CHAPTER 4

Quercetin in vesicular delivery systems: Evaluation in combating arsenic-induced acute liver toxicity associated gene expression in rat model
Quercetin in vesicular delivery systems: Evaluation in combating arsenic-induced acute liver toxicity associated gene expression in rat model

INTRODUCTION

Arsenic is one of the most important global environmental toxicants and its exposure in humans comes mainly from consumption of drinking water contaminated with inorganic arsenic (Celik et al., 2008).

Acute exposure to arsenite has been reported to induce death and / or multiple organ failure with symptoms including nausea, vomiting, diarrhoea, gastrointestinal haemorrhage, cerebral edema, tachycardia, dysrhythmias and hypovolemic shock.

Arsenic induced hepatic injury is known to be exerted through excess production of reactive oxygen species, namely superoxide (O$_2^-$), hydroxyl (·OH), and peroxyl (ROO·) radicals and hydrogen peroxide (H$_2$O$_2$) (Mittal & Flora, 2007). The harmful expressions of arsenic are primarily due to an imbalance between pro-oxidant and anti-oxidant homeostasis in physiological system and also due to its fascination to bind sulphhydryl groups of proteins and thiols of glutathione (GSH). Arsenic initiates a rapid depletion of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD) and catalase (Wang et al., 1996).

Arsenic-generated oxidative insult eventually leads to fatty liver and hepatic fibrosis in rats as well as human (Majumder, 2005; Sarin et al., 1999). Peroxidative damage of membrane lipids plays an important role in free radical-induced excess
collagen deposition and fibrogenesis. Molecular evaluation of procollagen gene expression of arsenic induced liver toxicity leading to fibrogenesis is very important for providing perspective on therapy development. Upregulation of transforming growth factor β (TGF β) has been implicated in the pathogenesis of fibrosis in rats (Armendariz-Borunda et al., 1990). TGF β stimulate fibroblast and produce collagen and inhibit collagen degradation. Arsenic exposure also accelerates type I pro-collagen mRNA expression, responsible for liver fibrogenesis (Wu et al., 2008). However, exact mechanism(s) by which arsenic induces hepatic fibrosis still remains unclear. 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). All these effects have been verified in rats. Rats, unlike other mammals, retain the dimethyl arsenic acid, one of the key intermediate metabolites of NaAsO₂ in the blood. Studies were performed in this report on rats because this species is known to be superior to others in forming monomethyl arsenous acid, the most toxic metabolic intermediates of NaAsO₂. Within 2 hrs after NaAsO₂ injection (50 µM/kg b.wt, i.v.), rats excreted 30% of the injected arsenite dose, whereas mice excreted only 21% of the dose (Csanaky & Gregus, 2002). Thus arsenic metabolic rate is high in rat compared to mice. Monomethyl arsenous acid is produced as intermediate during arsenic metabolism in rats as well as human (Styblo et al., 2000). Monomethyl arsenous acid (MMAs³) has been found in the urine of 48% of human chronically consuming arsenic-contaminated water in West Bengal, India (Mandal et al., 2001).

Quercetin, a polyphenolic flavonoid, present in large amounts in vegetable, fruits, tea and olive oil, has been used to serve as a stress protectant for its ability to interact
with free radicals involved in oxidative damage and progression of fibrogenesis in liver (Lee et al., 2003; Peres et al., 2000).

But simple flavonoidal compound therapy is not an effective approach to counter the oxidative damage, for poor availability of antioxidant to interact with hepatocellular membranes. For optimum efficacy, antioxidant should be targeted to either hepatocytes or Kupffer cells of liver. To maximize the therapeutic effect and minimize systemic toxicity, the sufficient dose of antioxidant should be specifically delivered to a target with minimal exposure to non-target cells (Mandal et al., 2002).

Hence, it is essential to develop a delivery system for vectoring antioxidants to hepatic cells (Das et al., 1987). In this context, liposomes are accepted as a potent drug vehicle not only for increasing the efficacy of the drug and decreasing the toxicity of the same but also due to the feasibility of incorporating both hydrophobic and hydrophilic substances, can possess a surface charge, have biocompatible nature and have non immunogenic property in the biological system. Nanoparticles are accepted as effective drug carriers because of several important technological advantages over liposomes, e.g., long shelf life, high carrier capacity and feasibility of variable routes of administration including oral route.

The aim of our work is to optimise the dose of flavonoidal compound QC in liposome or nanocapsule drug delivery system and to compare their biological effectiveness in combating acute arsenite induced hepatocellular oxidative damage related to upregulation of cytokine TGF β and fibrosis associated gene expression in rat liver.
MATERIALS AND METHODS

Materials

Poly-D-L-lactide, phosphatidyl ethanolamine (PE), diphenyl hexatriene (DPH), Triton x-100, glutathione reductase, quercetin were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium arsenite from E.Merck, Germany (Suprapure, A.R. Grade) 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. All other reagents were of analytical grade.

Preparation of liposomal Quercetin (LQC)

Multilamellar liposomes were prepared with Phosphatidyl Ethanolamine (PE), Cholesterol, Dicetyl Phosphate (DCP) and Quercetin (QC) in molar ratio 7:1:1:1 (Mitra et al, 2005). 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 60 s. The suspension was centrifuged (10,000xg) for 1h and washed twice in PBS.

Preparation of nanoencapsulated Quercetin (NQC)

In brief, 18 mg polylactide was dissolved in 10 ml of dichloromethane. Phosphatidyl ethanolamine (26 mg) dissolved in 250 μl of isopropylmyristate along with quercetin (4.5 mg) and was added to dichloromethane phase. The organic phase was added slowly into 50 ml of 0.025 M PBS (pH 7.2) containing 0.4% of the non-ionic surfactant (Tween 80), with moderate magnetic stirring for approximately 3 h and the traces left, if any, removed by passing gaseous nitrogen for 5 min. The suspension was
ultracentrifuged at 105,000 g in Sorval RC 5B Plus using the rotor Sorval T-865 for 1 h. The pellet of nanocapsules was washed with PBS and collected and resuspended in 2 ml PBS (Lala et al., 2006).

**Determination of encapsulation efficiency of liposomes & nanocapsules**

To estimate incorporation into liposomes, the pellet was dissolved in methanol and an aliquot of 100 μl was taken and diluted to 1 ml with PBS. The O.D. was measured at 369 nm. The total amount of quercetin ($E_m=0.03825 \text{ L mol}^{-1} \text{ cm}^{-1}$) in the liposomal form was calculated from the concentration of the drug in the dissolved pellet divided by the total amount of drug added during the liposome preparation. The incorporation of quercetin in the liposome was 89.9%.

To estimate the intercalation of nanocapsules, the pellets were then dissolved in 2 ml of dichloromethane and kept for 3 days at 4 °C. The O.D. was measured at $\lambda_{\text{max}}$ 369 nm and % of incorporation was calculated by following the above procedures. The percent intercalation of QC in nanocapsule was found to be 45% of the added amount.

**Determination of size of quercetin-encapsulated liposomes / nanocapsules**

The size determination of QC-encapsulated liposomes and nanocapsules was done through dynamic light scattering using a mean laser wavelength of 632.2 nm and a 90°-angle measurement.

**Animals and Treatment**

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 (commercial pelleted rat chow, purchased from Hindustan Unilever Limited,
Maharashtra, India) and drinking water ad libitum. Food and water were checked to be arsenic free before giving to animals (mentioned under section 2.13). Animals were randomly selected for groups and arsenic and drug were administered as per individual body weight of rat. Rats were divided into seven groups (seven cages) with 5 animals in each group. Cages were of polycarbonate type with husk type bedding. Rats in the normal group were fed a single oral gavage dose of physiological saline (5 ml/kg b.wt). NQC (2.71 mg/kg b.wt) suspension (5 ml/kg b.wt) was injected into tail vein of each rat of second normal group. Rats in the arsenic treated groups were fed with a single oral gavage dose of NaAsO$_2$ (13 mg/kg b.wt.). Only arsenic fed group of animals were kept as control without any drug treatment. Free QC (2.71 mg / kg b. wt.) suspension (5 ml/kg b.wt) of 0.2 % Tween 80 was injected into tail vein of second arsenic treated group of rats (1 h after NaAsO$_2$ treatment). Empty nanocapsules (5 ml/kg b.wt suspension), LQC and NQC (5 ml/kg b.wt suspension containing 2.71 mg QC/kg b.wt.) were injected in the tail vein of rats in other three arsenic fed groups (1 h after NaAsO$_2$ treatment). All the rats used in this study received proper care and handling in compliance with Animal Ethics Committee, India, Registration No.147/99/CPC SEA, India. QC uptake by liver was estimated after 2 h of free QC, liposomal and nanoencapsulated QC treatment.

### General Procedures

Twenty-four hours after NaAsO$_2$ administration the rats were anaesthetized with ether and blood was collected by cardiac puncture (Hoff, 2000). One part of the blood was taken for serum and the other part for plasma. Serum and plasma were made. Serum was isolated by following standard protocol. Blood was centrifuged at 2000xg for 10 mins at 4 ºC to assay the activity of serum enzymes. Plasma was made by adding the
anticoagulant heparin followed by centrifugation. Serum aspartate transaminase (AST), alkaline phosphatase (AP) (Karim et al., 2001; Tyagi et al., 2005) and serum urea and creatinine (mod.Berthelot method and alkaline picrate method) were determined using a standard kit manufactured by Coral Clinical Systems; India. The plasma was used for TGF β measurement. After collection of blood, all rats were decapitated and their livers were isolated promptly and washed with cold physiological saline. A part of the organ was immediately fixed in Bouin’s fixative and processed for histological examination. The other part was kept at -80 °C for further experiments. Liver histochemistry for collagen and liver tissue histology were studied by microscopic examination (Lin et al., 2005).

Assay of enzyme activities

A portion of the liver was homogenized in 0.25 M sucrose solution. The homogenate was centrifuged at 8,200xg for 10 min. using a Sorvall SS34 rotor. The supernatant obtained was again spun at 105,000xg for 1 h in an OTD-50B Sorvall ultracentrifuge (4 °C). The supernatant from the second centrifugation was collected as the cytosolic fraction of liver cells.

The assay of superoxide dismutase (EC1.15.1.1) in cytosolic fractions of liver homogenate was estimated by following the methods of Beyer and Fridovich, 1987 using spectrophotometer with slight modifications. The activity of SOD was expressed in unit by assuming that the activity of the enzyme as one unit which inhibited the initial rate of reduction of 10 mM ferricytochrome C by 50% detected in Rayleigh UV 2601 double beam spectrophotometer (Mandal et al., 2008).
A part of the cytosolic fraction was used for the assay of catalase activity spectrophotometrically by the method of Moragon *et al.*, 2005 with slight modifications. The reaction mixture contained sodium phosphate buffer (0.05 M, pH 7.0), 50 mM H$_2$O$_2$, 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$_2$O$_2$ consumption and enzyme activity expressed as amount of H$_2$O$_2$ decomposed per minute per milligram of protein.

GPx activity from cytosolic fraction of liver was measured spectrophotometrically by the method of Sarkar and Das (Sarkar & Das, 2006). Cytosol containing the enzyme source was mixed with 0.25 M potassium phosphate buffer, 25 mM EDTA, glutathione reductase, 40 mM glutathione GSH and 20 mM NADPH. The activity was determined and expressed as µmol NADPH oxidized/min/protein.

GR was assayed by the method of Castro *et al.*, 1990 with slight modification. A 3-ml mixture contained 100 mM phosphate buffer (pH 7), 1 mM GSSG, 1 mM EDTA, and 0.1 mM NADPH. 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 µmol of NADPH oxidation/min/mg protein.

GST activity was determined spectrophotometrically using 1-Chloro-2,4-dinitrobenzene (CDNB) by the method of Maiti & Chatterjee, 2000) using a spectrophotometer. The rate of formation of CDNB-GSH complex was monitored at 340 nm and used to express the enzyme activity.

G-6-PDH activity was determined using a Sigma Diagnostics Kit by the method of Iimuro *et al.*, 1997 using a spectrophotometer with slight modifications. The rate of formation of NADPH is proportional to G-6-PDH activity and was measured
spectrophotometrically as an increase in absorbance at 340 nm. One unit of G-6-PDH activity was defined as 1 µM NADPH produced/min.

**Measurement of ROS level**

Intracellular ROS level was measured in liver tissue (Betainder et al., 2002). Briefly, liver tissue was homogenized (10%) in PBS (pH 7.2). The homogenized cells (0.4 mg/ml) were loaded with the cell permeant probe CM-H<sub>2</sub>DCFDA (5-(and-6)-chloromethyl-2′, 7′-dichlorodihydro-fluorescein diacetate acetyl ester) (2µM) for 15 min at 30 ºC in dark, and fluorescence was monitored. H<sub>2</sub>DCFDA, an uncharged, cell-permeable fluorescent probe readily diffuses into cells and gets hydrolyzed by intracellular esterases to yield H<sub>2</sub>DCF, which is trapped inside the cell. Then it is oxidized from the non-fluorescent form to a highly fluorescent compound dichlorofluorescein primarily by hydroxyl radical (•OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or other low-molecular-weight peroxides produced in the cells. Thus the fluorescence intensity is proportional to the amount to the ROS produced by the cells. Fluorescence was measured through a spectrofluorometer (LS 3B, Perkin Elmer, USA) by using 499 nm as excitation and 520 nm as emission wavelengths. Data were expressed as relative fluorescence intensity taking the value for normal as 100%.

**Lipid peroxidation assay**

Lipid peroxidation in the whole liver membrane was determined by measuring the amount of conjugated diene by the method of Mandal et al using a spectrophotometer (Mandal et al., 2002). Lipids were extracted from liver homogenate in a chloroform-methanol mixture (2:1 v/v). The extract was evaporated to dryness under nitrogen atmosphere at 25ºC, and redissolved in n- cyclohexane. Lipids in cyclohexane solvent
were assayed at 234 nm and the results were expressed as µmol of Lipohydroperoxide/mg protein by using \( \varepsilon_m \) of 2.52 \( \times 10^4 \) M\(^{-1}\) cm\(^{-1}\). Total protein was measured by the method of Lowry et al., 1951.

**Quantitation of Quercetin level in liver**

Quantitation of QC in liver homogenate was performed spectrophotometrically by the method of Mandal & Das, 2005. Liver homogenates of normal and experimental rats were diluted with an equal volume of absolute ethanol containing 1 µgm/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 48 °C. The heptane layer was removed and another 1 ml of fresh heptane was 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 ml was injected onto a high performance liquid chromatography (HPLC) ODS–2 column and quantitation of QC in liver homogenate detected spectrophotometrically at 370 nm.

**Fluorescence depolarization measurements of the fluidity of liver cell membrane**

The fluorescence depolarization, associated with the hydrophobic fluorescence probe diphenyl hexatriene DPH, was estimated in hepatic cell membrane by spectrofluorimeter by the method of Mandal & Das, 2005. The fluorescence depolarization, 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. Plasma 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

$$r = \frac{I_{II} - I_{I}}{I_{II} + 2I_{I}}$$

where $I_{II}$ and $I_{I}$ 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.

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

4-HP and collagen were estimated following the Mandal et al using a spectrophotometer (Mandal et al., 2007). The liver was cut into small pieces and homogenized 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 hydrolyzed in Teflon–capped vials at 105 ºC for 18 h. 4-Hydroxyproline (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.

Hepatic collagen was determined by 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; simultaneously, the absorbance obtained at 550 and 625 nm can be used to determine the amount of collagen and total protein, respectively.

**Determination of total arsenic content**

Arsenic in drinking water given to the animals in the cages was determined by the following method. 1 ml of water was taken. It was treated with 6 ml of 10% HCl, 1 ml of
5% KI, 1 ml of 5% ascorbic acid and 1 ml of conc HCl. It was allowed to stand at room temperature for 45 min. Reading was taken using As$^{+3}$ as standard using flow injection hydride generation atomic absorption spectrometer (Perkin Elmer, AA 700) fitted with a graphite furnace. Arsenic quantification in rodent chow was done (Mandal et al., 2007; Flora et al., 1995). Food pellets 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 flow injection hydride generation atomic absorption spectrometer (Perkin Elmer, AA 700) fitted with a graphite furnace. The detection limit for arsenic is (1-10) ppb.

Total arsenic accumulation in liver homogenate was also measured following the method of Mandal et al., 2007 by flow injection atomic absorption spectrometer (spectra AA 30/40; Varian, Inc., Palo Alto, CA) fitted with a graphite furnace. Subcellular fractions were digested with acid mixture prior to quantification.

**Reduced glutathione (GSH) level**

Glutathione level of a part of tissue homogenate was determined by the method of Davila et al., 1991 with the help of a spectrophotometer by using tetrachloroacetic acid with EDTA as protein precipitating reagent. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200xg at 4 °C. The mixture was then transferred to a new set of test tubes and 0.3 M phosphate buffer and Ellmen Reagent (5, 5’-dithiobis-2 nitrobenzoic acid in 1% Na-citrate) were added. After completion of the total reaction,
solutions were read at 412 nm. Absorbance values were compared with a standard curve generated from known GSH concentration to evaluate liver homogenate GSH levels.

**Western blot analysis**

Liver tissues were homogenized (1:10, W/V) in 10mM Tris-HCl, pH 7.4, 150mM NaCl, containing freshly added protease inhibitors and cytosols were prepared by centrifugation at 15,000x g for 10 min at 4 °C. Protein concentrations were determined (Lowry *et al.*, 1951). SDS/PAGE was performed by subjecting 30 μg total protein under reducing conditions on 10% polyacrylamide gels, followed by electrophoretic transfer to PVDF membrane (Sigma) at 15 V for 20 min by using Semi dry (Bio-Rad) transblot apparatus. Membrane was blocked in 4% BSA in PBS (overnight) at 4 °C, followed by incubation with the primary rabbit anti Cytochrome c (cyt c) antibody (1:100) in blocking solution (0.2% Tween 20 in PBS and 2% BSA) for 3 h at room temperature. After 5 washes with 0.2% Tween 20 in PBS, the membrane was incubated in secondary alkaline phosphatase conjugated anti rabbit goat IgG antibody (1:1000) for 1 h 30 min. After 5 washes with 0.2% T-20 in PBS and 2% BSA, proteins were visualized by the development of colour using Sigma premixed BCIP/NBT substrate solution. Their density was analysed with Image J software system.

**Silver staining**

Silver staining of total proteins in liver cytosolic fraction subjected to SDS PAGE gel was done (Wray *et al.*, 1981).

**RNA extraction and RT-PCR analysis**

The total RNA of liver tissue was extracted by SV Total RNA Isolation System of Promega. It was treated with RNase-free DNase1 (Promega) and 2.5 μg of RNA was
reverse transcribed into cDNA using the Promega Kit at a final volume of 20 µl. β-actin was used as an internal reference gene. The sense and anti-sense primers for β-actin were 5'-ACATCTGCTGGAAGGTGGAC-3' and 5'-GGTACCACCATGTACCCAGG-3' (the expected product size 163 bp) respectively (Li et al., 1995). The sense and anti-sense primers for type I pro-collagen α1 (I): 5'-CACCCCTCAAGGCCTGAGTC-3' and 5'-GTTCCGGCTGATGTACCAGT-3' (the expected product size 253 bp) (Li et al., 1995).

The RT-PCR reaction was carried on with Access Promega RT-PCR Kit. The mixture was heated at 42 °C for 60 min in an Eppendorf PCR system Master Cycler. Type I pro-collagen α1 (I) and β-actin were amplified in 40 sequential cycles, the program was denaturation (94 °C, 50 sec), annealing (49 °C, 45 sec), and extension (72 °C, 1 min). The PCR product was fractionated in 2% agarose gel electrophoresis containing ethidium bromide (EB) 0.5 mg/L and photographed under UV transilluminator. Their density was semiquantitatively analysed with Image J software system.

Measurement of Plasma TGF β1

Plasma TGF β1 was measured using an ELISA kit (R & D Systems, Catalog MB 100B).

Statistical analysis

Statistical analysis was performed with one-way ANOVA with post hoc Tukey’s test. The software used was version 15.0; SPSS Inc: Chicago, IL. In all instances, $p < 0.05$ was considered as the minimum level of significance.
RESULTS

Size determination of QC encapsulated liposomes and nanoparticles

The average diameters of QC-encapsulated liposomes and nanocapsules were found to be 200 and 100 nm, respectively as measured through dynamic light scattering using a mean laser wavelength of 632.2 nm and a 90°-angle measurement.

NQC on NaAsO$_2$-induced accumulation of inorganic arsenic in rat liver

A single oral application of NaAsO$_2$ (13 mg/kg b.wt) induced a substantial amount of accumulation of inorganic arsenic in liver cells. No significant protections were observed in rats treated with empty nanocapsules or free QC 1 h after arsenic consumption. Liposomal QC treatment significantly reduced arsenic level in liver. However maximum reduction was achieved through nanocapsulated QC treatment. No detectable amount of arsenic was present in the liver homogenate of normal and nanocapsulated QC treated rats not receiving arsenic (Fig. 1). This signifies that arsenic accumulation in the arsenic administered rats is solely due to the oral gavage dosing of arsenic and not due to any contamination from food and drinking water given to rats.

![Fig. 1. Values of arsenic levels in liver tissue from normal, sodium arsenite and nanocapsulated QC treated rats. The groups are Normal(A), Normal+Nanoencapsulated QC treated (B), Arsenite treated (C), C+Empty nanocapsule treated (D), C+Free QC treated (E), C+Liposomal QC treated (F) and C+Nanoencapsulated QC treated (G). Values are mean ± S.E. of 5 rats. *p<0.001 is significantly different from normal. #p<0.001 is significantly different from sodium arsenite treated rats.](image-url)
NQC on NaAsO$_2$-induced GSSG/GSH ratio levels in the liver tissue of rats

The GSSG/GSH ratio, an important marker of oxidative stress, decreased in normal rats treated with nanocapsulated QC as compared to normal. A single oral application of NaAsO$_2$ (13 mg/kg b.wt) induced considerable increase in the ratio. The ratio was significantly reduced in rats treated with liposomal QC but maximum reduction was observed in rats treated with nanocapsulated QC. However no noticeable reduction was observed in case of rats treated with empty nanocapsules or free QC (Fig. 2).

Fig. 2. Values of GSSG/GSH ratio in liver tissue from normal, sodium arsenite and nanocapsulated QC treated rats. The groups are Normal (A), Normal+Nanoencapsulated QC treated (B), Arsenite treated (C), C+Empty nanocapsule treated (D), C+Free QC treated (E), C+Liposomal QC treated (F) and C+Nanoencapsulated QC treated (G). Values are mean ± S.E. of 5 rats. *$p<0.001$ is significantly different from normal. #$p<0.001$ is significantly different from sodium arsenite treated rats.

NQC on NaAsO$_2$-induced membrane microviscosity rat liver

A single oral dose of arsenic (13 mg/kg b.wt) induced a significant decrease in the membrane microviscosity of liver tissue as compared with the rats of the normal group and normal rats treated with nanocapsulated QC. Free QC treated and empty nanocapsules treated groups also showed a significant decrease in the membrane microviscosity similar to the arsenic fed control group. However liposomal QC treated rats showed a significant elevation but maximum increase was observed in the rats treated with nanocapsulated QC (Fig. 3)
**Fig. 3.** Microviscosity ([r°/r –1]-1) of rat liver cell membrane from normal, sodium arsenite and nanocapsulated QC treated rats. The groups are Normal (A), Normal+Nanoencapsulated QC treated (B), Arsenite treated (C), C+Empty nanocapsule treated (D), C+Free QC treated (E), C+Liposomal QC treated (F) and C+Nanoencapsulated QC treated (G). Values are mean ± S.E. of 5 rats. *p<0.001 is significantly different from normal; **p & #p<0.001 are significantly different from sodium arsenite treated rats.

**NQC on NaAsO₂-induced ROS and lipid peroxidation levels in the whole liver tissue of rats**

Normal rats treated with nanocapsulated QC showed a decrease in ROS generation and conjugated diene level when compared to normal rats. A single oral application of NaAsO₂ (13 mg/kg b.wt) induced a marked increase in those levels. No significant reduction was observed in the case of rats treated with free QC or empty nanocapsules 1 h after arsenic consumption. Although liposomal QC treatment induced a significant reduction of the ROS and conjugated diene levels, maximum reduction was observed in the case of rats treated with nanocapsulated QC (Table 1).

**Table 1. Effect of QC in free, liposomal and nanoencapsulated forms on the changes in the generation of reactive oxygen species (ROS) and lipid peroxidation levels in rat liver by the induction of sodium arsenite**

<table>
<thead>
<tr>
<th>Groups</th>
<th>DCF-Fluorescence (% of normal)</th>
<th>Lipohydroperoxide (µM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A)</td>
<td>100 ± 6.32</td>
<td>1.45 ± 0.01</td>
</tr>
<tr>
<td>(A) + Nanoencapsulated QC treated</td>
<td>103 ± 2.73</td>
<td>1.36 ± 0.06</td>
</tr>
<tr>
<td>NaAsO₂-treated (B)</td>
<td>300 ± 15.30*</td>
<td>2.75 ± 0.04*</td>
</tr>
<tr>
<td>(B) + Empty nanocapsule treated</td>
<td>290 ± 14.72*</td>
<td>2.63 ± 0.02*</td>
</tr>
<tr>
<td>(B) + Free QC treated</td>
<td>270 ± 13.55 *</td>
<td>2.60 ± 0.03*</td>
</tr>
<tr>
<td>(B) + Liposomal QC treated</td>
<td>200 ± 10.02*</td>
<td>2.20 ± 0.04*</td>
</tr>
<tr>
<td>(B) + Nanoencapsulated QC treated</td>
<td>110 ± 7.11*</td>
<td>1.50 ± 0.01*</td>
</tr>
</tbody>
</table>

Results are expressed as % of normal cells, n=5; mean ± SE. *p<0.001 represent significant differences from normal values (A). **p<0.001 represent significant differences from sodium arsenite treated (B) values.
NQC on NaAsO\(_2\)-induced antioxidant enzymes

Normal rats injected with NQC showed an increase of different antioxidant enzyme activities in liver cells. A single oral application of NaAsO\(_2\) (13 mg /kg b.wt) resulted in a significant depletion of those enzyme levels. No significant protection from arsenic insult was provided with free QC or empty nanocapsules. Although liposomal QC treatment checked the depletion of those enzyme levels, nanoencapsulated QC treatment prevented the depletion completely in liver cells (Table-2).

Table 2. Effect of QC in free, liposomal and nanoencapsulated forms on the changes in SOD, catalase, GPx, GR, GST and G-6-PDH activities in rat liver by the induction of sodium arsenite

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>Catalase (U/mg protein/min)</th>
<th>GPx ((\mu)mol NADPH oxidation/min/mg protein)</th>
<th>GR ((\mu)mol NADPH oxidation/min/mg protein)</th>
<th>GST (nmol produced/min/mg protein)</th>
<th>G6PDH (nmol NADP reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A)</td>
<td>66.20±2.09</td>
<td>8.67±0.45</td>
<td>10.06±1.12</td>
<td>32.16±1.79</td>
<td>107.35±1.80</td>
<td>13.02±1.28</td>
</tr>
<tr>
<td>(A) + NQC treated</td>
<td>71.81±3.41</td>
<td>9.32±0.31</td>
<td>11.68±2.17</td>
<td>33.72±2.18</td>
<td>110.81±2.18</td>
<td>15.92±2.11</td>
</tr>
<tr>
<td>NaAsO(_2) treated (B)</td>
<td>23.65±0.57*</td>
<td>3.33±0.12*</td>
<td>4.06±0.13*</td>
<td>11.24±0.88*</td>
<td>53.04±0.53*</td>
<td>4.67±0.27*</td>
</tr>
<tr>
<td>(B) + Empty nanocapsule</td>
<td>24.34±0.70*</td>
<td>3.56±0.09*</td>
<td>5.25±0.33*</td>
<td>13.44±0.99*</td>
<td>56.84±0.64*</td>
<td>5.45±0.35*</td>
</tr>
<tr>
<td>treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B) + Free QC treated</td>
<td>26.73±1.20*</td>
<td>4.65±0.23*</td>
<td>6.62±0.66*</td>
<td>16.27±1.23*</td>
<td>63.12±0.71*</td>
<td>7.36±0.77*</td>
</tr>
<tr>
<td>(B) + LQC treated</td>
<td>42.68±1.51#</td>
<td>5.49±0.33#</td>
<td>7.28±0.72#</td>
<td>21.45±1.44#</td>
<td>72.66±1.04#</td>
<td>9.13±0.88#</td>
</tr>
<tr>
<td>(B) + NQC treated</td>
<td>59.98±1.80#</td>
<td>8.33±0.48#</td>
<td>9.68±0.84#</td>
<td>30.96±1.68#</td>
<td>96.17±1.66#</td>
<td>12.47±1.15#</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE of 5 rats. *p<0.001 is significantly different from normal. #p<0.001 is significantly different from sodium arsenite treated (B).
Chapter 4  Nano Quercetin in arsenic-induced liver toxicity

QC in nanocapsules on NaAsO₂-induced hepatic 4-HP and collagen contents in rat liver

Normal rats injected with nanocapsulated QC showed no such change in the hepatic 4-HP and collagen contents when compared to normal. A single oral application of NaAsO₂ (13 mg/kg b.wt) induced a marked increase in those levels (Table 3). But this increase was not inhibited much by the treatment of free QC or empty nanocapsules whereas QC in liposomes and nanocapsules injection inhibited partially and completely the collagen protein deposition and 4-HP increase respectively in rat liver.

Table 3. Effect of free QC, liposomal and nanoencapsulated QC treatments on 4-HP and collagen levels in liver following NaAsO₂ treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hepatic 4-HP (µg protein)</th>
<th>Hepatic Collagen (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A)</td>
<td>21.96±0.99</td>
<td>12.08±1.32</td>
</tr>
<tr>
<td>(A) + Nanoencapsulated QC treated</td>
<td>19.62 ± 0.78</td>
<td>11.24 ± 0.92</td>
</tr>
<tr>
<td>NaAsO₂-treated (B)</td>
<td>85.63±4.46*</td>
<td>21.69±1.75*</td>
</tr>
<tr>
<td>(B) + Empty nanocapsule treated</td>
<td>82.48±3.73*</td>
<td>20.13±1.67*</td>
</tr>
<tr>
<td>(B) + Free QC treated</td>
<td>74.67±3.08*</td>
<td>17.35±1.55*</td>
</tr>
<tr>
<td>(B) + Liposomal QC treated</td>
<td>58.54±2.81#</td>
<td>15.27±1.36#</td>
</tr>
<tr>
<td>(B) + Nanoencapsulated QC treated</td>
<td>22.45±1.44#</td>
<td>11.98±1.0#</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE of 5 rats. *p<0.001 is significantly different from normal for hepatic 4-HP and collagen. #p<0.001 is significantly different from NaAsO₂-treated (B) for hepatic 4-HP and collagen.

QC-entrapped nanocapsules on NaAsO₂-mediated hepatocellular and nephro toxicity

Normal rats injected with nanocapsulated QC showed similar results with the normal group. Rats treated with a single dose of NaAsO₂ developed significant hepatic and nephro damages as observed from elevated AST, AP and urea, creatinine in serum. A single injection (i.v.) of empty nanocapsules or free QC exerted no significant
protection against NaAsO$_2$ induced liver and nephro toxicity although liposomal QC provided a significant depletion of those levels. The degree of protection was observed close to normal levels when arsenic fed groups were injected with nanocapsulated QC (Table 4).

**Table 4. Effect of liposomal and nanoencapsulated QC injections (i.v.) on blood serum biochemical parameters in NaAsO$_2$-induced hepatocellular injury**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AP (U/I)</th>
<th>AST (IU/I)</th>
<th>Urea (g/l)</th>
<th>Creatinine (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A)</td>
<td>270±10.92</td>
<td>33.86±1.72</td>
<td>0.46±0.03</td>
<td>13.56±1.45</td>
</tr>
<tr>
<td>(A)+Nanoencapsulated QC treated</td>
<td>269±13.71</td>
<td>31.48±2.33</td>
<td>0.45±0.02</td>
<td>12.91±2.17</td>
</tr>
<tr>
<td>NaAsO$_2$-treated (B)</td>
<td>768±21.03*</td>
<td>98.71±5.07*</td>
<td>1.31±0.06*</td>
<td>46.80±4.63*</td>
</tr>
<tr>
<td>(B)+Empty nanocapsule treated</td>
<td>741±14.54*</td>
<td>95.62±4.33*</td>
<td>1.24±0.05*</td>
<td>44.60±3.66*</td>
</tr>
<tr>
<td>(B) +Free QC treated</td>
<td>724±8.55*</td>
<td>89.71±3.64*</td>
<td>1.18±0.05*</td>
<td>40.17±4.34*</td>
</tr>
<tr>
<td>(B) +Liposomal QC treated</td>
<td>510.25±7.20#</td>
<td>65.23±3.03#</td>
<td>0.69±0.05#</td>
<td>25.12±3.26#</td>
</tr>
<tr>
<td>(B)+Nanoencapsulated QC treated</td>
<td>292.44±4.60#</td>
<td>36.43±2.20#</td>
<td>0.47±0.04#</td>
<td>13.91±1.63*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE of five rats. *p<0.001 is significantly different from normal. #p<0.001 is significantly different from NaAsO$_2$-treated (B).

**Quantification 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 liposomes and nanocapsules by liver were calculated. Normal rats injected with nanocapsulated QC showed the highest uptake of 91.32% in the liver as compared with the other experimental groups. Only 25.30% of the injected dose was detected in the liver when free QC was injected. Arsenic fed rats injected with nanoencapsulated QC showed the second highest uptake in the liver which was 83.92% whereas the uptake of liposomal QC in the liver was only 49.98%.
Table 5. Quercetin level in liver homogenate from rats treated with QC (in Tween 80), liposomal and nanoencapsulated QC.

<table>
<thead>
<tr>
<th>Groups</th>
<th>QC concentration in liver homogenate (nM/mg protein)</th>
<th>Injected QC in whole liver (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (A)</td>
<td>0.04±0.02</td>
<td>-</td>
</tr>
<tr>
<td>(A) + NQC treated</td>
<td>2.96 ± 0.49*</td>
<td>91.32</td>
</tr>
<tr>
<td>(B) + Empty nanocapsule treated</td>
<td>0.06±0.04</td>
<td>-</td>
</tr>
<tr>
<td>(B) + Free QC treated</td>
<td>0.82±0.13*</td>
<td>25.30</td>
</tr>
<tr>
<td>(B) + LQC treated</td>
<td>1.62±0.25**</td>
<td>49.98</td>
</tr>
<tr>
<td>(B) + NQC treated</td>
<td>2.72±0.33**</td>
<td>83.92</td>
</tr>
</tbody>
</table>

Rats received 2.71 mg/Kg b.wt. intravenous injection of free QC and nanoencapsulated QC. Values are mean ± SE (n=5). *p <0.001 is significantly different from normal. #Value is significantly different (p<0.001) from free QC.

**NQC against NaAsO₂ induced release of cyt c in rat liver cytosolic fraction**

Rats when treated with a single dose of NaAsO₂ developed 54.77% increment of cyt c release in cytosol above the level of normal group of rats as detected by western blot. Nanoencapsulated QC decreased the release to 53.19% with respect to the NaAsO₂ treated control group of rats (Fig. 4a3).

![Figure 4a1](image1.png)

**Fig. 4. a1.** Silver staining of total protein in cytosolic fraction subjected to SDS - PAGE. Lane 1: normal (A), Lane 2: arsenite treated (B), Lane 3: nanoencapsulated QC treated 1 h after arsenite intake (C), Lane 4: Cyt c marker.

**a2.** The effect of nanoencapsulated QC on cyt c protein expression in liver cytosolic fraction. Western Blot was used to analyze the expression of the cyt c protein. Lane 1: normal (A), Lane 2: arsenite treated (B), Lane 3: nanoencapsulated QC treated 1 h after arsenite intake (C), Lane 4: cyt c marker.

**a3.** Densitometric analysis of cyt c bands obtained in Western blot: normal (A), Arsenite treated (B) and Nanoencapsulated QC treated 1 h after arsenite intake (C). Results were statistically significant from normal with *p<0.001 and from NaAsO₂ treated rats with #p<0.001.
NQC against NaAsO\textsubscript{2} induced type I procollagen $\alpha_1$(I) expression in rat liver

RT–PCR analysis revealed that a single oral application of NaAsO\textsubscript{2} (13 mg/kg b.wt.) induced 85.7% increment of type I pro-collagen $\alpha_1$ (I) mRNA expression in comparison to normal rats whereas nanoencapsulated QC reduced it to 49.24% in contrast to NaAsO\textsubscript{2} treated rats (Fig. 5a2).

**Fig. 5.** a1. Effect of nanoencapsulated QC on the gene expression of type I procollagen of liver tissue. RT-PCR product 6 \(\mu\)l was run on 2% agarose gel stained with ethidium bromide. Lane 1, 2 and 3: $\alpha_1$(I) procollagen of normal, arsenite treated and nanoencapsulated QC treated 1 h after arsenite administration of rat liver. Lane 4: PCR marker, Lane 5, 6 and 7: $\beta$ actin of normal, arsenite treated and nanoencapsulated QC treated 1 h after arsenite administration of rat liver respectively.

a2. Densitometric analysis of $\alpha_1$(I) procollagen mRNA expression in: Normal (A), Arsenite treated (B) and nanoencapsulated QC treated 1 h after arsenite intake (C). Results were statistically significant from normal with *$p<0.001$ and from NaAsO\textsubscript{2} treated rats with #$p<0.001$.

**Nanocapsulated QC treatment on NaAsO\textsubscript{2} induced up regulation of TGF $\beta$ concentration in rat blood plasma**

Fig. 6 shows the blood TGF $\beta$ concentration of both the control and experimental animals exposed to arsenic. Oral feeding of NaAsO\textsubscript{2} (13 mg/kg b.wt.) caused a significant increase of TGF $\beta$ in blood plasma (From normal 75.2 ± 8.67 ng/ml to 196.2 ± 12.07 ng/ml). Free QC treatment resulted in no significant change in TGF $\beta$ level in
blood plasma. But nanocapsulated QC treatment caused a substantial decrease in TGF \( \beta \) level in blood plasma of NaAsO\(_2\) fed rats (87.6 ± 7.23 ng/ml).

![Graph showing TGF \( \beta \) concentration in plasma of rats.](image)

**Fig. 6.** Effect of nanoencapsulated QC treatment on TGF \( \beta \) concentration in plasma of rats. Normal(A), NaAsO\(_2\) treated (B), (B) + Free QC treated (C), (B) + Nanoencapsulated QC treated (D). Values are mean ± S.E. of 5 rats. *\( p < 0.001 \) significantly different from normal. #\( p < 0.001 \) significantly different from sodium arsenite treated rats.

**Pathomorphology and histochemistry of the liver**

Haematoxylin-Eosin-stained liver sections of normal rats showed (Fig. 7a) the cords of normal hepatocytes, normal looking sinusoids lined by Kupffer cells. 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 oral application of NaAsO\(_2\) (Fig. 7b). No such alterations appeared in the case of NaAsO\(_2\) intoxicated animals injected with nanoencapsulated QC (Fig. 7c).

Liver section of normal rats stained with Van Gieson exhibited very small amount of collagen in the periportal region (Fig. 7d). But periportal region showed mild to moderate amount of collagen tissues when fed with a single intake of NaAsO\(_2\) (Fig. 7e). The maximum protection from the deposition of collagen contents was observed when rats were treated with nanoencapsulated QC (Fig. 7f).
Fig. 7. Histopathological examination of eosin-hematoxylin stained liver section of normal and experimental rats with magnification x 400. (a) Physiological saline-treated normal, (b) NaAsO$_2$-treated, (c) NaAsO$_2$ + nanoencapsulated QC (2.71 mg/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 normal, (e) NaAsO$_2$-treated, (f) NaAsO$_2$ + nanoencapsulated QC (2.71 mg /kg b.wt.) treated. ↓indicates initiation of fibrosis, ↓↓indicates fattymetamorphosis, ↓↓↓indicates necrosis.
DISCUSSION

Arsenic generates ROS such as •OH, O₂^−, H₂O₂ which cause oxidative damage (Das et al., 2005) by increased lipid peroxidation and thiol depletion, and oxidative damage, in turn, initiates gene expression and liver fibrogenesis (Majumder, 2005) as evident by our results (Figs. 1-4 and Table 2). ROS interacts with a number of components of cell including lipid, protein, DNA, carbohydrate, thiols, and other low-molecular weight antioxidants causing ultimate oxidation of macromolecules and ultimately leading to pathogenesis.

Fibrosis indicates the critical stage of liver injury, where it may turn to cirrhosis, and even to liver cancer (Centeno et al., 2002). Hepatic fibrosis is a diseased state characterized by exuberant synthesis and deposition of collagen in the extracellular matrix. Fibrogenesis is expressed by an increase in the hepatic hydroxyproline level (Testa et al., 1993). Our observations indicate that acute arsenic toxicity by a single dose of NaAsO₂ (13 mg/kg b. wt) in oral route could initiate fibrogenesis in rat liver by an indication of increment of 4-HP and overexpression of type I procollagen mRNA (Table 2 & Fig. 5). TGF β has been implicated in the induction of synthesis and accumulation of collagen in the extracellular matrix (Milani et al., 1991). But a moderate increase of Pro-collagen and TGF β levels and an inadequate deposition and accumulation of collagen in the extracellular matrix in our observations could not be a positive indication of liver fibrosis.

Liver fibrosis is a disease where the rate of synthesis of scar tissue increases with a rapid imbalance in fibrogenesis and fibrolysis in liver. Prolonged treatment of arsenic causes liver fibrosis. Early fibrosis is difficult to diagnose because it is often
asymptomatic. Toxic manifestation of arsenic starts with the induction of lactic acid production because of an imbalance in cellular energy metabolism and the enol acid thus produced when utilized by cells significantly increase the intracellular proline pool along with 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 (Guha Majumder 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). Liposomal and nanoencapsulated QC with a high incorporation rate in hepatocytes have been formulated by us and tested in reducing NaAsO₂ induced liver fibrogenesis and hepatocellular damage. We observed that QC in liposomes and nanocapsules interacts with target cells at a much faster rate than that of free components. Clinical trial of the herbal flavonoidal compound against toxicant induced tissue damage is not possible because of its insoluble property. Quercetin in vesicle based drug delivery systems is expected to be an ideal formulation to protect cells against toxicant induced necrosis in cellular level.

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 depositions in whole liver cells from NaAsO₂ treated rats generate more ROS which in turn produce more lipohydroperoxides and ultimately upregulates gene expression (Table1 & Fig. 5). When arsenic accumulation in liver cell is prevented by nanoencapsulated QC treatment, hepatocellular levels of ROS, lipohydroperoxide and
collagen gene expression are maximally reduced (Table 1 & Fig. 5). The GSSG/GSH ratio was increased substantially by the induction of arsenic. On the other hand membrane microviscosity decreased considerably by arsenic induction. But vesicular QC treatments prevented the alteration of the ratio and membrane microviscosity confirming the protective role of QC against arsenic induced cell damage.

We observed that liver injury is accompanied by the accumulation of arsenic with impaired activity and depletion of hepatocellular antioxidant status in NaAsO$_2$ treated rats. Maximum reduction of liver injury with an improvement of antioxidant status and marked reduction of arsenic content in liver is noticed by the treatment of QC in nanocapsules compared to liposomal QC treatment (Fig. 1 & Table 2).

Our findings indicate that nanoencapsulated QC results in maximum prevention of arsenic deposition and protects liver from NaAsO$_2$ induced collagen deposition and fibrosis initiation in liver (Fig. 1, 5). Administration of QC in nanocapsules to rats protects those animals from arsenic induced acute liver toxicity while free QC does not. QC in nanocapsules may be more protective compared to liposome as enhanced intracellular accumulation of QC in hepatic cells is noticed only when rats are treated with nanoencapsulated QC (Table 5). A single oral dose of NaAsO$_2$ (13 mg/kg b.wt) to rats results in the impairment of the antioxidant status with a marked increase in arsenic content in liver. Our observation suggests that the mechanism of the protective effect of liposomal or nanoencapsulated QC against arsenic induced liver injury can be related primarily to the reduction of arsenic accumulation in liver and it may be because of arsenic chelation or/ and inhibition of liver arsenic entry as observed by Aherne and Brien’O, 2000).
Release of cyt c in cytosol indicates cellular damage (Tang et al., 1998). Our results indicate that NaAsO₂ induces an increase of 17 kd cyt c release in cytosol whereas nanoencapsulated QC treatment 1 h after NaAsO₂ treatment decreases the release significantly. This event suggests that targeted QC in hepatocytes can check the cyt c release in cytosol favouring the protective role of QC against hepatocellular damage.

Our results indicate that arsenite induces an upregulation of TGF β in plasma of rats and nanocapsulated QC treatment prevents it (Fig. 6). Nanoencapsulated QC treatment down regulates pro-collagen gene expression with a prevention of collagen deposition in hepatic cells. The mechanism how nanoencapsulated QC controls arsenic induced pro-collagen gene expression and upregulation of TGF β is not known, however, it may be presumed that nanocapsule mediated targeting of QC to hepatic cells may be a reason for better accumulation of QC in liver and then prevents procollagen mRNA activation with a control of TGF β synthesis in hepatic tissue.

The levels of AST and AP in blood plasma increase by the treatment of NaAsO₂. Targeting of QC prevents NaAsO₂ induced hepatic membrane damage and thus the leakage of AST and AP in the blood circulation is prevented (Table 4). The protective role of QC in nanocapsules against NaAsO₂ induced hepatotoxicity has also been examined by our histopathological and histochemical analysis (Fig. 7).

Whatever the exact mechanism is, judging from the efficiency in reducing oxidative damage, protection from fibrogenesis initiation, controlling of TGF β synthesis and procollagen gene expression in liver and capacity to reduce overall toxicity, quercetin could well be tried in the clinics and best result could be expected when it could be treated in nanoencapsulated form. Obviously, those biocompatible and biodegradable
polymeric nanocapsules have much longer circulation time in blood and they remain unaffected by circulating lipases, protecting the drug from bio-environment and play a key role to deliver QC in liver by following a mechanism of common colloidal particles clearance from circulation by liver.