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Original Article

Promising anti-oxidative therapeutic potentials of edible freshwater snail *Bellamya bengalensis* extract against arsenic-induced rat hepatic tissue and DNA damage

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Abstract: Epidemiological data suggest that arsenic ultimately results in cancer in different parts of the body. Several synthetic therapeutic agents manifest inadequate potency with severe side effects against arsenic toxicity. The flesh of *B. bengalensis*, has long been used as an ethno-medicine in case of arthritis, blood-impurities, impaired immune system, conjunctivitis and liver anomalies. This potent organism might be a natural choice against arsenic and several other toxicities. Our earlier studies on arsenic-exposed human can correlate carcinogenesis with DNA-damage. In an attempt to investigate the possible protective and therapeutic effect against arsenic induced hepatotoxicity, the extract of *B. bengalensis* was tested in arsenic intoxicated rat model. The time- and dose-dependent effect of arsenic toxicity was also tested in *B. bengalensis*. Sodium-meta-arsenite $NaAsO_2$ (0.6 ppm/100g bw/day for 28 days, as earlier reported) was treated alone or in combination with the *B. bengalensis* water extract (BBE, 100 mg/100g bw) to rat and compared with vehicle treated control. In a separate experiment, the *B. bengalensis* was exposed to high concentration of $NaAsO_2$ contaminated water (5 to 20 ppm for 1 to 9 days) in laboratory condition and their DNA quality was evaluated in relation to its possible oxidative threat. Any concentration of arsenic was incapable to initiate a significant DNA damage in *B. bengalensis*. Lipid peroxidation was increased in arsenic exposed *B. bengalensis* after longer duration of its exposure. Increase in reduced antioxidant like non-protein-soluble thiol (NPSH) is concordant with the decrease in lipid peroxidation and DNA stability in this organism. In rat experiment, the BBE supplementation strongly prevented arsenic-induced oxidative, necrotic and apoptotic damages to liver tissue/DNA by strengthening antioxidant systems, which has been shown in hepatic DNA-fragmentation, comet-assay, histo-architecture (hematoxylin/eosin), alkaline-phosphatase, serum-glutamate-pyruvate-transaminase and lactate-dehydrogenase (tissue-degeneration-marker) results. Only arsenic exposure decreased hepatic superoxide-dismutase (SOD) in-vivo and in-vitro (H_2O_2/arsenite redox-stress to dialyzed and concentrated, 6-8kd cutoff-Millipore liver cytosolic SOD), catalase, xanthine-oxidase, lactoperoxidase activities and the level of NPSH with a concomitant increase in malondialdehyde resulting in mutagenic DNA-breakage and apoptotic liver damage which has been decisively restrained in *B. bengalensis* extract. The present investigation offers strong evidence on the hepato-protective and medicinal efficiencies of BBE against oxidative stress induced by arsenic.

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Introduction

Clinical manifestations of the chronic arsenicosis are the skin lesions, keratosis, different metabolic disorders and finally cancer in several organs including liver, lung, and skin, of the exposed individuals. The liver is a major target tissue of arsenic toxicity. A few drugs like British Anti Lewsite (BAL) and dimercaptosuccinic acid (DMSA) are available and mundane in the market as arsenic chelating agents, but all these have severe side effects (Inns et al., 1990). Several components of herbal origin like quercetin (a flavonol, IUPAC

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name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4 *H*-chromen-4-one), combination of monoisoamyl dimercaptosuccinic acid (MiADMSA) and *Moringa oleifera* Lam (family Moringaceae), *Hippophae rhamnoides* L., curcumin ((a diarylheptanoid, IUPAC name: (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione)), *Emblica officinalis* Gaertn (a deciduous tree of the family Phyllanthaceae and synonym of *Phyllanthus emblica* L.) and some other phytochemicals have been demonstrated to show varied extent of protection against arsenic-induced oxidative stress, DNA breakage, hepatic damage, fibrosis and carcinogenesis (Ghosh et al., 2010; Chattopadhyay et al., 2011; Flora et al., 2012; Maiti et al., 2014, Srivastava et al., 2014). Recent studies in our laboratory elucidate the protective role of Green tea flavonoids (*Camellia sinensis*, family Theaceae) and vitamins (B12; cyanocobalamin + folate) against arsenic-induced mutagenic DNA-breakage/ intestinal damages in experimental rat model (Acharyya et al., 2015a, Acharyya et al., 2015b). Though several researches investigated on phyto-chemicals, synthetic/natural micronutrients, but focus has not been made on tissue extracts from some small organisms/animals of certain ethno-medicinal importance.

Some edible shell fishes are well-known to provide the economically important nutraceuticals, which are of great medicinal values. These are consumed by the indigenous people of the rural and urban areas of the different parts of the world (Deb and Emdad Haque, 2011; Kim and Pallela, 2012). Different species of these organisms are biologically diverse in nature and belongs to the genera of the *Paratelphusa*, *Macrobrachium*, *Bellamya*, *Pila*, *Achatina*, *Lamellidens*, *Novaculina* and *Parreysia* (Mahata, 2002; Westneat and Alfaro, 2005; Ma et al., 2010; Arul Prakash et al., 2011; Venugopal and Siva Kumar, 2014; Shabelnikov and Kiselev, 2015; Ma et al., 2016). Those are long been used as the traditional medicines and potent micronutrient supplier in a number of ailments such as rheumatism, cardiac diseases, hypertension, asthma, rickets, hypocalcemia and nervousness (Fenical, 1997; Mahata, 2002; Deb and Emdad Haque, 2011; Kim and Pallela, 2012). The foot of *Pila* sp., *Bellamya* sp., *Lamellidens* sp. and *Parreysia* sp. are large muscular and proteins/ micronutrient rich fat free part of these organisms (Prabhakar and Roy, 2009; Ma et al., 2010; Kim and Pallela, 2012; Venugopal and Siva Kumar, 2014). Secretion extract of *Bellamya bengalensis* constitute potent anti-neoplastic/ anti-proliferative agent which is cytotoxic and apoptogenic against human myeloid leukemia cells (Besra et al., 2013). The hepatoprotective activity of *B. bengalensis* flesh extract has been shown in carbon tetrachloride-induced liver damages in rat model (Gomes et al., 2011).

In this background, the present study was designed to elucidate the therapeutic effects of the water extract of *B. bengalensis* for the management of arsenic induced hepatic toxicity and its possible mechanistic approach in the experimental rat model. We also made effort to find a cheap non-invasive medicinal-protective source against arsenic-associated toxic health hazards.

Materials and Methods

Chemicals and Reagents: Sodium arsenite, Bovine serum albumin (BSA), thiobarbituric acid (TBA), reduced glutathione (GSH), 5-5´-dithiobis-2-nitro benzoic acid (DTNB), ammonium molybdate, ascorbic acid, nitro blue tetrazolium (NBT), agarose (low melting point), acrylamide, N,N'-methylene-bis-acrylamide, ammonium per sulphate, xanthine, and tetramethylethylenediamine (TEMED) were purchased from Sigma chemicals (St. Louis, MO). Sodium dihydrogen phosphate (NaH2PO4), disodium hydrogen phosphate (Na2HPO4), ethylenediamine tetraacetic acid (EDTA), and eosin and hematoxylin were supplied from SRL, India or MERCK, India. For Urea, Total protein, Serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and uric acid assay kits were purchased from either from Ranbaxy Laboratories Limited, India or MERCK India, Worli, Mumbai 400018.

Bellamya bengalensis extract (BBE) preparation: Mature *B. bengalensis* were collected from local control ponds at Midnapore (22.424°N, 87.319°E) region (animal sp. are nurtured in a standard condition) and carefully washed to remove clay particle then blotted on a soak paper to remove excess moisture. The flesh (edible part) of the organism was homogenized by electric homogenizer to prepare 40% *B. bengalensis* water extract (BBE). The total homogenate tissue was collected in cold condition and centrifuged at 4°C temperature for 10 min at 10,000 rpm. The supernatant was collected and used freshly or stored for couple of days at -4°C.

Estimation of non-protein soluble thiol from BBE: The Nonprotein Soluble Thiol (NPSH) in *B. bengalensis* tissue extract was determined by standard 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) method with a slight modification (Forman, 2009). In brief, the protein was precipitated by trichloro-acetic acid and clear cytosol was added to 0.1 M sodium phosphate buffer containing 5 mM DTNB. The level of NPSH was determined against a glutathione (GSH) standard curve.

Estimation of ascorbic acid, phosphorus and glycogen from 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).

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 (Barnes and Blackstock, 1973; Rodríguez-González et al., 2006) method. The total amino acid was estimated by ninhydrin method (Moore and Stein, 1954; Bergstrom et al., 1974).

Determination of calcium from BBE: Quantitative determination of calcium from BBE by arsenazo III method was done by Accucare assay kit. Calcium with Arsenazo III (1, 8- Dihydroxy-3, 6-disulpho-2, 7-naphthalene-bis (azo)-dibenzenearsonic acid) yielded a blue colored complex (Hazari et al., 2012). Absolute values were calculated from the calcium standard curve.

Time and dose dependant exposure of sodium arsenite ($NaAsO_2$) to B. bengalensis: Live adult *B. bengalensis* were separated in 6 groups and housed in laboratory environment. Those were exposed to $NaAsO_2$ with different doses (0, 10, 12.5, 15, 17.5 and 20 ppm). The animals were collected in 48, 72 and 96 hrs interval and tissue extract was prepared by standard laboratory protocol and used for following biochemical experiments. In another experiment, organisms were exposed to 15 ppm of $NaAsO_2$ for nine days and in regular interval their tissues were collected for DNA stability study.

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.

DNA fragmentation in arsenic exposed B. bengalensis: DNA fragmentation assay was performed in the tissues of *B. bengalensis* exposed to arsenic in a dose and time-dependent manner (Beltran et al., 2008). This assay is described in the preceding section.

Testing of protective potential of B. bengalensis in arsenic–induced hepatotoxic rat model: **Animal selection and arsenic treatment**: Female albino rats weighing 150-160 g 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 with 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 were maintained throughout the investigation. Rats were randomly distributed in three groups having six in each. Animals of group-II and group-III were fed with 0.5 ml drinking water containing sodium arsenite at a concentration of 0.6 ppm/100 g bw/day for 28 days. Initially, several dose response studies of arsenic were conducted on rat model. The present dose range usually does not cause animal mortality but exposure for a moderate time period (≥ 3weeks) increased the level of liver and kidney toxicity marker and other clinical marker suggesting significant level of cellular toxicity (Maiti et al., 2014). The group-I, designated as control was supplied with same amount of drinking water for stipulated duration. The Group-III animals were supplemented with *B. bengalensis* water extract by gavages at a concentration of 100 mg tissue of *B. bengalensis* /100 g body weight/ day for 28 days. On the day 29, animals were exposed to light anesthesia (by ether), blood was collected using a disposable syringe (21-gauge needle) and serum was separated and organs required for biochemical and histological examinations were dissected out, stored at -20ºC.

Evaluation of general toxicity: liver and kidney function tests: Serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), urea, uric acid and lactate dehydrogenase (LDH) were measured from the rats by standard protocol with the assay kits (Ranbaxy, India or other reputed company). Total protein (serum) was measured following Biuret method using standard kit from Ranbaxy Diagnostic India Limited, Mumbai, India.

Determination of catalase activities: The catalase activity was measured spectrophotometrically in hepatic cytosolic sample observing the rate of degradation of H_2O_2, the substrate of this enzyme (Aebi, 1984; Maiti and Chatterjee, 2001).

Estimation of liver xanthine oxidase activities: Xanthine Oxidase (XO) activity was measured spectrophotometrically from liver tissue homogenate by following the oxidation of xanthine to uric acid according to the method of 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). Liver homogenate was used as the enzyme source. The rate of uric acid production was recorded for 5 min at 290 nm (ε=12,200 $M^{-1}cm^{-1}$). The results were expressed as units of XO/mg protein, whereby 1 unit of XO was defined as the amount of enzyme converting 1.0 μmol xanthine to uric acid at 25°C.

Determination of lactoperoxidase activities: Lactoperoxidase (LPO) activity was measured in 0.1 M phosphate buffer (pH=7.4), containing 0.6 mM 2, 2´- azion- bis (3-ethylbenez- thiazoline-6- sulfonic acid (ABTS) and 0.1 mM H_2O_2 as described by (Barrett et al., 1999).

Estimation of Malondialdehyde (MDA) level: The MDA assay was conducted following the protocol of Buege and Aust (1978) with a slight modification. To chelate iron and reduce its interference in peroxidation reaction of unsaturated fatty acid, 1 mM EDTA was used in the reaction mixture. To reduce the interference caused by a yellow-orange color produced by some carbohydrates, the reaction mixture was heated at 80°C instead of 100°C. Finally, the MDA was measured and calculated using the molar extinction coefficient of MDA ($\varepsilon=1.56\times10^5$ cm^2/mmol) (Maiti and Chatterjee, 2000; Maiti et al., 2014).

Assay of super oxide dismutase activities: A tablet of nitro blue tetrazolium (NBT) was dissolved in 30 mL water and the non-denaturing (10%) acrylamide gel was soaked in it for 30 min with shaking. The gel was shaken in 40 mL superoxide dismutase (SOD) solution [0.028 M tetra methyl ethylene di amine (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 5-15 min. The gel became purple except at the position containing SOD (Ismail, 2006). The gel was scanned when the maximum contrast between the band and background has been

achieved. An identical gel was stained with coomassie brilliant blue to verify the liver protein of the corresponding SOD protein bands in different group of animals (Acharyya et al., 2014).

Estimation of non-protein soluble thiol: The Non-Protein Soluble Thiol (NPSH) in liver tissue homogenate and *B. bengalensis* extract (prepared in 0.1 M phosphate buffer, pH=7.4) was determined by standard 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) method with a slight modification (Forman, 2009). In brief, the protein was precipitated by trichloroacetic acid and clear cytosol was added to 0.1 M sodium phosphate buffer containing 5 mM DTNB. NPSH was measured and calculated using a GSH standard curve.

Histology and DNA fragmentation analysis: Liver tissue was embedded in paraffin, serially sectioned at 5 mM, stained with eosin and hematoxylin (Harris) and observed under a microscope (Nikon, Eclipse LV100, magnification X400) to study the histoarchitechture. The liver cells were used for DNA preparation, tissue was 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 was 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 tenth equivalence of sodium acetate. After spinning down and decanting, the precipitate was re-suspended in 30 mL of deionized water-RNase solution (0.4 mL water15 mL of RNase) and 5 mL of loading buffer for 30 min at 37°C. The 0.8% agarose gel with ethidium bromide was run in Bio-Rad submarine electrophoresis system at 5V for 5 min before increasing to 100 V and the band image was captured in a gel documentation system. The band intensity of DNA at its different relative positions on the agarose gel was evaluated by ImageJ software (Garcia-Martinez et al., 1993), expressed in relative/ normalized values and plotted in a graph.

Comet assay: The alkaline comet assay was conducted for the confirmation of DNA damage following the guidelines proposed by Singh et al. (1988) with slight modifications. The microscope slides were coated with 0.75% normal melting point agarose in PBS, 25 µl of liver cell suspension was mixed with 110 µl of 0.5% low-melting-point agarose in phosphate buffered saline (PBS) and applied to slides. After electrophoresis, the slides were neutralized with neutralizing buffer (Tris 0.4 M, pH=7.5) and then stained with ethidium bromide. Detection of DNA breakage was measured by using ImageJ software. Slides were read using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (Impuls Bildanalyse) software. A total of 100 comets/ slide were read for each experiment.

In vitro regulation of SOD activity: 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 *B. 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., 2014).

Results

Arsenic toxicity in B. bengalensis tissue: A dose and time dependant increase in MDA production is noticed in this tissue which has been ceased and further decreased in longer time at higher dose (Fig. 1). This suggests the possible acclimatization by the organism in the present toxic environment. This result is found to be related by the protecting antioxidant agent i.e. NPSH. A dose and time dependant increase of NPSH is evident (Fig. 1). No significant DNA-fragmentation is noticed in dose (5-20 PPM $NaAsO_2$) and time (1-9 days) dependent experiment (Fig. 2).

Components in B. bengalensis tissue extract: The

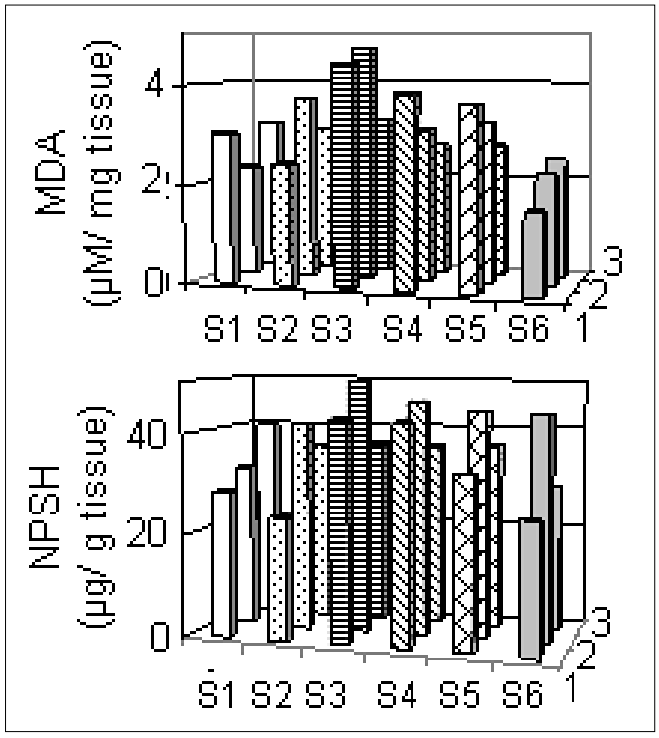

Figure 1. Non Protein Soluble Thiol (NPSH) and Malondialdehyde (MDA) level in *Bellamya bengalensis* tissues after its dose and time dependent exposure to arsenic ($NaAsO_2$). Bars in the figure represent as follows; S1 control, S2 10 ppm, S3 12.5 ppm, S4 15 ppm, S5 17.5 ppm, and S6 20 ppm $NaAsO_2$ exposed group and 1 denotes 48 hrs, 2 is 72 hrs and 3 is 96 hrs.

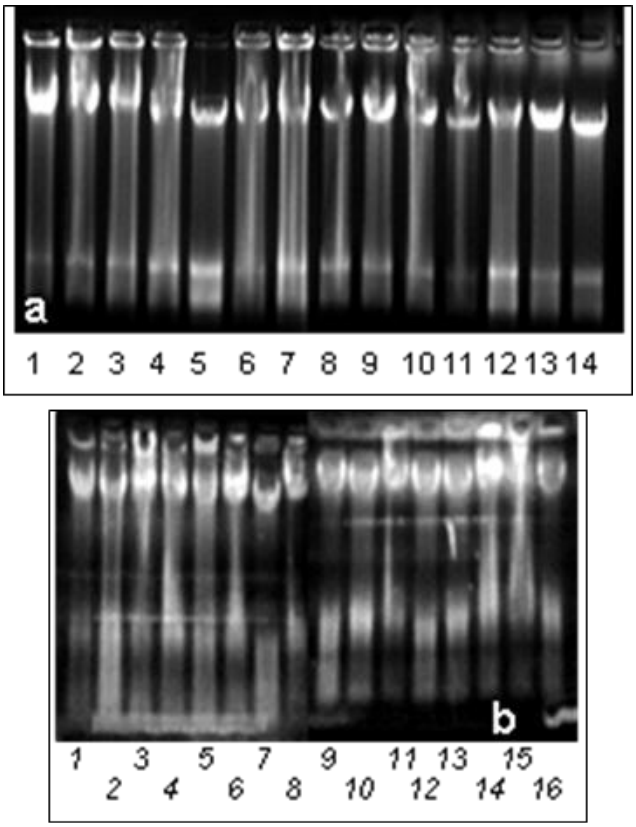

Figure 2. The effect of arsenic exposure on the quality and stability of DNA of *Bellamya bengalensis* tissue. Lane distribution; (panel a) Lane 1- 48 hrs control, 2- 48 hrs control, 3- 48 hrs 10 ppm, 4- 48 hrs 12.5 ppm, 5- 48 hrs 15 ppm, 6- 48 hrs 17.5 ppm, 7- 48 hrs 20 ppm, 8- 96 hrs control, 9- 96 hrs control, 10- 96 hrs 10 ppm, 11- 96 hrs 12.5 ppm, 12- 96 hrs 15 ppm, 13- 96 hrs 17.5 ppm and 14- 96 hrs 20 ppm of $NaAsO_2$ in the water where the animals were kept. (Panel b) The 15 ppm of $NaAsO_2$ was treated to the *B. bengalensis* in a time dependant manner. Lane distribution; Lane 1- 24 hrs control, 2- 24 hrs of $NaAsO_2$ exposed, 3- 48 hrs control, 4- 48 hrs of $NaAsO_2$ exposed 5- 72 hrs control, 6- 72 hrs of $NaAsO_2$ exposed 7- 120 hrs control, 8- 120 hrs of $NaAsO_2$ exposed, 9- 144 hrs control, 10- 144 hrs of $NaAsO_2$ exposed, 11- 168 hrs control, 12- 168 hrs of $NaAsO_2$ exposed, 13- 192 hrs control, 14- 192 hrs of $NaAsO_2$ exposed, 15- 216 hrs control, 16- 216 hrs of $NaAsO_2$ exposed. At every 48 hrs, the animal incubation-water was refreshed maintaining the stipulated concentration of $NaAsO_2$.

level of nutrients and micronutrients are presented in the Table 1. The protein content in the flesh of *B. bengalensis* was found to be 54.59 mg/g tissue. Flesh of *B. bengalensis* contains moderate amount of carbohydrate, 25.75 mg/g and lipid, 242 µg/g. Similarly, the micronutrient and non-protein soluble thiol (free -SH) levels are also described in Table 1.

Status of oxidative stress markers: The MDA content in liver homogenates significantly increased in the sodium arsenite-exposed rats ($P<0.05$) which is restored in the BBE supplemented group and this result is significantly reciprocated by the tissue NPSH level (Fig. 3). The strong antioxidant enzymes i.e. lactoperoxidase ($P<0.05$) and xanthine oxidase ($P<0.01$) and the oxidant-neutralizing molecule uric acid ($P<0.05$) have been found to be decreased significantly in arsenic treated group and restrained in the BBE supplemented group (Fig. 3).

General toxicity: Liver and kidney function test: Figure 4 suggests that the serum SGPT and ALP enzymatic activities are significantly increased ($P<0.05$) in arsenic intoxicated rat suggesting necrotic tissue damage which has been ceased and restored by the BBE supplementation. Similarly, the increase in the kidney function marker, urea and general tissue degeneration marker like LDH are also nullified by the BBE supplemented group in arsenic exposed rats (Fig. 4).

Hepatic tissue architecture, DNA fragmentation and

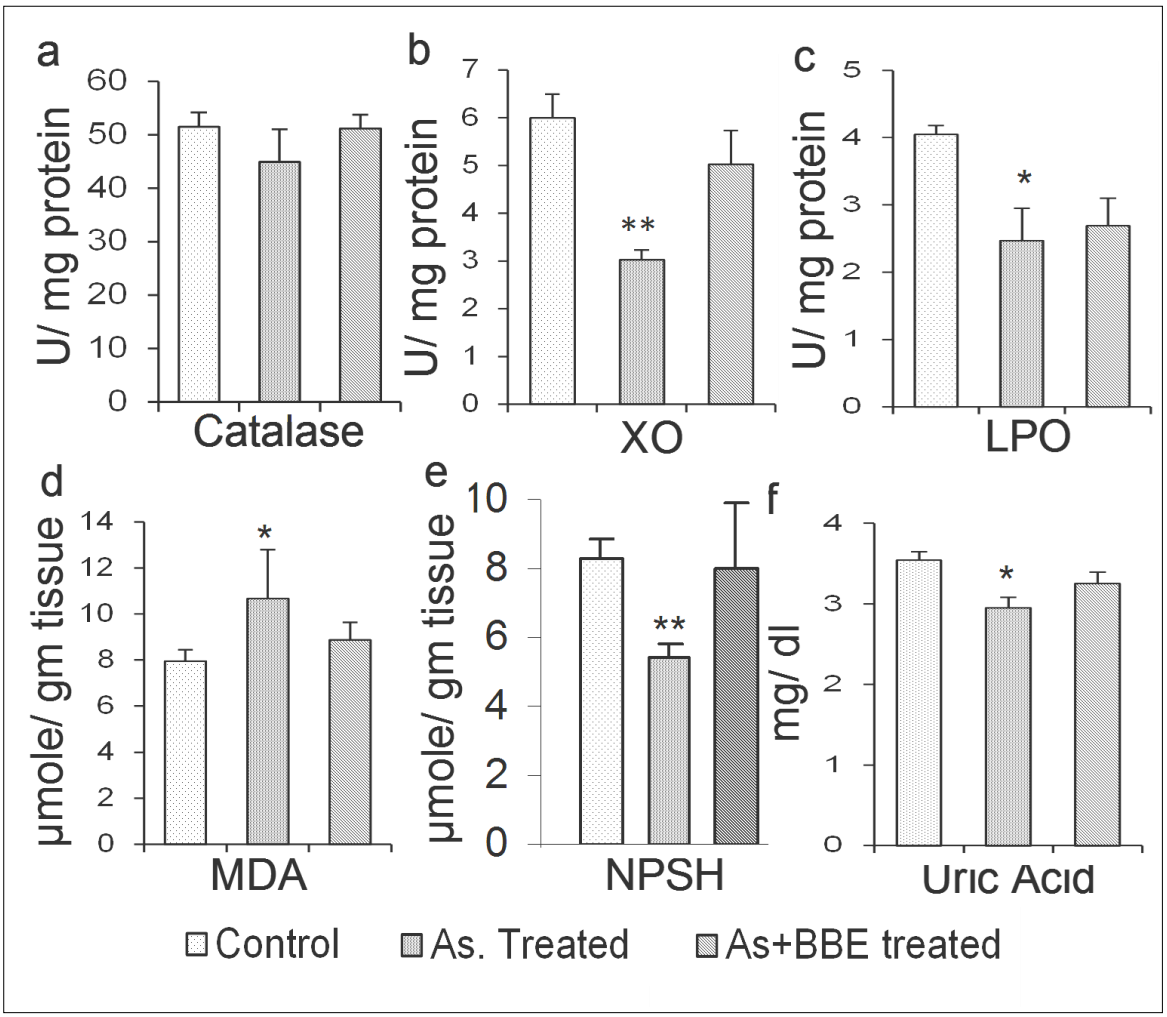

Figure 3. The effects of arsenic toxicity (0.6 ppm/100g bw/day for 28 days) and its possible preventive/ therapeutic effects of BBE (100 mg/100g bw) on different antioxidant enzymes and cellular components in female rat liver. The malondialdehyde (MDA) is regarded as the oxidative stress marker representing the deleterious effects of Reactive Oxygen Species (ROS) on cellular lipid components. All the parameters were measured in the hepatic tissues except the antioxidant uric acid, which was measured in serum sample. Results are mean ±SE (n=6 in each group). Data of treated group or supplemented group is compared to the corresponding vehicle treated group (Student 't' test). Levels of significances are denoted as; *=P<0.05; **=P<0.01.

Table 1. Composition of Nutrients and micro-nutrients in the *Bellamya Bengalensis* tissue.

Parameters	Concentration in BBE
Phosphorous (µg/g)	52.24±3.89
Vitamin C (µg/g)	71.85±4.74
Calcium (mg/g)	0.36±0.023
Protein (mg/g)	54.59±4.75
Total lipid (µg/g)	242±23.54
Total carbohydrate (mg/g)	25.75±2.94
Total amino acid (mg/g)	0.96±0.056
NPSH (µg/g)	50.8±3.7

comet assay result: Arsenic ingestion with the present dose and duration decisively resulted in appreciable amount of "ladder" of DNA fragments (lane 4, 5) in liver with comparison to the control rats (lane 1, 2, 3), whereas, the DNA is found to be partially but significantly protected from fragmentation in BBE co-administered group (Fig. 5). The DNA/ladder density (normalized value) is calculated for the different migrated locations on the lane in the gel and the mean values are plotted against the relative migrated location which clearly reveals the outcome noticed in the ladder image.

The single cell DNA status (comet assay) basically supports the DNA fragmentation results. The number of comet forming cells is found to be higher in arsenic exposed rat liver whereas, in BBE supplemented group the genetic materials of the cells are noticed to be highly protected (Fig. 6).

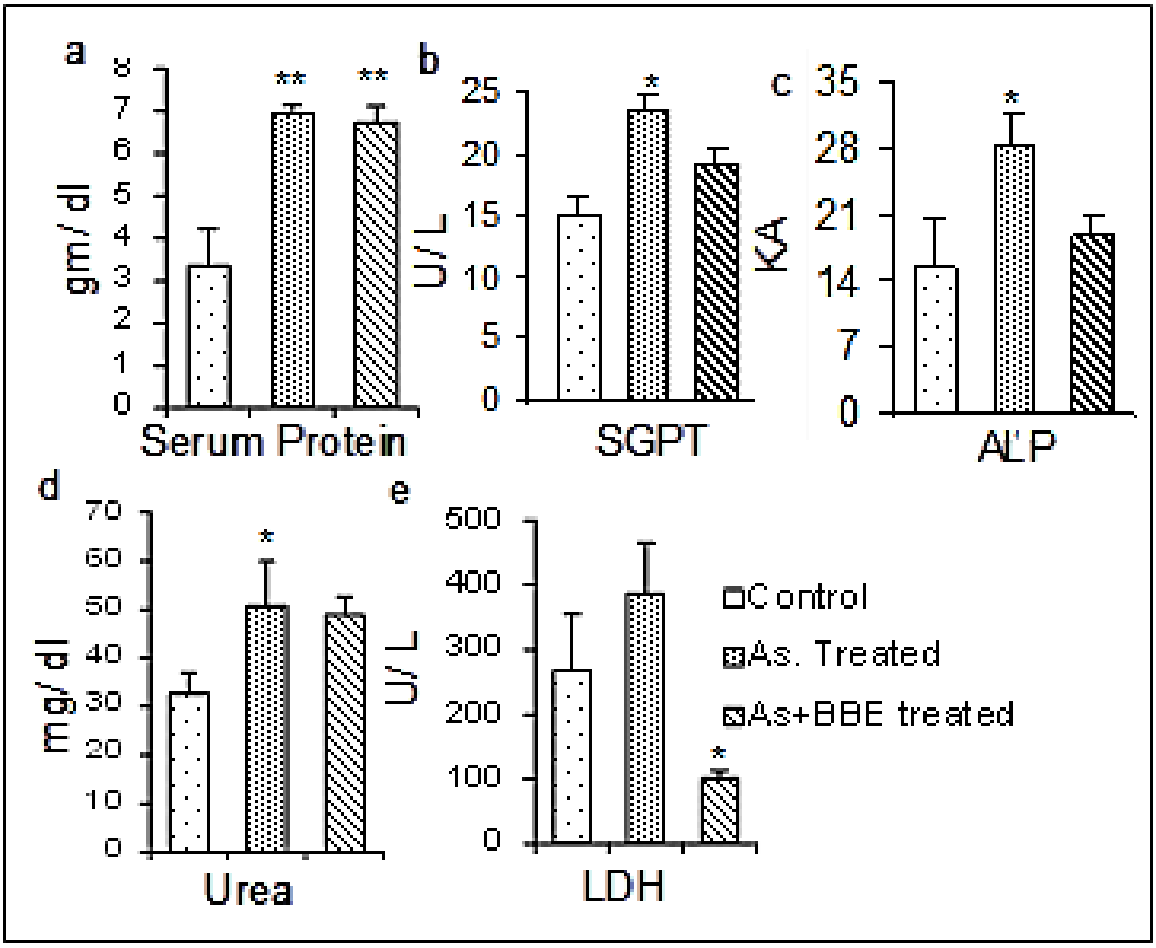

Figure 4. The effects of arsenic toxicity (0.6 ppm/100g bw/day for 28 days) and its possible preventive/ therapeutic effects of BBE (100 mg/100g bw) on different functional markers of liver and kidney, metabolic inflammatory, and tissue necrotic markers. The parameters were measured with the standard assay kit (refer to the materials and methods section). Result shows that arsenic induced impairment of liver and kidney functions are significantly restored by the BBE supplementation. Results are mean ±SE (n=6 in each group). Data of treated group or supplemented group is compared to the corresponding vehicle treated group (Student 't' test). Levels of significances are denoted as; *=P<0.05; **=P<0.01.

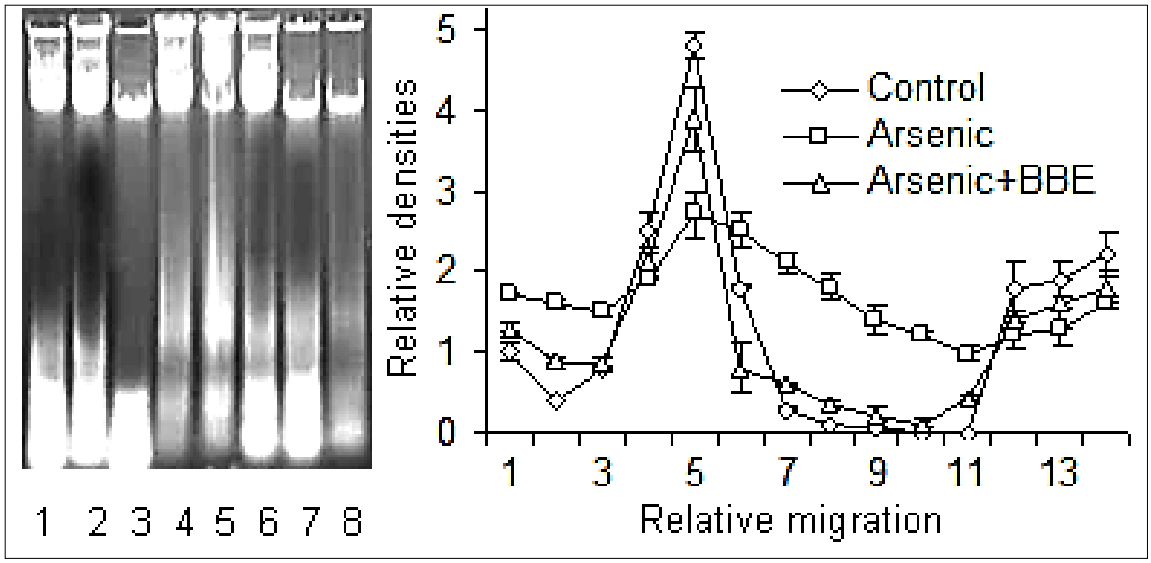

Figure 5. DNA fragmentation result is shown in liver of female rats treated with arsenic. Lane distribution; Lanes 1, 2, 3- control; lanes 4, 5- $NaAsO_2$ exposed and lanes 6, 7, 8- $NaAsO_2$ + BBE (left panel). Densitometry analysis of the different bands was done in ImageJ software and the mean normalized values are plotted at the position of their relative migration.

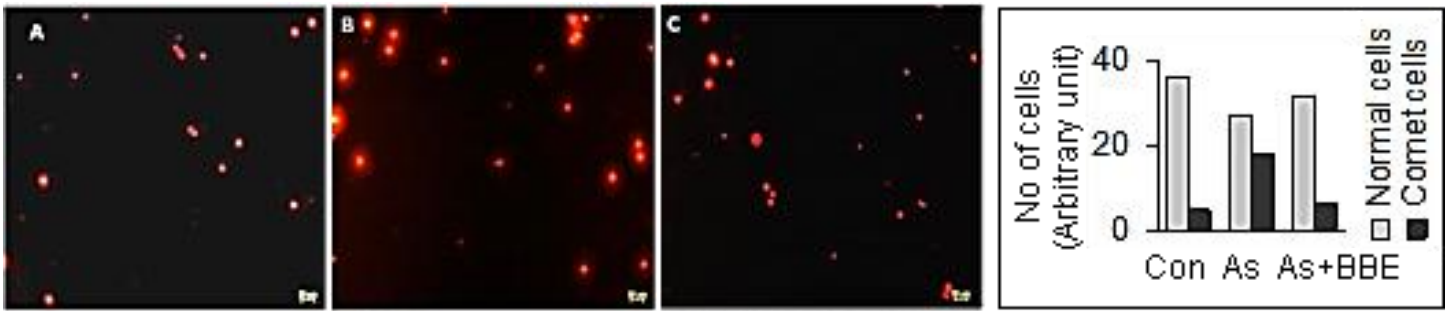

Figure 6. Arsenic induced hepatic DNA breakage in single cell apoptotic damage which is markedly prevented by the BBE administration. Panels are represented as control rat (a) $NaAsO_2$ exposed (b) $NaAsO_2$+BBE (c). The alkaline comet assay is done following the lyses of cells then electrophoresis and staining with ethidium bromide. (Please refer to the method section).

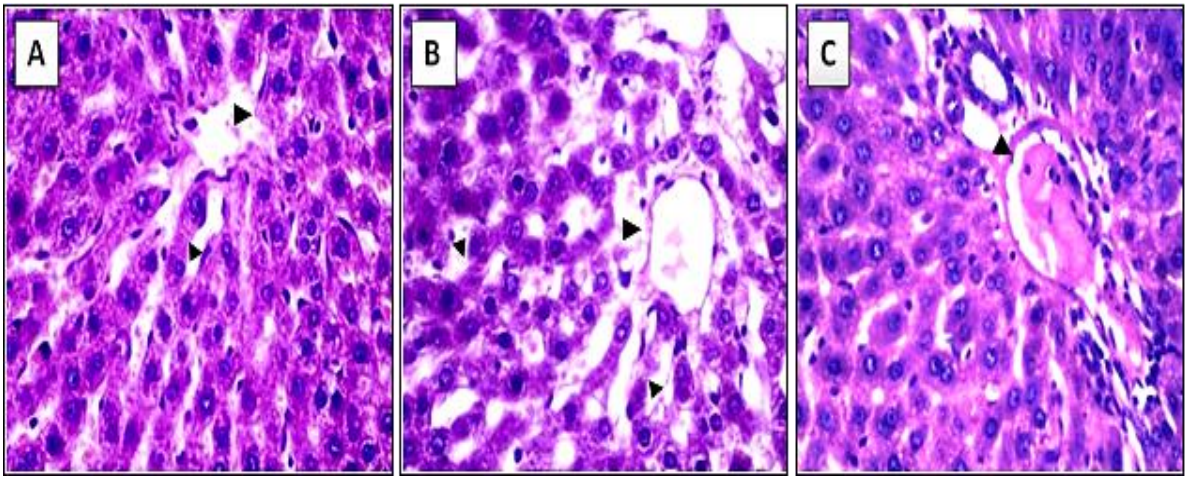

Figure 7. The hepatic histoarchitecture is shown by H&E staining (10X) of female rat treated with arsenic. Control rat (a) or treated with sodium arsenite (b) or sodium arsenite + BBE. The lumen lining of the central vein of liver is present. The hepatic lobules are comprised mostly of plates of hepatocytes. Tissue degeneration is clearly visible in slide b as the formation of a mesh like structure losing the eccentric feature of hepatocyte organization. The present histoarchitectural results are justified by the serum markers of liver and kidney functions and tissue degenerative marker i.e. LDH activity.

The results on the DNA stability in the Figures 5 and 6 are concordant with the histo-architecture picture (Fig. 7). We noticed a significant alteration in the liver functions of the experimental animal. This is supported by our present hepatic histological study. Arsenic caused hepatic injury due to the necrotic tissue lesions and disruption of the central canal and associated convergent lobular structure of the hepatic cells.

In vivo and in vitro SOD regulation: The result suggests that in the whole animal study, the SOD activity did not alter significantly in arsenic treated group (lane 4-7) with comparison to the control (Fig. 8a). But in the BBE supplemented group, this activity augmented significantly suggesting its protective role. In the in vitro study, a significant decrease in arsenic and H_2O_2 treated group (lane 3, Fig. 8b) is noticed which is markedly reversed BBE and BBV treated group (lane 4 and 5, respectively, Fig. 8b). Lane 6 and 7 served the control to test the possible SOD activity in the only BBE and BBV, respectively. Similarly, only H_2O_2 was used in oxidant-stress associated SOD inactivation and its reversal and even activation by the *B. bengalensis* extract and *B. bengalensis* venom (Fig. 8c).

Discussion

In the present study, we demonstrate that arsenic-induced hepatic toxicity with present treatment schedule made a significant alteration in the liver functions of the experimental animal. This is supported by our present hepatic histological study. Arsenic caused hepatic injury due to the necrotic tissue lesions and disruption of the central canal and

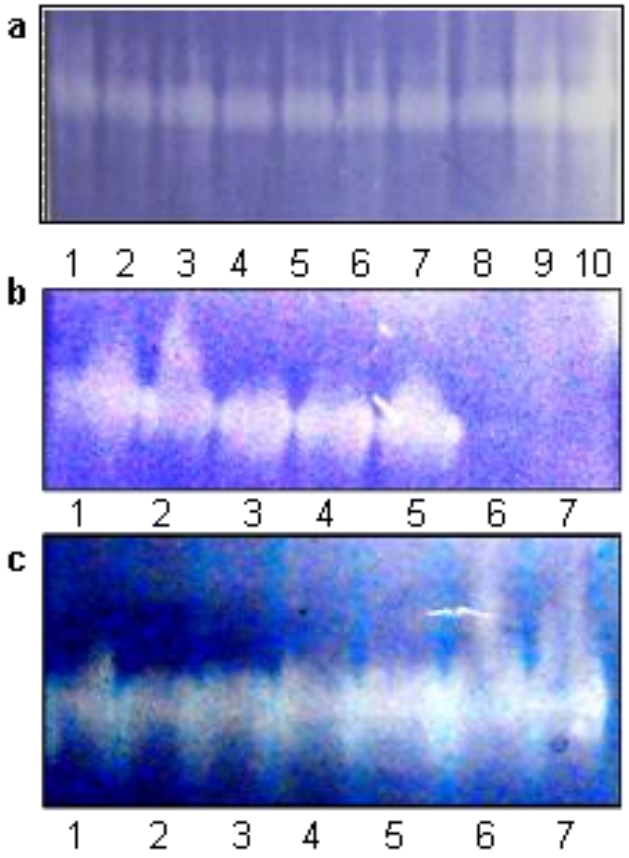

Figure 8. Cytosolic SOD (Cu-Zn SOD or SOD1) activity of hepatic cells is shown on a polyacrylamide gel. Upper panel a (in vivo experiment)- Lane distribution; 1, 2, 3 control; 4, 5, 6, 7 arsenic treated, and 8, 9, 10 arsenic + BBE treated. Middle panel b- In vitro H_2O_2 inactivation of rat cytosolic SOD1 and its protection by BBE and BBV. Lane distribution: Lane 1- control, 2- control (2 hrs), 3- H_2O_2 (100 mM) (2 hrs), 4- H_2O_2 (100 mM) + BBE 15 µl (25%) (2 hrs), 5- H_2O_2 (100 mM) + BBV 15 µl (2 hrs), 6- BBE 15 µl (25%) (2 hrs) and 7- BBV 15 µl (25%) (2 hrs). Lower panel c- In vitro H_2O_2 inactivation of rat cytosolic SOD1 and its protection by BBE and BBV. Lane 1- control., 2- con incubated 2 hrs., 3- H_2O_2 (1 M) 2h rs., 4- BBE 18 µl (25%) + H_2O_2 (1 M) 2 hrs., 5- BBE 18 µl (25%) + H2O2 (1M) 2 hrs., 6- BBV 18 µl + H2O2(1M) 2 hrs. and 7- BBV 18 µl + H_2O_2 (1M) 2 hrs.

associated convergent lobular structure of the hepatic cells. Report reveals that ingestion of arsenic-contaminated drinking water caused infiltration of inflammatory cells in the periportal area in the liver (Liu et al., 1999). During the investigation on arsenic speciation and their mechanism, Calatayud (2013) suggests that As (III) and its organic derivatives induce tissue death mainly by necrosis, apoptosis, cancers, and other abnormalities. It is evident that arsenic-induced liver damages could be the result from oxidative stress which is substantiated by the histological and biochemical manifestations i.e. an elevated level of serum transaminases (Karimov et al., 2002; Li et al., 2007) and lactate dehydrogenase. Not only in the experimental animals, has a similar increase of serum transaminases been reported in arsenic-exposed workers (Zaldivar et al., 1981). The present result on the BBE protection of liver and kidney functions indicates that the extract of this organism which has some traditional ethno-pharmacological effect, also possess a strong therapeutic potentials against arsenic-related toxicities. *Bellamya bengalensis* is a freshwater organism. Therapeutic potentials and drug development provisions from marine natural products have been extensively reviewed by Molinski (2009).

In the present study, a potent self-protective/ preventive ability are shown in *B. bengalensis* itself against very high arsenic concentration. This is interesting that, a very low dose of arsenic (0.6 ppm) become highly toxic and damaging to the rat tissues and several of its macromolecular structures nevertheless, the DNA of *B. bengalensis* manifested a strong stress withstanding ability against the continuous exposure to 10-20 ppm of arsenic for several days. The inability of arsenic to damage *B. bengalensis* DNA is paralleled with the higher increase of NPSH and decrease of MDA in this organism. This suggests that increase in NPSH result in minimizing the generation of lipid peroxidation products (MDA) and other free radicals. As a result, the organism's DNA remained highly protected. Arsenic produces reactive oxygen species (ROS) when react with H_2O_2 and/or metal cataions and produces high level of lipid peroxides and conjugated di-enes (Messarah et al., 2013; Acharyya et al., 2014). Arsenic exposure also exhibits oxidative stress through a significant reduction of GSH in liver, cultured lung epithelial cells and brain tissues (Li et al., 2002). The NPSH, an antioxidant itself and a precursor of GSH is significantly declined by arsenic but that is restored by the BBE supplementation in the present investigation. The impairment of the antioxidant enzymes such as SOD

and catalase activity has been reported to link with oxidative stress and DNA damage (Sinha et al., 2007; Maiti et al., 2012; Calatayud et al., 2013), which is demonstrated in the present investigation. The present protection of BBE has been demonstrated at the level of antioxidative efficacy of several enzymes that results in a protection in macromolecular structure like DNA and proteins. The possible anti-apoptotic role of this medicinally potent organism has been demonstrated in the comet assay results. Anti-inflammatory role of this organism extracts has been pointed out. Anti-cancer activity of some natural products from several marine organisms has been demonstrated. Commercial forms of the drugs prepared from these products are used in different physiological ailments (antitumor, analgesia, antiinflammatory, immunomodulation, allergy, and anti-viral) (Singh et al., 2008; Montaser and Luesch, 2011).

Arsenic is reported to induce cellular inflammatory responses by increasing the tumor necrosis factor α (TNFα), interferon γ (IFN-γ) and interleukin 6 (IL-6) (Dutta et al., 2015). Arsenic induced ROS aggravate the situation by furthering the NF-Kβ action (Kaul et al., 2014). The BBE has been shown to decrease the LPS-induced inflammatory and oedematous responses like TNFα and macrophage activation (Bhattacharya et al., 2014). BBE has also been shown to effectively nullify the LPS induced nuclear translocation of NF-Kβ and p65 (Bhattacharya et al., 2014). Possible anti-inflammatory action of BBE might be occurring in the protection mechanism in the present study, where a remarkable amount of ROS is produced by the arsenic. Further investigation is required for more conclusive remark.

Ramanathan et al. (2005) evaluated the molecular changes during arsenic exposure and possible therapeutic efficacy of antioxidants like Vitamin C and Vitamin E on arsenic induced apoptosis in rats. They reported that administration of Vitamin C and Vitamin E along with arsenic significantly reduced the apoptosis. Vitamin C was found in *B. bengalensis* at a satisfactory level i.e. 71.85±4.74 µg/g tissue. Vitamin C acts as a scavenger of free radicals and plays an important role in the regeneration of α-tocopherol (Young and Woodside, 2001). Moreover, supplementation of ascorbic acid and α-tocopherol has been known to minimize the DNA damage by reducing TNF-α level and disfavoring the activation of caspase cascade in arsenic intoxicated animals (Ramanathan et al., 2005). Anti-carcinogenic and anti-leukemic activity of BBE has been demonstrated. Selective degradation of cancerous cells by apoptogenic pathway by BBE-induced mitochondrial caspase cascade has been associated to these anti-cancer effects (Besra et al., 2013). Mitochondrial death signal is linked to the influences of the p38, p53, and c-myc regulations and DNA damages (Maiti, 2015). Arsenic-induced mitochondrial deregulation is evident. In the present study, arsenic induced DNA fragmentation has been restored by the BBE extract which has been demonstrated in ladder assay results and single cell DNA damage picture (comet assay). This data suggest the apoptotic cell death by arsenic is circumvented by the BBE extract.

The *B. bengalensis* extract has a higher amount of phosphate i.e. 52.24±3.89 µg/g tissue, which possibly offers a better protective effect against arsenic toxicity. Arsenic in the form of arsenate is chemically similar to phosphate. It uncouples oxidative phosphorylation by substituting the phosphate molecule in ATP synthesis (Mitchell et al., 1971; Gresser, 1981). It has been reported that arsenate and phosphate share the same transport mechanism and compete for the same sites with a moderate preference for arsenate adsorption (Maiti, 2015). The intestinal absorption of arsenic is significantly decreased with the phosphate infusion in rat. This transportation is an active carrier-mediated system depending on Na^+ and H^+ gradient (Gonzalez et al., 1995; Dixon, 1996). Furthermore, even in complex systems, simulating natural conditions like in ground water, phosphate showed dominance in regulating the arsenic concentrations (Stachowicz et al., 2008). Thus higher phosphate in BBE might play a better protective role more

efficiently than that what only nutrient dose.

Specific functional groups within enzymes, receptors or coenzymes, such as thiols have shown a major influence in the activity of these molecules. A significant amount of non-protein soluble thiols has been demonstrated in the *B. bengalensis* extract (50.8±3.7 µg/g). The trivalent arsenicals readily react with thiol-containing molecules such as GSH and cysteine in in vitro experimental condition (Scott et al., 1993; Delnomdedieu et al., 1994). The in vitro binding of MMAIII and DMAIII to proteins occurs at a greater extent than with their corresponding pentavalent forms (Styblo et al., 1995). Arsenite has a higher affinity for dithiols than monothiols, as shown by the highly favored transfer of arsenite from a (GSH)3-arsenic complex to the dithiol 2, 3-dimercaptosuccinic acid (Delnomdedieu et al., 1993). The binding of trivalent arsenic to a critical thiol group may inhibit important biochemical events which could lead to toxicity. However, binding of arsenite at nonessential sites in proteins may offer a detoxication mechanism (Aposhian, 1989). The inhibition of hepatic SOD in arsenic intoxicated rats and its protection by BBE support the role of thiol. This finding is further justified by our in vitro study, where $NaAsO_2$+H_2O_2-mediated SOD inactivation is circumvented by BBE or BBV (*B. bengalensis* venom). These fractions is reported and found in the present study to have high thiol content. Role of important Cys residues in the catalytic/substrate-binding domain of SOD is evident (Acharyya et al., 2014).

In most of the cases, the metal-binding site contains a Cys or a His residue (Kojima et al., 1999; Cobbett and Goldsbrough, 2002). Proteins and peptides functioning in the uptake, distribution and detoxification of metal ions possess one or several metal-binding sites. The -Cys-X-X-Cys- and -Cys-Cys- motifs of various proteins are well-known for their heavy metal binding properties (Kojima et al., 1999; Cobbett and Goldsbrough, 2002). It has long been acknowledged that sulfhydryl-containing compounds have the ability to chelate metals. The sulfur-containing amino acids methionine and cysteine, N-acetylcysteine, an acetylated analogue of cysteine, methionine metabolite S-adenosyl-methionine, α-lipoic acid, and tripeptide glutathione (GSH), all contribute to the chelation and excretion of metals from the human body. Gouri et al. (2011) reported an anti-microbial peptide of 1676 Da, purified from *B. bengalensis* venom (collected carefully beneath the lid and inside the mantle part of the organism) having, three cysteine residues (Cys3, Cys5 and Cys16) and may possess heavy metal binding/ chelating properties. We also noticed a high level of thiol containing substances in the *B. bengalensis* extract in our study. Cysteine-rich secretory protein from the snail *Achatina achatina* has been reported (Shabelnikov and Kiselev, 2015). BBE has been shown to be effective against the hepato-toxicity model in animal. In the light of the information regarding the ability of arsenic to develop hepato-carcinogenesis, the BBE potency is very significant. Anti-cancer potentials of marine organisms are reported (Singh et al., 2008). In the present study, we demonstrate for the first time, its strong hepato-protective role against a natural toxic contaminant like arsenic. Further exploration of this work might be helpful for the optimization of the protective/therapeutic effects of BBE against arsenic and other toxic heavy metals.

The aquatic edible snail *B. bengalensis* resides at the water-soil (muddy) interface. Notwithstanding, this aquatic environment is highly exposed to the toxic deposit that is naturally occurring in that habitat or accumulates during sewage, drainage and during natural/artificial water circulatory processes (Dutta et al., 2014). Possibly, due to the implication of a strong natural selection pressure generated from the sustained environmental exposure of this organism to different toxic deposits/ metals increase its adaptation and confronting ability against stress-associated tissue and macromolecular damages. It is clearly reflected in the present study. Not only against the arsenic-induced oxidative stress, *B. bengalensis* demonstrated a remarkable protection against heat-induced oxidant stress by buttressing the SOD, catalase activity and heat shock

protein 70 (HSP70) expression (Dutta et al., 2014). Arsenic is also reported to influence different HSPs (HSP70) in exposed individuals as well as in experimental laboratory animals (Maiti, 2015).

Here, we report for the first time on the effective medicinal role of any animal/ organism extract against the arsenic associated disorder and carcinogenesis. The clinical implications of this study focus on the immense therapeutic possibility of this organism against arsenic-induced organ toxicity/carcinogenesis. More investigations are necessary to explore the active constituent of *B. bengalensis* extract and its hepato-protective, antineoplastic-antioxidative activities.

Conclusion

Our study established that apparently banal, notwithstanding valuable this common edible snail (*B. bengalensis*) may provide a beneficial role against liver injury by arsenic toxicity. Not only as a nutrient material, several of its components (i.e. thiols, phosphate, calcium, vitamins) can perform via cellular signal-transduction, nuclear factors/ receptors regulations, SOD activity alteration, TNF-α,/caspase modification, direct DNA and cytoskeleton protection and possibly many more yet to be explored mechanistic approaches. A concept of folk medicine regarding the *B. bengalensis* is found here for the first time, unambiguously to be potent against the hepato-toxicity induced by arsenic which needs more detail study.

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