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Protective effects of *Corchorus olitorius* and *Butea monosperma* against Arsenic induced aberrant methylation and mitochondrial DNA damage in wistar rat model

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ABSTRACT

Millions of people around the world are chronically exposed to Arsenic (As) through food and drinking water. Studies revealed that Arsenic is genotoxic and causes damage to DNA. In this study, we evaluated *Corchorus olitorius* and *Butea monosperma* for their alleviative properties against Arsenic induced genotoxicity *in vivo* using Wistar Rat model. Arsenic exposed rats were given *C. olitorius* leaf powder and *B. monosperma* flower powder as supplementation with normal food. Methylation status of *p53* promoter was measured using Methylation Sensitive Restriction Endonuclease PCR (MSRE-PCR) assay and mitochondrial DNA (mtDNA) copy number as well as occurrence of a common deletion in mtDNA in liver and kidney tissue was determined through quantitative realtime PCR (qPCR). Arsenic exposed rats after supplementation showed relatively less severe effects of toxicity evident by significantly higher amount of ($p < 0.05$) mtDNA copy number and reduced occurrence of deletion containing mtDNA as well as lower levels of methylation in *p53* gene promoter. Histopathological analysis revealed less severe histopathological changes of liver and kidney and normal liver and kidney function parameters in supplemented rats. So, the protective properties of *B. monosperma* and *C. olitorius* against Arsenic toxicity is evident in molecular level.

1. Introduction

Arsenic (As) is a ubiquitous toxic metalloid present in the environment all over the world in organic and inorganic form [1]. It is one of the most epidemiologically important toxicant affecting millions of people around the world [2]. Beside severe skin pigmentation and keratosis, Arsenic causes respiratory disease, peripheral neuropathy, liver fibrosis, edema of legs, anemia and cancers. Chronic exposure to Arsenic especially through drinking water and food is associated with a number of different cancers (e.g., skin, bladder, liver and urinary tract) [3,4].

Arsenic is a proven carcinogen [5]. Though the exact mechanism is still not apparent it is understood that As is a potent genotoxic substance. It has the ability to directly affect the genetic material in numerous ways [6]. The genotoxic effects of Arsenic include induction of oxidative stress and DNA damage, inhibition of DNA repair enzymes, tumor promotion, cell proliferation, chromosomal aberrations (CA), signal transduction, [7] inhibition of DNA ligase [8], interference with tubulin polymerization in the mitotic spindle [9] and influencing telomere length through the stimulation of telomerase reverse transcriptase [10]. Also, exposure to heavy metal pollutants can influence neuro-developmental disorders such as Autism spectrum disorder (ASD) through various mechanisms [11]. Reports found association between the levels of heavy metals such as Arsenic, lead etc. in hair and severity of ASD symptoms [12]. In addition, prolonged inhalation of heavy metal polluted particulate matter (PM 2.5) can cause respiratory dysfunction and aggravated symptoms in respiratory viral infections such as influenza and COVID-19 [13]. Recent reports also suggest that Arsenic is an epi-mutagen [14]. That is Arsenic is capable of changing the methylation pattern of a single

Abbreviations: As, Arsenic; MtDNA, Mitochondrial DNA; ALT, Alanine aminotransferase; ASTAspartate, aminotransferase; ROS, Reactive oxygen species; MSRE-PCR, Methylation sensitive restriction enzyme digestion Polymerase Chain Reaction; qPCR, Quantitative Polymerase Chain Reaction; HG-AAS, Hydride Generation - Atomic Absorption Spectroscopy.

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gene to even whole genome [15]. Epigenetic changes are closely related with various diseases such as cancer [16], neurodegenerative diseases [17] and cardiovascular diseases [18].

Despite being a very concerning problem worldwide there is no dependable and safe treatment for arsenic toxicity [19]. Currently, Arsenic toxicity is treated with Sulfhydryl containing chelating agents like 2,3-dimercaptopropane-1-sulfonate, Meso-2,3-dimercaptosuccinic acid etc. [20]. These are prescribed in combination with natural anti-oxidants like vitamin C and E. But most of these chelating agents have severe side effects and are toxic in nature [21]. At this context, medicinal plants attracted much attention lately; largely due to the antioxidative properties of many plant derivatives. The free radical scavenging property of plant derivatives can reduce the oxidative damage induced by Arsenic [22].

Corchorus olitorius otherwise known as 'Tossa' jute contains antioxidants such as carotenoids, flavonoids and vitamin C and antitumor agents such as phytol and monogalactosyl-diacylglycerol [23]. Studies also provided evidence of *C. olitorius* reducing oxidative damage in rat renal and hepatic tissue due to Arsenic [24].

Butea monosperma is another medicinal plant used in folk medicine alike *C. olitorius*. According to Ayurvedic medicine and modern studies every part of *B. monosperma* contains medicinal properties. These include anti-oxidative, anti-inflammatory, anti-diarrheal, bactericidal and fungicidal properties [25]. Studies also reported that *B. monosperma* flower contains butein, butin, butrin, isobutrin, coreopsin etc., all of which have anti-oxidative attribute [26]. Therefore, evidences suggest that traditional herbal medicines such as *B. monosperma* and *C. olitorius* can potentially produce a protective effect against Arsenic and reduce its toxic effects, although this remains to be fully determined.

Drinking water is the most common route of exposure to Arsenic. But Arsenic exposure may also be through food, especially rice, via soil/water-crop-food transfer [27]. Many studies confirmed presence of Arsenic in rice in concentration ranging from 0.03 to 1.83 mg per kilogram of rice. Indeed, one of the most disturbing facts is that the Arsenic content of soil in rice cultivating areas, where Arsenic may be found in the ground water, is significantly increasing. This poses a potential severe hazard for countries such as Bangladesh where rice is a staple component of the everyday diet [28]. Recent studies discovered a strong positive correlation between amount of As contaminated rice intake and increase in As induced toxicity [29]. Arsenic exposure may also occur from other dietary sources such as fish and sea foods [30] or even from indoor dusts [31].

In this study, our objective was to ascertain the genotoxic and epigenetic effects of Arsenic contaminated rice induced toxicity in rat model alongside evaluating the potential protective effects of two medicinal plants *Butea monosperma* and *Corchorus olitorius* against the induced toxicity. This study will hopefully validate the deep laden health impacts of As contaminated rice and its prospective hazardous ability.

2. Materials and methods

2.1. Preparation of As contaminated rice and plant supplements

Rice grain of local BR-28 variety was collected and checked for background Arsenic contamination through flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS) according to Rahman et al. [32]. After confirming that no arsenic contamination is present, the rice grains were soaked in 20 mg/kg Sodium Arsenite solution for 36 h and subsequently tested for Arsenic concentration. Rice Arsenic content was 47.2 mg/kg. Then the grains were dried, grinded and mixed with pallet feed for the rats.

B. monosperma flowers were collected from University of Chittagong campus and *C. olitorius* leaves were collected from local plantations in the nearby Hathajari region. Both plant species were identified by Professor Dr. Sheikh Bakhtear Uddin, a taxonomist (Department of Botany, University of Chittagong, Bangladesh). Petals were separated from the flower and the leaves from the plant and washed thoroughly with distilled water. Then the petals and leaves were sundried and ground to powder. The powder was kept in sterile air tight containers and stored at a cool and dry place. As both the plant product is seasonal a single preparation was used through the study. After that *B. monosperma* flower powder and *C. olitorius* leaf powder were mixed with pellet feed at 4 % wt/wt ratio for supplementation.

2.2. Animal model and treatment

For this study 20 Female Wistar albino rats (*Rattus norvegicus*), weighing 160–170 gm were collected from the animal breeding center of Bangladesh Council of Scientific and Industrial Research (BCSIR), Chittagong. Single sex of rats was used in this study to reduce variability. The average weight of the rats was 167.35gm and the rats were provided with sufficient laboratory rodent pellet diet and water. Then the rats were divided into four groups randomly placing five rats in each group. Group I, the control group received normal diet (pellet feed), Group II received normal diet mixed with Arsenic contaminated rice, Group III and IV was fed with Arsenic contaminated rice with *C. olitorius* leaf powder and *B. monosperma* flower powder mixed pellet feed supplementation in their diet respectively for 150 days for simulating sub-chronic exposure. All the experiments were carried out according the institutional and national guidelines.

2.3. Collection of blood and tissue samples

The experimental animals were sacrificed on the 150th day after fasting them overnight. Blood was collected in K_2EDTA tubes for each rat and centrifuged at 1500xg for 15 min. After centrifugation blood plasma was collected in pre labeled microcentrifuge tubes and stored at −80 °C. Liver and kidney from sacrificed rats were removed carefully and after washing the organs with PBS a section was immersed in 10 % formalin solution for histology and the rest of the organs were immersed in absolute ethanol and preserved in an ultra-low temperature freezer (−86 ℃) for molecular analysis and Arsenic measurement.

2.4. Biochemical assay

Liver and kidney function tests were performed by measuring liver enzymes (AST, ALT) and urea level in serum respectively. These tests were performed with commercially available kits from Human GmbH using CHEM-5v3 analyzer (Erba, Mannheim, Germany) following manufacturer's instruction.

2.5. Histopathology

Preserved liver and kidney tissues were cut in longitudinal and transverse pieces and passed through ascending series of ethanol washes. Then the samples were cleared with toluene and embedded in paraffin. 4μM sections of the paraffin embedded tissues were fixed on a glass slide after staining with hematoxylene and eosin and observed under light microscope.

2.6. Arsenic measurement in tissue

The concentration of As in different organs (liver, kidney) was determined using FI-HG-AAS method. From each organ, 0.25gm sample was weighed and taken in beaker. The samples were digested with a mixture of $HClO_4$-HNO_3 solution (ratio 1:3 v/v) at 130 °C. After removal of HNO_3 by evaporation, the digested samples were diluted with deionized water up to 100 mL. The concentrations of As in digested samples were measured at 193.7 nm wave length and 10 mA current using Atomic Absorption Spectrophotometer equipped with As lamp. Vapor generation accessory (VGA) was used to produce hydride vapors

using 0.6 % sodium borohydride and 10 mM HCl [33].

2.7. DNA extraction

DNA from cryopreserved rat liver and kidney tissues were extracted following standard phenol-chloroform method [34]. After ethanol precipitation DNA was resuspended in nuclease free water. DNA was quantified and checked for purity in a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) and stored in −20 °C freezer.

2.8. Methylation of TP53 promoter region

Methylation Sensitive Restriction Endonuclease PCR (MSRE-PCR) technique was used to quantify the methylation level in the promoter region of the tumor suppressor gene *TP53* following a previously described technique [35]. The extracted DNA from liver and kidney tissue of the rats from each group were digested with a methylation sensitive restriction endonuclease BstUI. BstUI enzyme recognizes a CpG site 5′-CGCG-3′ within the 85bp basal promoter region of *TP53*. Methylation of the internal cytosine blocks cleavage. Thus the number of available template for amplification is dependent on the number of methylated and unmethylated copies of the template. After the restriction digestion only the unmethylated copies will be amplified.

Approximately 200 ng of genomic DNA was digested with 7 units of enzyme overnight (16 h) at 37 °C. Following the MSRE digestion the digested products were used as a template to amplify a 505bp region of the *TP53* promoter which includes the 85bp basal promoter with the following primers, forward: 5′-TCT GTT TCA AAA AGC CAA AAA GAT G-3′ and reverse: 5′-CAG TCT TCA GGG GAG CGT G-3′. PCR reactions were carried out in 25 µl volume and each reaction mixture contained 2x GoTaq Reaction Buffer (Promega Corp.), 500 nM of each forward and reverse primer, 200 mM of each dNTPs and 1 unit of GoTaq DNA Polymerase (Promega Corp.). The cycling conditions were an initial denaturation of 5 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, elongation at 72 °C for 1 min and a final elongation at 72 °C for 5 min. The reactions were carried out in a Q-Cycler (HAIN Life Science, UK). The amplified region of *p53* promoter region was detected by electrophoresis in 1.5 % agarose gel stained with ethidium bromide and visualized in a WiseDoc gel documentation apparatus (Daihan Scientific, Korea). After that, ImageJ software (National Institute of Health) was used to measure the relative intensity of each band for calculating the relative percentage of methylation in each group (Fig. 1).

2.9. mtDNA copy number and deletion analysis

mtDNA copy number and frequency of deletion in rat liver and kidney tissue was carried out following a method reported by Nicklas et al. [36]. For the quantification of mtDNA copy number and frequency of deletion, separate reactions were set up with 2x AddProbe qPCR Mastermix (ADDBIO Inc. Korea), 7.5 pM of D-Loop specific primer (Fwd 5′- GGT TCT TAC TTC AGG GCC ATC A-3′, Rev 5′- GAT TAG ACC CGT TAC CAT CGA GAT-3′) or Deletion specific primer (Fwd 5′- AAG GAC GAA CCT GAG CCC TAA TA-3′, Rev 5′- CGA AGT AGA TGA TCC GTA TGC TGT A-3′), 100 nM of each specific probe (D-loop- 56FAM- TTG GTT CAT /ZEN/ CGT CCA TAC GTT CCC CTT A- 3IABkFQ, Deletion- 56FAM- TCA CTT TAA /ZEN/ TCG CCA CAT CCA TAA CTG CTG T -3IABkFQ) and 50 ng of genomic DNA. The reactions were carried out in 25 µL volume. The PCR profile was 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s and hybridization and elongation at 60 °C for 30 s. Fluorescence was detected in the last step in a QuantStudio 3 system (Applied Biosystems, USA). Rat β-actin gene (Fwd 5′-GGG ATG TTT GCT CCA ACC AA-3′, Rev 5′- GCG CTT TTG ACT CAA GGA TTT AA-3′) was used as the internal control to calculate the relative copy number of mtDNA and relative frequency of deletion in mtDNA using the $2^{-\Delta\Delta CT}$ method.

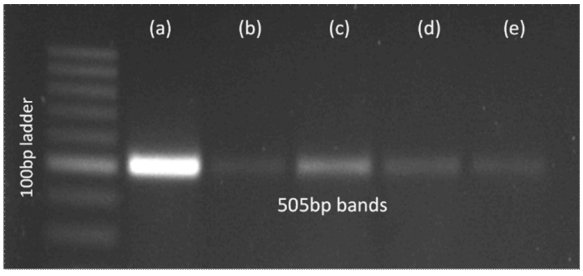

Fig. 1. 505bp bands of PCR amplified p53 promoter region of liver tissue. Visible under UV translumination after agarose gel electrophoresis and ethidium bromide staining. (a) PCR product amplified from undigested genomic DNA and PCR amplified product from digested genomic DNA of (b) Group I (c) Group II (d) Group III (e) Group IV. Bands are showing varied intensity corresponding to the level of point methylation.

2.10. Statistical analysis

Statistical analysis was performed with SPSS for Windows V.24. All data were analyzed by using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMART) with a p-value<0.05 considered to be statistically significant. All the values are expressed as mean ± SEM.

3. Results

3.1. Changes in morphology, food and water intake

All the activities were found normal in both control and arsenic-treated groups. No mortality was observed in any of the groups during the period of study. The albino rats of Group II developed 'Chromodacryorrhea' around their eyes after 80 days of Arsenic exposure. The food and water intake and body weight of the animals were monitored regularly although the study period. Change in body weight is used as an indicator for checking the animal health status. We observed significant ($P < 0.05$) changes in food and water intake after arsenic exposure. After treatment with *C. olitorius* and *B. monosperma* food and water intake both improved significantly ($P < 0.05$) (Table 1). The mean initial body weight of Group I (Normal diet for 150 days), Group-II (As cont. rice for 150 days), Group-III (As cont. rice plus 4 % *C. olitorius* for 150 days), Group-IV (As cont. rice plus 4 % *B. monosperma* for 150 days) were 166.1 ± 2.46 gm, 163.78 ± 3.08 gm, 160.56 ± 3.12 gm, 163.18 ± 3.66 respectively. After 150 days study, the mean final body weight was 205.7 ± 3.60 gm, 183.9 ± 2.61 gm, 197.14 ± 3.49 gm, 205.48 ± 3.47 gm respectively. The mean final body weight of Group-II significantly ($P < 0.05$) decreased compared to Group-I. On the other hand, the mean final body weight of Group-III and Group-IV significantly ($p < 0.05$) increased compared to Group-II.

Table 1
Effect of *C. olitorius* and *B. monosperma* on daily food and water consumption of albino rats. Group II rats consumed significantly ($p < 0.05$) less food and water compared to control group. Whereas, in Group III and IV food and water consumption increased significantly ($p < 0.05$) after *C. olitorius* and *B. monosperma* treatment compared to arsenic exposed rats of group II.

Groups	Food Intake (gm/rat/day)	Water Intake (ml/rat/day)
Group-I (Control)	17.17 ± 0.62	13.30 ± 0.20
Group-II (As cont. rice)	11.98 ± 0.35	7.23 ± 0.27
Group-III (As + *C. olitorius*)	17.39 ± 0.35	13.20 ± 0.46
Group-IV (As +*B. monosperma*)	21.08 ± 0.40	12.76 ± 0.32

Here, values are expressed as MEAN ± SEM;

3.2. Arsenic deposition in rat liver and kidney

Arsenic accumulation in the rat organ samples were determined through FI-HG-AAS analysis. Fig. 2 is showing the mean ± SEM values of arsenic deposition in liver and kidney tissues of the three experimental groups exposed to arsenic. In control rats (Group I) mean Arsenic accumulation in liver and kidney was 0.44 ± 0.15 μg/gm and 0.46 ± 0.04 μg/gm respectively. After Arsenic exposure overall arsenic accumulation was significantly higher in kidney than liver in all groups. Arsenic contaminated rice fed rats (Group II) had 33.872 μg/gm accumulated arsenic in kidney and 12.97 μg/gm accumulated arsenic in liver on average. However, rats of group III (*C. olitorius* leaf powder) and IV (*B. monosperma* flower powder) which received supplementation with arsenic contaminated rice had significantly less arsenic accumulation in both liver (5.27 μg/gm in GIII and 5.44 μg/gm in GIV) and kidney (18.34 μg/gm in GIII and 21.89 μg/gm in GIV) tissue compared to group II (no supplementation).

To further investigate the extent of protective capabilities of *Corchorus olitorius* and *Butea monosperma* histopathological analysis of liver and kidney tissue was done. The tissue samples from group II (only arsenic contaminated rice) exhibited severe changes in the histostructure (Fig. 3b). We observed necrosis and degenerative changes of varied severity in hepatocytes and central veins including sinusoidal dilation, venous congestion, increased lymphatic cell population, free nuclei and fatty degeneration. Similar degenerative changes were also seen in kidney tissue samples (Fig. 3f). Moderate to severe glomerulonephritis, coagulative and liquefactive necrosis along with epithelial damage and loss of nuclei was seen. Minor alterations in histostructure with mild degenerative changes in liver and kidney was observed in groups III and IV which received *Corchorus olitorius* (Fig. 3c, g) and *Butea monosperma* (Fig. 3d, h) supplementation respectively. The organ weight to body weight ratio was calculated to determine hypertrophy of organs in study animals. We observed mild liver hypertrophy in group II animals (Arsenic exposed) when compared to control rats (Group I) where the liver weight to body weight ratio was 3.13 ± 0.03 and 3.82 ± 0.09 in Group I and Group II respectively. In treatment groups the liver weight to body weight ratio was 2.97 ± 0.07 (Group IV- As + *C. olitorius*) and 2.89 ± 0.09 (Group III-As + *B. monosperma*). Kidney weight to body weight ratio was 0.29 ± 0.01, 0.37 ± 0.01, 0.31 ± 0.01, 0.29 ± 0.01 respectively in Group I, II, III and IV. However, none of these changes were statistically significant ($p>0.05$).

The evidence of Arsenic induced damage was also clear from the biochemical assays. As the data on Table 2 represents the Arsenic exposed rats had abnormal liver function due to significantly increased level of liver enzymes (AST and ALT) compared to control group of rats.

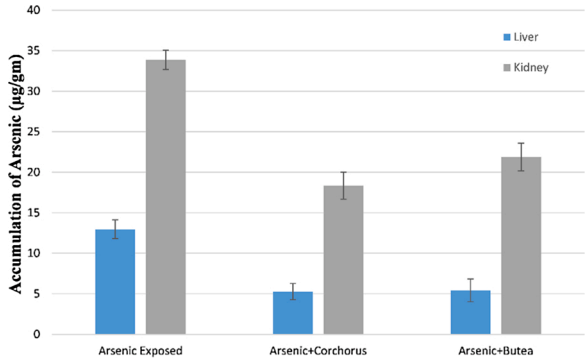

Fig. 2. Effects of *C. olitorius* and *B. monosperma* on As deposition pattern. Group III (Arsenic + Corchorus) and Group IV (Arsenic + Butea) both had significantly reduced ($p < 0.05$) Arsenic accumulation both in liver and kidney tissue than Arsenic exposed rats (Group II). Overall Arsenic accumulation was higher in kidney than liver tissue in experimental animals. Each bar represents the mean ± S.E.M.

These enzymes work as markers for liver abnormalities. Arsenic exposed rats also suffered from kidney abnormalities having significantly higher levels of serum urea. However, all the markers were maintained nearly within the normal levels with *C. olitorius* leaf powder and *B. monosperma* flower powder supplementation showing significant difference from arsenic exposed group of rats. In our study we did not find any significant difference in serum urea level between the treatment groups.

3.3. Methylation in the p53 promoter region

Methylation status of a CpG site within the 85bp basal promoter region of rat *TP53* gene was analyzed in liver and kidney tissue of rats from experimental groups. The semiquantitative analysis revealed control group of rats had about 10 % methylation at the CpG site (both in liver and kidney) but arsenic exposed rats had significantly higher percentage of methylated CpG (25.5 % in liver and 17 % in kidney) at the specific site of the *p53* promoter (Fig. 4). However, after *B. monosperma* flower powder and *C. olitorious* leaf powder supplementation despite arsenic exposure rats showed significantly lower site-specific methylation both in liver (12.55 % in GIII and 17.8 % in GIV) and kidney (12.6 % in GIII and 13.4 % in GIV) tissue. On the other hand, the mean percentage of methylation was higher in kidney than liver of arsenic exposed rats. Although DNA hypermethylation may precede histological changes but in this study no significant correlation was seen between hypermethylation of *p53* promoter and degree of histological damage both in liver and kidney tissue.

3.4. mtDNA copy number

The basic mechanism of arsenic induced toxicity is oxidative stress [37]. Therefore, mitochondria are the key target of arsenic induced oxidative damage. To ascertain the mitochondrial damage after arsenic exposure in liver and kidney tissue mtDNA number were measured through qPCR. The relative number of mtDNA in liver and kidney tissue of rats from experimental groups are shown in Fig. 5. The relative number of mtDNA was significantly ($p < 0.05$) lower in group II (72 % and 57 % of normal value in liver and kidney respectively) when compared to group I. Conversely, *B. monosperma* and *C. olitorious* helped to retain higher level of mtDNA despite arsenic exposure both in liver (79 % and 86 % of normal value in GIII and GIV respectively) and kidney (76 % and 70 % of normal value GIII and GIV respectively). Both the plants showed good nephroprotective activity but showed lesser potential in retaining hepatic mtDNA after arsenic exposure. *C. olitorius* supplementation in GIII did not have significant effect on liver tissue.

3.5. Relative frequency of deletion in mtDNA

Similar results (Fig. 6) were also found in case of deletion in mtDNAs. Deletions in mtDNA is seen as a marker of excessive oxidative stress and may cause mitochondrial dysfunction [38]. Arsenic exposure caused drastic increase in deletion containing mtDNA population in liver tissue (4.2-fold) compared to control rats. In kidney tissue however this increase in deleted mtDNA population was also significant (2.2-fold) but not as marked as in liver tissue. The nephroprotective potential of *B. monosperma* and *C. olitorious* was also evident in preserving mtDNA as the group of rats receiving supplementation retained near normal level (1.46-fold and 1.3-fold more than normal in GIII and GIV respectively) of deleted mtDNA population. This difference was significant ($p < 0.05$) compared to the arsenic exposed group. However, we observed contrasting result in liver tissue as neither plant supplementation produced significant difference between the arsenic exposed rats and treated rats.

4. Discussion

In our previous studies we have shown that *C. olitorius* [39] and *B. monosperma* [40] can alleviate the altered histological and

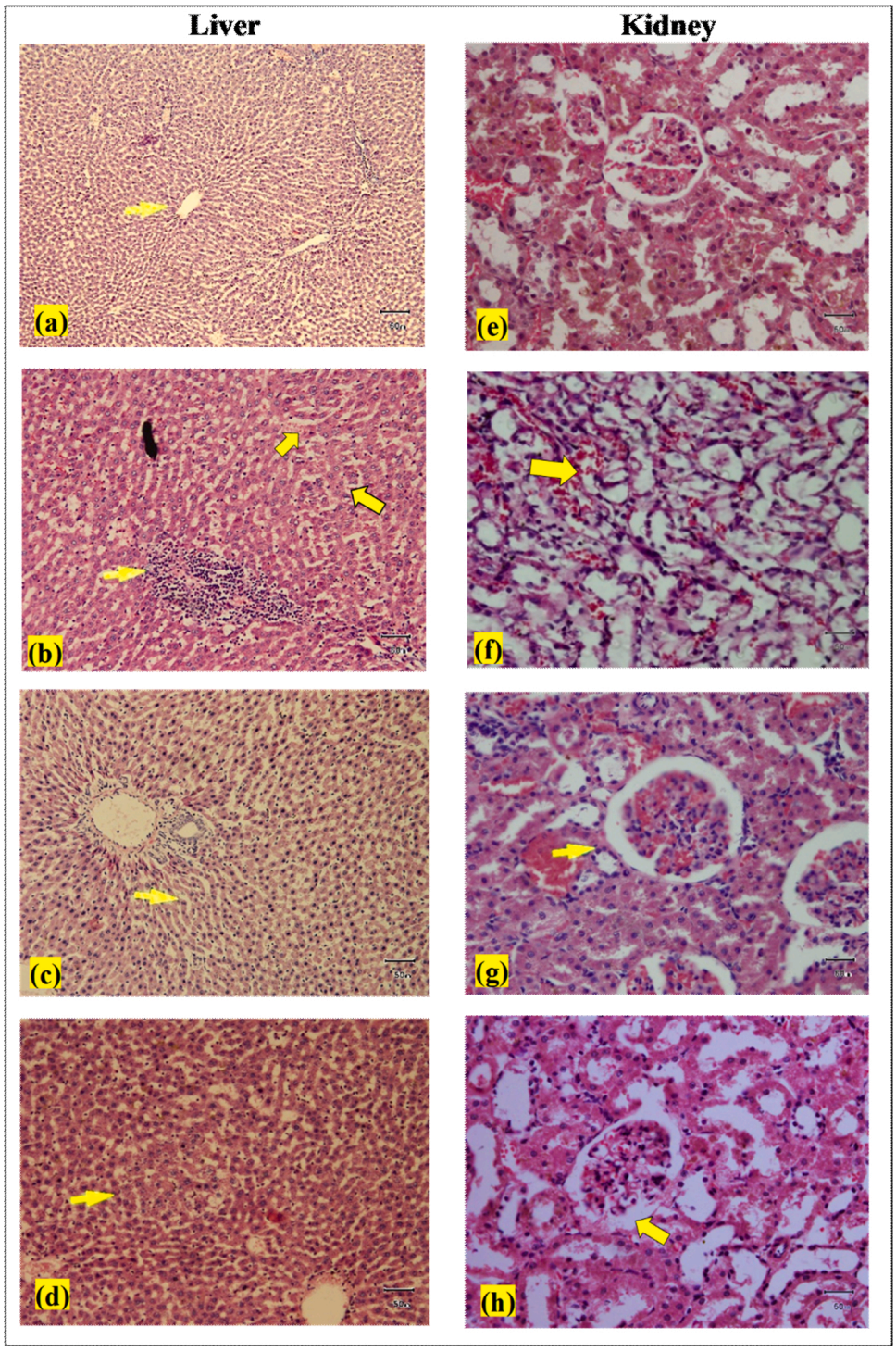

Fig. 3. Photomicrograph of liver and kidney tissue cross section of experimental groups. Features are indicated with arrow. Cross section of liver tissue of **(a)** control group showing normal histostructure (H&E 20x), **(b)** arsenic exposed group showing necrosis and mononuclear cell infiltration in liver tissue (H&E 40x), **(c)** group III (As+ *C. olitorius* leaf powder) showing mild congestion and edema (H&E 40x) and **(d)** Group IV (As+ *B. monosperma* flower powder) showing portal duct and mild necrosis (H&E 40x). Kidney tissue cross section of **(e)** control group depicting normal histostructure (H&E 200x), **(f)** arsenic treated group showing total tubular epithelium necrosis with hemorrhage, mononuclear cell infiltration (H&E 400x) besides **(g)** group III (As+ *C. olitorius* leaf powder) and **(h)** Group IV (As+ *B. monosperma* flower powder) showing near normal kidney structure with mild proximal tubular necrosis, mononuclear cell infiltration and mild hemorrhage (H&E 400x).

hepato-biochemical parameters caused by rice induced arsenic toxicity. In this study, we aimed to investigate the genotoxic effects of arsenic contaminated rice in rat model and assess the protective effects of *Butea monosperma* and *Corchorus olitorius* two plant species used in traditional herbal medicine, against arsenic induced toxicity.

Arsenic is known to cause both hypo and hypermethylation either in the promotor region or in whole genome [15]. We observed significantly increased methylation in *p53* promoter of the Arsenic exposed rats of Group II (As containing rice for 150 days) both in liver and kidney tissues (Fig. 4). Similar results confirming hypermethylated *p53* promoter were observed in other studies [41,42]. In other experiments, it was suggested that promoter regions of tumor suppressor genes are target of oxidative stress induced aberrant methylations [43]. As methylated promoters of tumor suppressor genes are frequently reported in malignant tissues, the findings of our study suggest that prolonged Arsenic exposure may result into malignant transformation in liver and kidney tissues.

Arsenic toxicity can cause mitochondrial dysfunction, mtDNA depletion and occurrence of deletions in mtDNA [38]. In our study we observed significant decrease in relative mtDNA copy number in arsenic exposed rat liver and kidney tissues (Fig. 5). There are different factors which could affect the number of mitochondrial DNA such as cellular

Table 2
Effect of *C. olitorius* and *B. monoperma* supplementation on biochemical markers. All three biochemical parameters were elevated significantly ($p < 0.05$) after arsenic exposure in group II compared to control rats. After treatment with *C. olitorius* and *B. monosperma* respectively in Group II and IV, biochemical parameters reduced significantly ($p < 0.05$) compared to arsenic exposed group II.

Parameters	Group-I (Control)	Group-II (Arsenic)	Group-III (Arsenic+ *C. olitorius*)	Group-IV (Arsenic+ *B. monosperma*)
AST	60.6 ± 3.61	99.4 ± 3.5	67.6 ± 2.5	70.00 ± 4.35
ALT	80.0 ± 2.98	122.4 ± 6.87	108.4 ± 5.57	97.2 ± 5.45
Urea	49.4 ± 2.08	64.4 ± 1.8	52.0 ± 1.61	59.2 ± 1.77

Values are expressed as mean ± SEM.

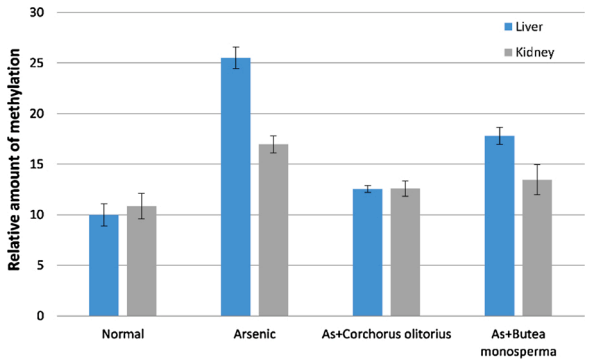

Fig. 4. Effect of *Corchorus olitorius* and Butea monosperma on *p53* promoter methylation in liver and kidney tissue of wistar albino rats. After arsenic exposure Arsenic exposed group is showing significantly increased methylation in liver and kidney tissue compared to control group. Both treatment groups, Group III (As + C. olitorius) and IV (As + B. monosperma) showing significantly ($p < 0.05$) reduced methylation compared to non-treatment arsenic exposed group II both in liver and kidney tissue. Each bar represents the mean ± S.E.M.

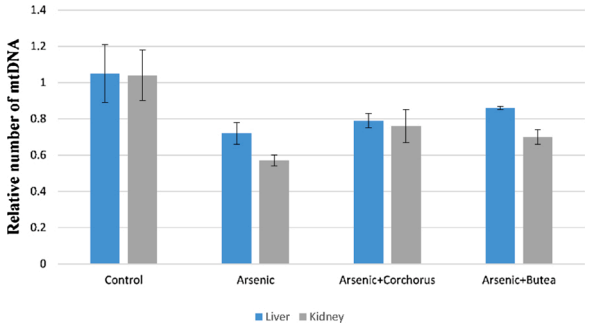

Fig. 5. Relative number of mtDNA copy in rat liver kidney tissue. Relative mtDNA count increased significantly ($p < 0.05$) in liver and kidney tissue in Group III (Arsenic + Corchorus) and IV (Arsenic + Butea) except in liver tissue of group III. Arsenic exposed group II had significantly ($p < 0.05$) decreased mtDNA count both in liver and kidney compared to control rats. Each bar represents the mean ± S.E.M.

ROS level, mitochondrial dysfunction and reduced expression of DNA polymerase and repair enzymes [38] Recent reports suggesting that Arsenic decreases the expression of DNA polymerase γ - the enzyme responsible for replication and repair of mtDNA could be the potential reason behind depletion of mtDNA [44,45]. The findings of our study certainly agree with these explanations. We also observed significantly increased number of deletion containing mtDNA (Fig. 6). Truncated expression and activity of mtDNA replicating enzymes might cause

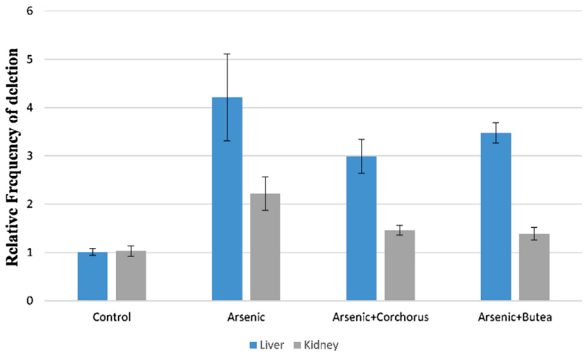

Fig. 6. Relative frequency of mtDNA with common deletion in rat liver and kidney tissue. Group II (Arsenic) is showing significant ($p < 0.05$) increase in the number of deleted mtDNA after Arsenic exposure compared to control group. Group III (Arsenic + Corchorus) and IV (Arsenic + Butea) is showing significant ($p < 0.05$) decrease in deleted mtDNA population after treatment in kidney tissue. However, the changes were not significant ($p > 0.05$) in liver tissue, both in group III (Arsenic + Corchorus) and IV (Arsenic + Butea). Data expressed as mean ± S.E.M.

gradual increase in deleted mtDNA percentage in arsenic exposed rat liver and kidney. In addition to that the deleted segment of DNA contains a subunit of COX enzyme and three subunits of NADH dehydrogenase enzyme [46]. As a result, arsenic exposed tissues exhibit diminished COX activity giving rise to ROS that further promotes Arsenic induced genotoxicity and carcinogenesis [47].

In our study, we observed marked adverse liver and kidney changes associated with Arsenic accumulation and mitochondrial damage in Group II animals. Varied degree of necrosis was present throughout liver and kidney tissues along with other histostructural changes (Fig. 3). A previous work of Santra et al. [48] also obtained similar results and attributed the cause of damage to mitochondrial dysfunction [48]. Several other studies also published similar reports that Arsenic causes mitochondrial induction of cellular apoptosis and necrosis through activating caspase cascade signaling and induction of mitochondrial membrane permeability transition (MMP) and inhibition of electron transport system [49,50]. Besides, Arsenic induced free radicals can promote lipid peroxidation which causes loss of membrane integrity and results in the leakage of cytosolic enzymes into the circulation [51]. This explains the increased level of liver enzymes (AST and ALT) in the serum in arsenic exposed rats compared to normal rats. Almost similarly, Group-II rats had significantly elevated serum urea level, which also indicates cellular damage in kidney. All these correspond with the evidence from histological analysis too.

As the major genotoxic mechanism of Arsenic is through activities of ROS it is understandable that antioxidants can significantly help to decrease the toxic effects of Arsenic. Many *in vitro* studies reported the capability of natural and chemical antioxidants that exhibits protective effects against Arsenic [52]. In our study we observed remarkable protective effect of *Corchorus olitorius* against Arsenic induced toxicity.

We observed significant decrease in methylation in rat liver and kidney of Group III (*C. olitorius* leaf powder supplementation) (Fig. 4). Arsenic induced hypermethylation of gene promoters originates from reactive oxygen species (ROS) induced stress [43]. Thus, the oxidative effects could be countered by administering antioxidants. In group III, mtDNA copy number increased and mtDNA deletion decreased compared to Arsenic exposed Group II rats. However, the changes in both mtDNA damage parameters were significant in kidney tissue. Group III also suffered less amount of Arsenic accumulation and less severe histological damage in both liver and kidney. Previous studies reported similar protective effects of *C. olitorius* in rat brain, liver and kidney against oxidative damage. [24] *C. olitorius* leaves are rich in polyphenolics and flavonoids which are active antioxidants [53]. The mechanism of protection against Arsenic toxicity might be through

prevention of lipid peroxidation by chelating Arsenic induced ROS. Heavy burden of ROS due to Arsenic can impair the activity of anti-oxidative enzymes such as super oxide dismutase (SOD) [54]. Thus *C. olitorius* may provide protection from the oxidative damage by inhibiting lipid peroxidation with phytophenolic compounds to prevent mitochondrial damage and preventing the ensuing damaging effects.

Similarly, rats of Group IV (*B. monosperma* flower powder for 150 days) showed lower *p53* promoter methylation along with significant retention of mtDNA in both liver and kidney after *B. monosperma* flower power supplementation in their feed. Even after Arsenic exposure the mtDNA number was almost similar to control animals and showed significant increase in mtDNA count from the Group II animals. Reduced mitochondrial damage in Group IV is also accompanied by low As accumulation and less severe histological damage. This protective effect against Arsenic induced toxicity might be due to presence of butein in *B. monosperma* flower. Previous studies reported about butein's antioxidant, free radical scavenging and anti-apoptotic properties [25]. Moreover, the presence of different flavonoids, alkaloids, butrin and isobutrin etc. attribute to the antioxidant property of *B. monosperma*. Butrin and isobutrin also have hepatoprotective and nephoprotective capability against oxidative damage [55]. Ethanolic extract of *B. monosperma* flower is a potent free radical scavenger and actively reduces super oxide anion *in vivo* [56]. Because creating oxidative stress is one of the prominent modes of action for Arsenic toxicity, presence of natural antioxidants and active ROS scavenger properties of *B. monosperma* can reduce the burden of ROS imposed by Arsenic biotransformation.

Overall, we found that protective effects of *Corchorus olitorius* and *Butea monospema* were more pronounced in kidney tissue than in liver. We have observed significant ($p < 0.05$) improvements in terms of both reduced methylation and mtDNA damage. This protective effect is exhibited through marked reduction in As accumulation, retention of normal level of mtDNA and lower amount of stress induced mtDNA deletion in both liver and kidney.

5. Conclusion

There are many plants that contain anti-oxidative properties like *B. monosperma* and *C. olitorius* which, as we have shown in this study, can alleviate the Arsenic induced toxicity in molecular level. But more knowledge is required to recommend a specific and safe use of these plant derived products. Potentially, this study can provide data that can initiate a pharmacological research to develop a remedy against Arsenic toxicity in Bangladesh as well as other Arsenic affected regions of the world.

Author Statement

Thank you again for the prompt review of the manuscript. In this revision text modifications have been done as per suggestion. We mainly changed the captions for all the tables and figures to eliminate the use of symbols describing statistical significance which seemed to lack cohesion across the literature. Now we reported the results in the captions of respective images and tables.

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Declaration of Competing Interest

The authors declare that there is no known conflict of interest.

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