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RESEARCH ARTICLE

Protection against Mitochondrial Oxidative-Stress by Flesh-Extract of Edible Fresh-water Snail *Bellamya bengalensis* Prevents Arsenic Induced DNA and Tissue Damage

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Abstract: ***Aims*:** Arsenic has carcinogenic properties because of the formation of Reactive Oxygen Species (ROS). ROS damages different macromolecules, tissues and organs, and severely exhausts cellular anti-oxidants.

***Background*:** Cytosolic and mitochondrial contribution of ROS production by arsenic are not well reported. In regard to the issues of therapy against arsenic or any other toxicity, natural product has gained its popularity due to its less side-effects and non-invasive nature.

***Objectives*:** Here, as an ethnomedicine, the flesh-extract (BBE; 100mg/100g bw) of *Bellamya bengalensis* (an aquatic mollusk) was applied in arsenic intoxicated (0.6 ppm/100g bw/for 28 days alone or in combination with BBE) experimental rats. Our objective was to study the anti-oxidative and anti-apoptotic role of BBE in hepato-gastrointestinal tissue damage by arsenic.

***Methods*:** DNA fragmentation assay, catalase activity (gel-zymogram assay) suggests that BBE has a strong protective role against arsenic toxicity, which is decisively demonstrated in hepatic histoarchitecture study by HE (hematoxylin and eosin) staining and by intestinal PAS (Periodic Acid Schiff) staining.

***Results*:** Measurement of mitochondrial-membrane-potential by fluorescent microcopy clearly demonstrated less membrane damage and lower release of the redox-active inner-membrane product (cytochrome-C, ubiquinone, *etc*.) in BBE supplemented group compared to that of the only arsenic fed group. The present study clearly suggests that mitochondrial disintegrity is one of the major causes of ROS mediated tissue damage by arsenic.

***Conclusion*:** This study also offers an option for prevention/treatment against arsenic toxicity and its carcinogenicity by widely available low-cost, non-invasive *Bellamya* extract by protecting cytoskeleton, DNA and mitochondria in the cell.

Keywords: Arsenic toxicity, mitochondrial oxidative damage, carcinogenesis, DNA damages, oxidative stress and apoptosis, chemotherapeutics by *Bellamya bengalensis*.

1. INTRODUCTION

Arsenic absorption occurs in the liver, gastrointestinal tract, kidneys, lungs and brain. Liver is the major target organ in arsenic-related carcinogenesis [1]. Orally taken arsenic by drinking water reaches firstly to liver from gastrointestinal tract [2] where glutathione, which is rich in this organ, helps in detoxification of arsenic by reducing reactive oxygen species [3]. In the course of detoxification processes, arsenic is methylated in the liver by the help of arsenic methyl transferase, where S-adenosylmethionine (SAM) is available as a substrate. Several studies stated a strong relationship between chronic arsenic exposure and liver abnormalities like liver fibrosis, hepatomegaly and liver cirrhosis. Arsenic induces gastrointestinal problems like ingestion, loss of appetite, abdominal pain, and finally, develops abnormal liver function [4]. Degeneration of intestinal epithelial tissue is also an important outcome of arsenic toxicity. At the time of metabolism, arsenate and arsenite undergo their organification steps to form dimethyl arsenic acid and monomethyl arsenic acid. These two forms of arsenic can be excreted through urine with higher rate clearance. Arsenite (AsIII) can bind with the pyruvate dehydrogenase and glyceroldehyde-3-phosphate dehydrogenase enzyme with the help of their sulfhydryl group and thus impair glucose and calorie metabolism which is an important step in the initiation of the pre-tumorigenic environment. By binding with lipoic acid, which is a component of pyruvate dehydrogenase complex, arsenic can interfere in the oxidative phosphorylation process [5]. Arsenite (AsV) can competitively substitute its ion for phosphate ion present in the body. Arsenic induced ROS production and tissue damage are reported, but the role of mitochondria to contribute ROS mediated damage is not well documented. Mitochondrial membrane damage could be devastating in two ways; by disintegrating the oxidative phosphorylation and calorie depletion, and by producing an excessive amount of cytosolic redox-active substances. The report reveals that uncontrolled depletion of high energy ATP by hydrolysis ultimately leads to cell death, which is another indication of the genesis of tumorigenic events [6]. Arsenic can induce ROS production, calcium overload and leads to apoptosis by releasing the cytochrome c from the mitochondria. Thus, arsenic causes mitochondrial dysfunction [6].

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Bellamya bengalensis has anti-apoptotic and anti-inflammatory properties. According to a report, the hepatoprotective activity of *Bellamya* has shown in a rat model where liver damage was initiated by carbon tetrachloride [7]. This organism, *Bellamya bengalensis* is widely available as low-cost protein-nutrients rich with a number of minerals and other nutritive substances. For the possible protective role of *Bellamya*, we were intended to use it as therapeutic substances in arsenic-induced toxicity. In our experiment, we have supplemented arsenic-induced rat by *Bellamya* extract for investigating its effect in reducing toxicity.

2. MATERIALS & METHODS

2.1. *Bellamya bengalensis* Extract (BBE) Preparation

Mature *Bellamya* sp. was collected from the local market and cleaned. The edible part (50g of flesh) of the organism was added to 125ml distilled water and homogenized carefully to prepare 40% of BBE. Then the total homogenate was centrifuged by cold centrifuge machine at 4°C temperature for 20min at 10,000 rpm. The supernatant was collected and used fresh or stored at -20°C.

2.2. Animal Selection and Treatment

Male albino rats weighing 150-160g were acclimatized for 10 days at 12h light-dark cycle, 25±2°C temperature with moderate humidity in the institutional animal house. Rats 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 norms 3.i ec2014). Rats were randomly segregated into three groups having six in each. Rats of group-II and group-III were fed with drinking water containing sodium arsenite at a concentration of 0.6 ppm (600µg/L)/100g body weight/day for 28 days. The present dose did not result in animal mortality, but exposure for the period (≥ 3 weeks) initiated intestinal toxicity and other clinical marker suggesting a significant level of cellular toxicity [8]. The control rats were fed the same amount of drinking water for the exposure period.

Group-III was supplemented with BB extract at a dose of 100mg tissue of *Bellamya bengalensis*/100g body weight/day for 28 days. On day 29, animals were exposed to light anesthesia, blood was collected and serum was separated and liver and intestinal epithelial cells/tissues were collected and stored at -20°C. Cytosol was prepared for further analysis, as described earlier [8].

2.3. Isolation of Mitochondria and Evaluation of its Membrane Potential

Mitochondria from liver tissues were isolated after the animal-sacrifice by decapitation of the anaesthecia-treated rats [9]. The tissue homogenate (0.22M mannitol, 0.02M HEPES, 0.07M sucrose, 0.1mM K-EGTA, 1mM K-EDTA, pH 7.4 containing 0.4% albumin) was centrifuged at 2000 rpm for 10min to discard nuclei and plasma membrane fractions. The supernatant was filtered and centrifuged at 10,000 rpm for 10min in a cold centrifuge to obtain the mitochondrial pellet. Mitochondria were washed in a washing buffer containing 0.25M sucrose, 1mM K-EDTA, 0.02M HEPES and 0.1mM K-EGTA, pH 7.4 and resuspended in the same buffer.

Mitochondrial potential ($\Delta\psi_{mit}$) changes have been evaluated by measuring RH-123 fluorescence quenching under the as described by Baracca *et al*. 2003 [10]. Before rhodamine (50nM) addition, samples were incubated with 33nM cyclosporin A, 1µg/ml rotenone, and 0.1mM ADP. Finally, mitochondrial membrane potential was measured by using a fluorescence microscope (Nikon, Eclipse LV100 POL). Time courses of RH-123 fluorescence decay were analysed by means of an exponential decay best fitting using the GraphPad Prism 3.0 software (GraphPad Software).

2.4. Determination of Catalase Activities gel-zymography

Catalase activity assay was done using 8% native gels. After running the gel, it was incubated in the reaction mixture containing 0.003% H_2O_2 (30% solution), 2% ferric chloride and 2% potassium ferricyanide. After staining, the gel was developed, a green-blue color with the white band where the enzyme catalysis occurred [8, 11].

2.5. Determination of Biochemical Parameters from BBE

Several components like total protein, lipid, vitamin C, non-protein thiols, calcium, phosphorus and other biomolecules, were measured in the *Bellamya* muscle extract by standard kit or manual biochemical methods.

2.6. Histology and DNA Fragmentation Analysis

Liver and intestine tissues (intact and fresh, just after sacrifice) were poured in fixative. Those tissues were embedded in paraffin, serially sectioned at 5 micron, stained with eosin and hematoxylin (liver tissues, Harris) and PAS staining was done for intestinal tissues. Those were observed under a microscope (Nikon, Eclipse LV100, magnification × 50 ×) to study the histoarchitecture.

Liver tissues were used for DNA preparation; tissue was treated with 500µL of lysis buffer (50mM Tris pH 8.0, 20mM EDTA, 10mM NaCl, 1% SDS, 0.5mg/mL proteinase K) for 20min on ice (4°C) and centrifuged in cold at 12,000g for 30min. The supernatant was extracted for DNA isolation with standardized method [12]. Extracted DNA was run in 1% agarose gel and the band was scanned in a gel doc system.

3. RESULTS AND DISCUSSION

Hematoxylin and eosin staining (for liver tissues, Fig. **1**) and Periodic Acid Schiff staining (for intestinal tissues, Fig. **2**) were done in control, arsenic and arsenic + *Bellamya* supplemented groups to analyze the histo-architecture and carbohydrate/glycogen deposition/accumulation in the tissue. The mitochondrial membrane was assessed by monitoring the fluorescence quenching of RH-123 (Fig. **3**). Protons were extruded from mitochondria by the respiratory complexes and easily diffused through the F_0 component of the ATP synthase complex. Other redox-active substances like cytochrome C were released from the mitochondrial inner membrane to the cell cytosol. In this study, the arsenic group has shown a significant decrease of mitochondrial membrane-stability and BBE treated group demonstrated significant protection against it. Catalase, an anti-oxidant enzyme that converts H_2O_2 to H_2O, was assessed in gel zymographic assay (Fig. **4**). The arsenic group has given a stronger band than that of control, but *Bellamya* treated group has shown a 3-4 fold stronger band than that of control, which suggests the strongest H_2O_2 cleaning effects of this group. DNA fragmentation assay was done for investigating the damage at DNA level. Arsenic-induced DNA damage was noticed to be significantly protected in BBE supplemented group after the different duration of its exposure (24-96 hrs). The schematic diagram (Fig. **5**) depicts the possible pathways of mitochondrial and cytosolic sources of ROS generation in response to arsenic exposure. It also demonstrates the possible steps of BBE protection against arsenic-induced toxicity. BBE has shown a strong protective effect against arsenic-induced hepatic and intestinal toxicity.

Several economically important nutraceuticals are produced from aquatic organisms. Some edible shellfishes are used for different therapeutic and disease prevention purposes. The indigenous and the tribal people, mainly from the rural origin, use this kind of products from ancient times in different parts of the world [13]. *Bellamya*, Pila, Achatina, Lamellidens and many more are this kind of organism which produces bioactive components as home-made products or commercial preparation [14]. The hepatoprotective

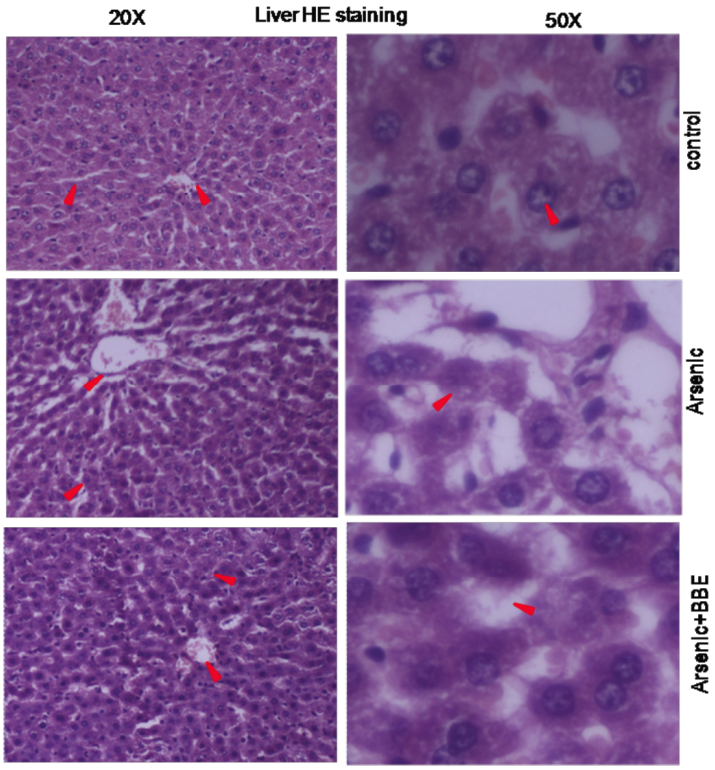

Fig. (1). Histoarchitectural analysis of liver tissue of rat exposed to arsenic and supplemented by BBE has shown strong protective effects of BBE. Hepatic lobular and cytoskeletal structure, central canels are decisively protected by BBE. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

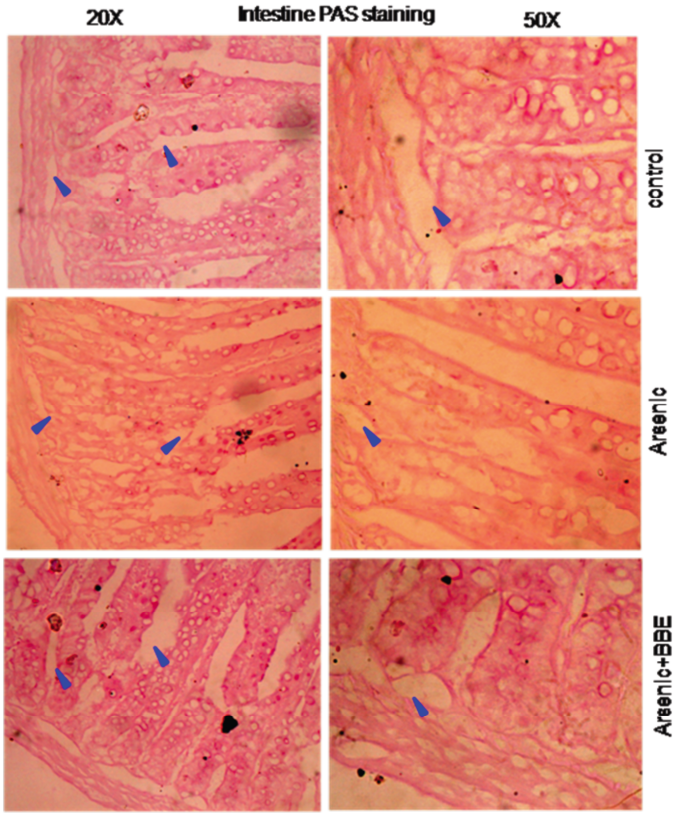

Fig. (2). In arsenic induced damage condition abnormal polysaccharide staining is noticed in the middle panel picture. Degenerated crypt and lumen structure is visible with glycogen or glycoproteins localization. But intact crypt and lumen structure with no polysaccharide is noticed in control and BBE supplemented group. This suggests that BBE is strongly protective against arsenic induced cryptic and lumen damage and abnormal polysaccharide metabolism/localization. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

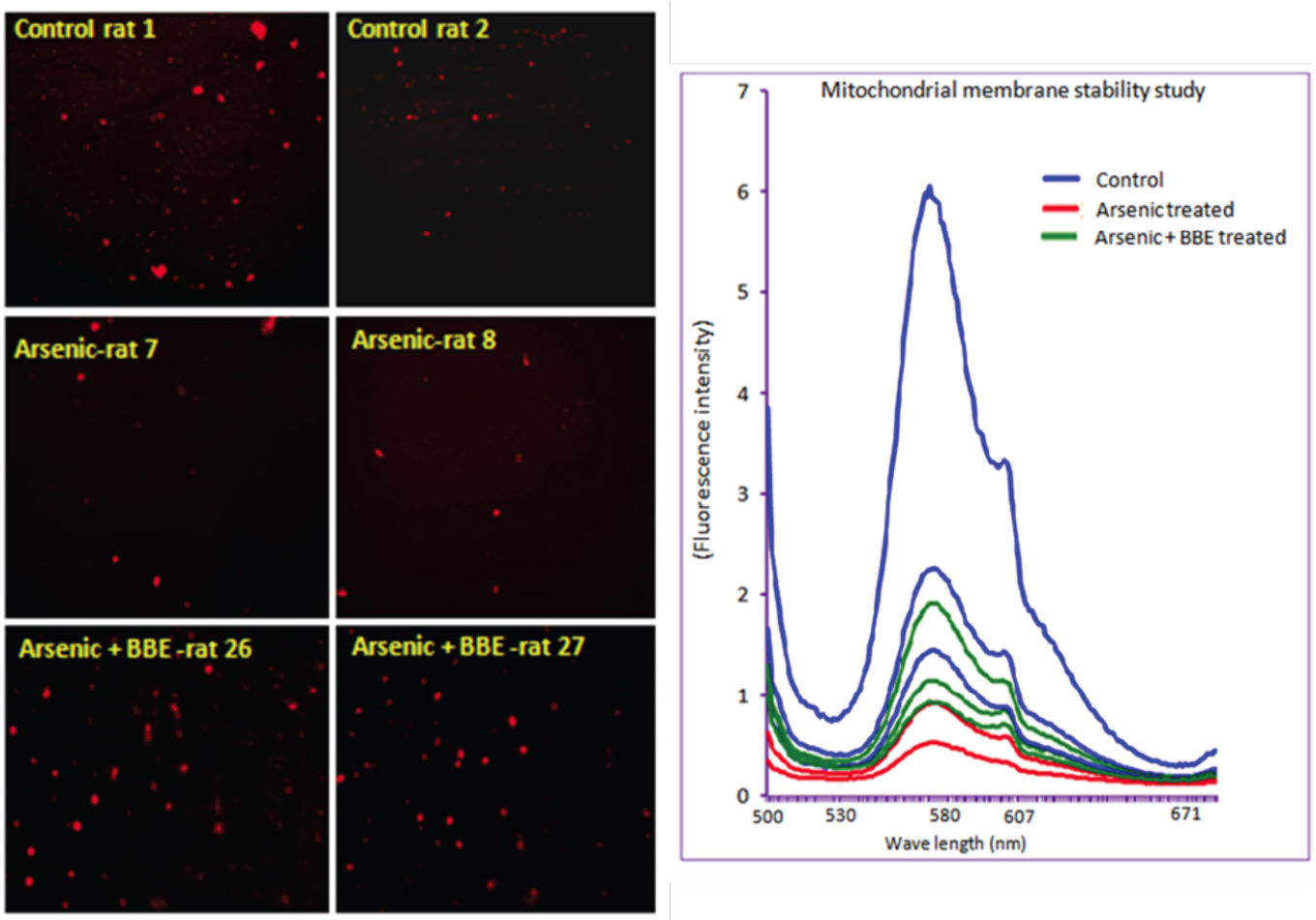

Fig. (3). Mitochondrial membrane stability is severely compromised in response to arsenic in rat liver. The membrane stability is strongly prevented by BBE. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

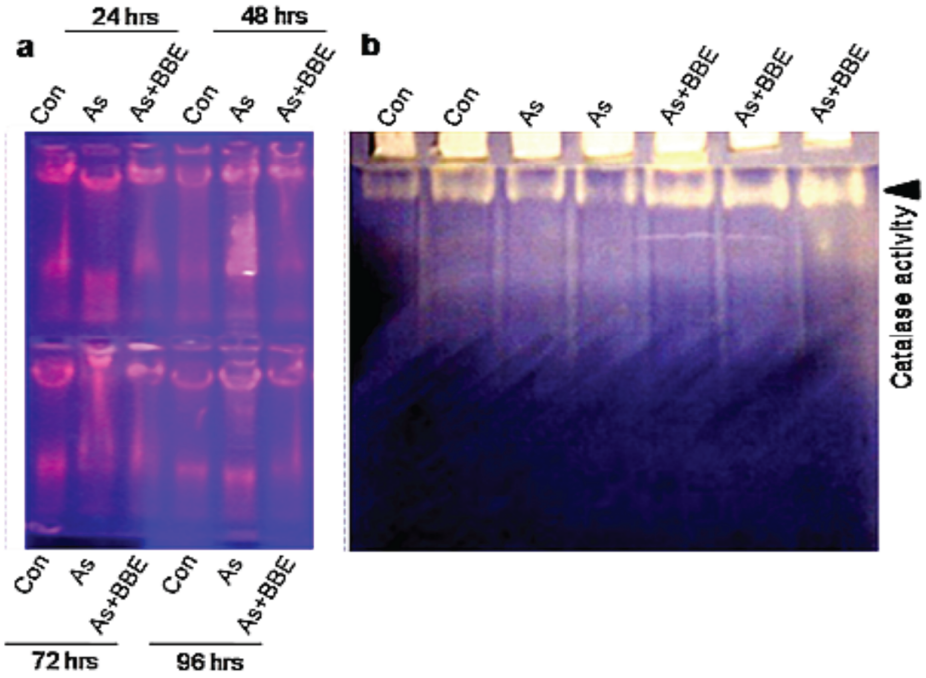

Fig. (4). DNA stability and catalase activity is shown in arsenic toxicity and BBE supplementation effect to it in arsenic exposed rat liver tissues. BBE has demonstrated strong DNA protection effect. BBE not only restored but also augmented the catalase activity by few fold in response to arsenic toxicity. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

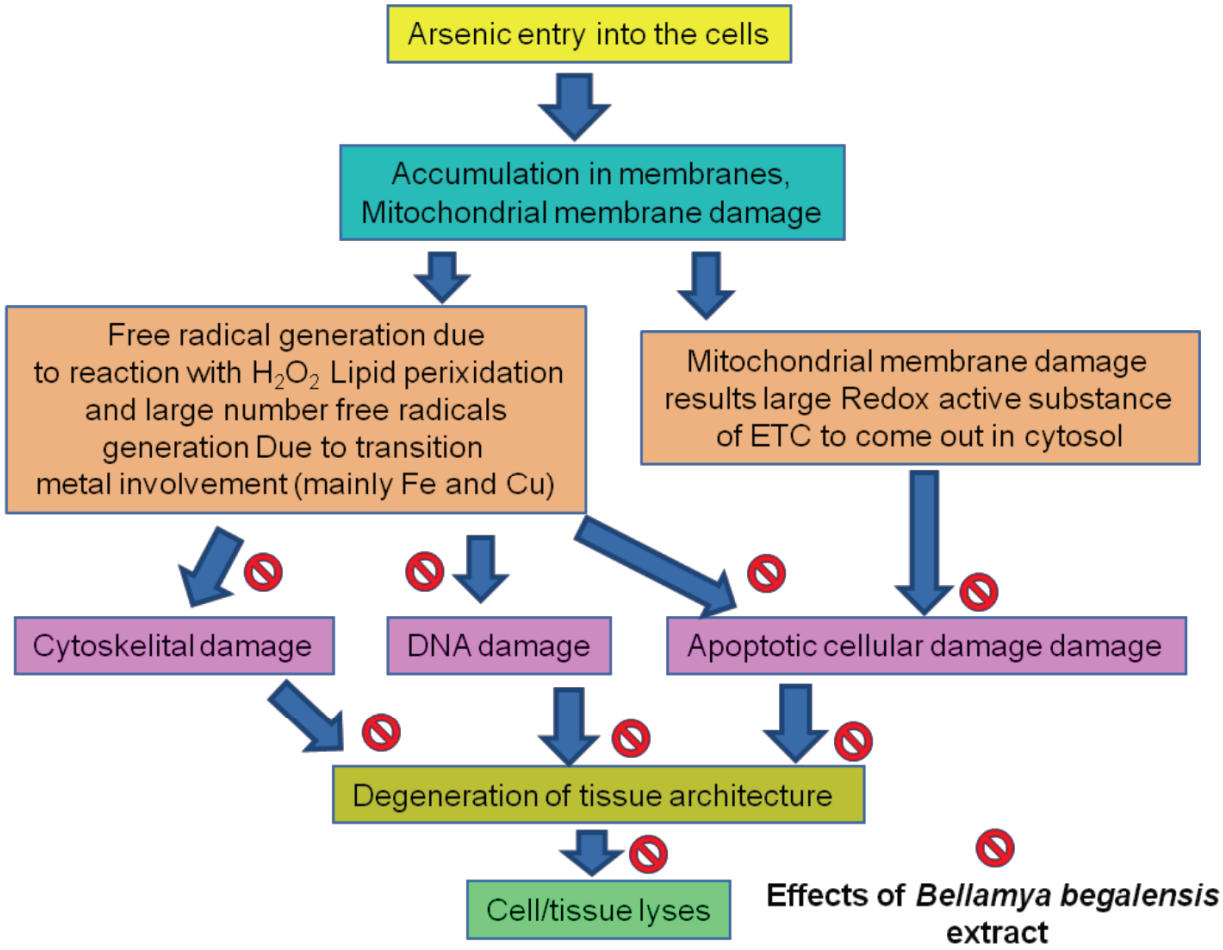

Fig. (5). Schematic diagram shows the possible mechanistic layout of BBE protection against mitochondrial and cytosolic ROS production, tissue and DNA damage. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

role of *B. bengalensis* extract has been demonstrated in common toxicant-induced liver damages in rats [7]. Mucilage like extract of *Bellamya bengalensis* constitutes a potent anti-necrotic and anti-infective agent that is degenerative and apoptogenic against human leukemia cells [15].

Intestinal tissues are the first anatomical entry point of all ingested materials. Intestinal microsomal enzymes participate in the metabolism of these materials. The results of the PAS-staining of the present intestinal tissues suggest a strong role of BBE in the protection of the arsenic-induced intestinal epithelial damages. Both un-metabolized and partially metabolized substances (if any) exert toxicity on the intestinal tissues. On the other hand, the liver extends this metabolism up to the next level and a large number of drug-metabolizing enzymes help for the organification of the toxicants and increase the polarization and excretion of those materials.

In the current study, the BBE is found to have a higher amount of phosphate *i.e.,* 76.24±4.12µg/g tissue, which offered a better protective response against arsenic toxicity. The arsenate is chemically similar to phosphate. It uncouples oxidative phosphorylation by substituting the phosphate molecule during the ATP synthesis process [16, 17]. A very significant amount of cholesterol, total fatty acids and phospholipids were noticed in the *Bellamya* flesh extract. The report reveals that this flesh extract contains balanced proportions of almost all essential and non-essential amino acids. Amongst those, threonine and lysine remain with the highest amount (>15mg/g protein) [18]. Alanine, glutamate and aspartate remain ~7mg/g proteins [18]. This makes this extract a much enriched pool for protein synthesis steps, which is very much helpful for the prevention of necrosis and post-degeneration healing. It is known that arsenate and phosphate compete to the same transporter with a moderate preference for arsenate adsorption [19]. The intestinal absorption of arsenic is significantly decreased with the phosphate infusion in the rat. However, a high amount of phosphate in the BBE can help compete for arsenic and replace it during the intestinal absorption. In the present study, a high rate of absorption of arsenic increased the rate of cellular lipid peroxidation and MDA production that is reflected in the present results of hepatic DNA fragmentation and damage of the tissue and cytoskeletal architecture.

MDA can be measured as an indirect indicator of oxidative stress [20]. In this study, we found that the levels of ROS products such as MDA in hepatic and intestinal epithelial tissues increased after arsenite exposure. And this ROS production is attributed to the mitochondrial membrane damage, which is demonstrated in this study. Mitochondrial damage increases membrane degeneration and release of a large amount of redox-active substances, which initiated cytosolic free-radical cascade reactions. But the treatment of BBE significantly decreased arsenite-induced ROS and MDA production both in mitochondria and cytosols. In this regard, it is noticed arsenic-induced catalase inhibition is not only restored by the BBE, but this value also augmented by 3 to 4 fold than that of the control. This suggests that the synergistic effect of both cytosolic and mitochondrial ROS production and further radical-cascade reactions were the igniting events for the flare-up response of catalase in the supplemented group. Not only catalase, Glutathione Peroxidase (GPX)

expression and activity have also been a protective response against arsenic-toxicity [21]. The report reveals that BBE are the sources for different bioactive ingredients like thiol-containing amino acid, ascorbic acid, phosphorous *etc* [22]. The phosphate influenced the arsenic concentration and its regulations inside the cells [23]. Thus, higher phosphate in BBE might play a better protective role. The report reveals that arsenite has a higher affinity for dithiols than monothiols [24]. Very high vitamin C content was also noticed in the BBE. Nullification of certain free radicals by vitamin C is also an important strategy for anti-cancer treatment. All these compounds may have strong anti-oxidative potentials in the case of arsenic-induced cytotoxicity. The influences of the p38/p53, and c-myc in the generation of mitochondrial death signal and DNA damages have been reviewed and can be correlated to the present findings [19]. In the current study, mitochondrial protection by BBE against arsenic-induced free radical cascade is being demonstrated for the first time. A high amount of non-protein thiol (precursor of GSH) and vitamin C are the main anti-oxidants against mitochondrial free-radical cascade.

Selective degenerative responses against cancerous cells *via* the apoptogenic pathway by BBE have been shown earlier. This effect is implicated by arsenic-induced mitochondrial caspase-cascade being the main feature of the anti-cancer effects of BBE [15]. Arsenic-induced mitochondrial instability may be linked here to the hepatic DNA damage and has been shown to be prevented by the BBE extract (Fig. **4**). This data suggests that the apoptotic cell-death *via* mitochondrial membrane instability by arsenic is circumvented by the BBE. Several effects of *B. bengalensis* venom from its body parts and its amino acid composition (high Cys containing) have been reported by Gouri *et al.* (2011) [25]. This compound is assumed for its anti-microbial activity. Besides this activity, we explain that its high Cys content was able to perform a good anti-oxidant for its thiol source and the scavenging potentials of unauthorized-electron. This anti-oxidant protein might deserve heavy metal binding/ chelating properties [14]. For the verification of the histo-architecture of the liver, HE staining was prepared. Microscopic image of the liver tissue indicates significant damage in the arsenic-treated group than that of control. But the *Bellamya* supplemented arsenic-treated group clearly demonstrates restored and intact cellular organization and cytoskeletal components. In the arsenic-treated group, liver sinusoids are inflamed, junctional gaping among cells are increased and matrix-associated intactness is significantly reduced. But all the normal features are restored in the *Bellamya* supplemented group.

One study focusing on arsenic-related hepatotoxicity has suggested that impairment of liver mitochondria can be a major cause of hepatic anomalies [19]. The current study on the mitochondrial membrane potential shows a relatively low peak in the arsenic group than the control. Low mitochondrial membrane potential due to arsenic treatment was decisively restored by *Bellamya* extract. Mitochondrial and DNA protection by live organism extract is being demonstrated here for the first time against arsenic-induced toxicity. It has immense applicability in relation to the recent scenario of a large incidence of natural arsenic toxicity in different parts of the world. Further studies are necessary in this regard.

It may be hypothesized that a large number of efficient components [22] are available in *Bellamya* extract. Some of those are very strong and have diverse acceptability of free electrons from different types of free radicals. This diversity in free radical-acceptability is not available in single isolated constituent. This compatibility of whole *Bellamya* extract makes it a more efficient electron-scavengers compared to vitamin C (scavenger of superoxide radical) [26], vitamin E (scavenger of peroxyl radical) [27], DMSO (scavenger of hydroxyl radical) [28]. In conclusion, our finding clearly demonstrates, for the first time, the protective and curative mechanisms of *Bellamya* against arsenic-induced DNA and mitochondrial damages.

The nature and the therapeutic efficacy of a molecule against a disease depend on the mechanism or the pathway of that disease. It is well-known that arsenic induces oxidative stress-related toxicity and its chronic eventuality generates tumorigenesis/carcinogenesis. Free radical related damages are the main key factors that induce DNA damages and mitochondrial degenerations. There are certain cancers which are caused by some definite mechanism like proto-oncogene activation, growth factors/cell-cycle dysregulation, specific DNA mutations, retroviral infections or by some specific tumorigenic factor. When it is postulated that the cancer is developed by a single defining factor, then it is easier to successfully treat this by a more purified or single compound. But when the disease is caused due to large scale toxicity initiating a variety of metabolic deregulations, then the therapeutic mode of the drugs expected to be different. In this regard, it may be pointed out that some carcinogenesis is a multi-factorial and multi-step process. Nevertheless, the multi-factorial process includes the impairment at the metabolic level, DNA-stability level and immunological levels. Free radical damages are related to the cascade generation of large number (and types) of free radicals those cause biomolecular damage.

It may be unlikely that one single material will be effective as a therapeutic agent against the multi-factorial mode of carcinogene-

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

Parameters	Concentration	Refs.
Total carbohydrate (mg/g)	29.63±3.14	[31]
Total lipid (mg/g)	268±19.75	[32]
Total Protein (mg/g)	52.67±4.76	[33]
Total amino acid (mg/g)	2.45±0.09	[34]
Phosphorous (µg/g)	76.24±4.12	[35]
Vitamin C (µg/g)	98.56±5.43	[36]
Calcium (mg/g)	1.76±0.54	[37]
Non protein soluble thiol (µg/g)	67.78±5.32	[38]
Lipid fraction (standard kit method)		
Phospholipid (mg/g)	49.74±3.87	-
Trihlyceride (mg/g)	9.57±1.56	-
Free fatty acid (mg/g)	7.88±0.87	-
Cholesterol (mg/g)	17.68±2.34	-

sis. When different free radicals initiate cellular and DNA damage, then the combination of anti-oxidant molecules will be effective against a variety of free radicals. Actually, arsenic affects a number of metabolic systems when it is metabolized to variety of its derivatives. Because, arsenic has multi-valence state and it undergoes a number of organification state. So this large number of toxic and potential mutagenic forms affects different biomolecules/biological functions like cell membrane, cytoskeleton, enzymatic reactions, mitochondrial functions and DNA stability. In some of our previous and recent investigation we noticed that purified compound is less effective than combined substances or extracts against arsenic-induced damage. When individual falvonoid (ECG or EGCG) or specific free radical scavenger (see previous section) is used against arsenic-induced DNA damage, the protection was found to be lesser than that of whole flavonoids mixtures or green tea extract [29]. Similarly, when specific solvent extract preparation from the whole *Bellamya*-flesh was used the protection becomes lesser than that of whole extract. When we used the BBE semi-purified or BBV (*Bellamya* venom), 1.6 kD antibacterial peptide [25], fractioned and single-band cut-triturated sample in single cell-preparation, we found that none is more effective than that of the combined form of extract (data not shown). For example, when whole extract is dialyzed with a 2-3 kD MW cut-off cellulose acetate column, it loses its phosphate, thiol and ascorbate and amino acid contents and simply showed 2-3 fold lower protective/ therapeutic effects. Purified BBV fraction has been shown to be a good anti-infective agent. Anti-inflammatory role of BBE and anti-mutagenic role of whole BBE has been demonstrated earlier [7, 25, 30]. Therefore, the therapeutic nutraceuticals (*i.e.* phosphate, thiols, ascorbate and amino acids) of BBE exerted beneficial effects (please see the Table **1** for reference values and the protocol).

In this regard, it can be argued in favor of combined chemotherapeutic agents and their synergistic role in cancer treatment likely to be more effective in certain advanced disease stages. In some other diseases like TB, chronic gastroenteritis and cancers, multi-drug therapy is more effective than single-drug treatment. Drug combinations can more effectively treat cancer caused by multi-step processes of anti-oxidants, anti-mutagen, anti-inflammatory substances, apoptotic molecules and anti-infective components. In this regard, further research is necessary.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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