

**Hepatoprotective activity of liposomal flavonoid against  
arsenite induced liver fibrosis**

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**Running title :** Liposomal flavonoid protects arsenite induced liver fibrosis

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### **Abbreviations list**

Quercetin, QC; Sodium arsenite, NaAsO<sub>2</sub>; Reactive oxygen species, ROS; Superoxide dismutase, SOD; Phosphatidyl ethanolamine, PE; Dicyetyl phosphate, DCP; Diphenyl hexatrine, DPH; Serum aspartate transaminase, AST; Alkaline phosphatase, AP; High-performance liquid chromatography, HPLC; Glutathione peroxidase, GPx; Glutathione, GSH; Glutathione reductase, GR; Glutathione-S-transferase, GST; Glucose-6-phosphate dehydrogenase, G6PDH; 4-hydroxy proline, 4-HP.

**Section:** Toxicology

**Abstract**

Arsenic, the environmental metalloid toxicant, is known to induce oxidative damage to liver and produces hepatic fibrosis. The theme of our study was to optimise and evaluate the therapeutic efficacy of galactosylated liposomal flavonoidal antioxidant [Quercetin (QC)] in combating arsenic induced hepatic fibrogenesis. The rats of the hepatic damage group were injected (s c) a single dose of sodium arsenite ( $\text{NaAsO}_2$ ) ( $100.06 \mu\text{M} / \text{kg b wt}$  in 0.5 ml of physiological saline). Hepatocytes and stellate cells were separated. Mitochondrial membranes were isolated from all those separated cells. Oxidative damage was monitored at different isolated sub-cellular parts of different hepatic cells. Liver fibrosis was also induced by the injection of  $\text{NaAsO}_2$ . Galactosylated liposomal QC injection prior to  $\text{NaAsO}_2$  treatment checked fibrogenesis completely by protecting the liver from oxidative attack in cellular and subcellular levels. The maximum protection from hepatocellular and fatty metamorphosis, necrosis, Kupffer cell hyperplasia, fibrosis and in the deposition of collagen contents were observed and reconfirmed by our histopathological and histochemical analysis when rats were treated with galactosylated liposomal QC prior to  $\text{NaAsO}_2$  injection. Application of galactosylated liposomal QC may be a potent therapeutic approach for  $\text{NaAsO}_2$  induced fibrogenesis through a complete protection against oxidative attack in cellular and subcellular parts of rat liver.

## **Introduction**

Arsenic the naturally occurring metalloid has been recognized as environmental toxicant and its contamination of drinking water is a serious environmental calamity worldwide because of the million of people at risk, particularly in developing countries such as West Bengal in India and Bangladesh (Chatterjee et al., 1995). An important feature in arsenic toxicity is in a form of hepatic fibrosis that causes portal hypertension (Guha Mazumder et al., 1998). Although mechanism(s) by which arsenic induces hepatic fibrosis remains poorly understood, however, the common theme that was emerged is the role of reactive oxygen species (ROS) in the pathogenesis of arsenic-induced hepatic fibrosis. Hepatocyte necrosis itself may activate lipocytes. Such activation could be mediated by the lipid peroxides formed after the membrane of the hepatocyte is injured (Greenwel et al., 2000). The critical event in hepatic fibrosis is the activation of lipocytes (Stellate, fat storing or Ito cell), the main source of extracellular matrix in fibrosis formation (Du et al., 1999).

Complementary approach that delineates the  $\text{NaAsO}_2$  induced contribution of ROS includes the use of antioxidants, superoxide dismutase (SOD) and catalase. But introduction of either SOD or catalase can only suppress a little part of toxic oxygen species that is generated by the induction of  $\text{NaAsO}_2$  (Kessel et al., 2002).

Non-enzymatic supplementation as a protective strategy has also resulted in conflicting outcomes in in vivo experiments. Administration of D- $\alpha$ -tocopherol to rats provided no protection against ROS in liver diseases. So the present challenge is to counter  $\text{NaAsO}_2$  induced oxidative attack and to isolate a non-toxic antioxidant that can be selectively targeted to liver, which provide an even distribution in intra hepatic membrane.

Liposomes are accepted as potent drug carriers not only for its biocompatible nature but also those phospholipid vesicles do not elicit negative biological responses that generally occur when a foreign material is introduced in the system. Furthermore, owing to the presence of galactosyl receptor on the surface of hepatocytes, galactosylated liposomes are effective in the site-specific drug delivery to hepatic tissue with a homogenous intrahepatic membrane distribution of its intercalated components (Sinha et al., 2000). The mammalian liver consists primarily of hepatocytes and stellate cells. The membrane composition of liposome is crucial for its targeting and function. It was observed that galactosylated liposomes entrapped material was largely taken up by hepatocytes. The high endocytic activity of sinusoids lining cells makes them most competent to internalise colloidal particles like liposomes. So, natural targeting of liposomes takes place by those cells and it is reasonable to assume that galactosylated liposome administration increases intracellular accumulation of vesicular content in hepatocytes (Manajit et al., 2005).

Quercetin, the most abundant flavonoid in nature, presents in large amounts in vegetable, fruits, tea and olive oil and as it contains a numbers of phenolic hydroxyl groups, it exhibits its therapeutic potential against many diseases, including ischemic heart diseases, atherosclerosis, liver fibrosis, renal injury and in chronically biliary obstruction (Peres et al., 2000; Sing et al., 2004; Tokyol et al., 2006; Lee et al., 2003). In the light of the above observations it seems reasonable to expect that administration of QC in galactosylated liposomes might contribute to reduce NaAsO<sub>2</sub> induced oxidative damage in cellular or sub-cellular parts of hepatic tissue and provide a protective mechanism against liver fibrosis.

Despite the fact that rats unlike other mammals retain the dimethyl arsinic acid, one of the intermediate metabolites of  $\text{NaAsO}_2$  in the blood, studies were performed in this report on rats because this species is known superior to others in forming monomethyl arsonous acid (Csanaky and Gregus, 2002). Monomethyl arsonous acid is most toxic among other  $\text{NaAsO}_2$  metabolic intermediates to rat as well as human hepatocytes (Styblo et al., 2000).

The purpose of our in vivo studies is to ascertain whether treatment with QC intercalated in galactosylated liposomes exerts any hepatoprotective effect against  $\text{NaAsO}_2$  induced fibrosis in liver.

**Materials and methods*****Materials***

Phosphatidyl ethanolamine (PE), cholesterol, dicetyl phosphate (DCP), p-amino phenyl  $\alpha$ -D-galactoside, glutaraldehyde, collagenase, diphenyl hexatriene (DPH), triton x-100, glutathione reductase, were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium arsenite from E.Merck, Germany was used for experimental purpose. Chloramine-T, Fast Green FCF and Sirius Rose BB were acquired from Loba-Chemie and Fluka respectively whereas chloroform and methanol were from E.Merck (India) Ltd. Quercetin was isolated and purified from Fagopyrum esculentum (buck wheat). All other reagents were of analytical grade.

***Preparation of liposomal QC and Coupling of p-aminophenyl  $\alpha$ -D galacto-pyranoside with liposomes intercalated with QC***

Multilamellar liposomes were prepared with Phosphatidyl Ethanolamine (PE), Cholesterol, Dicetyl Phosphate (DCP) and Quercetin (QC) in molar ratio 7:1:1:1 (Budai and Szoqvi, 2001). In short PE, Cholesterol, DCP and QC are dissolved in chloroform and methanol mixture (2:1, v/v) in a round bottom flask. The lipid film was made by drying the organic solvents and was desiccated over night. The thin film was swollen in PBS (pH 7.2) for 1 h and sonicated in a probe type sonicator for 30 s.

Liposomal QC was coupled with p-aminophenyl  $\alpha$ -D galacto-pyranoside by using glutaraldehyde as the coupling agent (Mitra et al., 2005).

***Animal and Experimental design***

Adult male Swiss Albino rats, each weighing 120-150g were acclimatized to conditions in the laboratory (26-28°C, 60-80% relative humidity, 12 h light/dark cycle) for 10 days prior to the commencement of the treatment during which they received food (purchased from Hindustan Lever) and drinking water. Rats were divided into six groups with 5 animals in each group. Rats in the normal group were injected subcutaneously (s c) with a single dose of physiological saline (0.5ml) at multiple sites. Rats in the NaAsO<sub>2</sub> treated group were injected (s c) with NaAsO<sub>2</sub> (100.06 µM/kg body weight in 0.5 ml physiological saline) at five different sites of its abdominal skin by making a 10 s time gap between one injection to its next. Free drug (0.5 ml suspension of 0.2% tween 80 aqueous solution containing 0.898 µM QC) was injected into tail vein of third group of rats 2 h before NaAsO<sub>2</sub> treatment. Liposome with QC (0.5 ml suspension containing 0.898 µM QC), empty galactosylated liposome or galactosylated liposome intercalated with QC (0.5 ml suspension contain 0.222, 0.445, 0.898 or 1.335 µM QC) was injected into tail vein of rats in those different experimental groups 2 h before NaAsO<sub>2</sub> treatment. QC uptake by liver was estimated in the other groups of rats after 2 h of injection of free QC, liposomal QC or galactosylated liposomal QC.

For LD<sub>50</sub> determination, a single dose of NaAsO<sub>2</sub> (84.67-130.8 µM/kg body weight) was injected to each group of rats subcutaneously.

Twenty-four hours after NaAsO<sub>2</sub> administration the rats of all groups were anaesthetized with ether and blood was collected from heart. Serum aspartate transaminase (AST), alkaline phosphatase (AP) (Karim et al., 2001; Tyagi et al., 2005) and serum urea and creatinine were determined using a standard kit. Animals were



sacrificed immediately after collecting blood. The liver was promptly removed and a part of the organ was immediately fixed in Bouin's fixative and processed for histological examination. All the rats used in this study received proper care in compliance with animal Ethics Committee, India. Liver histochemistry for collagen and liver tissue histology were studied by microscopic examination (Lin et al., 2005).

Perfusion of liver and separation of sub-cellular fractionations were done (Sinha et al., 2000). Mitochondria were separated and purified with Percoll gradient. The purity and yield of the mitochondrial membrane fractions were assayed using standard subcellular markers (Tirmenstein and Nelson, 1989).

### ***Biochemical analysis and enzyme assays***

#### *Lipid peroxidation Assay*

Lipid peroxidation in the mitochondrial membrane was determined by measuring the amount of conjugated diene. The mitochondrial membrane was extracted twice in chloroform-methanol mixture (2:1 v/v). The pooled extract was evaporated to dryness under nitrogen atmosphere at 25°C and redissolved in cyclohexane. Lipids in cyclohexane solvent was assayed at 234 nm and the results were expressed as  $\mu\text{mol}$  of lipohydroperoxide / mg protein by using an  $\epsilon$  m of  $2.52 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$  (Mandal et al., 2002).

#### *Quantitation of Quercetin level in liver*

Liver homogenates of normal and experimental rats were diluted with an equal volume of absolute ethanol containing 1  $\mu\text{g/ml}$  butylated hydroxyanisole. One ml of n-heptane

was added and the whole suspension was vortexed. This sample was centrifuged at 1000 rpm for 5 min at 4°C. The heptane layer was removed and another 1 ml of fresh n-heptane added. The sample was centrifuged as earlier until three volumes of heptane had been added. These volumes were combined and dried under nitrogen atmosphere. The residue was dissolved in 0.2 ml of methanol and 20 µl was injected onto a high-performance liquid chromatography (HPLC) column and quantitation of Quercetin in liver homogenate was performed spectrophotometrically (Mandal and Das, 2005).

#### *Different enzyme activities*

Rat liver was perfused with the perfusion buffer (125 mM NaCl, 2.47 mM KH<sub>2</sub>PO<sub>4</sub>, 15 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NH<sub>4</sub>Cl, 34.01mM CaCl<sub>2</sub>.2H<sub>2</sub>O, 5mM glucose, 0.2% BSA having a pH 7.2) containing collagenase and CaCl<sub>2</sub> at 37°C through the portal vein of the animal for 10 min at a rate of 10 to 15 ml/min. The tissue was chopped, homogenized in ice-cold perfusion buffer containing BSA (2g/L) and filtered through a nylon gauge (110 mesh). The cell suspension was centrifuged at 50xg for 4 min. The pellet was resuspended, washed and collected as hepatocytes. The supernatant was then centrifuged at 500xg for 9 min to precipitate stellate cells. The isolated stellate cells were further purified with Percoll gradient. The cells were resuspended in 9.3 ml of perfusion buffer and mixed with 12.7 ml of Percoll containing 1/10 volume of 10-fold concentrated perfusion buffer. After centrifugation at 20,000xg for 10 min at 4°C, the cell layer above the 1.07 gradient was carefully recovered. Thus, stellate cells were collected and resuspended in buffer. The hepatocytes and stellate cell suspensions were homogenized at 4°C with 10 up and down

strokes in a glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 105,000 g for 60 min to get cytosolic fractions.

#### *Catalase activity*

The cytosolic fractions, thus obtained, were used for enzyme activities. Catalase activity was assayed (Moragon et al., 2005). The reaction mixture contained sodium phosphate buffer (0.05 M, pH 7.0), 50 mmol/L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and 50 µl of enzyme extract in a 3 ml volume. The activity was assayed by monitoring the decrease in absorbance at 240 nm as a consequence of H<sub>2</sub>O<sub>2</sub> consumption and enzyme activity expressed as amount of H<sub>2</sub>O<sub>2</sub> decomposed per min per mg of protein.

#### *Glutathione peroxidase (GPx) activity*

The GPx activity was determined (Sarkar and Das, 2006). The cytosol, containing the enzyme source was mixed with 0.25 M potassium phosphate buffer, 25 mM EDTA, glutathione reductase, 40 mM glutathione (GSH), 20mM NADPH. The mixture was mixed and then incubated for 2 min at 37°C. The reaction was initiated by adding t-butyl hydroperoxide at the final concentration of 0.3mM. The mixture was stirred and the absorbance was read immediately at 340 nm at 1-min intervals for 4 min. The absorbance change during the 2 to 4 min interval was used to calculate enzyme activity. The activity was determined and expressed as µmol NADPH oxidized/min/mg protein.

#### *Glutathione reductase (GR) activity*

GR was assayed (Castro et al., 1990). A 3 ml reaction mixture contained 100 mM phosphate buffer (pH 7), 1 mM GSSG, 1mM EDTA, 0.1 mM NADPH, and 25 to 50 µl

enzyme extract. The reaction was started by adding the enzyme extract. The rate of NADPH oxidation was followed by monitoring the decrease in absorbance at 340 nm with a recording spectrophotometer. The activity was expressed as  $\mu\text{mol}$  of NADPH oxidation/min/mg protein.

*Glutathione – S – transferase (GST) activity*

GST activity was determined in a total volume of 1.0 ml, containing 100mM potassium phosphate buffer (pH 6.5) and 2 mM each of GSH and 1-chloro-2,4-dinitrobenzene (final concentration). The rate of formation of S-2,4-dinitrophenylglutathione (a GSH-1-chloro-2,4-dinitrobenzene conjugate) by enzyme extract was quantified at 340nm using the extinction coefficient of  $9.6 \text{ L mmol}^{-1} \text{ cm}^{-1}$  (Maiti and Chatterjee, 2000) and the activity was expressed as  $\mu\text{mol/min/mg protein}$ .

*Glucose – 6-phosphate dehydrogenase (G6PDH) activity*

The G6PDH activity was determined using a Sigma Diagnostics Kit which is based on a modification of Spectrophotometric method (Maiti and Chatterjee, 2000). G6PDH catalyses the first step in the pentose phosphate pathway, oxidising glucose-6-phosphate to 6-phosphogluconate and reducing nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. The rate of formation of NADPH is proportional to G6PDH activity and was measured spectrophotometrically as an increase in absorbance at 340 nm. One unit of G6PDH activity was defined as  $1\mu\text{M}$  NADPH produced per min.

***Fluorescence Depolarisation Measurements of the fluidity of mitochondrial membrane***

The fluorescence depolarisation, associated with the hydrophobic fluorescence probe DPH, was used to monitor the changes in the fluidity of the lipid matrix accompanying the gel to liquid crystalline phase transition. Mitochondrial membrane fractions of hepatic cells were incubated at 37°C by the addition of DPH dissolved in tetrahydrofuran (DPH/lipid molar ratio 1:500). The excitation and the emission maxima were kept at 365 and 430 nm respectively. The fluorescence anisotropy was calculated by using the equation

$$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp}) \dots\dots\dots(1)$$

where  $I_{\parallel}$  and  $I_{\perp}$  are the fluorescence intensities in parallel and perpendicular to the direction of polarization of the excited light. The micro viscosity parameters  $[(r_0/r)-1]^{-1}$  were calculated in each case, knowing the maximal limiting fluorescence anisotropy  $r_0$  which for DPH is 0.362 (Sarkar et al., 2002).

***Estimation of hepatic 4-hydroxyproline (4-HP)***

The liver was cut into small pieces and homogenised in sufficient deionized water to yield 10% homogenate (W/V). Aliquots (2 ml) of the homogenate were added to an equal volume of 12 N HCl and hydrolysed in Teflon capped vials at 105°C for 18 h. 4-HP levels from those hydrolysates were measured (Sarin et al., 1999). The absorbance at 558 nm was read and values were plotted against a standard graph using known amounts of 4-HP.

***Estimation of total hepatic collagen***

The technique (Sarin et al., 1999) was used which is based on selective binding of Sirius Rose BB and Fast Green FCF to collagen and non-collagen components, respectively. When the sections are stained with both dyes dissolved in aqueous saturated picric acid, they are eluted readily with NaOH and simultaneously the absorbance obtained at 550 and 625 nm can be used to determine the amount of collagen and total protein respectively.

***Arsenic analysis***

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 (Flora et al., 1995).

***Statistical analysis***

Statistical analysis was performed with student's t-test. Linear correlation between two data was calculated by means of Pearson's correlation coefficient  $r$ . In all instances,  $P < 0.05$  was considered as the minimum level of significance.

## **Results**

### ***Acute NaAsO<sub>2</sub> induced mortality in rats***

A single injection of NaAsO<sub>2</sub> produced a dose dependent mortality in rats (Fig.1). Arsenite induced mortality at a dose of arsenic higher than 84.67µM/kg b wt and no rats survived the dose of 130.8 µM/kg. The LD<sub>50</sub> value for arsenic-induced lethality in the rats was 107.75µM/kg.

### ***Effect of QC in galactosylated liposome on arsenic deposition in mitochondria of different hepatic cells***

A single dose of NaAsO<sub>2</sub> injection (s.c.) (100.06µM/kg b wt) resulted a deposition of arsenic in mitochondria of hepatocytes and stellate cells in the level of 264±24 and 252±18 µg/g mitochondrial protein. The elevated arsenic level in experimental animals was found reduced maximally by the treatment with QC in galactosylated liposomes (Fig.2a&2b) where arsenic content was not detected by atomic absorption spectrophotometer in normal rats.

### ***Correlation of mitochondrial arsenic content of hepatocytes and stellate cells with lipid peroxidation***

Figure 3a shows positive correlation ( $r = 0.97$ ,  $P < 0.001$ ) between hepatocytic mitochondrial arsenic level and its conjugated diene level. The increased stellate mitochondrial arsenic level was also found to be associated with its increased lipid

peroxidation as was evident from a significant positive correlation ( $r = 0.93$ ,  $P < 0.001$ )

(Fig.3b) between its arsenic and conjugated diene content.

***Effect of QC in galactosylated liposomes on NaAsO<sub>2</sub> induced antioxidant enzymes, protection in hepatocytes and stellate cells***

A single injection of NaAsO<sub>2</sub> (100.06µM/kg b wt) resulted significant depletion of antioxidant level both in hepatocytes and stellate cells (Table-1). No significant protection was observed in the case of rats treated with free QC prior to arsenic treatment. Liposomal QC treatment prevented thiol depletion in stellate cells completely. But QC in galactosylated liposomes treatment prevented the depletion completely both in hepatocytes and stellate cells.

***Effect of QC in galactosylated liposome treatment on NaAsO<sub>2</sub> induced hepatic 4-hydroxy proline (4-HP) and collagen contents in rat liver***

A single injection of NaAsO<sub>2</sub> (100.06µM/kg b wt) induced an appreciable increase in hepatic collagen protein and 4-HP (Table-2). These increments of collagen and 4-HP were not reduced more by the treatment of free QC whereas these levels were decreased appreciably by the treatment of liposomal QC. QC in galactosylated liposome injection inhibited completely the collagen protein deposition and 4-HP increase in rat liver.



***Effect of QC entrapped galactosylated liposomes on NaAsO<sub>2</sub> mediated hepatocellular toxicity***

Rats treated with a single dose of NaAsO<sub>2</sub> developed significant hepatic damage as observed from elevated hepatospecific enzymes in serum. The activity of AST and AP in serum was increased in NaAsO<sub>2</sub> intoxicated animals. The serum urea and creatinine levels were also increased upon NaAsO<sub>2</sub> administration. A single injection (i v) of liposome entrapped QC exerted a significant protection compared with free QC against NaAsO<sub>2</sub> induced liver toxicity. The degree of protection was observed maximally when galactose coated liposomal QC was injected (Table-3).

***Effect of galactosylated liposomal QC on NaAsO<sub>2</sub> induced mitochondrial membrane microviscosity of hepatocytes and stellate cells***

Arsenite induced a decrease of mitochondrial membrane microviscosity both in hepatocytes and stellate cells of rat liver. Liposomal QC treatment prevented the alteration in mitochondrial membrane microviscosity of stellate cells whereas QC in galactosylated liposome treatment protected mitochondrial membrane of both hepatocytes and stellate cells completely from any decrease mediated by NaAsO<sub>2</sub> treatment (Table-4).

***Quantitation of Quercetin in liver homogenate***

In Table-5 QC levels were expressed per mg protein. By determining the amount of protein present in total liver homogenate the amount of uptake of QC and QC entrapped in different types of liposomes by liver was calculated. For liposomal QC, the uptake of

QC in the liver was found to be 50.29% of the injected dose where as only 25.61% of the injected dose was detected in the liver when free QC was injected. For galactosylated liposomal QC the uptake in the liver was to be 85.16%.

### ***Pathomorphology and histochemistry of the liver***

Haematoxylin-Eosin-stained liver sections of normal rats showed (Fig.4a) the cords of normal hepatocytes, normal looking sinusoids lined by Kupffer cells. Central veins were normal. The histology was within normal limit. But the positive histological changes in the areas of hepatocellular and fatty metamorphosis, few focal areas of necrosis, Kupffer cell hyperplasia and localized fibrosis in the periportal region resulted from a single injection of NaAsO<sub>2</sub> (Fig.4b). The induction for mild hyperplasia of Kupffer cell and the histology within normal limit appeared in the case of NaAsO<sub>2</sub> intoxicated animals injected with galactosylated liposomal QC (Fig.4c).

Liver section of normal rats stained with Van Gieson exhibited very small amount of collagen in the periportal region (Fig.4d). But periportal region showed mild to moderate amount of collagen tissues when injected with a single injection of NaAsO<sub>2</sub> (Fig.4e). The maximum protection from the deposition of collagen contents was observed when rats were treated with galactosylated liposomal QC prior to NaAsO<sub>2</sub> injection (Fig.4f).

## Discussion

Exposure to the metalloid arsenic has become an increasingly recognized source of illness worldwide. Arsenic has a direct toxic effect on cellular respiration in liver mitochondria with an evidence of oxidative stress and hepatic collagenesis in human (Pi J, Yamauchi et al., 2002; Das et al., 2005). Hepatic fibrosis is a disease state characterized by exuberant synthesis and deposition of collagen in the extracellular matrix. Fibrogenesis is expressed by an increase in the hepatic hydroxyproline levels (Testa et al., 1993). The present observation also indicates that fibrogenesis could be induced with an increment of 4-HP by a single injection (s c) of NaAsO<sub>2</sub> (100.06µM/kg b wt) (Table –2).

The exact mechanism of fibrogenesis by arsenic is not known. Arsenic induces the production of lactic acid because of an imbalance in cellular energy metabolism and the enol acid thus utilized by cells significantly increases the intracellular proline pool and collagen synthesis by stimulating the activity of prolyl hydroxylase.

A greater number of drugs including arsenic specific antidotes have been tested to reduce hepatic damage or necrosis and to inhibit liver fibrogenesis. However, none of them is liver specific or cell type specific (Guha Mazumder et al., 2001). Quercetin, a polyphenolic flavonoidal compound has been suggested in preventing the development of hepatic fibrosis (Lee et al., 2003). It is also known to reduce toxicant induced liver damage (Peres et al., 2000). Protective role of QC in galactosylated liposomes against carbontetrachloride induced hepatocellular damage have been shown at our previous observation (Mandal and Das, 2005). Hepatospecific liposomal QC (galactosylated liposome) with a high incorporation rate in hepatocytes has been formulated by us and

tested in reducing NaAsO<sub>2</sub> induced liver fibrogenesis and hepatocellular damage. The increased hepatoprotective effect of galactosylated liposomes encapsulated QC compared to its free form that has been shown in this report against NaAsO<sub>2</sub> induced liver damage could be explained by the observation of another group (Datta et al., 2003). They demonstrated that liposome entrapped compound interact with target cells at a much faster rate than that of free components. Moreover, by utilizing the galactosyl receptor of hepatocytes, galactosylated liposomes could be targeted to those non- phagocytic cells (Kawakami et al., 2001) with a simultaneous delivery to other types of hepatic cells of natural phagocytic activity.

Arsenic toxicity was reported as an inhibitory effect on cellular respiration at the level of mitochondria (Stanton et al., 2006). In our observations, the increased arsenic deposition in hepatocytes and stellate cells mitochondria from NaAsO<sub>2</sub> treated rats are generating more ROS than normal animals (Fig.2; Table-1). When arsenic deposition in mitochondria is prevented by galactosylated liposomal QC treatment, NaAsO<sub>2</sub> induced fibrogenesis is markedly reduced. Maintenance of mitochondrial membrane microviscosity i.e. reciprocal of membrane fluidity of hepatocytes and stellate cells could be achieved by the protective action of QC in galactosylated liposomes in cases where significant fall of mitochondrial membrane microviscosity takes place by the induction of NaAsO<sub>2</sub> (Table-4).

Previous investigators have shown a deviation of the pro-oxidant/antioxidant balance in NaAsO<sub>2</sub> treated rats with development of oxidative damage and a reduction in antioxidant status (Das et al., 2005). We also observed that liver injury was accompanied by the accumulation of arsenic with impaired activity and depletion of antioxidant status

in NaAsO<sub>2</sub> induced rats. Reduction of liver injury was noticed by the improvement of antioxidant status with an increased concentration of QC and a marked reduction of arsenic content in liver by QC in galactosylated liposomes.

Our results indicate that galactosylated liposomal QC prevents arsenic deposition and protects liver from NaAsO<sub>2</sub> induced collagen deposition and fibrogenesis (Fig.2, Table-2). Administration of QC in galactosylated liposomes to rats protects those animals from arsenic induced liver fibrosis while free QC does not. It is expected that QC in galactosylated liposomes may be more protective than free QC or liposomal QC because of the enhanced intracellular accumulation of QC by selective tissue targeted delivery of galactosylated liposomes (Table-5). We demonstrated a positive correlation between arsenic accumulation and lipid peroxidation level in liver (Fig.3) as earlier report (Ramos et al., 1995). A single injection (s c) of NaAsO<sub>2</sub> (100.06µM/kg) to rats resulted impairment in antioxidant status with a marked increase in arsenic content in liver. Our observation suggests that the mechanism of the protective effect of galactosylated liposomal QC against arsenic induced liver injury could be related primarily to the reduction of arsenic accumulation in liver.

As the levels of AST and AP increase in NaAsO<sub>2</sub> induced hepatocellular injury, it appears that targeting of QC prevents hepatic cells against NaAsO<sub>2</sub> induced membrane damage by decreasing the leakage of AST and AP in the circulation (Table-3). The protective role of QC in galactosylated liposomes against NaAsO<sub>2</sub> induced hepatotoxicity has also been confirmed by our histopathological and histochemical analysis (Fig.4). This approach of delivering a non-toxic herb origin polyphenolic compound QC selectively to

the liver might be useful in therapeutic application to prevent NaAsO<sub>2</sub> induced liver fibrogenesis.

Further studies are planned to perform sub-chronic and chronic studies, in order to substantiate our claims that QC might be useful in therapeutic application in combating NaAsO<sub>2</sub> induced fibrogenesis resultant from chronic arsenic exposure.

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## References

- Budai M and Szoqvi M (2001) Liposomes as drug carrier systems. Preparation, classification and therapeutic advantages of liposomes. *Acta Pharm Hung* **71**: 114-118.
- Castro VM, Soderstrom M, Carlberg I, Widersten M, Platz A and Mannervik B (1990) Differences among human tumor cell lines in the expression of glutathione transferases and other glutathione-linked enzymes. *Carcinogen* **11**: 1569-1576.
- Chatterjee A, Das D, Mandal BK, Chowdhury TR, Samanta G and Chakraborti D (1995) Arsenic in ground water in six districts of West Bengal, India: the biggest arsenic calamity in the world. Part 1. Arsenic species in drinking water and urine of the affected people. *Analyst* **120**: 643-650.
- Csanaky I and Gregus Z (2002) Species variations in the biliary and urinary excretion of arsenate, arsenite and their metabolites. *Comp Biochem Physiol C Toxicol Pharmacol* **131**: 355-365.
- Datta N, Mukherjee S, Das L and Das PK (2003) Targeting of immunostimulatory DNA cures experimental visceral leishmaniasis through nitric oxide up regulation and T-cell activation. *Eur J. Immunol* **33**: 1508-1518.
- Das S, Santra A, Lahiri S and Guha Mazumder DN (2005) Implications of oxidative stress and hepatic cytokine (TNF-alpha and IL-6) response in the pathogenesis of hepatic collagenesis in chronic arsenic toxicity. *Toxicol Appl Pharmacol* **204**: 18-26.

- Du WD, Zhang YE, Zhai WR and Zhou XM (1999) Dynamic changes of type  $\alpha_1(I)$  collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride induced rat liver fibrosis. *World J Gastroenterol* **5**: 397-403.
- Flora SJS, Dube SN, Arora U, Kannan GM, Shukla MK and Malhotra PR (1995) Therapeutic potential of meso 2,3-dimer-captosuccinic acid or 2,3-dimercaptopropane 1-sulfonate in chronic arsenic intoxication in rats. *Biometals* **8**: 11.
- Greenwel P, Dominguez-Rosals JA, Mavi G, Rivas-Estilla AM and Rojkind M (2000) Hydrogen peroxide: a link between acetaldehyde-elicited  $\alpha_1(I)$  collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells. *Hepatology* **31**: 109-116.
- Guha Mazumder DN, Das Gupta J, Santra A, Pal A, Ghosh A and Sarker S (1998) Chronic arsenic toxicity in West Bengal – The worst calamity in the World. *Ind Med Assoc* **96**: 4-7.
- Guha Mazumder DN, De BK, Santra A, Ghosh N, Das S, Lahiri S, et al. (2001) Randomized placebo-controlled trial of 2,3-dimercapto-1-propanesulfonate (DMPS) in therapy of chronic arsenicosis due to drinking arsenic-contaminated water. *Clinical Toxicol* **39**: 665-674.
- Karim S, Bhandari U, Kumar H, Salam A, Siddiqui MAA and Pillai KK (2001) Doxorubicin induced cardiotoxicity and its modulation by drugs. *Ind J Pharm* **33**: 203-207.
- Kawakami S, Munakata C, Fumoto S, Yamashita F and Hashida M (2001) Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs. *J Pharm Sci* **90**: 105-113.



- Kessel M, Liu SX, Xu A, Santella R and Hei TK (2002) Arsenic induces oxidative DNA damage in mammalian cells. *Mol Cell Biochem* **234-235**:301-308.
- Lee ES, Lee HE, Shin JY, Yoon S and Moon JK (2003) The flavonoid Quercetin inhibits dimethyl nitrosamine-induced liver damage in rats. *J Pharm Pharmacol* **55**: 1169-1174.
- Lin Y, Li HB and Huang JT (2005) Following the fate of murine epidermal Stem cells in a syngeneic dermal equivalent in vivo. *Zhong Zheng Xing Wai Ke Za Zhi* **21**: 452-456.
- Maiti S and Chatterjee AK (2000) Differential response of cellular antioxidant mechanism of liver and kidney to arsenic exposure and its relation to dietary protein deficiency. *Environ Tox Pharm* **8**: 227-235.
- Manajit C, Kawakami S, Yamashita F and Hashida M (2005) Effect of galactose density on asialoglycoprotein receptor-mediated uptake of galactosylated liposomes. *J Pharm Sci* **94**: 2266–2275.
- Mandal AK and Das N (2005) Sugar coated liposomal flavonoid: A unique formulation in combating carbontetrachloride induced hepatic oxidative damage. *J Drug Target* **13**: 305-315.
- Mandal AK, Sinha J, Mandal S, Mukhopadhyay S and Das N (2002) Targeting of liposomal flavonoid to liver in combating hepatocellular oxidative damage. *Drug Deliv* **9**: 181-185.
- Mitra M, Mandal AK, Chatterjee TK and Das N (2005) Targeting of mannosylated liposome incorporated benzyl derivative of *Penicillium nigricans* derived compound

MT81 to reticuloendothelial systems for the treatment of visceral leishmaniasis. *J*

*Drug Target* **13**: 285-293.

Moragon AC, De Lucas Garcia N, Encarnacion Lopez Fernandez M, Rodriguez-Manzaneque AS and Jimenez Fraile JA (2005) Antioxidant enzymes, occupational stress and burnout in workers of a prehospitalary emergency service. *Eur J Emerg Med* **12**: 111-115.

Peres W, Tunon MJ, Collado PS, Herrmann S, Marroni N and Gonzalez-Gallego J (2000) The flavonoid quercetin ameliorates liver damage in rats with biliary obstruction. *J Hepatol* **33**: 742-750.

Pi J Yamauchi H, Kumagai Y, Sun G, Yoshida T, Aikawa H, et al. (2002) Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. *Env Health Perspect* **110**: 331-336.

Ramos O, Carrizales L, Yanez L, Mejia L, Batres L, Ortiz D, et al. (1995) Arsenic increased lipid peroxidation in rat tissues by a mechanism independent of glutathione levels. *Environ Health Perspect* **103**: 85-88.

Sarin SK, Sharma G, Banerjee S, Kathayat R and Malhotra V (1999) Hepatic fibrogenesis using chronic arsenic ingestion : Studies in a murine model. *Ind J Expt Biol* **37**: 147-151.

Sarkar S and Das N (2006) Mannosylated liposomal flavonoid in combating age-related ischemia-reperfusion induced oxidative damage in rat brain. *Mech Age Develop* **127**: 391-397.

Sarkar S, Mandal S, Sinha J, Mukhopadhyay S, Das N and Basu MK (2002) Quercetin:

Critical evaluation as an anti leishmanial agent in vivo in hamsters using different vesicular delivery modes. *J Drug Target* **10**: 573-578.

Sing D, Chander V and Chopra K (2004) The effect of Quercetin, a biflavonoid on ischaemia /reperfusion induced renal injury in rats. *Arch Med Res* **35**: 484-494.

Sinha J, Das N and Basu MK (2000) Targeting of glycoside grafted liposomes to specific hepatocellular sites. *J Surface Sci Technol* **16**: 200-209.

Stanton CR, Thibodeau R, Lankowski A, Shaw JR, Hamilton JW and Stanton BA (2006) Arsenic inhibits CFTR-mediated chloride secretion by killifish (*Fundulus heteroclitus*) opercular membrane. *Cell Physiol Biochem* **17**: 269-278.

Styblo M, Del Razo LM, Vega L, Germolec DR, Le Cluyse EL, Hamilton GA, et al. (2000) Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Arch Toxicol* **74**: 289-299.

Testa R, Bindi P, Risso D, Borzone S, Cagliaris S, Bardellini E, et al. (1993) *Eur J Gastro Hepatol* **5**: 103.

Tirmenstein MA and Nelson SD (1989) Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3<sup>1</sup>-hydroxyacetanilide, in mouse liver. *J Biol Chem* **264**: 9814-9819.

Tokyol C, Yilmaz S, Kahraman A, Cakar H and Polat C (2006) The effects of Desferrioxamine and Quercetin on liver injury induced by hepatic ischaemia-reperfusion in rats. *Acta chir belg* **106**: 68-72.

Tyagi R, Lala S, Verma AK, Nandy AK, Mahato SB, Maitra A, et al. (2005) Targeted

delivery of arjunglucoside I using surface hydrophilic and hydrophobic nanocarriers

to combat experimental leishmaniasis. *J Drug Target* **13**: 161-171.

**Footnotes**

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## Legends for Figures

**Fig.1** Acute arsenic-induced lethality in the Albino Swiss rats. Rats (6 per group per dose) were given NaAsO<sub>2</sub> s c at the doses of 84.67 - 130.8μM/kg and the mortality was determined 24 h later.

**Fig.2** Arsenic concentrarion in mitochondria of hepatocytes (a) and stellate cells (b) of hepatic tissue. Values are mean ± SD for 5 rats. #P<0.001 significantly different from NaAsO<sub>2</sub> treated. A. Normal, B. NaAsO<sub>2</sub>-treated, C. B + empty galactosylated liposome treated, D. B + free drug treated, E. B + liposomal QC treated, F. B + galactosylated liposomal QC treated.

**Fig.3** Correlation of mitochondrial arsenic content of hepatocytes (a) and stellate cells (b) with lipid peroxidation. For a,  $Y = 0.1352X + 12.357$ ,  $r = 0.97$ ,  $P < 0.001$  whereas for b,  $Y = 0.2191X + 20.733$ ,  $r = 0.93$ ,  $P < 0.001$ . For a and b, □ NaAsO<sub>2</sub> treated (A), ■ (A) + empty galactosylated liposome treated, ◆ (A) + free drug treated, ◇ (A) + liposomal QC treated, ▲ (A) + galactosylated liposomal QC treated.

**Fig.4** Histopathological examination of eosin-hematoxylin stained liver section of normal and experimental rats with magnification x 400. (a) Physiological saline-treated control, (b) NaAsO<sub>2</sub>-treated, (c) NaAsO<sub>2</sub> + galactosylated liposomal QC (8.9μM/kg b wt) treated.

Histochemical examination of Van Gieson stained liver section of normal and experimental rats with magnification x 400. (d) Physiological saline-treated control, (e) NaAsO<sub>2</sub>-treated, (f) NaAsO<sub>2</sub> + galactosylated liposomal QC (8.9μM/kg b wt) treated.

↓ indicates fibrosis, ↓↓ indicates fatty metamorphosis, ↓↓↓ indicates necrosis.

Table-1. Effect of QC in free, liposome encapsulated and galactosylated liposome encapsulated forms on the changes in various components of antioxidant defence system in hepatocytes (HP) and Stellate (ST) cells of rat liver by the induction of NaAsO<sub>2</sub>.

	GPx $\mu$ -mol NADPH oxidation/mg protein		G6PDH n-mol NADP reduced/min/mg protein		GR $\mu$ -mol of NADPH oxidation/min/mg protein		Catalase $\mu$ mol H <sub>2</sub> O <sub>2</sub> reduced/min/mg protein		GST n-mol produced/min/mg protein	
	HP	ST	HP	ST	HP	ST	HP	ST	HP	ST
Normal	7.69 $\pm$ 0.31	9.25 $\pm$ 0.57	8.25 $\pm$ 0.41	12.31 $\pm$ 0.61	23.17 $\pm$ 1.56	34.32 $\pm$ 1.38	6.81 $\pm$ 0.13	9.22 $\pm$ 0.21	117.82 $\pm$ 19.23	134.92 $\pm$ 21.71
NaAsO <sub>2</sub> treated (A)	3.78 $\pm$ 0.17 *	4.55 $\pm$ 0.29 *	4.02 $\pm$ 0.22 *	6.05 $\pm$ 0.30 *	11.29 $\pm$ 0.92 *	16.71 $\pm$ 0.87 *	3.31 $\pm$ 0.08 *	4.49 $\pm$ 0.13 *	56.79 $\pm$ 9.09 *	68.25 $\pm$ 10.08 *
(A) + Empty galactosylated liposome treated	3.92 $\pm$ 0.12	4.76 $\pm$ 0.23	4.16 $\pm$ 0.24	6.35 $\pm$ 0.33	11.48 $\pm$ 0.36	16.92 $\pm$ 0.54	3.40 $\pm$ 0.02	4.61 $\pm$ 0.15	57.85 $\pm$ 7.31	69.62 $\pm$ 6.56
(A) + Free QC treated	4.47 $\pm$ 0.23	5.37 $\pm$ 0.26	4.78 $\pm$ 0.23	7.14 $\pm$ 0.38	13.38 $\pm$ 1.71	19.73 $\pm$ 1.05	3.57 $\pm$ 0.09	5.29 $\pm$ 0.18	61.42 $\pm$ 11.28	76.88 $\pm$ 13.17
(A) + QC-entrapped liposome treated	5.12 $\pm$ 0.26 *	8.82 $\pm$ 0.48 *	5.95 $\pm$ 0.41 *	11.96 $\pm$ 0.59 *	18.32 $\pm$ 1.07 *	33.34 $\pm$ 1.72 *	4.06 $\pm$ 0.12 *	8.69 $\pm$ 0.27 *	70.27 $\pm$ 13.76 #	126.29 $\pm$ 17.81 *
(A) + QC-entrapped in galactosylated liposome treated	7.27 $\pm$ 0.44 *	9.07 $\pm$ 0.45 *	8.05 $\pm$ 0.40 *	11.86 $\pm$ 0.59 *	22.46 $\pm$ 1.21 *	32.07 $\pm$ 1.65 *	5.96 $\pm$ 0.31 *	8.72 $\pm$ 0.29 *	102.39 $\pm$ 17.83 *	129.73 $\pm$ 19.93 *

Results are expressed as mean  $\pm$  SD. NaAsO<sub>2</sub> treated group was compared with normal and the value was significantly different where \* = P<0.001. Experimental groups QC entrapped in liposome and QC in galactosylated liposomal group were also compared with NaAsO<sub>2</sub> treated group and in all those cases \* = P<0.001 and # = P<0.01.

Table-2. Effect of QC and liposomal QC treatment on collagen and 4-hydroxy proline (4-HP) levels in liver following NaAsO<sub>2</sub> treatment.

Groups	Hepatic collagen ( $\mu\text{g}/\text{mg}$ protein)	Hepatic 4-HP ( $\mu\text{g}$ protein)
Normal	10.93 $\pm$ 1.86	22.18 $\pm$ 3.14
NaAsO <sub>2</sub> treated (A)	19.27 $\pm$ 2.13 *	83.21 $\pm$ 7.19 *
(A) + Empty galactosylated liposome treated	19.02 $\pm$ 1.14	81.78 $\pm$ 5.45
(A) + Free QC treated	18.52 $\pm$ 1.96	76.52 $\pm$ 5.77
(A) + Liposomal QC treated	15.19 $\pm$ 1.67 *	61.73 $\pm$ 4.88 *
(A) + QC in galactosylated liposome treated	11.21 $\pm$ 1.73 *	26.36 $\pm$ 4.11 *

Results are expressed as mean  $\pm$  SD. NaAsO<sub>2</sub> treated group was compared with normal and the values were significantly different where \* = P<0.001 for hepatic collagen and 4-HP. Experimental groups QC entrapped in liposome and QC in galactosylated liposomal group were also compared with NaAsO<sub>2</sub> treated group and in all those cases \* = P<0.001 for hepatic collagen and 4-HP.



Table-3. Effect of liposomal-encapsulated QC injection (i.v) on blood serum

biochemical parameters in NaAsO<sub>2</sub>-induced hepatocellular injury.

Parameter	Normal	NaAsO <sub>2</sub> - treated group (A)	Experimental groups			
			(A)+empty galactosylated liposome treated	(A)+free QC treated (8.9µM/Kg b wt)	(A)+liposomal QC treated (8.9µM/Kg b wt)	(A)+ galactosylated liposomal QC treated (8.9µM/Kg b wt)
Alkaline phosphatase (U/l)	280±11.70	818±36.60 *	808±16.50	824±33.81	513.55±11 *	307.78±14.43 *
SGPT (IU/l)	36.86±2.18	89.71±6.42 *	85.52±4.51	94.11±4.61	67.43±3.97 *	49.14±2.21 *
Urea (g/l)	0.45±0.04	1.48±0.08 *	1.40±0.02	1.61±0.08	0.71±0.09 *	0.46±0.03 *
Creatinine (mg/l)	13.4±1.84	74.17±5.49 *	72.43±2.67	76.8±4.86	44.7±3.18 *	14.7±3.18 *

Results are expressed as mean ± SD of five animals. NaAsO<sub>2</sub> treated group was compared with normal and the value was significantly different where \* = P<0.001.

Experimental groups (galactosylated liposomal QC and liposomal QC group) were also compared with NaAsO<sub>2</sub> treated group and in all those cases \* =P<0.001.

Table-4. Effect of QC in galactosylated liposome treatment on the NaAsO<sub>2</sub> induced mitochondrial membrane microviscosity, ( $[r_0/r-1]^{-1}$ ) of rat hepatocytes (HP) and stellate (ST) cells.

	Membrane microviscosity $[r_0/r-1]^{-1}$	
	HP	ST
Normal	0.677±0.073	0.687±0.024
NaAsO <sub>2</sub> treated (A)	0.216±0.021*	0.274±0.019*
(A)+empty galactosylated liposome treated	0.231±0.018*	0.286±0.027*
(A)+free QC treated	0.340±0.089*	0.382±0.046*
(A)+ liposomal QC treated	0.312±0.099	0.606±0.052#
(A)+ QC in galactosylated liposome treated	0.661±0.045#	0.666±0.061#

Values are mean ± SD for 5 rats. \*P<0.001 significantly different from normal.

#P<0.001 significantly different from NaAsO<sub>2</sub> treated.

Table-5. Quercetin level in liver homogenate from rats treated with QC (in tween 80), liposomal QC and galactosylated liposomal QC.

	Free QC	Liposomal QC	Galactosylated liposomal QC			
			QC1	QC2	QC3	QC4
QC concentration in liver homogenate (nM/mg protein)	$0.83 \pm 0.17^a$	$1.63 \pm 0.32^{a,b}$	$0.36 \pm 0.09^{a,b}$	$0.92 \pm 0.18^{a,b}$	$2.76 \pm 0.54^{a,b}$	$1.92 \pm 0.19^{a,b}$
% of injected QC in whole liver	25.61	50.29	52.17	57.18	85.16	48.65

Rats received  $8.9\mu\text{M}/\text{kg}$  b.wt intravenous injection of both of the free QC and liposomal QC, and galactosylated liposomal QC (QC1→  $2.22\mu\text{M}/\text{Kg}$  b wt, QC2→  $4.45\mu\text{M}/\text{Kg}$  b wt, QC3→  $8.90\mu\text{M}/\text{Kg}$  b wt, QC4→  $13.35\mu\text{M}/\text{Kg}$  b wt). <sup>a</sup> values expressed as mean  $\pm$  SD (n = 5). <sup>b</sup> values are significantly different (p <0.001) from free QC <sup>a</sup>.

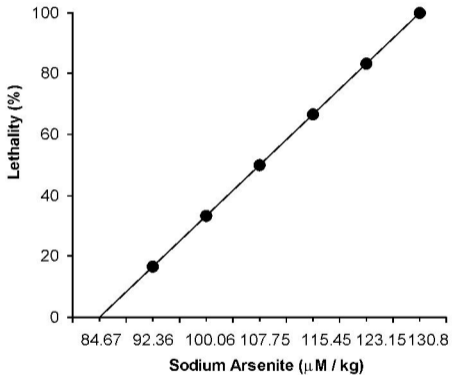


Fig. 1

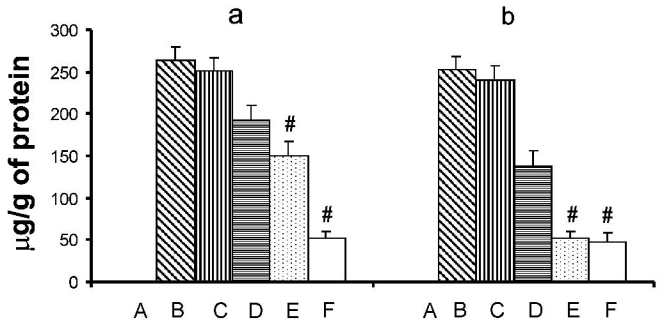


Fig. 2

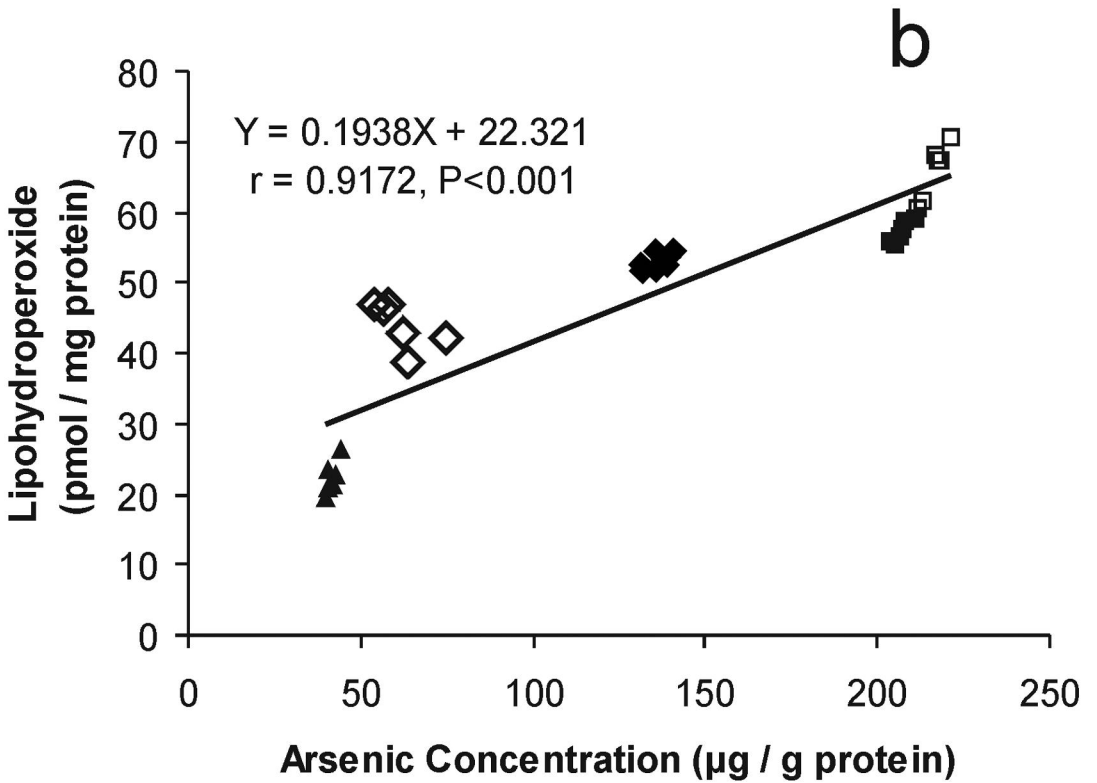
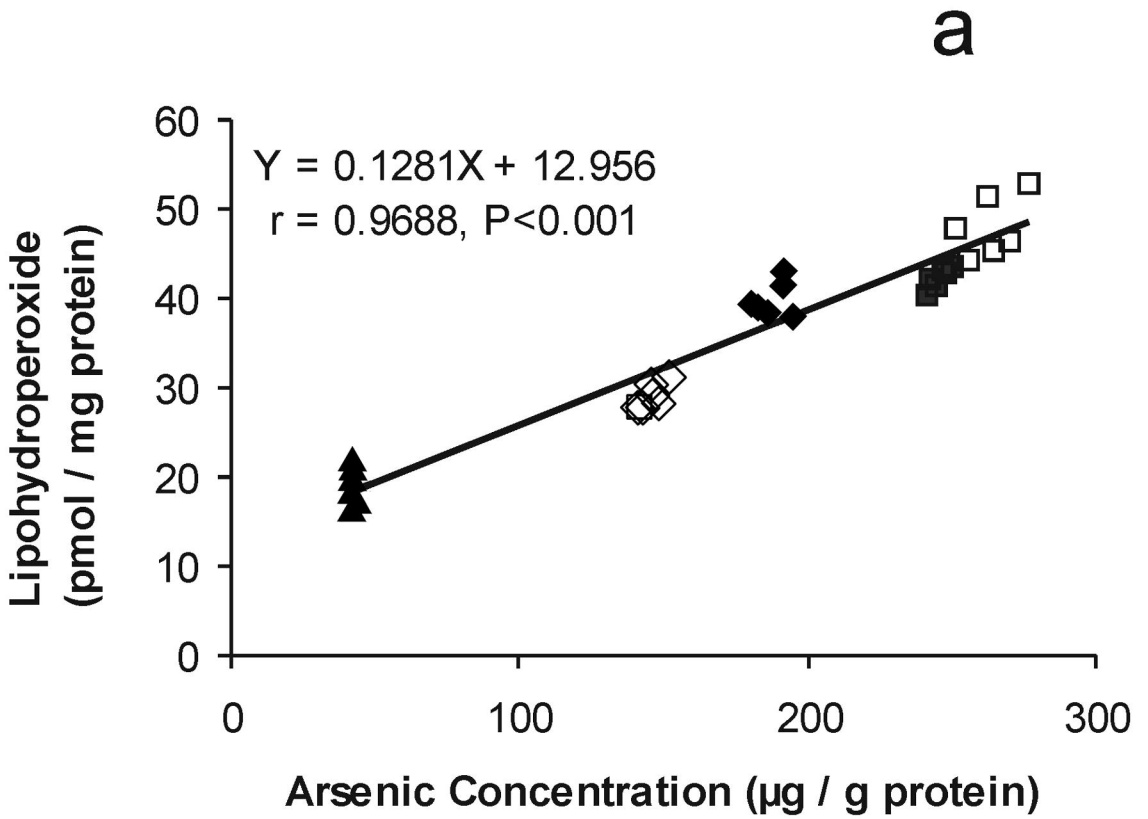
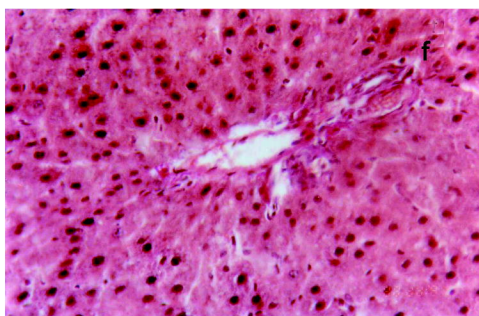
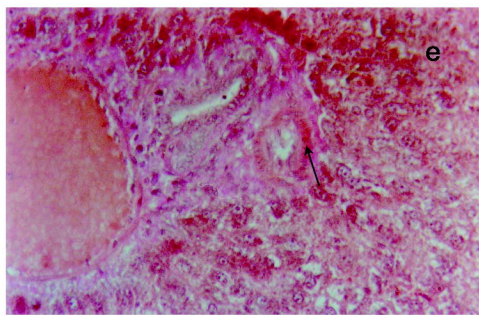
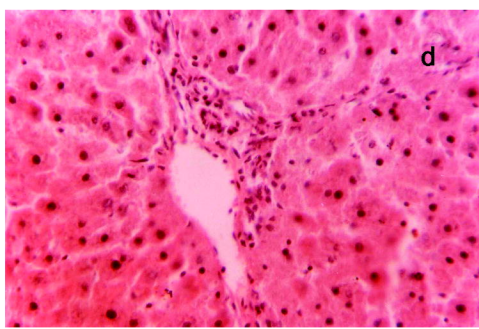
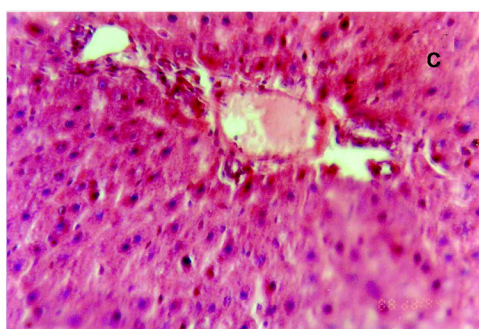
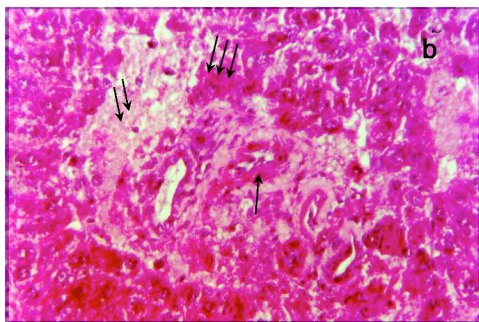
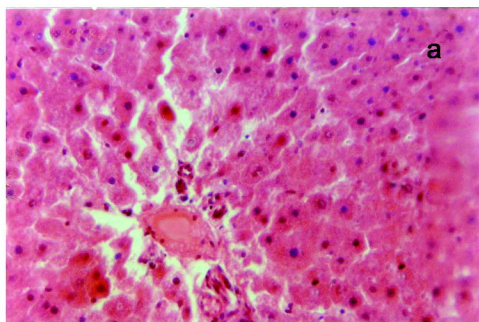


Fig 3



**Fig. 4**