6. Discussion

In the traditional folk medicine and therapeutic sciences, it has been mentioned that snail flesh is used as medicines for the cure of a number of health anomalies such as conjunctivitis and gastro intestinal disorders (Prabhakar and Roy, 2009).In several locations of our country and south East Asia it has been used for a long time. In the north Bihar, the flesh of *Bellamya bengalensis* is used as a traditional medicine against arthritis and other joint disorders (Prabhakar and Roy, 2009). In the Southern Italy, the most popular use of slug (*Arian hortensis*) is to treat gastritis enteric diseases or stomach ulcer (Quave et al., 2008). *Bellamya bengalensis* has a very high nutritive value. It is affordable, easily available and may be used as food of high protein source in a large number of population of different economic conditions. The habit of this mollusk is also, impressive that is these organisms mostly reside in the muddy layer of water body. That is just upper surface of the soil. This soil is an important depository of several environmental, industrial wastes, sewages, effluents. This soil and water are enriched with heavy metals, pesticides, herbicides, organic effluents, polyaromatic hydrocarbon etc. So, for a sustained contact with all these pollutants, these organisms are more stress/toxicity resistant. Small aquatic animals, *Bellamya* extract (BBE) are the sources for a wide variety of compounds like ascorbic acid, thiol containing amino acid, phosphorous etc (Ali et al., 2016). These compounds may be responsible for increasing antioxidant status. All of their micronutrients have wide varieties of implication in biological systems. High nutritive values are also one cause that a large member of people are inclined to consume it.

In the present study, a potent self-protective ability is shown by the *B. bengalensis* against arsenic. This is due to partly by increasing its NPSH (nonprotein soluble thiol) level which resisted tissue lipid peroxidation (LPX). Lipid peroxidation is the cell damaging event.

NPSH can resist LPX effect. A very low dose of arsenite (0.6ppm) became highly toxic and damaging to the rat tissues and several of its macromolecular structures (like DNA and SOD1 protein) nevertheless, the DNA of *B. bengalensis* manifested a strong stress withstanding effects against a high concentration of arsenic. Arsenic can produce reactive oxygen species (ROS) when react with H_2O_2 and/or metal cations (especially transition element) and produces high level of lipid peroxides and conjugated di-enes (Messarah et al., 2013; Acharyya et al., 2014b). Arsenic exposure also exhibits oxidative stress via a significant reduction of GSH in hepatic tissue, cultured lung epithelial cells and brain tissues (Li et al. 2002). The NPSH, an antioxidant itself and a precursor of GSH is significantly decreased by arsenic. But that is restored by the flesh extract of *B. bengalensis*. The impairment of the antioxidant enzymes such as SOD1 (cytosolic Cu-Zn SOD) and catalase activity has been reported to link with oxidative stress and DNA/protein damage (Sinha et al., 2007; Maiti et al., 2012; Calatayud et al., 2013), This is demonstrated in the present investigation. The present protection by BBE has been demonstrated at the level of its antioxidative function in the restoration of several enzymes. Zaman et al., (1995) pointed out that arsenic could inhibit the super oxide dismutase (SOD) activity in the larvae of an insect model. As because SOD catalyzes the dismutation reaction of superoxide anion radical ($O^{\cdot-}_2$) to form hydrogen peroxide (H_2O_2), the inhibition of this enzyme SOD activity might indicate the excess occurrence of $O^{\cdot-}_2$.Yamanka (1989,1990) also suggested that free radicals are produced by arsenic treatment and this group pointed out that free radical species (ROS) are generated by the reaction of molecular oxygen with dimethylarsine, a metabolite of dimethylarsinic acid. Yamanka et al.,(1989) considered one of these radicals to be superoxide anion radical produced by one electron reduction of molecular oxygen by dimethylarsine. The cytotoxicity of lipid peroxidation may also be manifested by the arsenic induced inhibition of

mitochondrial aldehyde dehydrogenase by excess production of malonaldehyde (Hjelle, 1982). In the present study, however, the unaffected SOD activity and lipid peroxidation in liver following exposure to arsenic indicated no accumulation of superoxide anion. Experimental finding with rat liver preparation suggested that two different enzymatic activities are involved in the methylation of inorganic arsenic in mammals (Buchet and lauwerys, 1981). Furthermore, observation on human , repeatedly ingesting small inorganic arsenic doses or acutely intoxicated (suicide attempts) by As_2O_3, also suggest a different rate for two methylation steps and an inhibitory effect of the trivalent inorganic form on the second methylation step leading to dimethylarsine. In the current study also, the dimethylarsine was not probably produced sufficiently to interact with molecular oxygen in liver resulting lack of formation of $O^{\cdot}_2$. Hydrogen peroxide, a deleterious oxidizing agent, is formed mainly $O^{\cdot}_2$ by the catalytic action of SOD and catalase, A major primary antioxidant defense components, which catalysis the decomposition of H_2O_2 to H_2O (Chen CJ et al., 1982). A similar type of reaction is catalyzed by sharing the function with glutathione peroxidase. Our current study suggests that the exposure to sodium arsenite decreases the catalase activity in experimental rat liver and intestine. The ability of arsenic to inhibit the catalase activity is also evident from the studies on human fibroblast cells by Lee and Ho (1995) and on chinese hamster ovary cell line by Wang and Huang (1994). It was also suggested that the enhancement of the genotoxicity caused by arsenic may be the result of micronuclei induction due to the over production of H_2O_2 (wang and Huang, 1994). Nordenson and Beckman, (1991) suggested that arsenic could induce chromosomal aberrations by sister chromatid exchanges (SCE). It was also reported that arsenic can induce cytotoxicity by direct inhibition of DNA repair mechanism (Lee- Chen et al., 1992). Arsenic could inhibit DNA ligases I and II, the enzymes playing a major role in DNA repair process,

has been demonstrated by Rudel et al., (1996). They also showed that arsenic enhanced clastogenecity and mutagenicity of other DNA damaging agents. So, it can be hypothesized that arsenic has a direct mutagenic effect and also indirect effect by promoting the mutagenicity of other chemical. In this experiment, after arsenic exposure for 28 days, the inhibited catalase activity could also initiate oxidative stress. Chen et al., (1998) reported that arsenite is able to induce cellular apoptosis which is triggered by the generation of hydrogen peroxide through activation of flavoprotein- dependent superoxide producing enzymes (such as NADPH oxidase). Hydrogen peroxide might also play a role as a mediator to induced apoptosis through release of cytochrome c to cytosol. After 28 days exposure, in the present study the possible accumulation of H_2O_2 in the liver and intestine due to the diminished activity of catalase on arsenic treated group is probably circumvented by the increased GSH concentration stimulating the glutathione peroxidase –mediated reduction of H_2O_2 and organic hydroperoxides (Yu, 1994). Scott et al., (1993) demonstrated that glutathione can inactivate arsenic through conjugation. The potential protective activity of GSH is implicated by the antioxidant role of GSH. That finally results in a protection in the macromolecular structures like DNA and proteins in rat. One earlier study suggested that DNA damage caused by arsenic in intestinal epithelial tissue could be reversed by supplementation of phytochemicals (Roy et al., 2008). Protective role of other phytochemicals have been focused by several investigators. Arsenic toxicity caused considerable involvement to the depletion of GSH content in liver, cultured lung epithelial cells, and in discrete brain areas (Maiti et al., 2001; Shila et al., 2005). This finding coincides with our present result outcome. In reaction to the present finding of hepatic impairment, recently, it is shown that arsenic-negotiate liver injury is related to the increased oxidative stress in the hepatic mitochondria via an alteration of mitochondrial permeability (Santra et al., 2007). Our present result also suggests that

mitochondrial membrane experience excessive stressful condition due to high concentration of arsenic exposure. Membrane destabilization and damage is shown in arsenic exposed rat mitochondria. Moreover, this damage was reversed and mitochondria remain protected by the exposure of BBE. This event results in the release of cytochrome c and damage the mitochondrial membrane followed by the hepatic tissues damage (Bustamante et al., 2005). Like hepatic tissue, arsenic may also be deposited in the pancreas and initiates its degeneration (Xue et al., 2007). Pancreatic degeneration result in an impairment of glucose homeostasis and abnormal lipid digestion. Investigations on arsenic-mediated intestinal toxicity mechanism are inadequate. Inflammatory responses have been implicated in several toxicological pathogenesis processes. In this case some specific immunological marker, that is, TNF-α or IL-6 has been increased (Acharya et al., 2010; Das et al., 2009). The possible anti-apoptotic role of this therapeutically potent organism has been demonstrated in our comet assay results. Anti-inflammatory role of this organism extracts has been shown. Arsenic induced induction of proinflammatory cytokines (i.e. IL-6 and TNF-α) is evident in some previous investigation (Singh et al., 2014). This is noticed in the present study. Here, the BBE notably restored the TNF-α level. The increase of metabolic inflammatory component like, C-reactive protein (CRP) has been noticed in our previous studies *in vivo* in mouse and *in vitro* in human in response to arsenite exposure (Druwe et al., 2012). Arsenic-induced alterations in nitric oxide (NO) in the blood and tissues have been demonstrated and also evident in the current study (Zarazúa et al., 2006).

The decrease in NO level and nitric oxide synthase (NOS) activity may result in an insufficient NO signalling which have been correlated to abnormal cellular metabolism. Moreover, the generation of several free-radicals (ROS) and nitrogen species during arsenic exposure may result in tissue degeneration, necrosis and carcinogenesis (Maiti, 2015;

Zarazúa et al., 2006). Anti-cancer activity of some natural substances from marine organisms has been demonstrated. Commercial forms of the drugs prepared from these products are used in different physiological ailments (antitumor, analgesia, anti-inflammatory, immunomodulation, allergy, anti-viral) (Singh et al., 2008; Montaser and Luesch, 2011).

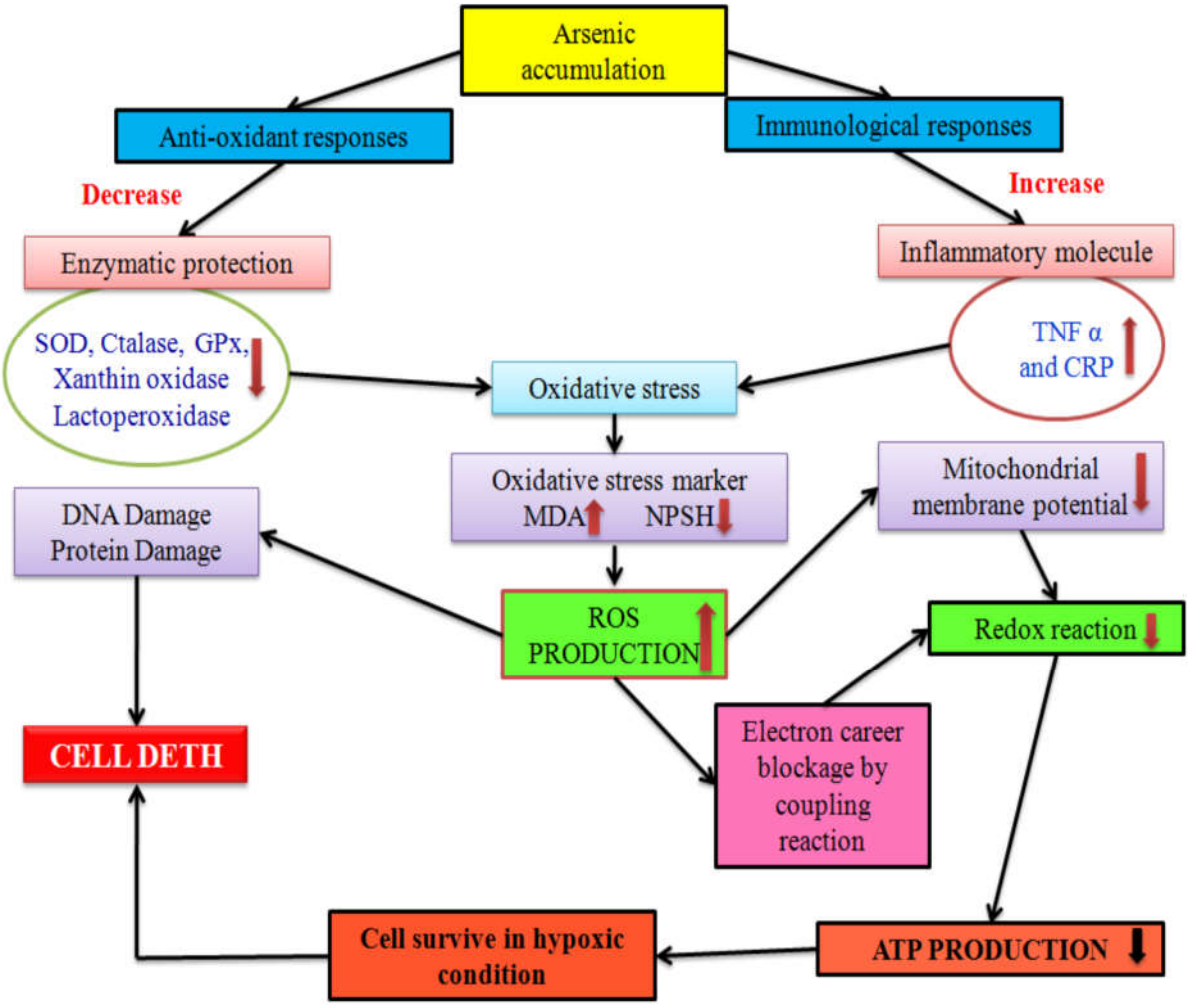

Fig. 1. Mechanism of arsenic toxicity and cell death

Arsenic is reported to induce cellular inflammatory signaling by increasing the tumor interferon γ (IFN-γ), necrosis factor α (TNF-α), and interleukin 6 (IL-6) (Dutta et al. 2015). Arsenic-induced ROS deteriorates the situation by promoting the NF-*K*β action (Kaul et al., 2014). The BBE has been demonstrated to decrease the LPS-induced inflammatory and

edematous processes like TNF-α and macrophage activation (Bhattacharya et al., 2014). This is also shown in the present study against arsenic exposure. BBE has been shown to effectively nullify the LPS-induced nuclear translocation of NF-*K*β and p65 molecules (Bhattacharya et al., 2014). These are known to regulate several. It can be assumed that an anti-inflammatory action of BBE might be occurring in the protection mechanism in the present study. Arsenic exposure results an elevation in the number of total white blood cells (WBC) with a higher neutrophil count and reduction in lymphocyte number (Table 3). Exposure of rats to arsenic in this study to sodium arsenite produced leukocytosis that evident in the current result. Increased number of WBCs is generally known to be a normal reaction to foreign substances. Leukocytosis, mainly due to leukocyte mobilization into the circulation can be an event of stimulation of the immune system against infectious agents or chemicals. (Adebayo et al., 2010) On the other way, the trend of leukocytosis has been restored by BBE supplemented group (Table 4). From the results presented in, there is a significant increase in the activities of ALP, AST, and ALT in the serum of arsenic treated rats when compared to that of the control group. This might have resulted from sodium arsenite-induced oxidative threat and related stress-related damages to hepatocytes membrane. The situation promotes leakage of hepatic transaminases into extracellular spaces. (Chattopadhyay et al., 2001)However, there was a decrease ($P > 0.05$) in serum activities of ALP, AST, and ALT in the $NaAsO_2$ + BBE pre-treated group compared to that of $NaAsO_2$ treated group. This suggests that hepatocytes are protected from arsenic by BBE application. This can be attributed to the antioxidant present in BBE. In this experiment, sodium arsenite treated in rat interfered with kidney functions as seen by elevation of these values. Urea, excretory martial of protein catabolism, can rise when the kidney is defective. In renal diseases, urea accumulates in the serum because the rate of production serum urea exceeds the rate of its

clearance. (Mayne, 1994) Increased urea and creatinine levels observed may be an indication of nephrotoxicity by arsenite compound. This is in pretty with the findings of that arsenite toxicity induces several metabolic syndromes including urea and creatinine elevation following proximal tubular damages and glomerular injuries.(Anwar et al., 1999)(Nandi et al., 2006) Pretreatment with BBE had a reversal effects on these parameters. This study, therefore, showed the nephron-protective effects of BBE in arsenite-induced toxicity. The present investigation, demonstrates that arsenite treatment clearly induced the degeneration hepatic tissues. Metabolites of arsenite can interfere with cell-division via tubulin disruption and cytoskeletal degeneration (Kligerman et al., 2005). Hepatic necrosis and lobular structural disintegration, pointed in the present study is supported by this result. Similarly, damages in the renal tissues and nephritic damages are evident in the current study. This disintegration is obviously protected by the present supplementation schedule.in some other supplementation study it is observed that the methylated As (III) compounds are more genotoxic than methylated arsenicals in its (V) state. MMA (III) and DMA (III) are the terminal genotoxic forms of arsenic and they are regarded as the clastogens. Their intracellular levels are regulated by folate and B12 (Kligerman et al., 2003). Here in the current study BBE has been shown to contain high level of vitamin c and the micronutrients. The oxidation of more toxic DMA (III) to less toxic DMA (V) requires some strong oxidant like H_2O_2. The present result of XO-inhibition suggests the possible inhibition in production of H_2O_2 thus the unavailability of H_2O_2 which could have been utilized to catalyze more toxic As (III) to less toxic As (V) state keeps the internal cellular environment more toxic. In addition, the urate, product of the XO-catalyzed reactions might have played as a good antioxidant component. This might inhibit cellular oxidant stress burden. One earlier report on rat model suggests that arsenic-induced DNA-damage can be inhibited by the treatment

uric acid (Gurr et al., 1998). DNA damage has been proposed to induce apoptotic tissue damage (Vermeulen et al., 2005). In this study, a significant decrease in uric acid and the increase in free radical production like MDA level may promote the cellular toxicity and tissue damages. Ramanathan et al. 2005 reported that administration of Vitamin C and Vitamin E to rat along with arsenic significantly reduced cellular apoptotic responses. Vitamin C was found in *B. bengalensis* with a satisfactory level i.e. 71.85 ± 4.74 µg/gm tissue. This is advantageous for the tissue protection against oxidative stress. Vitamin C acts as a scavenger of free radicals and plays an important role in the regeneration of α-tocopherol (Young et al., 2001). Moreover, supplementation of α-tocopherol and ascorbic acid has been known to decrease the DNA damage by reducing the level of TNF-α and disfavouring the activation of caspase-cascade reaction in arsenic intoxicated animals (Ramanathan et al., 2005). Anti-carcinogenic and anti-leukemic activity of BBE has been demonstrated in previous report. Selective degeneration of cancerous cells through apoptogenic pathway by BBE- promoted mitochondrial caspase pathway has been associated to these anti-cancer effects (Besra et al., 2013). Mitochondrial death-signal due to arsenic toxicity has been shown and it is linked to the influences of the p38, p53, and c-*myc* regulations and DNA damages. These are extensively reviewed in previous publication (Maiti, 2015). In the present study, arsenic-induced DNA fragmentation has been restored by the BBE extract having been demonstrated in the DNA ladder-assay results. This result is also by supported by the single cell DNA damage assay (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/gm tissue, which offers a better protective responses against arsenic toxicity. Arsenic, in the form of arsenate is chemically similar to phosphate molecule. It uncouples oxidative phosphorylation

during the metabolic processes of ATP synthesis (Gresser, 1981). It has been reviewed that arsenate and phosphate share the same transport mechanism and compete for the same binding sites (Maiti, 2015). The intestinal absorption of arsenic is significantly inhibited with the phosphate infusion in rat model (Dixon et al., 1997). Furthermore, even in complex systems, simulating natural conditions like in ground water, phosphate demonstrated distinct ways in regulating the arsenic concentrations in the living systems (Stachowicz et al., 2008). Thus, higher phosphate in BBE might play a better protective role against systemic arsenic toxicity. And it is hypothesized BBE may a good choice for therapeutic materials against arsenic toxicity. But this obviously needs standardization and optimization study.

Specific functional groups such as thiols have shown a major influence on receptors enzymes or receptors coenzymes functions. A significant amount of non-protein soluble thiols has been demonstrated in the *B. bengalensis* extract. The trivalent arsenicals (AsIII) readily react with thiol-containing molecules such as cysteine and GSH in *in vitro* experimental condition (Delnomdedieu et al., 1994). The binding of trivalent arsenic to a critical thiol group may inhibit important biochemical events which would lead to toxicity. However, binding of arsenite molecules at non-essential sites in proteins might offer a detoxification mechanisms (Aposhian et al., 1989) and this could promote some extent of cellular protection.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 NaAsO2+H2O2-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., 2014b). It has long been acknowledged that sulfhydryl-containing compounds have the ability to chelate metals. Gauri et al., 2011 reported an anti-microbial

peptide of 1676 Da, purified from *Bellamya bengalensis* venom (BBV, 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 Bellamya extract in our study. Cysteine-rich secreted protein from the snail *Achatina achatina* has been reported (Shabelnikov and Kiselev, 2015). 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 hepatoprotective 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.

In the present study the cytosolic super oxidase dismutase activity was tested in gel zymogram study where it was noticed that several $NaAsO_2$ treated group moderately decrease its activity in the liver cell that has been extended higher in long duration incubation group but this impairment of SOD activity was successfully prevented by the BBE treated group and even this activity increased then the control. Increased SOD activity is generally produces higher amount of H_2O_2 which is an inducing factor for catalase activity. In the present study similar pattern of response like SOD activity was notice in catalase gel zymogram. The moderate decrease of catalase activity by arsenic was efficiently restored and even increased in BBE treated group. This suggests that the combination protection of SOD & catalase in addition to thiol increase was more efficient for hepatic DNA protection.

From all these experimented results we can conclude that BBE has significant role of cellular macromolecular protection like DNA and protein and that was able to prevent against arsenic induced oxidative stress.