4. Materials and Methods

4.1.Chemicals and Reagents

Sodium dihydrogen phosphate (NaH_2PO4), disodium hydrogen phosphate (Na_2HPO_4), thiobarbituric acid (TBA), reduced glutathione (GSH), 5-5′-dithiobis-2- nitro benzoic acid (DTNB), agarose (low melting point), cyclohexane, sodium meta arsenite ($NaAsO_2$), potassium di-chromate, acetic acid, ethylene diamine tetra acetic acid (EDTA), Ether, Nitroblue tetrazolium (NBT), Tetramethylethylenediamine (TMEDA or TEMED), Tris, or trisaminomethane, Potassium chloride (KCl), Isoamyl alchol, phenol, Xanthine, Potassium iodide, Eosin and Hematoxylin, Periodic acid- Schiff (PAS), Sodium dodecyl sulphate (SDS), Isopropanol, Riboflavin, Sulfuric acid (H_2SO_4), Hydrochloric acid (HCL), Vanillin, Copper sulphate ($CuSO_4$), Ninhydrin, Free Cysteine, Chloroform, Methanol (molecular grade), Ethanol (molecular grade), Ethidium Bromide, Sodium Chloride (NaCl) , Dimethyl sulfoxide (DMSO), Triton, Sodium lauryl sarcosinate, Trizma base, Sodium hydroxide (NaOH), Hydrogen per oxide (H_2O_2), Ferric chloride, Potassium ferricyanide, tri chloroacetic acid (TCA) and all the reagents (if not mentioned otherwise) used for biochemical assay and DNA electrophoresis were purchased from Merck ind. pvt ltd. Proteinase-K and RNAse were purchased from Life Technologies. All the biochemical kits were collected from Ranbaxy Diagnostic India Limited.

4.2.*Bellamya bengalensis* as a therapeutic agent

In West Bengal fresh water snail market related with mainly *Bellamya bengalensis* which is locally called “Genri”or “Gugly”. We used *Bellamya bengalensis* extract, an edible-snail has long been used by some tribes as traditional medicine in several health-anomalies/liver disorders, as a therapeutic agent with sodium arsenite. These aquatic extract are co administered with arsenite for the same time period to examine its therapeutic efficacy on experimental rat model.

4.3.*Bellamya bengalensis* extract (BBE) preparation

Mature *Bellamya bengalensis* were collected from local control ponds at Midnapore (22.424°N 87.319°E) region (animal sp. are nurtured in a standard condition). The foot flesh (edible part and traditional used for therapeutic purpose) of the organism was homogenized by electric homogenizer to prepare 40% *Bellamya bengalensis* water extract (BBE). The total homogenate tissue was collected in cold condition and centrifuged at 4°C temperature for 45 mints at 12,000 x g. The supernatant was collected and used freshly or stored for couple of days at -20°C.

4.4. Estimation of nutrient and micronutrient content in fresh *Bellamya bengalensis*

4.4.1. Estimation of non-protein soluble thiol in BBE

The NPSH in 10,000×g supernatant from *Bellamya bengalensis* tissue homogenate (prepared in 0.1 M phosphate buffer, pH 7.4) was determined by standard DTNB (5,5′-dithiobis- 2-nitrobenzoic acid) method with a slight modification (Forman 2009). In brief, the protein was precipitated by sulfosalisialic acid and clear cytosol was added to 0.1M sodium phosphate buffer containing 5 μM DTNB. The level of NPSH was determined against a GSH standard curve.

4.4.2. Estimation of ascorbic acid, phosphorus and glycogen in BBE

The reaction of ascorbic acid with ammonium molybdate was initiated to generate molybdenum blue which was estimated to evaluate this vitamin in the extract. The method is appreciably sensitive (2 μg/ml) and specific for having no interference from common reducing sugars, antioxidants and degradation products of the vitamin (Sayed Elnenaey and Soliman, 1979; Barrows et al., 1985). Total phosphorus was estimated spectro photometrically by the method of Chen et al., (1956) with some modifications (Motomizu et al., 1984; Barrows et al., 1985). The glycogen content in *B. bengalensis* tissues was determined by the method of Seifter et al., (1950) with slight modifications (Muriel and Deheza, 2003).

4.4.3. Estimation of total protein, lipid and amino acids in BBE

The total protein was estimated from *B. bengalensis* tissue extract by the method of Lowry et al., (1951). Total Lipid was estimated by an established (González et al., 2006) method. The total amino acid was estimated by ninhydrin method (Bergström et al., 1974).

4.4.4. Determination of calcium in BBE

Quantitative determination of Calcium from BBE by arsenazo lll method was done by Accucare assay kit (Hazari et al., 2012). Absolute values were calculated from the calcium standard curve.

4.5.Arsenic exposure to *Bellamya bengalensis* and its impact

Fresh *Bellamya bengalnsis* were exposed to $NaAsO_2$ at different dose (0 ppm or control, 10 ppm, 12.5 ppm, 15 ppm, 17.5 ppm, 20 ppm) and different duration (48 hours, 72 hours, 96 hours). In another experimental group *Bellamya bengalnsis* were exposed to a fixed dose of $NaAsO_2$ (15ppm) for different duration consisting of 24 hrs gap (24 hrs to 216 hrs) along

with corresponding controls. The animal incubation water was refreshed maintaining the stipulated concentration of NaAsO2.

4.5.1. Estimation of non-protein soluble thiol (NPSH) and malondialdehyde (MDA) level in arsenic exposed *B. bengalensis*

The NPSH assay has been described previous section (Forman, 2009; Maiti et al., 2012). The MDA assay was conducted following the protocol of Buege and Aust, (1978) and Maiti et al., (2014) with a slight modification from control and arsenic exposed *Bellemaya* tissue extract.

4.5.2. DNA fragmentation in sodium arsenite exposed *Bellamya bengalensis*

Fresh *B. bengalensis* muscle tissue is used for DNA extraction, muscle tissue is the best source for DNA in molluscs. Digestive gland may contain parasite and hence the foreign particle, DNA can contaminated a sample.100- 200 mg of muscle tissue was found to produce enough DNA for most application. We routinely slice 100-200 mg of muscle on 2-3 mm thick pieces and squash them between two sheets of clean aluminium foil. Slices tissue are added to 2 ml plastic tube containing 1 ml of lysis buffer (50mM Trish- HCL, pH 7.5, 100mM NaCl, 10 mM EDTA, 1% sodium dodecyl suphate (SDS), 0.2-0.4 mg/ml proteinase K), briefly vortex and incubated 1-2 hrs at 55^0 C for complete digestion. 100 µl saturated KCL is added to the clear lysate and mixed well by repeated tube inverting. The solution is incubated on ice for 5 minutes. At this stage most of the polysaccharides and some proteins are precipitated along with the insoluble potassium dodecyl sulphate. Centrifuged the solution at 16000 rpm for 15 minutes at 4^0 C, collect the supernatant in a sterile tube and extract twice with an equal volume of phenol/chloroform / isoamyl alcohol (25:24:1) mixture. Transfer the clear supernatant to another tube, add an equal volume of isopropanol, mix by inversion and incubate for 10 minutes at room temperature. Then centrifuge for 20 minutes at 15000Xg, discarded the supernatant and wash the DNA pellet in 70 % alcohol. Dry the pellet under

vacuum and dissolve in 100 µl of TE buffer (10 mM tris – HCL, 1 mM EDTA), Add to solution RNAse and incubate at 37^0 C for 60 minutes. The 1% agarose gel with ethidium bromide is run at 5 V for 5 min before increasing to 100 V and documented in gel documentation system (The 0.8% agarose gel with ethidium bromide is run at 5 V for 5 min before increasing to 100 V and documented in gel documentation system (Eugene and Sokolov, 2000).

4.6.Animal Selection and Group distribution

Female albino rats weighing 150-160 gm. were acclimatized for 10 days at 12-h light-dark cycle, 32 ± 2°C temperature, and 50–70% humidity in the institutional animal resource facility. Those were fed a standard pellet diet (Hindustan Lever, Mumbai, India) and water ad libitum. Studies were carried out in accordance with the National Institutes of Health, USA guidelines and the institutional ethical concerns (no. 3.i ec2014) were maintained throughout the investigation. Rats were randomly distributed into three groups having six in each. Animals of group-II and group-III are fed with 0.5 ml drinking water containing sodium arsenite at a concentration of 0.6 ppm (600 µg/L) /100 g body weight / day for 28 days. Initially, several dose-response studies of arsenic were conducted on a rat model. The present dose range usually does not cause animal mortality but exposure for a moderate time period (≥ 3 weeks) increased the level of hepatic and intestinal toxicity and other clinical marker suggesting a significant level of cellular toxicity (Maiti et al., 2014).

4.7.Supplementation with BBE to arsenic-exposed Rats

The group-I designated as control is supplied with the same amount of drinking water for stipulated duration. Group-III is supplemented with *Bellamya bengalensis* extract by gavages at a concentration of 100 mg tissue of *Bellamya bengalensis* /100 g body weight/ day for 28 days. On the day 29, animals are exposed to light anaesthesia (by ether), blood is collected

using a disposable syringe (21-gauge needle) and serum is separated and organs required for biochemical and histological examinations were dissected out. In the in-vitro experimentation, rat liver slices were incubated with arsenite or different oxidant/reductant alone or with BBE or BBV in Kreb's buffer. Post centrifuged (12,000 × g) cytosolic fraction was prepared from tissue homogenate and that was cleaned by dialysis membrane (Spectrum Lab, USA) and concentrated with the amicon centrifugal filter units (Millipore, USA, 6-8kd MWCO) to negate small molecules interferences. The concentrated fraction was tested for SOD1 activity by NBT *(*Nitro blue tetrazolium*)* test in polyacrylamide gel as described in the previous section.

4.7.1. Preparation of rat-tissue and preservation for further use

After 28 days of arsenic treatment and supplement, on the day 29, animals are exposed to light anaesthesia (by ether), blood is collected using a disposable syringe (21-gauge needle) and serum is separated. The small intestinal lumen is washed carefully with slow passages of chilled sterile saline (0.9%) and the epithelial cells are scrapped by a Teflon scrapper after cutting the intestine longitudinally and opening (inside out) the inner surface of the lumen. The tissues/cells are stored at - 40°C until use.

4.7.2. Biochemical estimation of xanthine oxidase and uric acid level

The xanthine oxidase (XO) activity was measured spectrophotometrically from liver homogenate by following the oxidation of xanthine to uric acid (UA) according to the method of Terada et al. 1990 (Terada et al., 1990) with some modifications. The reaction was started by adding 0.15 mM xanthine in 100 mM phosphate buffer (pH 7.5). The rate of uric acid production was recorded for 5 min at 290 nm (ε=12,200 M^{-1} cm^{-1}). Results were expressed as units of XO/mg protein, whereby, 1 unit of activity was defined as the amount of enzyme converting 1.0 μmol xanthine to uric acid at 25°C.

Serum uric acid level is examined by using standard kit (manufactured by crest biosystems).

4.7.3. Biochemical estimation of Lacto peroxidase

The reaction mixture was prepared by diluting 0.15 mL of 0.09 M hydrogen peroxide to 30 mL of 0.005 M potassium iodide. The reaction mixture was stable with no longer than 30 min at room temperature. The reaction mixture was taken into a cuvette and incubated at 25 °C for 3–4 min to achieve temperature equilibrium and establish a blank rate. By adding 0.01-mL diluted tissue homogenate, the test reaction was initiated and the increase in reading was recorded at A 350 for 3 min and calculated as reported earlier (Barrett et al., 1999).

4.7.4. Estimation of oxidative stress markers by spectrophotometric

The tissues where oxidative stress markers measured are homogenized (10 % w / v) in ice-cold phosphate buffer (0.1 mol / L, pH 7.4) and the homogenate is centrifuged at 10,000 rpm at 4ºC for 20 min. The supernatant is used for the estimation of MDA and CD.

4.7.4.1.Malondialdehyde (MDA) estimation by biochemical method

Tissues are homogenized (10% w/v) in ice-cold phosphate buffer (0.1 mol/L, pH 7.4) and the homogenate is centrifuged at 10,000 rpm at 4˚C for 20 min. The supernatant is used for the estimation of malondialdehyde (MDA). The MDA assay is conducted following the protocol of Buege et al. (1978) with a slight modification. To chelate iron and reduce its interference in peroxidation reaction of unsaturated fatty acid, 1 mM EDTA is used in the reaction mixture. To reduce the interference caused by a yellow-orange colour produced by some carbohydrates, the reaction mixture is heated at 80˚C instead of 100˚C. Finally, the MDA is measured and calculated using the molar extinction coefficient of MDA (1.56×10^5 cm^2/mmol) (Maiti and Chatterjee, 2000).

4.7.5. Assay of antioxidant enzymes

4.7.5.1.Superoxide dismutase assay by gel-zymography

A tablet of nitroblue tetrazolium (NBT) was dissolved in 30-mL water and the non-denaturing (10 %) acrylamide gel (Bio-rad, PROTEAN II) was soaked with it for 30 min with shaking. The gel was then shaken in 40-mL super oxide dismutase (SOD) solution (0.028 M tetramethylethylenediamine (TEMED)), 2.8×10−5 M riboflavin, and 0.036 M potassium phosphate at pH 7.8 for 15 min. The soaked gel was placed on a clean acetate sheet and illuminated for 5 to 15 min. The gel became purple except in the position containing SOD (Steinman 1978). The gel was scanned when the maximum contrast between the band and background has been achieved.

4.7.5.2.Catalase assay by spectrophotometric method

Catalase activity was assayed by a colorimetric method (Sinha, 1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of a dichromate-acetic acid mixture and the remaining H_2O_2 was determined as chromic acetate. One unit of activity was expressed as a mole of H_2O_2 consumed/min/mg protein.

4.7.5.3.Estimation of Conjugated Di-ene (CD) Levels

CDs are determined by a standard method (Jendryczko et al., 1988). In brief, lipids are extracted with chloroform– methanol (2:1), followed by centrifugation at 3000 rpm for 5 min, lipid residues are then dissolved in 1.5 mL of cyclohexane and the absorbance is measured at 233 nm in a UV spectrophotometry to determine the amount of hydroperoxide formed.

4.7.6. Estimation of non-protein soluble thiol (NPSH)

The nonprotein soluble thiol (NPSH) in tissue homogenate (prepared in 0.1 M phosphate buffer, pH 7.4) is determined by standard 5, 50-dithiobis-2-nitrobenzoic acid (DTNB) method with a slight modification (Forman et al., 2009). In brief, the protein is precipitated by trichloroacetic acid and clear cytosol is added to 0.1 M sodium phosphate buffer containing 5 mM DTNB. The level of NPSH is determined against a glutathione (GSH) standard curve.

4.7.7. Estimation of serum nitric oxide (NO) level by spectrophotometry method

Nitric oxide was assayed by use of the methemoglobin method, following a procedure described elsewhere, by use of a Beckman spectrophotometer (model DU6). Assay validity was confirmed by use of an independent chemiluminescence method (Karmohapatra et al., 2007).

4.7.8. Estimation of serum TNF-α level by ELISA

Serum TNF-α level was quantified by ELISA using monoclonal antibody according to the method described previously (Engvall and Perlmann, 1972).

4.7.9. Biochemical assays of transaminase, phosphatase and total protein

In the present investigation, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed each with 0.1 mL of plasma and utilizing Lalanine and α-ketoglutarate as substrate for ALT and, L-aspertate and α- ketoglutarate as substrate for AST. Enzyme activities were measured at 340 nm (Bergmeyer et al., 1986; Schumann et al., 2002). To measure the activity of alkaline phosphatise (ALP), 0.1 mL of plasma was incubated at 37ºC in the presence of a mixture of Tris-HCl (pH 8.0) and p-nitrophenyl phosphate. The activity was measured at 405 nm using a visible spectrophotometer (Copeland et al., 1985). Total protein was measured following Biuret method using standard kit from Ranbaxy Diagnostic India Limited, Mumbai, India (Gornall et al., 1949).

4.7.10. Detection of urea and creatinine level by biochemical method

The urea was determined by modified method where 10 µL of plasma was treated with urease at 37ºC and finally chromogen and hypochlorite induced green colour was measured at 570 nm (Fawcett and Scott, 1960). Following the modified Jaffe's kinetic method, creatinine was measured using alkaline picrate with 0.1 mL plasma at 37ºC and reading was taken at a wavelength of 520 nm (Bowers, 1980).

4.7.11. Analysis of Hematopoietic Profile

Total count and differential count (DC) were done manually by using a Spencer Bright-Line haemocytometer blood test kit (American Optical, Scientific Instrumental Division, Buffalo, NY).

4.7.12. Studies on histoarchitecture of rat tissue

The liver and intestine were embedded in paraffin, serially sectioned at 5 µM by an automated cryostat slicing machine (Leica Biosystems), stained with eosin and haematoxylin (Harris) and observed under a microscope (Nikon, Eclipse LV100, magnification ×40) to study the histoarchitecture. A separate set of slides were stained by standard protocol with periodic acid-schiff (PAS) reagent for the study of micronecrosis and presence of polysaccharides, glycolipids, and mucins in intestinal tissues. Both the Nikon microscopy and Leica cryo-cutting facilities were provided by the central laboratory facility (USIC), Vidyasagar University.

4.7.13. DNA fragmentation analysis in rat tissue by ladder assay

Liver tissues and small intestinal epithelial cells are used for DNA preparation, tissue is treated with 500 mL of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg/mL proteinase K) for 20 min on ice (4˚C) and centrifuged in cold at 12,000 g for 30 min. The supernatant is extracted with 1:1 mixture of phenol: chloroform with gentle

agitation for 5 min followed by centrifugation and precipitated in two equivalence of cold ethanol and one equivalence of sodium acetate. After spinning down and decanting, the precipitate is resuspended in 30 µL of deionized water-RNAse solution (0.4 mL water + 5 µl of RNAse) and 5 µl of loading buffer for 30 min at 37˚C. The 0.8% agarose gel with ethidium bromide is run at 5 V for 5 min before increasing to 100 V and documented in gel documentation system (Garcia-Martinez et al., 1993). The band intensity of DNA at its different relative positions on the agarose gel is evaluated by Image (J) software, expressed in relative/normalized values and plotted in a graph.

4.7.14. Single- cell DNA damage estimation in rat tissue by COMET assay

Cell suspension is prepared after it is scrapped and collected by a Teflon scrapper from the intestinal parts of animals after sacrifice and immediately before the assay procedure. It is performed in a cold room and under dimmed light to avoid any possible exposure to UV light. The chilled 1X PBS [calcium and magnesium free and Dimethyl sulfoxide (DMSO) added] is used to minimize endoglucanase activities and minimize the iron of hem (from haemoglobin) associated free radical interference. Using blunt-ended tips or thick syringe, cells are prepared homogeneous suspension and clump free. After the cell count cell number is adjusted to 10^5 cells/mL and kept in microcentrifuge. The alkaline Comet assay is done according to the method of Singh et al. with some minor modifications (Singh et al., 1988). A total of 75 µl of low melting point agarose (0.5%) in PBS at 37˚C is added to a 25 mL cell suspension. The mixture is then dropped onto a microscope slide precoated with 1% agarose and a cover slip is placed on it. Once the agarose solidifies the cover slips are removed and the slides are immersed in ice-cold lysis buffer (2.5 mM NaCl, 85 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, and 1% sodium lauryl sarcosinate, adjusted to pH 10) for 1 h at 4˚C. After lysis the slides are washed three times in

PBS at room temperature. Slides are then placed in a submarine gel electrophoresis chamber filled with alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 25 min. Then the electrophoresis is done for 30 min at 25 V and the current is adjusted to 300 mA by raising the buffer level. Slides are then neutralized with PBS and stained with a solution of 2 mg/mL ethidium bromide for 3- 4 min. Excess stain is removed by washing in water. Slides are read using a fluorescence microscope. A total of 100 comets /slide are read for each experiment. The alkaline Comet assay is done according to the method of Singh et al. with some minor modifications (Singh et al., 1988). A total of 100 comets /slide are read for each experiment.

4.7.15. Measurement of Mitochondrial Membrane Potential in rat liver

The alteration of mitochondrial membrane potential was evaluated using the spectro-fluorometric (M'Bemba-Meka et al., 2006) with some modifications. Rhodamine 123, a cationic fluorescent dye whose mitochondrial fluorescence intensity decreases quantitatively in response to the degeneration of the mitochondrial membrane potential, was estimated in terms of the perturbations in mitochondrial membrane potential (Wu et al., 1990). Mitochondria from experimental rat liver were isolated first (Scaduto, 1994). Incubations were conducted at either 28 or 37°C in a medium composed of 135 mM KCl, 20 mM MOPS, 5 mM K_2HPO_4, and 5 mM $MgCl_2$ at pH 7.00. Incubations recipe also contained Rhodamine 123 solution at concentrations indicated. The cellular fluorescence intensity of Rhodamine 123 was monitored for 2 min using a Hitachi F-7000 Fluorescence Spectrophotometer. An aliquot of cell suspension was also used for microscopic observations (Nikon Eclipse LV100POL) for the visual evaluation of the membrane stability. The mitochondrial membrane-potential was plotted as line diagram at an excitation wavelength of 493 nm and

an emission wavelength of 522 nm. Both the excitation and emission slit width were set to 5.0 mm.

4.8.In-vitro studies in tissue slices

4.8.1. In-vitro experiment in rat liver slices and intestinal tissue

Liver slices from albino female rat were incubated under five different experimental condition in Krebs ringer buffer for 2 and 4 hours accordingly, group -1 control (1A ,1B, 1C), group -2 sodium arsenite ($NaAsO_2$) (1mM) +H_2O_2(100 mM) (2A,2B,2C), group -3 NaAsO2 (1mM) (3A, 3B, 3C), group -4 NaAsO2 (1mM) +H_2O_2(100 mM) +100ul BBE(40%) (4A, 4B, 4C), group -5 $NaAsO_2$ (1mM) +100ul BBE (40%) (4A, 4B, 4C). Post centrifuged (12,000 x g) cytosolic fraction was prepared from tissue homogenate and that was cleaned by dialysis membrane (Spectrum Lab, USA) and concentrated with the Amicon centrifugal filter units (Millipore, USA, 6-8kd MWCO) to negate small molecules interferences.

4.8.2. In-vitro regulation of SOD activity in liver slices

Post-centrifuged (12,000 × g) cytosolic fraction was prepared from control rat hepatic tissue homogenate, and that was cleaned by dialysis membrane (Spectrum Lab, USA) and concentrated with the amicon centrifugal filter units (Millipore, USA, 6–8 KD MWCO) to negate small molecules interferences. The concentrated fraction was incubated with different concentration of arsenite-H_2O_2 combination or with BBE and *Bellamya bengalensis* venom, BBV (which is reported to be enriched with thiol containing protein) in Kreb's buffer. The SOD activity was tested by NBT test in polyacrylamide gel as described earlier (Acharyya et al. 2014b).

4.8.3. Catalase activity assay by gel-zymography from in-vitro studies

Catalase activity assay done by gel-zymography using 8% native gel. Chemicals used 0.003% H_2O_2 (30% solution vol/vol), 2% ferric chloride (wt/vol), 2% potassium ferricyanide (wt/vol). After staining developed a green-blue in colour with white band where the enzyme is present. Following separation of native protein, the catalase enzyme removes the peroxides from the area of the gel it occupies. Removal of peroxide does not allow for the potassium ferricyanide (a yellow substance) to be reduced to potassium ferrocyanide that reacts with ferric chloride to form a Prussian blue precipitate (Treadwell., 1948).

4.8.4. Estimation of malondialdehyde (MDA) level from in-vitro studies

The MDA assay was conducted following the protocol as describe earlier (Buege et al., 1978) with a slight modification.

4.8.5. Estimation of non-protein soluble thiol (NPSH) from in-vitro studies

The NPSH assay was conducted following the protocol as describe earlier. (Forman et al., 2009)

4.8.6. Comet assay in liver slice from in-vitro studies

The alkaline comet assay was conducted for the confirmation of DNA damage following the guidelines (Singh et al., 1988) with slight modifications.

4.8.7. In vitro study of Catalase and SOD (Cu-Zn SOD or SOD1) activity by sodium arsenite with free phosphorus and cysteine.

Liver slices from albino female rat were incubated under five different experimental condition in Krebs ringer buffer for 2 and 4 hours accordingly, and its protection by Na_2HPO_4 and L-cysteine. Group1-control, 2- $NaAsO_2$ (250µM), 3 -$NaAsO_2$ (250µM) +H_2O_2

(100mM), 4- $NaAsO_2$ (250µM) + Na_2HPO_4, 5- $NaAsO_2$ (250µM) + L- Cysteine (100mM), 6- $NaAsO_2$ (250µM) +H_2O_2 (100mM) + Na_2HPO_4, 7- $NaAsO_2$ (250µM) +H_2O_2 (100mM) + L-Cysteine (100mM). Post centrifuged (12,000 x g) cytosolic fraction was prepared from tissue homogenate and that was cleaned by dialysis membrane (Spectrum Lab, USA) and concentrated with the Amicon centrifugal filter units (Millipore, USA, 6-8kd MWCO) to negate small molecules interferences.

Catalase and SOD (Cu-Zn SOD or SOD1) activity by gel-zymography were conducted following the protocol as describe earlier. (Treadwell., 1948) (Acharyya et al., 2014b).

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