 700 × g for 20 min and then the supernatants obtained were centrifuged at 9,000 × g for 15 min. Then, the pellets were washed with 10 mM Tris–HCl (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer.

Estimation of heart mitochondrial oxidative stress markers and antioxidant Defense marker enzymes

The levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides in the heart mitochondria were estimated by the methods of Fraga[11] and Jiang[12] respectively. The activities of mitochondrial antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRx) and glutathione-S-transferase (GST)

were assayed by the methods of Kakkar, [13] Sinha, [14] Rotruck, [15] Horn and Burns[16] and Habig and Jakoby, [17] respectively. The concentration of reduced glutathione (GSH) was estimated by the method of Ellman.[18]

Assay of Kreb's Cycle and Respiratory Chain Enzymes in the heart Mitochondria

The activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), a-ketoglutarate dehydrogenase (a-KGDH), reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase and cytochrome-c-oxidase were assayed by the methods of King,[19] Slater and Borner,[20] Mehler,[21] Reed and Mukherjee,[22] Minakami[23] and Pearl,[24] respectively.

Estimation of heart mitochondrial lipids

From the mitochondrial fraction, the lipids were extracted by the method of Folch.[25] The concentration of cholesterol in the mitochondrial lipid fraction was estimated by the method of Zlatkis.[26] The concentration of free fatty acid (FFA) in the mitochondrial lipid fraction was estimated by the method of Falholt.[27] The levels of triglycerides in the mitochondrial lipid fraction were estimated by a reagent kit from Accurex Bio Pvt. Ltd, Mumbai. Phospholipids content in the mitochondrial lipid fraction was estimated by the method of Zilversmit and Davis.[28]

Estimation of heart mitochondrial ATP and Ca^{2+} level

The levels of ATP were determined by the method of Williams and Coorkey.[29] The levels of Ca^{2+} were measured by the O-Cresolphthalein Complexone method by a reagent kit purchased from Span Diagnostic Limited, India. The content of protein in the heart mitochondrial fraction was determined by the method of Lowry.[30]

Mitochondrial swelling

Mitochondrial volume changes were followed by the decrease of optical density measured at 540 nm with a Beckman DU 7400 spectrophotometer equipped with a magnetic stirrer assembly. The assays were performed in 2 ml of 200 mM sucrose, 10 mM TRISMOPS, 10 μM EGTA, 5 mM KH2PO4 (pH 7.4, 25 °C), and 2 μM rotenone to which was added 0.5 mg of mitochondrial protein. Succinate was added after the calcium addition. Swelling amplitude was calculated as the difference between initial (pre-succinate addition) and final optical density.

Estimation of nitric oxide concentration in mitochondria

Nitric oxide concentrations in mitochondria of the cardiac tissues were measured separately spectrophotometrically at 548 nm according to the method of Fiddler[31] by using Griess reagent. The reaction mixture in a spectrophotometer cuvette (1 cm path length) contained 100 μL of Griess Reagent, 700 μL of the sample and 700 μL of distilled water. The nitric oxide concentration was expressed as μM/mg of protein.

Mitochondrial H_2O_2 Production

To measure the release of H_2O_2 from isolated cardiac mitochondria, the Amplex Red (Invitrogen) protocol of Mohanty[32] was used with some modification. Briefly, 200 μg/30 μL of a mitochondrial suspension was added to the wells of a microplate and pre-warmed to 37°C for 10 min. One hundred μL of phosphate buffer containing 50 μM Amplex Red (10 acetyl-3,7-dihydroxyphenoxazine, Invitrogen) and 0.1 U/mL horseradish peroxidase was subsequently added to each well. To measure stimulated H_2O_2 release, 20 μL of 25 mM succinate was added to the reaction mixture. To account for background absorbance in the sample, un-stimulated samples were run in parallel for which succinate was substituted with an equal volume of respiratory buffer. Absorbance at 550 nm was measured after 30 min of incubation at 37°C and

background absorbance was subtracted from sample absorbance. H_2O_2 concentration was determined from a standard curve and expressed as pmol/min/mg protein.

Determination of oxygen consumption by rat heart mitochondria

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Spring, OH, USA). Oxygen consumption in the rat heart mitochondrial fraction was determined in a 3-ml reaction vessel containing 750 µmol of sucrose, 60 µmol of HEPES, pH 7.4, 300 nmol of EDTA, 600 µg of mitochondrial protein and 3 µmol of NADH. For standard 12 nmol of rotenone was used and mixed with 750 µmol of sucrose, 60 µmol of HEPES, pH 7.4, 300 nmol of EDTA, 600 µg of mitochondrial protein and 3 µmol of NADH. Oxygen consumption was then initiated by the addition of NADH. The electrode was then inserted and the linear control rate of oxygen consumption was determined for 10 min thereafter.The rate of oxygen consumption was based on a value of 597 nmol for the total dissolved oxygen content of the reaction mixture.[33]

Detection of cardiac mitochondrial membrane potential

Cardiac mitochondrial membrane potential change was determined by incubating cardiac mitochondria with 5-mM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra ethylbenzimidazolcarbocyanine iodide (JC-1) dye at 37°C for 30 min. Cardiac mitochondrial membrane potential changes were detected using a fluorescent microplate reader. JC-1 monomer form (green) fluorescence was excited at λex 485 nm and detected at λem 590 nm. JC-1 aggregate form (red) fluorescence was excited at λex 485 nm and detected at λem 530 nm. A decrease in the red/green fluorescence intensity ratio was considered an indicator of cardiac mitochondrial membrane depolarization.[34]

Determination of Arsenic Accumulation in the cardiac tissue and cardiac mitochondria

The arsenic contents in cardiac tissues of all rats were analyzed following the method in the literature with an atomic fluorescence spectrometry system (AFS930; Beijing Jitian Instrument Co. Ltd., Beijing, China).[35] Sub-cellular fractions were digested with acid mixture containing nitric acid, sulfuric acid and perchloric acid in the ratio of 6:1:1, over a regulated heater. After the digestion, the acid mixture was evaporated with occasional addition of triple distilled water and the solution thus obtained was employed for estimation of arsenic content. Estimation was carried out using the atomic absorption spectrophotometer (Spectra AA 30/40; Varian, Australia) fitted with a graphite furnace .[36]

Transmission electron microscopic (TEM) analysis

The mitochondrial pellet obtained was fixed in 2.5% glutaraldehyde with 50mM-cacodylate buffer, pH 7.4, post-fixed in 2% (W/V) osmium tetroxide ($Os0_{4)}$ and embedded in Epon 812 resin for electron microscopy. The grids containing sections were stained with 2% uranyl acetate and 0.2% lead acetate. Then, the sections were examined under a transmission electron microscope.

Statistical analysis

All the data were expressed as mean ± SD of a number of experiments (n = 6). The statistical significance was evaluated by one-way analysis of variance using SPSS version 13.0 (SPSS, Cary, NC, USA) and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Follow by the post- hoc test, least significant difference (LSD). Values were considered as statistically significant when $p < 0.05$.

Results

The effect of Diallyl trisulfide on arsenic induced changes in different pro and anti-oxidative markers in the heart mitochondria of rats.

Results indicate that, compared with control, heart TBARS and LOOH level was increased significantly ($p<0.05$) in arsenic-treated rats. Pre-administration of DATS in the arsenic-exposed rats' significantly reduced the TBARS and LOOH level compared with arsenic alone induced response (Fig, 1A). Similarly, compared with control, GSH, GST, SOD, CAT, GPx and GR activity in isolated heart mitochondria were significantly ($p<0.05$) inhibited in arsenic-treated rats. Pre-treatment with DATS in arsenic intoxicated rats' significantly restored the arsenic-induced GSH, GST, SOD, CAT, GPx and GR activity (Fig, 1B). No significant difference was observed in DATS alone treated rats when compared to the control rats.

The activities of heart mitochondrial enzymes and cytochrome c-oxidase

Figure 2(A) demonstrated the activities of heart mitochondrial citric acid cycle enzymes isocitrate dehydrogenase (ICD), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KGDH) and respiratory marker enzyme (NADH dehydrogenase and figure 2(B) demonstrated the activities of heart mitochondrial cytochrome c-oxidase in the cardiac mitochondria of control and experimental rats. In rats intoxicated with As, there was a significant ($p<0.05$) reduction in the activities of TCA cycle enzymes, NADH dehydrogenase and cytochrome c-oxidase when compared with the control rats. Interestingly, pre-supplementation of DATS in As treated rats significantly increased the activities of TCA cycle enzymes, NADH dehydrogenase and cytochrome c-oxidase when compared with As alone treated rats. DATS supplementation to rats shows no significant ($p<0.05$) difference in the

activities of mitochondrial citric acid cycle enzymes, NADH dehydrogenase and cytochrome c-oxidase when compared with the control rats.

The levels of heart mitochondrial lipid profile

The levels of mitochondrial cholesterol, FFA and triglycerides were significantly ($p<0.05$) increased and the level of phospholipidss was significantly ($p<0.05$) decreased in arsenic intoxicated rats when compared with control rats. Pre-treatment with DATS (80 mg/kg body weight) significantly ($p<0.05$) decreased the levels of cholesterol, FFA, triglycerides and significantly ($p<0.05$) increased the levels of phospholipidss in the heart mitochondria fractions of As-induced rats when compared with As-intoxicated rats (Fig. 3). No significant difference was observed in DATS alone treated rats when compared to the control rats.

The effect of Diallyl trisulfide on the levels of ATP in the heart mitochondria

As-intoxicated rats showed significant ($p<0.05$) decreased levels of ATP when compared with the control rats. Pre-treatment of DATS in arsenic intoxicated showed significant ($p<0.05$) increased levels of ATP in the heart mitochondria when compared with As alone exposed rats (Fig. 4). DATS supplementation to rats shows no significant ($p<0.05$) difference in the levels of ATP in the heart mitochondria when compared with the control rats.

The effect of Diallyl trisulfide on heart mitochondrial calcium content

The levels of $Ca2^{+}$ significantly ($p<0.05$) increased in arsenic exposed rats when compared with control rats. Pre-treatment with DATS (80 mg/kg) showed significant ($p<0.05$) decreased levels of $Ca2^{+}$ in the heart mitochondria when compared with As alone induced rats (Fig. 5). There is no significant difference between DATS alone treated rats and the control rats.

The effect of Diallyl trisulfide on heart mitochondrial swelling

Mitochondrial swelling is indicative of susceptibility to calcium-induced membrane potential (MPT). As illustrated in Fig. 6, cardiac mitochondria from the As exposed rats were more susceptible to calcium-induced mitochondrial swelling than being in controls rats. Pre-administration of DATS prevented the increased calcium-induced swelling caused by As treatment when compared with As alone intoxicated rats. DATS alone treated rats showed significant ($p<0.05$) effect when compared with the control rats.

The effect of Diallyl trisulfide on heart mitochondria NO concentration

Results indicate that, compared with control, concentration of NO in isolated heart mitochondria, increased significantly ($p<0.05$) in arsenic treated rats. Prior administration of DATS in the arsenic-exposed rats significantly reduced the concentration of NO when compared to arsenic alone treated rats (Fig 7). There is no significant difference between DATS alone treated rats and the control rats.

Effect of Diallyl trisulfide on H_2O_2 production in cardiac mitochondria of As intoxicated rats

The H_2O_2 production significantly increased in heart mitochondria of the rats treated with As (Fig 8). However, the pre-treatment of DATS to As treated rats significantly ($p<0.05$) decreased H_2O_2 production, when compared to As alone treated rats. DATS shows a more significant effect in reducing the H_2O_2 generation when compared with As alone intoxicated rats (Fig 8) which confirms the antioxidant efficacy of DATS in abrogating As induced cardiac mitochondrial oxidative stress. DATS alone treated rats showed no significant difference when compared with the control rats.

Effect of Diallyl trisulfide on oxygen consumption in cardiac mitochondria of As intoxicated rats

In arsenic intoxicated rats the rate of oxygen consumption significantly ($p<0.05$) decreased when compared with the control rats and standard (rotenone). Pre-treatment of DATS in arsenic intoxicated rats showed significant ($p<0.05$) increased rate of oxygen consumption in the heart mitochondria when compared with As alone exposed rats and standard (Fig. 9). DATS treated rats shows significant ($p<0.05$) difference when compared with the control rats.

Effects of Diallyl trisulfide on heart mitochondria membrane potential of As intoxicated rats

Administration of arsenic decreased the heart mitochondria membrane potential when compared with the control rats (Fig. 10). Pre-administration of DATS in arsenic intoxicated rats significantly increased the mitochondria membrane potential when compared with the As treated rats (Fig. 10). DATS alone treated rats' shows significant difference when compared with the control rats (Fig. 10).

Effects of Diallyl trisulfide on As concentration in heart tissue and heart mitochondria of arsenic treated rats

The concentration of arsenic increased in the heart tissue (Fig, 11A) and heart mitochondria (Fig, 11B) of arsenic treated rats when compared with the control rats. Pre-administration of DATS in arsenic intoxicated rats significantly decreased the concentration of arsenic increased in the heart tissue and heart mitochondria when compared with the As treated rats. DATS alone treated rats' shows significant difference when compared with the control rats (Fig, 11A).

Effect of Diallyl trisulfide on heart mitochondria Ultra structure

The electron microscopic structure of the mitochondrial pellet from the heart of control rats showed normal structural design and it is well preserved (Fig 12 A). Heart mitochondria isolated from the rat treated with As shows swollen mitochondria and disrupted cristae with vacuolation (Fig 12 B). Pre-treatment of DATS to As intoxicated rats show mild swollen and vacuolated heart mitochondria with mild separated cristae when compared with As alone treated rats (Fig 12 C). In DATS alone treated rats the heart mitochondria shows well preserved and normal architecture of the heart mitochondria (Fig 12 D).

Discussion

Heart is the major target organ in arsenic toxicity and carcinogenesis. Arsenic toxicity involves oxidative damage that plays a vital role for biochemical alteration.[37] A particular target in the cell is the mitochondria, which accumulates arsenic. As to the mechanism of toxicity, inorganic arsenic has been shown to cause impaired tissue respiration *in vivo*. It inhibits enzyme activity by reacting with the sulfhydryl groups of proteins. In particular, suppression of nicotinamide adenine dinucleotide-linked substrates (pyruvate, glutamate, and a-ketoglutarate) appears to play a crucial part in the toxicity of As. Pentavalent and trivalent forms of arsenic exert similar effects in the inhibition of mitochondrial respiration and uncoupling of mitochondrial oxidative phosphorylation. The mechanism of this inhibition is not clear: one possibility is that arsenate is reduced by the mitochondria to As (III) and that inhibition occurs through the formation of a complex with the lipoic acid cofactor that is necessary for oxidation of the substrate.[38] The purpose of the current study was to test the hypothesis that the supplementation of DATS reduces the oxidative mitochondrial toxicity of arsenic and thereby

plays a significant protective role against arsenic-induced cardiotoxicity. Figure 13 shows a summary of arsenic induced oxidative cardiac mitochondrial injury and its prevention by DATS.

The results of experiments with cardiac mitochondria pro-oxidative and anti-oxidative markers yielded identical results as observed previously with liver cell mitochondria.[9] The increase level of TBARs and LOOH, and decrease level of anti-oxidative enzymes (GSH, GST, SOD, CAT, GPx and GR) were observed in As treated rats. The elevated levels of lipid peroxidation products such as TBARs in the mitochondria may decrease mitochondrial membrane fluidity, increase the negative surface charge distribution and alter membrane ionic permeability including proton permeability, which uncouple oxidative phosphorylation.[2] Thus, accelerated lipid peroxidation damages both the mitochondrial structure and function of As treated rats. While pre-treatment of DATS in As exposed rat alter all these changes.

Pre-treatment with DATS lowered the levels of lipid peroxidation in As-intoxicated rats. The organosulfur compounds have been reported for their non-enzymatic antioxidant action, which is mainly from their reducing power and interactions with biological membranes and/or other antioxidant agents. In this perspective, DATS have the non-enzymatic antioxidant actions that might be responsible for its ability to inhibit lipid peroxidation. Fascinatingly, the fact that DATS could noticeably restore the destruction of antioxidant defense system in the heart mitochondria of As-treated rats might be attributed to its antioxidant and chelating properties, which could be due to the orientation of -SH groups present in DATS.[9] Data generated from pre-supplementation studies with DATS hold out further support to our claim that DATS has efficacy in preventing and reducing arsenic-induced mitochondrial damage and cellular injury.[9,39]

To evaluate the extent of cellular damage and functional alteration in cardiac cells that might have occurred on exposure to arsenic, functional status of cardiac mitochondrial enzymes was examined. Arsenic administration to rats also resulted in reduced activities of mitochondrial citric acid cycle enzymes ICDH, SDH, MDH and α –KGDH and respiratory marker enzyme NADH dehydrogenase.[9] These dehydrogenases are situated in the outer membrane of the mitochondria and pretentious by increased levels of free radicals generated on As intoxication.[40] It has been reported that, the marked deficiency in one or more electron transport chain resulted in decreased activities of mitochondrial citric acid cycle enzymes.[41] The decreased activities of these dehydrogenases will decrease the aerobic oxidation of pyruvate and reduce the production of ATP-molecules. The decreased activities of respiratory marker enzymes such as NADH-dehydrogenase and cytochrome-c-oxidase observed in As exposed rats might be due to enhanced phospholipids degradation resulting in the non-availability of Cardiolipin for their functional activity.[42] Increased free radicals produced by As also resulted in decreased activities of these enzymes. Pre-treatment of DATS enhanced the activities of TCA cycle and respiratory marker enzymes in the mitochondrial fraction of the heart in As treated rats. Since, the reason for such actions of DATS might be the free radical scavenging activity and the anti-oxidative properties of DATS.[43]

As intoxication considerably altered the mitochondrial lipids, lipid peroxides, antioxidants, Kreb's cycle and respiratory marker enzymes, Ca^{2+} and ATP. We observed an increased level of heart mitochondrial cholesterol, TGs and FFAs and decrease the level of phospholipids in As exposed rats.[9] Altered cholesterol levels in the mitochondrial membrane affect the fluidity, permeability of ions and activities of membrane bound enzymes. An increase in the mitochondrial cholesterol levels suggests the redistribution of cholesterol in the ischemic

cell. Further, the accumulation of FFAs is a consequence of changes in myocardial lipid metabolism. These changes in the metabolism of the subcellular fractions may lead to damage of the membranes of the cardiac mitochondria, which may be the cause of disorders of electrolyte metabolism and contractile properties of the myocardium.[44] Oral pre-treatment with DATS lowered the levels of mitochondrial cholesterol, TGs and FFAs and elevate the level of phospholipids, indicating the activity of DATS in maintaining the stability and integrity of the mitochondrial membrane in As intoxicated rats.[9]

In this study, decreased levels of ATP and Ca^{2+} in the heart mitochondria were observed in As-induced rats. Decreased levels of mitochondrial membrane Ca^{2+} is related to the altered mitochondrial membrane potential, thereby affecting ATP production in As induced rats.[9] This might be the reason for the decreased levels of ATP in As-induced rats. Pre-treatment with DATS (80 mg/kg) increased the levels of Ca^{2+} and ATP in As-induced cardio-toxic rats. Thus, DATS improve cardiac mitochondrial function by maintaining Ca^{2+} and ATP levels. This may be due to the ability of DATS to protect the SH groups from oxidative damage through the inhibition of peroxidation of membrane lipids and stabilization of the membrane.

Mitochondrion swelling is an indicator of MPT (mitochondrial permeability transition). Arsenic treatment is characterized by increased mitochondrial swelling and disruption of the mitochondria membrane leading to the loss of mitochondrial enzymes and eventually tissue death.[9,44] Hence, such altered MPT, which was indicated by an increase in mitochondrial swelling, could be significantly reduced by DATS. Results suggest that change in MPT which possibly initiated the apoptotic process in cardiac cell of exposure to arsenic could be effectively minimized by DATS.[39]

Nitric oxide acts as a multisite inhibitor of the mitochondrial electron transfer chain. Mitochondrial respiration and its regulation by nitric oxide are important in the heart for several reasons such as generation of ATP, which is required for muscle contraction in the heart, so that inhibition of mitochondrial respiration results in an inhibition of contractility. NO interacts with the mitochondrial respiratory chain by different means: (A) NO itself causes rapid, selective, potent, but reversible inhibition of cytochrome oxidase, and (B) reactive nitrogen species (RNS, which include $ONOO^-$ (peroxynitrite), NO_2, N_2O_3 and S-nitrosothiols) cause slow, non-selective, weak, but irreversible inhibition of mitochondrial components.[45] In our present finding, the concentration of NO in As treated rats was found higher than the control rat. Increase concentration of NO in As intoxicated rats is related to a multiple inhibition of mitochondrial electron transfer chain and have been generally related to toxicity or damage and even to the pathogenesis induced by.[46] Interestingly DATS pre-treatment, alter the concentration of NO. The main reason for the alteration of NO concentration in As intoxicated rats include an increase of anti-oxidative activity, a decrease of peroxidized lipids and its free radicals scavenging property. DAT has already been reported to have a beneficial effect on the cardiovascular diseases.[9,47]

We considered that interruption of mitochondria activity of arsenic may divert electrons from the respiratory chain into the formation of the kinds of ROS known to be concerned in the activation of transcription factors and production of cytokines. It has been demonstrated earlier that, the most primitive intracellular events following As treatment is the generation of ROS by mitochondria followed by activation of transcription factors, which confirms mitochondria as an important target in As toxicity.[48] The H_2O_2 production in the heart mitochondria of As exposed rats increased significantly when compared with the control rat and it is in accordance with all the biochemical changes observed in this study. In this study, we observed that, the H_2O_2

production was markedly reduced in DATS pretreated rats intoxicated with As. The reason behind this action of the DATS is due to its direct free radicals scavenging activity, ability to activate Nrf2 mechanisms and its ability to inhibit thiol group oxidation.[43,49]

Arsenic toxicity was reported as an inhibitory effect on cellular respiration at the level of mitochondria. Out of the known variety of different respiratory chain inhibitors, we have used the rotenone to confirm the effect of arsenic in inhibiting the cardiac mitochondrial respiration. In our study, consequently to the bioenergetics principle, arsenic shows inhibition of respiration process in rat heart mitochondria as it is confirm with the decreased rate of oxygen consumption rate in heart mitochondria of arsenic intoxicated rats and it is in accordance with the findindings of Paul[50] and the comparison was made with the known respiratory inhibitor (rotenone). Rotenone is known to inhibit complex I substrate mediated respiration and from this point we found to know that arsenic also inhibit cardiac mitochondrial complex I substrate mediated respiration and reduced the oxygen consumption. Surprisingly pre-administration of DATS in arsenic exposed rats significantly restored the rate of oxygen consumption in rat mitochondria. Such possible protective action of DATS may be due to its direct free radicals scavenging activity and the ability to activate the Nrf2 mechanisms and its ability to inhibit thiol group oxidation. [43,49]

In the present study, we observed the mitochondrial membrane potential, which could reflect the integrity and function, to evaluate the effects of DATS on cardiac mitochondrial dysfunction caused by As exposure. Alterations in the mitochondrial membrane potential and membrane permeability are now thought to be a central regulatory mechanism for cell death induction.[9] Cardiac mitochondrial dysfunction induced by arsenic as shown by increased mitochondrial ROS production and mitochondrial swelling and decreased mitochondrial

membrane potential.[9] Because it is known that increased ROS production is mainly contributed to mitochondrial depolarization and mitochondrial swelling, the major effect that DATS increased mitochondrial membrane potential could be due to their ability to decrease the mitochondrial ROS production and restored mitochondrial calcium content.[9,39]

In our observations, there was a significant increase in arsenic concentration in heart tissue and heart mitochondria of arsenic treated rats. While DATS pre- treatment significantly reduced the concentration of arsenic in heart tissue and heart mitochondria. The chelating efficacy of DATS possibly contributed in reducing As load in heart tissue and heart mitochondria and facilitated the excretion of As through urine.[51]

Alteration in the fine structure of mitochondria is the most prominent TEM finding in cardiac damage induced by As. DATS pre-treatment showed mild separation of cristae without swelling. These observations agree closely with the results obtained by biochemical parameters in the study.

Conclusion

Thus DATS reduced the extent of mitochondrial damage and improved the mitochondrial structure and function in As-intoxicated rats. TEM study also supported the protective efficacy of DATS on heart mitochondria. This findings strengthened the cardio-protective nature of DATS. We suggest that the possible mechanism for the observed effects of DATS could be due to its quenching of free radicals, lowering of lipid peroxides, lipids and improving the antioxidant-enzyme activities, Ca^{2+} and ATP and thereby improves the cardiac mitochondrial function in As-intoxicated rats. Restoration of cellular normalcy accredits the Cyto-protective role of DATS, as DATS possessed protective effects on mitochondria, which is a crucial element involved in both triggering and mediating cardioprotective responses in myocardial cells. Thus, this study may

have a significant impact for the treatment of As induced cardiotoxicity. On the basis of the present results, we considered that DATS is an effective antioxidant phyto-chemical entity against As-induced cardiac damage and oxidative stress.

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Figure legends

Fig 1 Effect of Diallyl trisulfide on arsenic induced changes in pro-oxidative markers TBARS and LOOH (Fig 1A) and antioxidative markers GSH, GST,SOD, CAT, GPx, and GR (Fig 1B) in the heart mitochondria of rats. Activity is expressed as- TBARS (n mol/mg of protein); LOOH (n mol/ 100 mg of protein); GSH (n M/ 100 mg of protein); GST (n mol CNDB conjugated /min/ 100 mg protein); SOD (*units/100 mg protein); Catalase (nM of H_2O_2 consumed/ min/ 100 mg protein); GPx (nM of GSH oxidized/ min/ 100 mg protein); GR (n mol of NADH oxidized/ min/ 100 mg protein). SOD *Units: One unit is defined as the enzyme concentration required inhibiting the optical density at 560 nm of chromogen production by 50% in 1 min. Values were mean ± SD (n=6); Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 2 Effect of Diallyl trisulfide on arsenic induced changes in the activities of heart mitochondrial enzymes (Fig. 2A) and cytochrome c-oxidase (Fig. 2B). Activity is expressed as nM of NADH oxidized/h/mg protein for ICDH; nM of succinate oxidized/min/mg protein for SDH; nM of NADH oxidized/min/mg protein for MDH; nM of ferrocyanide formed/h/mg protein for α -KGDH; nM of NADH oxidized/min/mg protein for NADH dehydrogenase; nM/min/mg protein for cytochrome-c-oxidase. Values were mean ± SD (n=6); Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 3 Effect of Diallyl trisulfide on arsenic induced changes in the levels of heart mitochondrial lipid profile. The levels is expressed as- TGs (nM/mg protein); Cholesterol (nM/mg protein); FFAs (nM/mg protein); Phospholipids (nM/mg protein). Values were mean ± SD (n=6); Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 4 Effects of DATS and As for heart mitochondria ATP level. Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 5 Effects of DATS and As for heart mitochondrial calcium content. Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 6 Effects of DATS and As on heart mitochondrial swelling an index of MPT (mitochondrial membrane permeability transition). Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 7 Effects of DATS and As on heart mitochondria NO concentration. Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 8 Effects of DATS and As on heart mitochondria H_2O_2 production. Values not sharing a common superscript letter ($^{a–c}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 9 Effects of DATS and As on the rate of heart mitochondria oxygen consumption. Values not sharing a common superscript letter ($^{a–d}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 10 Effects of DATS and As on heart mitochondria membrane potential. Values not sharing a common superscript letter ($^{a–d}$) differ significantly at $p < 0.05$ (DMRT).

Fig. 11 Effects of DATS on As concentration in heart tissue (Fig. 11A) and heart mitochondria (Fig. 11B) of arsenic treated rats . Values not sharing a common superscript letter ($^{a–d}$) differ significantly at $p < 0.05$ (DMRT). ND mean non-detectable.

Fig. 12 Effects of DATS and As on the ultra structure of heart mitochondria. Heart of control rats showed normal structural design and it is well preserved (Fig. 12 A). Heart mitochondria isolated from the rat treated with As shows swollen mitochondria and disrupted cristae with vacuolation (Fig. 12 B). Pre-treatment of DATS to As intoxicated rats show mild

swollen and vacuolated heart mitochondria with mild separated cristae when compared with As alone treated rats (Fig. 12 C). In DATS alone treated rats the heart mitochondria shows well preserved and normal architecture of the heart mitochondria (Fig. 12 D).

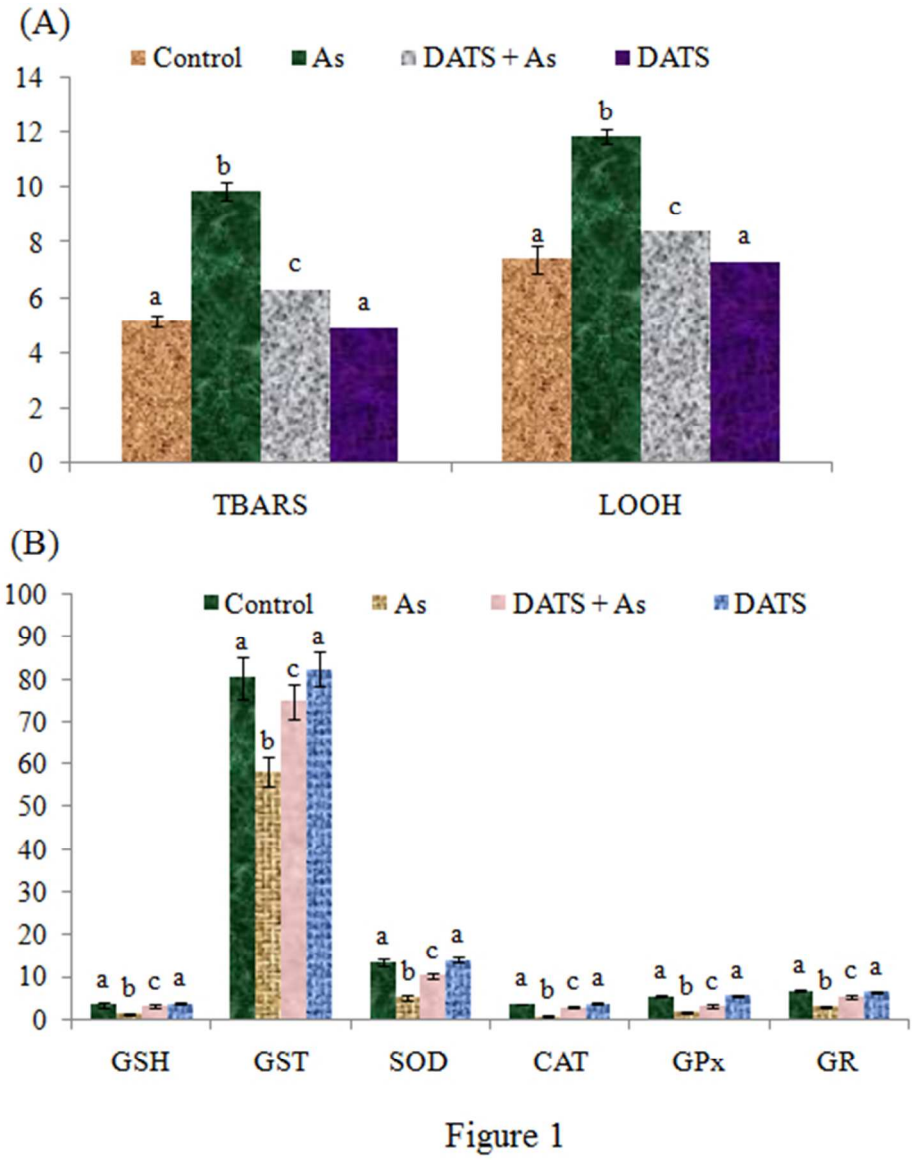

Figure 1

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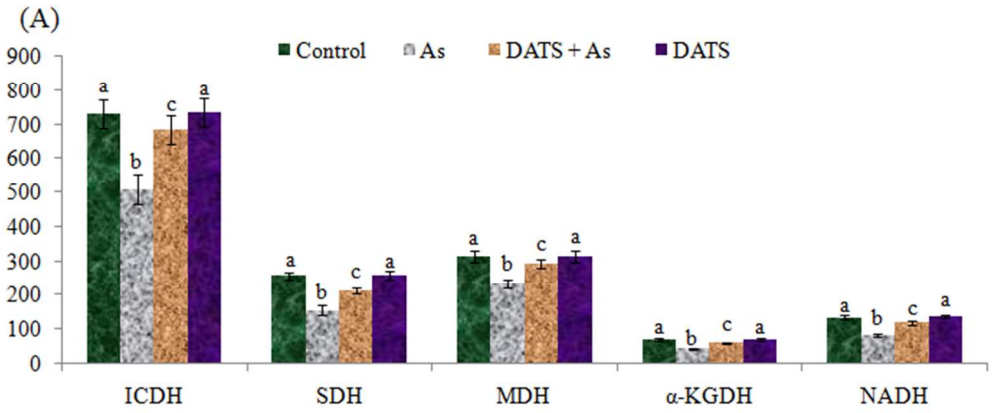

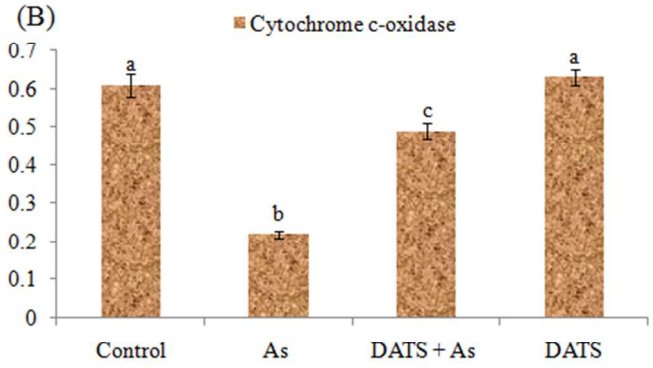

Figure 2

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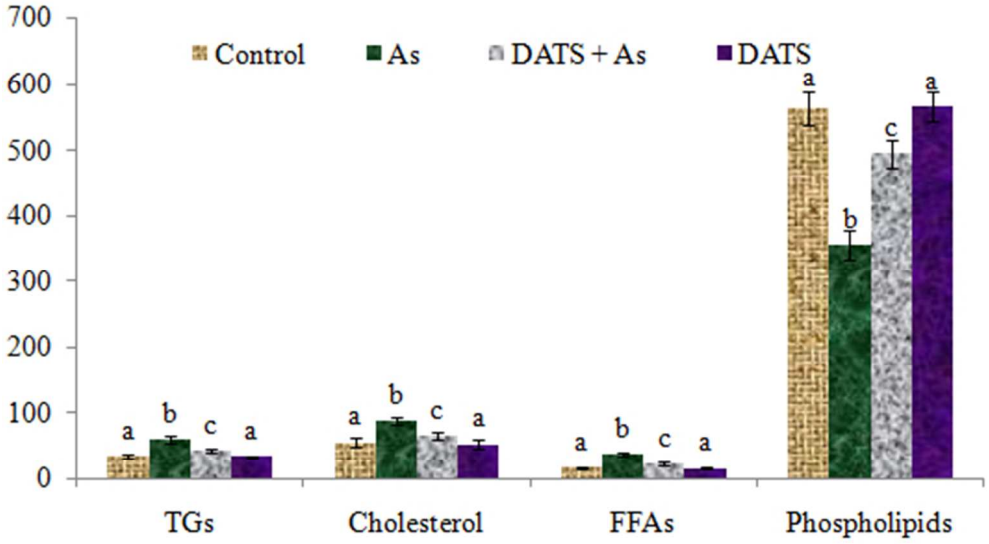

Figure 3

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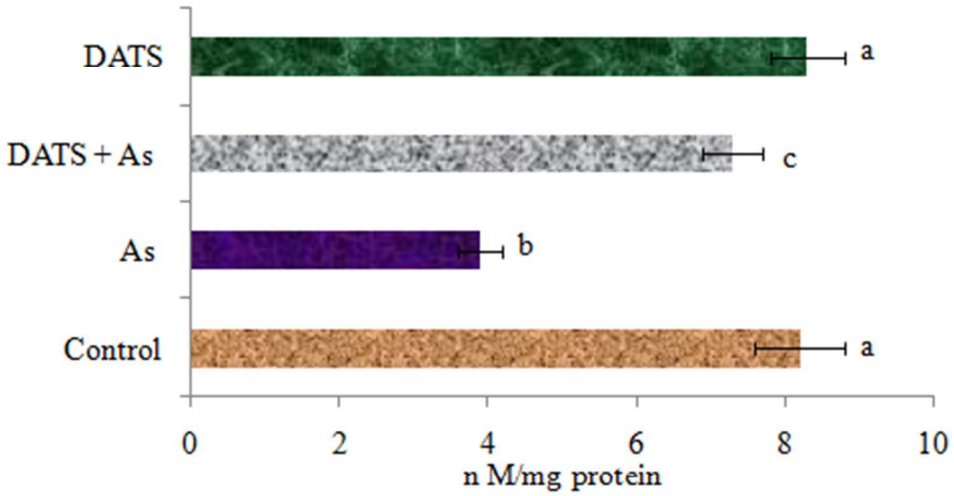

Figure 4

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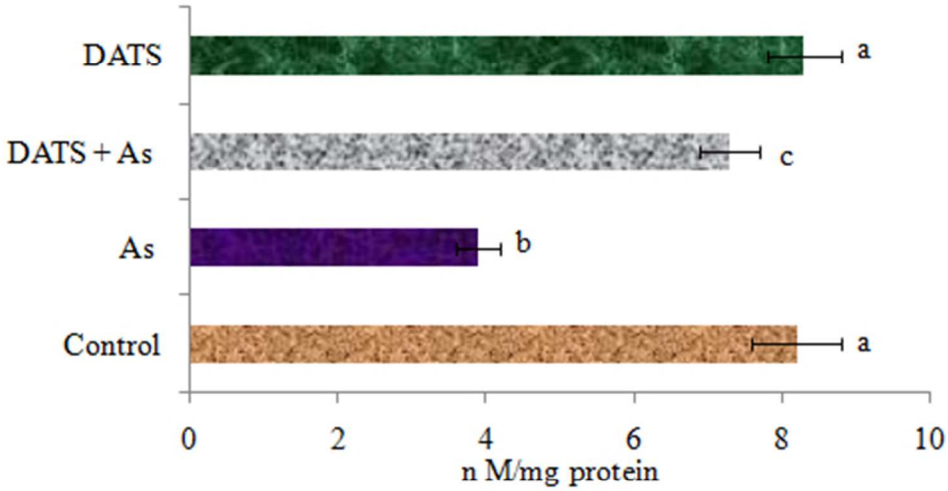

Figure 5

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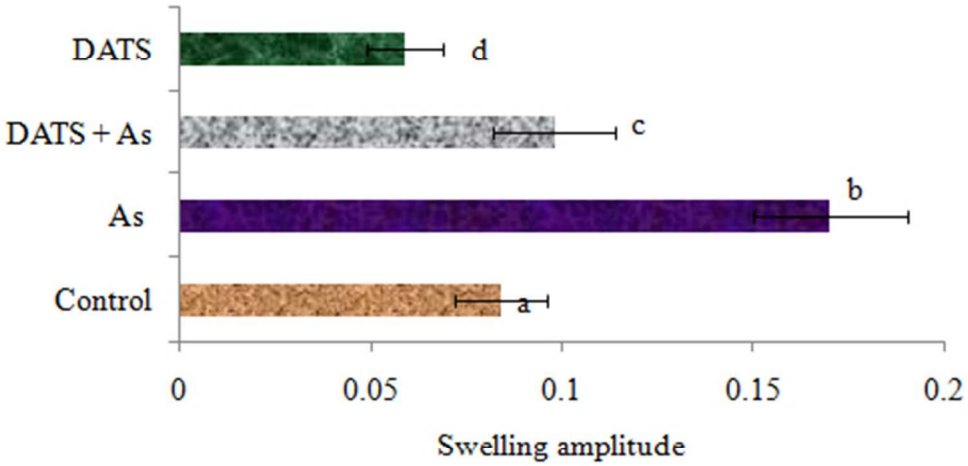

Figure 6

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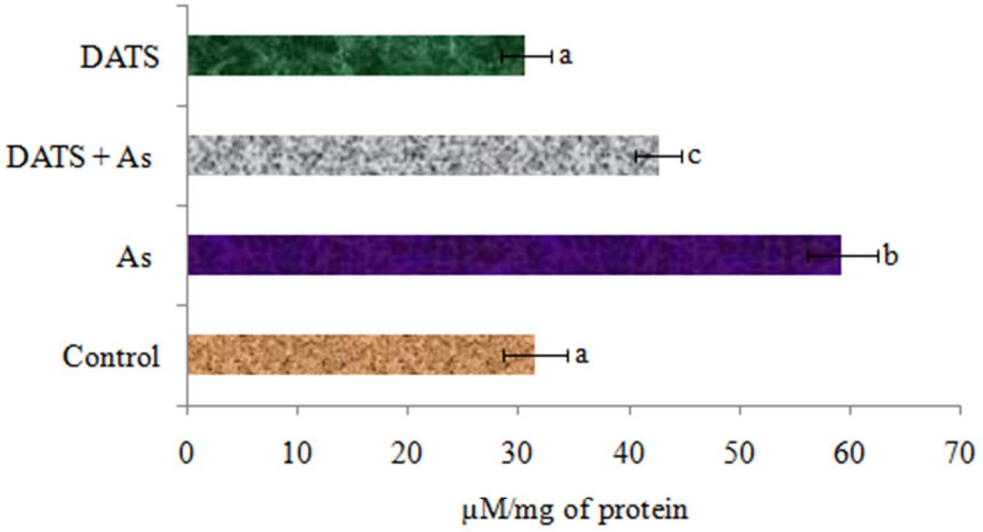

Figure 7

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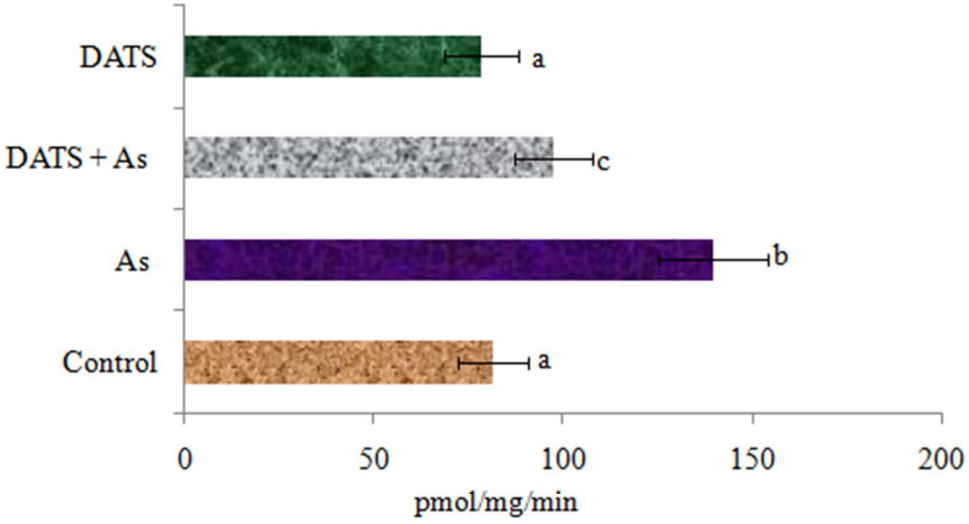

Figure 8

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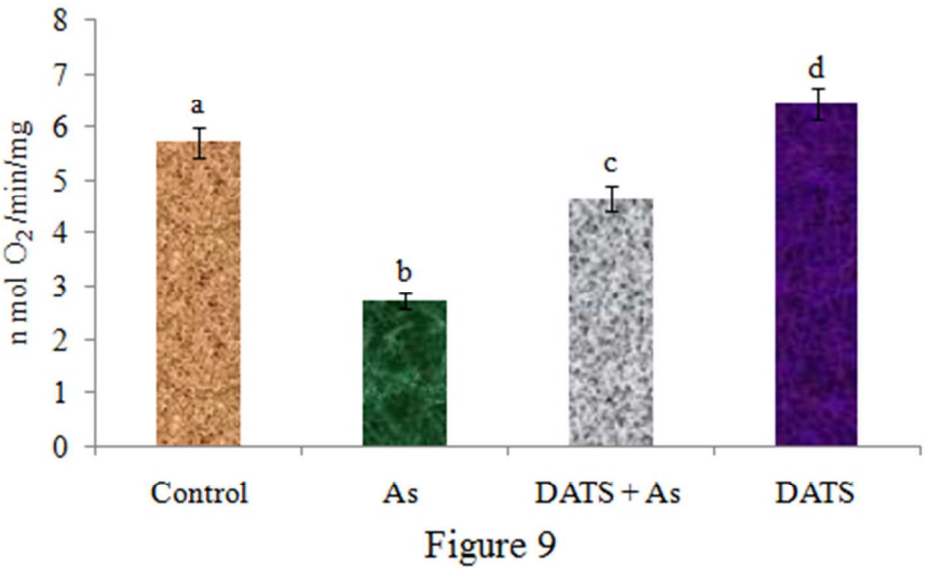

Figure 9

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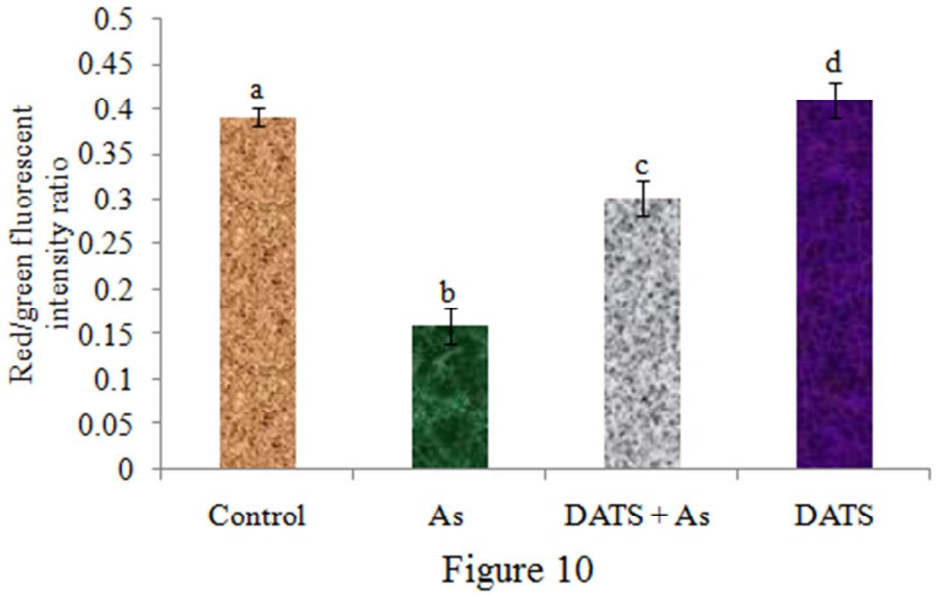

Figure 10

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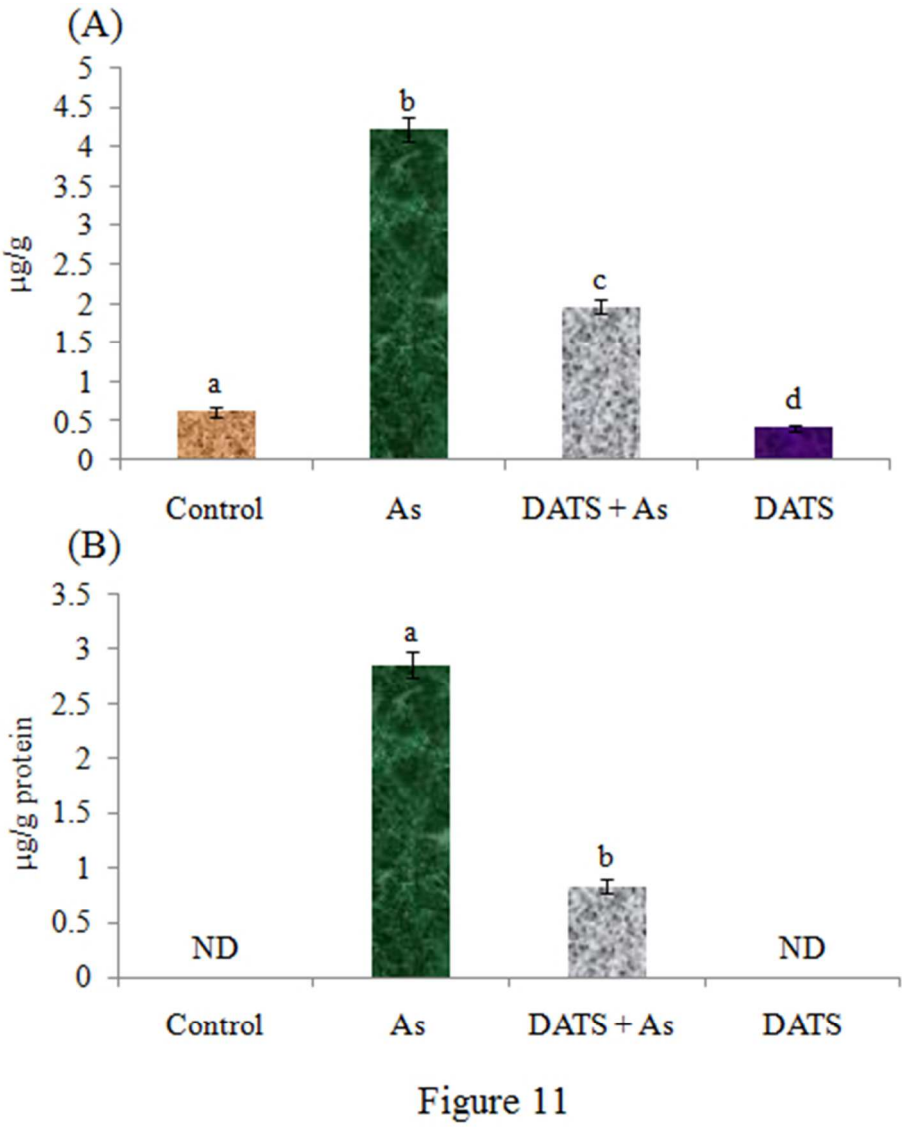

Figure 11

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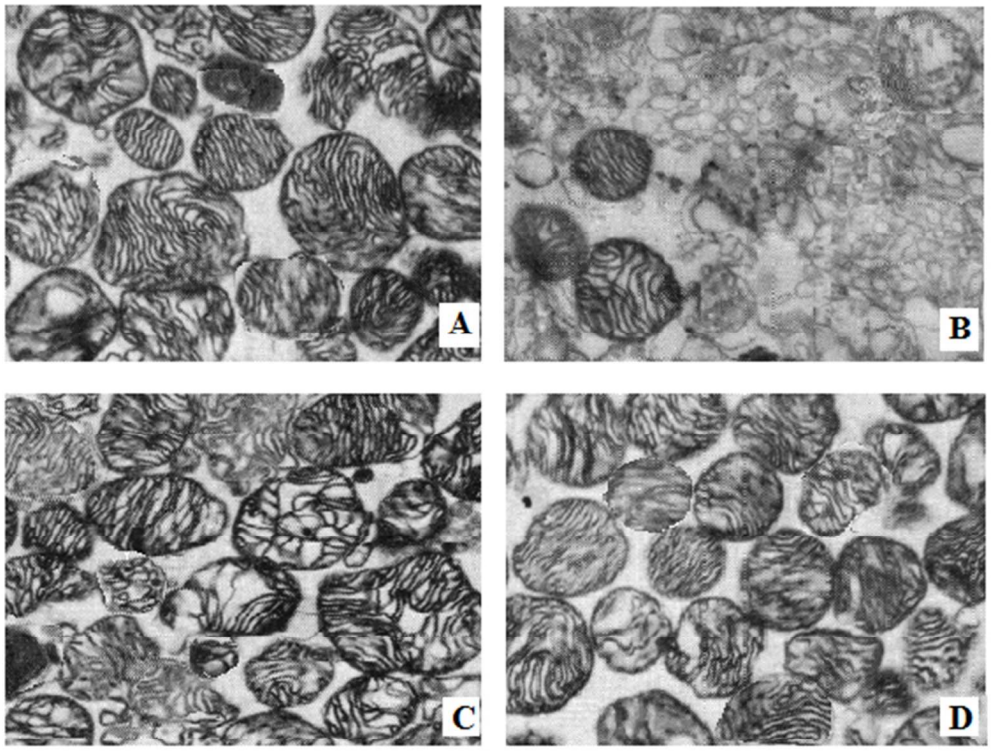

Figure 12

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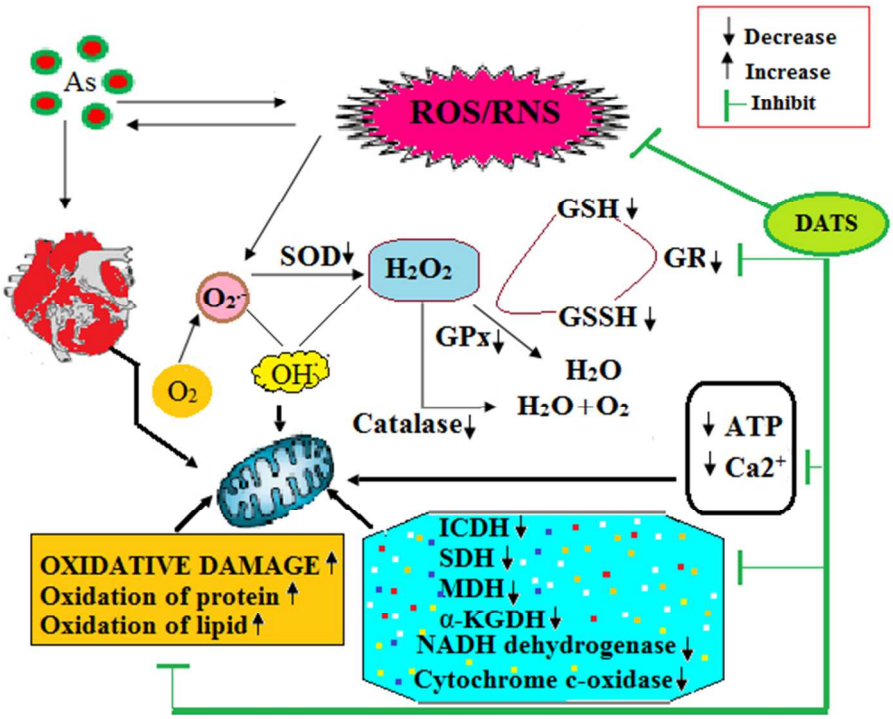

Figure 13