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PhD Thesis

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Investigation of the Ameliorating Effects of Dietary Supplementations of Plant Materials on Sodium Arsenite-Induced Biochemical and Molecular Perturbation in Mice

Dilruba, Sayada

University of Rajshahi

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**INVESTIGATION OF THE AMELIORATING EFFECTS OF DIETARY
SUPPLEMENTATIONS OF PLANT MATERIALS ON SODIUM
ARSENITE-INDUCED BIOCHEMICAL AND MOLECULAR
PERTURBATION IN MICE**



PhD Thesis

*A dissertation submitted to the University of Rajshahi in conformity
with the requirements for the degree of Doctor of Philosophy in
Biochemistry and Molecular Biology*

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June, 2015

Certificate

I certify that the thesis entitled **“INVESTIGATION OF THE AMELIORATING EFFECTS OF DIETARY SUPPLEMENTATIONS OF PLANT MATERIALS ON SODIUM ARSENITE-INDUCED BIOCHEMICAL AND MOLECULAR PERTURBATION IN MICE”** submitted by **Sayada Dilruba**, incorporates the original research work carried out by her in the Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh under my supervision. I am forwarding her thesis being submitted for the award of the degree of Doctor of Philosophy of the University of Rajshahi. This work has not been submitted previously anywhere for the awards of any degree.

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Declaration

I hereby declare that the material embodied in this entitle “**INVESTIGATION OF THE AMELIORATING EFFECTS OF DIETARY SUPPLEMENTATIONS OF PLANT MATERIALS ON SODIUM ARSENITE-INDUCED BIOCHEMICAL AND MOLECULAR PERTURBATION IN MICE**” prepared for submission in the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh, for the degree of Doctor of Philosophy. The work contained in this thesis is original and have not been previously submitted anywhere for the awards of any degree.

(Sayada Dilruba)

ACKNOWLEDGEMENTS

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible to complete as well as to submit the thesis for the degree of Doctor of philosophy.

I wish much pleasure to express my heartiest gratitude and deep appreciation to my reverend supervisor Professor Dr. Md. Khaled Hossain, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for his genius guidance, encouragement, valuable suggestions, critical and constructive discussions during the whole course of this research work and preparing this thesis.

I have honor to express my deepest sense of gratitude to Dr. Abu Shadat M. Noman, Chairman and Associate Professor, Department of Biochemistry and Molecular Biology, University of Chittagong for his kind supports in performing the RT-PCR and real time PCR related experiments in his laboratory.

I am highly indebted to Professor Dr. Md. Tofazzal Hossain, Chairman, Department of Biochemistry and Molecular Biology, University of Rajshahi for providing me privileges to carry out this research work. My immense gratitude also goes to Dr. Md. Zahangir Alam Saud, Professor, Department of Biochemistry and Molecular Biology, University of Rajshahi for his moral support, constructive advice and active help during the entire course of my research.

I would like to convey my deepest acknowledgement especially to Dr. Md Mashiur Rahman, Sharmin Aktar and Nayan Chandra Mohanto for their consistent help in statistical analysis, explanation, and presentation as well of their friendly attitude, encouragement and positive criticism.

I am also thankful to the members of Laboratory of Environmental Health Sciences, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh, Shofikul, Atiqur, Shahnur, Afroza, Momotaj, Laboni, Hasib and Tonu. They never hesitated to spare their valuable time and assisted me whenever required.

I would like to express my deepest gratitude to my beloved parents and other family members for their moral support, cooperation and encouragement, which enabled me to achieve this excellent goal.

At the end I would like to express my huge appreciations for my beloved husband and lovely daughter for their all sorts of supports during the entire period of this research.

Author

Sayada Dilruba

June, 2015

Abstract

Major cause of arsenic poisoning is the drinking of water contaminated by arsenic. Recently high levels of arsenic have been found in the several food items in the arsenic-endemic areas suggesting that exposure to arsenic is unavoidable. In this situation, phytobioremediation may be a plausible way to reduce arsenic toxicity. This study was designed to investigate the protective effects of plant materials having antioxidant and free radical scavenging activity against sodium arsenite (Sa)-induced toxic effects through mice model. Mice were divided into eight equal groups: control, *Raphanus sativus* leaves (RSL), *Momordica charantia* fruits (MCF), *Brassica nigra* leaves (BNL), sodium arsenite (Sa), RSL plus Sa, MCF plus Sa and BNL plus Sa. Sa (10 mg/kg body weight/day) was given orally, and plant materials (50 mg/Kg body weight/day) were given as food supplement. Results showed that serum lactate dehydrogenase (LDH) activity was significantly ($p < 0.05$) higher in Sa-treated mice than that in the control group. RSL and MCF supplementation decreased Sa-induced serum LDH activity significantly ($p < 0.05$). Serum butyryl cholinesterase activity (BChE) in Sa-treated mice was significantly ($p < 0.05$) lower than the control group, and food supplementation of RSL but not MCF and BNL could significantly ($p < 0.05$) prevent the reduction of Sa-mediated serum BChE activity. Sa administration increased the serum biomarkers used for liver function test that include alkaline phosphatase (ALP), Aspartate aminotransferase (AST), and Alanine aminotransferase (ALT) activities. RSL was found to reduce the Sa-induced elevation of these enzyme activities in serum significantly ($p < 0.05$). MCF was found to significantly ($p < 0.05$) inhibit only Sa-induced elevation of ALP activity, but not AST and ALT activities. BNL did not show any significant protective effect on Sa-induced elevation of these three enzymes. High density lipoproteins cholesterol (HDL-C), a serum biomarker of cardiovascular risk was found to be significantly lower in Sa-treated mice than that in the control group. RSL but not other two plant materials (MCF and BNL) showed significant ($p < 0.05$) protection against Sa-mediated perturbation of serum HDL-C levels. Finally Sa treatment increased the serum urea levels significantly ($p < 0.05$). RSL could reduce the Sa-induced elevation of serum urea level significantly ($p < 0.05$). However, MCF and BNL could not show any significant protective effects on Sa-induced elevation of serum urea levels. All these results explicitly stated that Sa

treatment caused the perturbation of blood indices in mice associated with hepatic, cardiovascular and renal dysfunctions, and RSL showed protection against Sa-induced perturbation of the blood indices more effectively than the two other plant materials (MCF and BNL). Since RSL showed the highest protection against Sa-induced perturbation of blood indices among the three plant materials tested, efficacies of RSL were tested through molecular approaches. Molecular part of this study targeted on the gene expression of heat shock proteins (HSPs). HSPs are stress sensitive molecular chaperon that can be expressed by heat, oxidative stress, heavy metals etc. In regular reverse transcription polymerase chain reaction (RT-PCR), it was observed that Sa treatment could increase the expression of several forms of hepatic and renal HSP genes such as HSP90 α , HSP90 β and HSP70. Intriguingly, RSL supplementation inhibited the Sa-induced hepatic expression of HSP90 α , HSP90 β and HSP70 genes. In kidney, RSL reduced the expression of Sa-induced HSP90 β , while it showed almost no protective effect on Sa-induced HSP90 α and HSP70 expression. For the further confirmation of the effects of RSL on Sa-induced expression of HSP genes, real time PCR was performed. Based on the real time PCR data, Sa treatment significantly ($p < 0.05$) enhanced the expression of hepatic HSP90 α , HSP90 β and HSP70. RSL showed significant protective effect on Sa-induced hepatic expression of HSP genes. In kidney, Sa treatment significantly ($p < 0.05$) increased the expression of HSP90 α and HSP90 β genes. Although Sa treatment up-regulated the expression of HSP70 gene compared to the control, this up-regulation was not statistically significant. RSL showed to have a general trend in the inhibition of Sa-induced renal expression of all three HSP genes; however, RSL-mediated inhibition was significant ($p < 0.05$) only in the Sa-induced up-regulation of HSP90 α gene, but not in HSP90 β and HSP70 genes. All these results indicated that RSL could be useful to reduce or prevent arsenic toxicity in human in future.

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ACRONYMS, ABBREVIATIONS AND SYMBOLS

ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AMP	2-amino-2-methyl-1 propanol
As	Arsenic
AST	Aspartate Transaminase
ATSDR	<i>Agency for Toxic Substances and Disease Registry</i>
BChE	Butyryl Cholinesterase
BFD	Black Foot Disease
BGS	British Geological Survey
big ET-1	big Endothelin-1
BNL	<i>Brassica nigra</i> leaves
CAT	Chronic Arsenic Toxicity
CCA	Copper Chrome Arsenate
cDNA	Complementary DNA
CRP	C-reactive protein
CVD	Cardiovascular Disease
DMA	Dimethylarsinic Acid
DNA	Deoxyribonucleic Acid
DTNB	Dithiobis Nitrobenzoate
EPA	Environmental Protection Agency
GDWQ	Guidelines for Drinking-Water Quality
HDL-C	High Density Lipoprotein-Cholesterol
HSDB	Hazardous Substance Data Bank
HSP	Heat Shock Protein
IARC	International Agency for Research on Cancer
ICAM-1	Intercellular Adhesion Molecule-1
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
LDH	Lactate Dehydrogenase
MCF	<i>Momordica charantia</i> fruit

MCL	Maximum Contamination Level
MCLG	Maximum Contaminant Level Goal
MCP	Membrane Cofactor Protein
MCP-1	Monocyte Chemotractant Protein-1
mg	Milligram
ml	Milliliter
MMA	Monomethylarsonic Acid
MRL	Minimum Risk Levels
NADH	Nicotinamide Adenine Dinucleotide
ng	Nanogram
NRC	National Research Council
Ox-LDL	Oxidized-low Density Lipoprotein
PML	Promyelocytic Leukemia
ppb	Parts Per Billions
ppm	Parts Per Millions
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSL	<i>Raphanus sativus</i> leaves
Sa	Sodium Arsenite
SD	Standard Deviation
SE	Standard Error
SPSS	Statistical Package for the Social Sciences
UNICEF	United Nations International Children's Emergency Fund

US EPA	United Nations Environmental Protection Agency
USA	United States of America
UV	Ultraviolet
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
µg	Microgram
8-hydroxy guanine	8-OH-G

Dedicated
to
my parents

CHAPTER I
General Introduction
(Literature Review)

General Introduction (Literature Review)

1.1 Background

Arsenic is a potent environmental pollutant that has caused an environmental tragedy in some parts in the world especially in Bangladesh where millions of people have been affected because of the drinking of water contaminated by arsenic. The general population is exposed to inorganic and organic arsenic through drinking water, air, food, occupation and other environmental sources (Mandal and Suzuki, 2002; Nordstrom, 2002; Roy and Saha, 2002; Tchounwou et al., 1999). Arsenic is found in food, frequently as organic forms (arsenobetaine, arsenosugars etc.), which are less toxic than inorganic arsenic (Duxbury et al., 2003; Meharg and Rahman, 2003; Sun et al., 2008). Inorganic form of arsenic is most damaging which is found in contaminated drinking water in many regions of the world (IARC, 2004; Zheng et al., 2004). It has been estimated that 200 million people worldwide are at risk from health effects associated with high concentrations of arsenic in their drinking water, a number which is expected to further increase due to the recent lowering of limits of arsenic concentration in drinking water to 10 $\mu\text{g/L}$ (NRC, 2001; Ravenscroft et al., 2009). This value has already been adopted by many countries, and some authorities are even considering decreasing this value further. As a consequence of the biggest arsenic calamity has emerged in several parts of the world, the situation of arsenic toxicity is alarming and severe health problems are reported amongst the inhabitants relying on ground water as sources of drinking water. Every year, new areas with arsenic occurrences in ground water exceeding the maximum contamination levels set by the World Health Organization (WHO, 10 $\mu\text{g/L}$) and other national and international regulatory organizations are identified. The use of ground water for irrigation and the bioavailability of arsenic to food crops and the uptake by humans and livestock through the food chain have opened additional pathways for arsenic exposure all over the world. The magnitude of the arsenic catastrophe has projected to be the largest in history of environmental disaster that will be more serious than those at Chernobyl, Ukraine in 1986 and Bhopal, India in 1984 (Khan et al., 2003; Smith et al., 2000; Watanabe et al., 2001). Long-term or chronic exposure to arsenic is related to severe adverse health effects including dermatitis, cardiovascular diseases, diabetes mellitus, chronic bronchitis, immune disorders, peripheral neuropathy, liver damage,

renal failure, adverse reproductive outcomes, hematological effects, and other ailments (Ali et al., 2010; Argos et al., 2010; Chen et al., 2007; Mazumder et al., 1998, 2000, Mazumder, 2005; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche, 2006; Vahidnia et al., 2008; Wang et al., 2002). Infact, arsenic affects almost all vital organs of human body causing the damage or dysfunction. Along with the adverse health effects, arsenic toxicity has also created social and economic problems for the population of the endemic countries.

1.2 Physico-chemical properties of arsenic

Arsenic is a chemical element with symbol As and atomic number 33. It is found to exist in many minerals, usually in conjunction with oxygen, chlorine, sulphur and metals, and also as a pure elemental crystal with an atomic weight of 74.92. Arsenic is a metalloid as it has properties of both metals and non-metals. It can exist as powder, amorphous or vitreous forms. Elemental arsenic has a specific gravity of 5.73 sublimates at 615°C and has a very low vapour pressure of 1 mm Hg at 373°C. Many of the inorganic arsenic compounds occur as white, odorless solids with specific gravities ranging from about 1.9 to more than 5. Arsenic trioxide, the most common arsenic compound in commerce, melts at 312°C and boils at 465°C. Elemental arsenic does not dissolve in water; however some salts of arsenic dissolve in water. Further arsenic trioxide, arsenic pentoxide and other arsenical compounds are soluble, depending on the pH and the ionic environment of the solution. When heated to decompose, arsenic compounds emit toxic arsenic fumes (HSDB, 2003). Arsenic can exist in four valence states: -3, 0, +3 and +5. Under reducing conditions, the +3 valence state as arsenite is the dominant form; the +5 valence state as arsenate is generally the more stable form in oxygenized environments (NRC, 1999). Inorganic arsenite and arsenate are the major arsenic species in natural water. Arsenic does react with hot acids to form arsenous acid (H_3AsO_3) or arsenic acid (H_3AsO_4).

Table 1.1 Chemical nature of arsenic

Atomic number	33
Atomic mass	74.92 g/mol
Electronegativity (according to Pauling)	2.0
Density	5.7 g/cm ³ at 14°C
Melting point	814 °C (36 atm)
Boiling point	615 °C (sublimation)
Atomic radius	0.139 nm
Oxidation states	-3, +3, +5
Key isotope	⁷⁵ As
Electronic shell	[Ar] 3d ¹⁰ 4s ² 4p ³
Energy of first ionization	947 kJ/mol
Energy of second ionization	1798 kJ/mol
Energy of third ionization	2736 kJ/mol
Standard potential	- 0.3 V (As ³⁺ / As)

Source: [Lenntech, Netherlands: Alumni from the Technical University of Delft.

Available at: <http://www.lenntech.com/periodic/elements/as.htm>]

1.3 Sources of arsenic

Arsenic is ubiquitously present in food, soil, water and air, and it is released into the environment from both natural and man-made sources. Globally, natural emissions of arsenical compounds have been estimated at about 8,000 tons each year whereas anthropogenic emissions are about 3 times higher (NRC, 1999, 2001). Arsenic is found to exist in several forms in different foods and environmental media such as soil, air, and water. Inorganic arsenic is the predominant form in drinking water, which is both highly toxic and readily bio-available (NRC, 1999, 2001). Arsenicals have been used in various purposes such as medicines, electronics, agriculture, military and metallurgy (Nriagu and Azcue, 1990). Arsenic is also found to exist in the combination with other elements such as sulphur, oxygen and different metals.

1.3.1 Natural sources of arsenic

Arsenic is widely dispersed element in the Earth's crust and found to exist at an average concentration of approximately 5 mg/kg (Garellick et al., 2008). It occurs in trace quantities in all rock, soil, water and air. More than 200 mineral species contain arsenic, the most common of which is arsenopyrite. About one-third of the atmospheric flux of arsenic is of natural origin. Volcanic action is the most important natural source of arsenic followed by arsenic-containing vapour that is generated from solid or liquid forms of arsenic salts at low temperatures. Arsenic is usually concentrated in sulphide-bearing mineral deposits especially those associated with gold mineralization and has a strong affinity for pyrite, one of the more ubiquitous minerals in the earth's crust. It is also concentrated in hydrous iron oxides. Weathering of rocks converts arsenic sulphides to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, river or groundwater. Arsenic can also enter into the food chain, causing widespread distribution throughout the plant and animal kingdoms. Elevated concentrations of inorganic arsenic (>1 mg/L) in ground water of geochemical origins have also been found in Taiwan, India and in major parts of Bangladesh (Biswas et al., 1998; Chen et al., 1985; Das et al., 1996; Dhar et al., 1997; Mandal et al., 1996). Organic arsenic compounds such as arsenobetaine, arsenocholine, arsenosugars, tetramethylarsonium salts, and arsenic-containing lipids are mainly found in marine organisms although some of these compounds have also been found in terrestrial species (Francesconi and Edmonds, 1997; Grotti et al., 2008).

1.3.2 Man-made sources of arsenic

Commercially elemental arsenic is produced from arsenic trioxide (As_2O_3) by the reduction with charcoal. Arsenic trioxide is a by-product of metal smelting operations. About 70% of the world production of arsenic is used in the timber treatment as copper chrome arsenate (CCA), 22% in agricultural chemicals and the remainder in glass, pharmaceuticals and metallic alloys (WHO, 2001). Mining, non-ferrous metals smelting and burning of fossil fuels are the major industrial processes that contribute to arsenic contamination of air, water and soil. Historically, use of arsenic-containing pesticides has left large areas of agricultural land contaminated. Since arsenic is used in the preservation of timber, it also leads to the contamination of the environment. In addition, the use of arsenic-contaminated ground water for irrigation leads to

widespread contamination of land and additional exposure to human and livestock via food all over the world (Kile et al., 2007; Lindberg et al., 2006; Meharg and Rahman, 2003).

1.4 Transformation and mobilization of arsenic in the environment: the arsenic cycle

The principal natural reservoirs of arsenic are rocks where arsenic remains as arsenopyrite (FeAsS). Release and mobilization of arsenic from these sources constitute the availability of this element in soil, water and air in various forms. Under normal ecological conditions, soils may contain arsenic levels between 0.1 and 40 ppm, if the underlying bedrock is not disturbed or redistributed by natural or pedogenic processes (Yan-Chu, 1994). Chemical reactions (i.e. oxidation reduction and methylation) in the soil–water and sediment–rock systems influence the environmental transport, distribution and availability of arsenic. Slow release of arsenic from rocks and sediments or oxidative dissolution of arsenopyrite (FeAsS) from sediments contributes flux of arsenic in the environment. Oxygen availability controls the arsenate–arsenite redox reactions. Adsorption and precipitation of arsenate and arsenite immobilize the soluble arsenic. Methylation of arsenite to monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA) followed by other organoarsenic compounds, constitute the major biological reactions in the arsenic cycle (Figure 1.1) (Bhumbla and Keefer, 1994; Carter and Fairlamb, 1993; Ferguson and Gavis, 1972; Knowles and Benson, 1983; Yan-Chu, 1994)

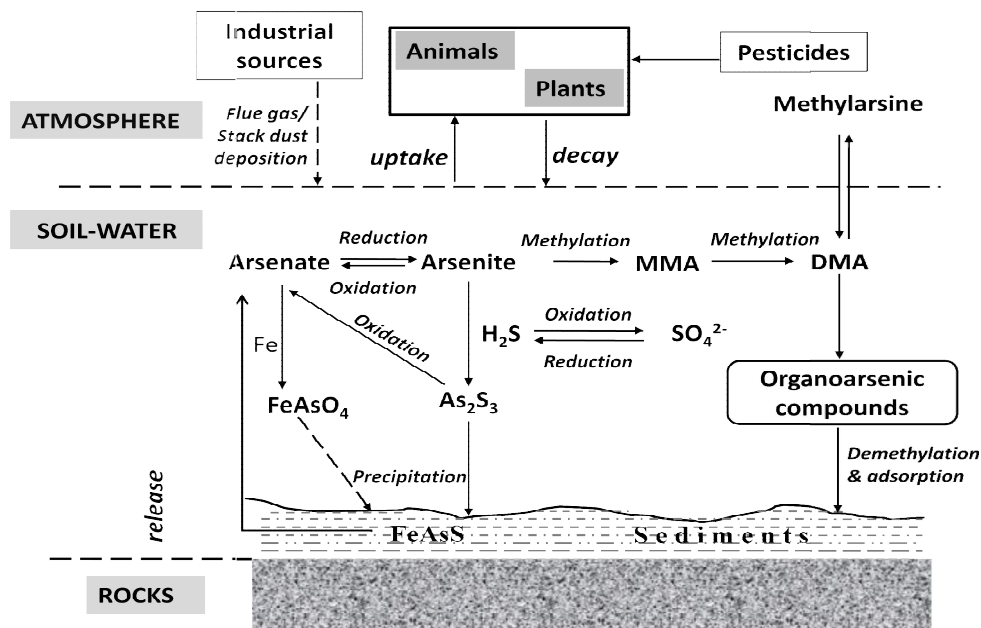


Figure 1.1: Transformation and mobilization of arsenic in the environment

Source: [Roy and Saha, 2002]

1.5 Exposure to arsenic

Human exposure to inorganic arsenic occurs mainly through the consumption of ground water containing high levels of inorganic arsenic, food prepared with this water and food crops irrigated with high-arsenic water sources (WHO, 2010). Occupational exposure to arsenic occurs in the copper or lead smelting, wood treating, or pesticide application. Workers involved in the production or application of arsenic containing pesticides may also be exposed to higher levels of arsenic. Since arsenic is a natural part of our environment, environmental exposure to arsenic is unavoidable. Various routes of human exposure to arsenic have shown in Figure 1.2. A person can come in contact with arsenic in any of the following ways-

1.5.1 General exposure

Consumption of arsenic through drinking water is the main cause of human exposure to arsenic worldwide. However, high levels of arsenic have been found in the food items collected from the highly arsenic-contaminated areas in the world, which have been recognized as another potential source of human exposure to arsenic (Huq et al., 2006).

1.5.2 Exposure from drinking water

Drinking water is an important source of exposure to the inorganic arsenic for the people living in areas where arsenic is naturally high in drinking water. In fact, drinking water accounts for most human arsenic exposures worldwide. Arsenic may enter lakes, rivers or underground water naturally, when mineral deposits or rocks containing arsenic dissolve. Arsenic may also get into water through the discharge of industrial wastes and by the deposit of arsenic particles in dust, or dissolved in rain or snow. Arsenic contamination of ground water became a high-profile problem in recent years due to the use of underground water (tube well water) for drinking purposes, causing serious arsenic poisoning to a large number of people in the world especially in Bangladesh and West Bengal of India (Biswas et al., 1998; Chen et al., 1985; Das et al., 1996; Dhar et al., 1997).

1.5.3 Exposure from food

Recently exposure to arsenic through food has created an attention as high concentrations of arsenic have been found in the different kind of vegetables, dairy products, meats, grains and other food materials (Al Rmalli et al., 2005; Grotti et al., 2008; Meharg and Rahman, 2003). Previously it was believed that food in arsenic was not so much harmful since food contains organic forms of arsenic that is relatively less toxic to human. However, high levels of inorganic arsenic are found in the foods or agricultural products that have been cultivated by arsenic contaminated ground water (Rahman and Hasegawa 2011; Smith et al., 2006). Recently high concentration of arsenic was found in the rice of Bangladesh (Al Rmalli et al., 2005; Duxbury et al., 2011; Smith et al., 2006; Williams et al., 2006). Rice is a staple food in many countries including Bangladesh. Several studies have confirmed that contaminated ground water used to cultivate vegetables and rice consumed by people may be an important pathway of ingesting arsenic (Chakraborti et al., 2003). Al Rmalli et al. (2005) have investigated arsenic levels in food imported from Bangladesh to the United Kingdom. Results of this study has suggested that imported vegetables from Bangladesh have 2-100 fold higher concentrations of arsenic than vegetables cultivated in the United Kingdom, European Union, and North America. Further, Huq et al. (2006) conducted a study in Bangladesh with 2,500 water, soil and vegetable

samples from arsenic-endemic and non-endemic areas and found that some commonly-grown vegetables, which would usually be suitable as good sources of nourishment, accumulate substantially-elevated amounts of arsenic. Although it has been established that arsenic enters the food chain but there is great uncertainty about the bioavailability and associated toxicity of arsenic from different foods.

1.5.4 Occupational exposure

Exposure to arsenic occurs occupationally in several industries, including mining, wood preservation, pesticide, pharmaceutical, glass, ceramic and microelectronics (IARC, 1980; NRC, 1999). Exposure to arsenic occurs via the oral route (ingestion), inhalation, dermal contact, and the parenteral route to some extent. Inhalation is the principal route of arsenic exposure in occupational settings. Workers who produce or use arsenic compounds in such occupations as vineyards, ceramics, glass-making, smelting, pharmaceuticals, refining of metallic ores, pesticide manufacturing and application, herbicides, fungicides, algaecides, sheep dips, wood preservation, or semiconductor manufacturing may be exposed to substantially higher levels of arsenic (Jones, 2007; Tchounwou et al., 1999). Arsenic is well absorbed by oral and inhalation routes, primarily metabolized in liver and excreted through urine within a few days after consuming any form of inorganic arsenic.

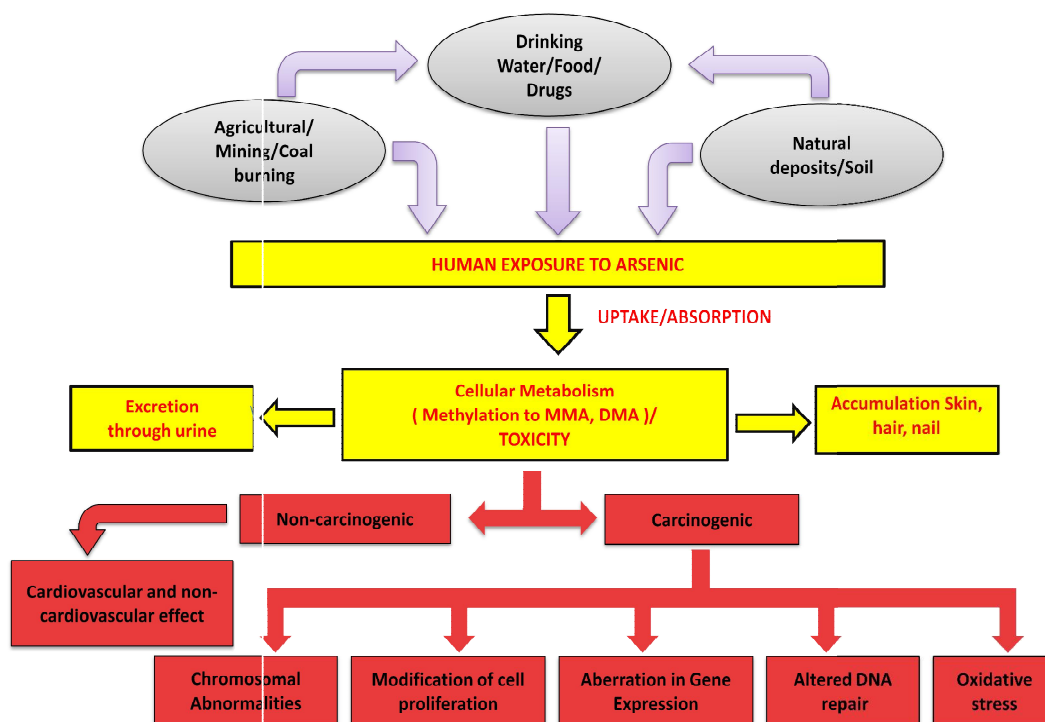


Figure 1.2: Arsenic exposure, metabolism and toxicity in human

Sources: [Roy and Saha, 2002]

1.6 Metabolism of arsenic

The liver is the primary target organ for the metabolism of arsenic compound. The absorption of arsenic into the blood stream occurs at the cellular level and is taken up by red blood cells, white blood cells and other cells that can reduce arsenate to arsenite (Winski and Carter, 1995). Before methylation arsenate is reduced to arsenite form (Miller et al., 2002; Vahter and Marafante, 1983; Vahter, 2002). The primary metabolic step of inorganic arsenic in human is its methylation in the liver (Figure 1.3). The methylation of arsenic has been demonstrated by the presence of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the urine and bile (Cui et al., 2004; Li et al., 2008). Inorganic arsenic i.e. arsenic (III) is methylated in liver by arsenic methyltransferase (AS3MT) to generate monomethylarsonic acid (MMA) which is reduced to monomethylarsonous acid (MMA) and then further methylated to dimethylarsinic acid (DMA), followed by reduction to dimethylarsinous acid. In both step of methylation, S-adenosyl methionine provides methyl group (Figure 1.3). These metabolites are more readily excreted through urine. Some other less important routes of elimination of arsenic include feces, skin, sweat, hair and

nails. Humans excrete a mixture of inorganic, monomethylated and dimethylated forms of arsenic. The pentavalent metabolites MMA (V) and DMA (V) are less toxic than arsenite or arsenate (Marafante and Vahter, 1987; Vater and Concha, 2001). Inorganic arsenic and its methylated metabolites are mostly excreted through urine within 2–4 days. Approximately 50% of excreted arsenic in human urine is dimethylated and 25% is monomethylated, rest of them is inorganic (Buchet et al., 1981). Some studies related to arsenic metabolism have also suggested that methylation of inorganic arsenic may be a toxification, rather than a detoxification pathway since the trivalent methylated arsenic metabolites, particularly monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA III) are unusually capable of interacting with cellular targets such as proteins and DNA (Goering et al., 1999; Kitchin, 2001). Methylation capacity in human appears to decrease at high arsenic doses. The types and patterns of methylated arsenic species in urine are similar between siblings and parents that indicates that arsenic methylation is genetically linked (Chung et al., 2002). When the methylating capacity of the liver is exceeded, exposure to excess levels of inorganic arsenic results in increased retention of arsenic in soft tissues.

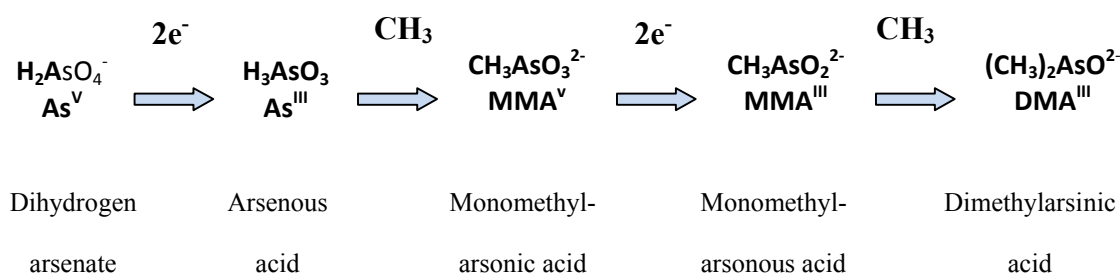


Figure 1.3: Arsenic methylation

Source: [Vahter and Concha, 2001]

1.7 Arsenic in different regions of the world

Arsenic toxicity is a global health problem affecting many millions of people. Over the past two or three decades, occurrence of high concentrations of arsenic in drinking-water has been recognized as a major public-health concern in several parts of the world. With the discovery of newer sites in the recent past, the arsenic-contamination scenario around the world, especially in Asian countries, has changed

considerably (Mukherjee et al., 2006). Ground water in different regions of the world is contaminated with arsenic and there are a number of regions including both developing and developed countries where arsenic contamination of drinking water is significant. As a result, arsenic toxicity has created a major public health concern throughout the world. A major and possibly the dominant source of arsenic exposure to human and livestock are drinking of arsenic-contaminated ground water. Millions of people are exposed to elevated levels of toxic inorganic arsenic through the drinking of contaminated ground water and food (Meharg et al., 2008; Ng et al., 2003; Smith et al., 2000). Most severely affected countries that include Bangladesh, China, Nepal, India, Myanmar, Pakistan, Cambodia, Vietnam, Argentina, Australia, Canada, Greece, Chile, Hungary, Japan, Mexico, Mongolia, New Zealand, South Africa, Philippines, Taiwan, Thailand, USA, etc. where the main source of human exposure is the mass use of arsenic-contaminated ground water for drinking and irrigation for agricultural production (Berg et al., 2007; IARC, 2004; Nicolli et al., 1989; Nordstrom, 2002; Razo et al., 1990; Tseng, 1999).

In Asia, arsenic menace is more devastating in some countries specially the arsenic poisoning in alluvial deltas of Ganges River in India and Bangladesh (Dhar et al., 1997; Mandal et al., 1996; Rahman et al., 2001). Sun, (2004) reported that the groundwater of the large areas of China and Inner Mongolia has also been contaminated with arsenic resulting in the toxicity of millions of people. Residents in the large alluvial deltas of the Mekong River in southern Vietnam and Cambodia and the Red River in northern Vietnam have been exposed to arsenic mainly through drinking water reported by Berg et al. (2007). Recently, ground water contamination by arsenic has also been reported in Iran (Mosaferi et al., 2008).

In Europe, the arsenic problem is most alarming in Hungary, Serbia and Croatia where in Hungary an inventory of ground water quality conducted which demonstrated that arsenic concentrations in drinking water for 400 towns and villages in the Great Hungarian Plain are several times higher than the guidelines of WHO and European Union (EU) (Csalagovits, 1999; Gurzau and Gurzau, 2001; Sancha and Castro, 2001).

In the American region, Mexico, United States, Chile and Argentina are the most arsenic affected areas where in some wells in Latin America including Bolivia and Peru, extremely high concentrations of arsenic were found (Nicolli et al., 1989; Razo et al., 1990; Welch et al., 1988). Different regions of the world having arsenic contaminated ground water are shown in Figure 1.4

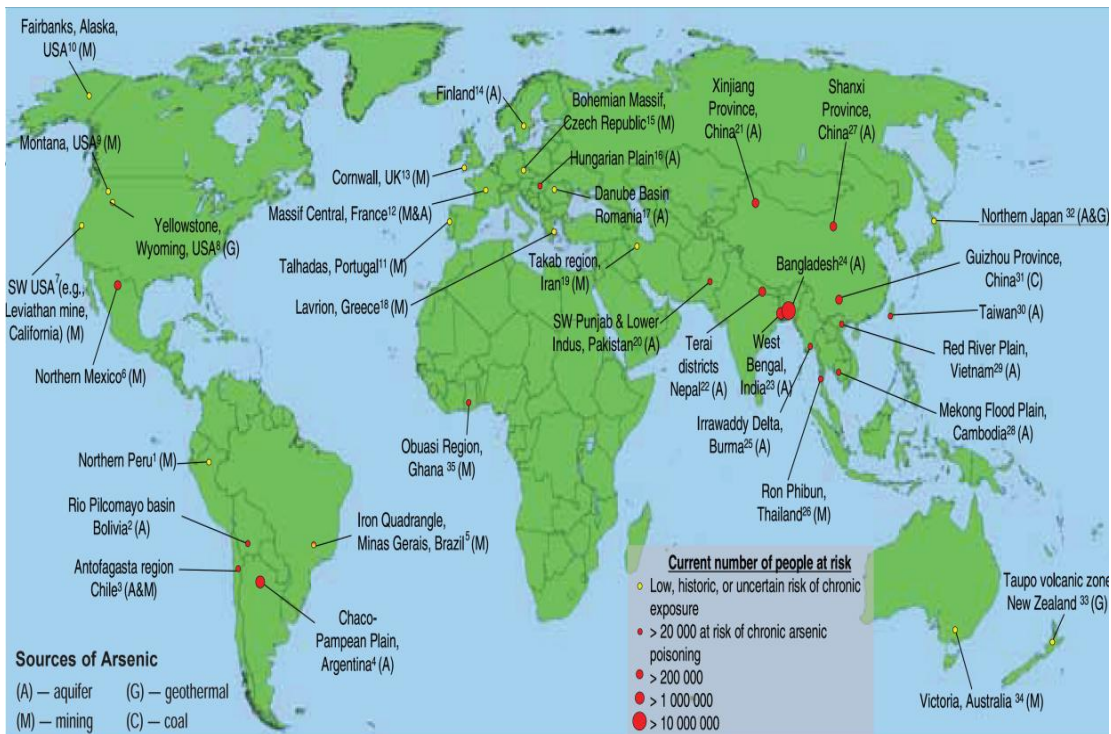


Figure 1.4: Worldwide distributions of arsenic contaminated regions, showing source of arsenic and numbers of people at risk of chronic exposure

Source: [Garelick and Jones, 2008]

1.8 Arsenic contamination in Bangladesh

Bangladesh is a small and densely populated country with an area of 1, 47,570 square kilometers having a population of around 160 millions. This is a common phenomenon here to cope with various natural disasters such as floods, cyclones, tidal bores, and droughts etc. in every year. There is an abundance of surface and ground water because of its flat deltaic land formed by the mechanical action of the great Himalayan Rivers the Ganges, Brahmaputra and Meghna (Safiullah, 2006). Bangladesh is grappling with the largest mass poisoning of a population in history

because groundwater used for drinking purposes has been contaminated with naturally occurring inorganic arsenic. According to the Bangladesh Bureau of Statistics, among 160 million inhabitants of Bangladesh 77 million people are affected by arsenic contaminated water (Flanagan et al., 2012). World Health Organization described the arsenic crisis in Bangladesh as the largest mass poisoning in the human history (Smith et al., 2000). The scale of this environmental disaster is greater than any seen before; it is beyond the accidents at Bhopal, India, in 1984, and Chernobyl, Ukraine, in 1986.

In 1993, in the Nawabganj district in Bangladesh, naturally-occurring arsenic contaminated water in tube-wells was first confirmed (Khan et al., 1997). As a part of arsenic mitigation programme in Bangladesh, UNICEF tested 4.7 million tube wells for arsenic and 1.4 million of those were found to contain arsenic above the Government drinking water limit of 50 parts per billion (ppb). Combined with another 200,000 unscreened tube wells, which are estimated drinking water to also exceed this limit, it means that almost one in five tube wells is not providing safe drinking water. Nationwide, approximately 20 percent of shallow tube-wells are contaminated. There are more than 8,000 villages where 80 percent of all tube wells are contaminated. Recent report indicated that about 20 million people in Bangladesh are using tube wells with more than 50 ppb of arsenic (UNICEF, 2009).

Historically, surface water sources in Bangladesh have been contaminated with microorganisms, causing a significant burden of disease and mortality. Infants and children suffered from acute gastrointestinal disease resulting from bacterial contamination of stagnant pond water. Consequently, during the 1970s the United Nations Children's Fund (UNICEF) worked with the Department of Public Health Engineering to install tube-wells to provide what was presumably a safe source of drinking-water for the population. At the time the wells were installed, arsenic was not recognized as a problem in water supplies, and therefore standard water testing procedures did not include tests for arsenic (UNICEF, 1999). It was considered as a huge success for concern agencies since the primary goal of hand-pumped tube well was achieved. Unfortunately the situation became complex when in 1993, a substantial proportion of the tube wells yielding with high levels of soluble arsenic contaminated water were found (Smith et al., 2000). After then a series of surveys were conducted in between 1995-1998 which revealed that the groundwater of

southern and north-eastern Bangladesh has been extensively contaminated with arsenic (BGS, 1999; Khan et al., 2003; Smith et al., 2000; Watanabe et al., 2001). In 1998, British Geological Survey (BGS) collected more than 2000 water samples from 41 of the worst-affected districts. This project tested one tube well in every 37 Km² in the two third of the country's most affected areas and found that, 51% of the tube wells were contaminated with at least 0.01 mg arsenic/L, 35% with at least 0.05 mg arsenic/L, 25% with at least 0.1 mg arsenic/L, 8% with 0.3 mg arsenic/L or more and 0.1% with 1.0 mg arsenic/L or more (BGS, 1999). In case of arsenic in drinking water, the current WHO recommended guideline is 10µg/L whereas some developing countries including Bangladesh still have a value of 50µg/L which is five times higher than the WHO guideline (Ng et al., 2003). Already an enormous number of toxicity cases have been reported in the north-west region of Bangladesh and approximately in between 80 to 100 millions of additional people are at risk for arsenic toxicity in the country (Caldwell et al., 2003; Chowdhury, 2004). The situation is deteriorating as the new cases of toxicity are still being reported in different parts of the country. There are 61 out of 64 districts (administrative blocks) has been affected by arsenic in Bangladesh (Khan et al., 2006). The entire districts in Bangladesh having arsenic contaminated drinking water exceeding WHO guidelines are shown in Figure 1.5

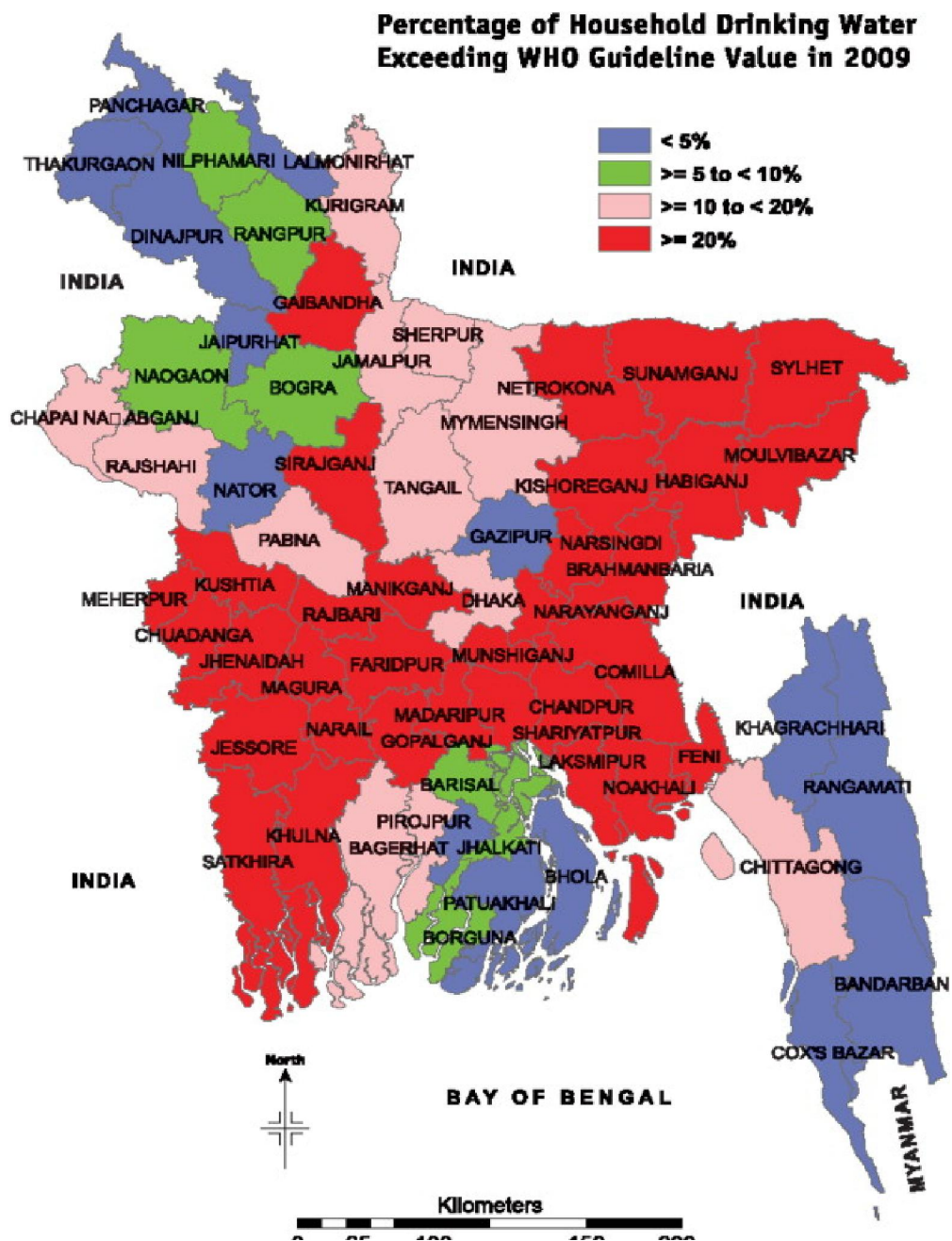


Figure 1.5: Arsenic polluted areas in Bangladesh.

Source: [Executive Summary World Water Day 22 March, 2010
http://www.unicef.org/bangladesh/Towards_an_arsenic_safe_enviro_n_summary%28english%29_22Mar2010.pdf]

1.9 Causes of ground water arsenic contamination in Bangladesh

There is a great temporal and spatial variation of ground water arsenic levels in different regions of Bangladesh. The precise reasons for the high level of arsenic in ground water in Bangladesh is controversial issue and that have not yet been clearly established but several theories have been proposed including role of microbial mobilization, anthropogenic activities, etc. (Harvey et al., 2002; Hossain et al., 2011; Islam et al., 2004; Polizzotto et al., 2006; Sutton et al., 2009). It is now widely believed that the elevated level of arsenic in the ground water in Bangladesh have a natural geological source which may be due to the abstraction of water from quaternary confined and semi-confined alluvial or deltaic aquifers.

There are a huge number of diverse chemical and biological reactions such oxidation, reduction, adsorption, precipitation, methylation and volatilization participate actively in the cycling of arsenicals in the groundwater. A geochemical survey was conducted in six districts of west Bengal bordering the western part of Bangladesh by Das et al. (1996) and the results indicates that the source of arsenic in ground water and soil is the arsenopyrite mineral. However, it is not understood yet clearly about how arsenic is released in ground water from arsenopyrite. In spite of being insoluble in water, pyrite decomposes when exposed to air or aerated water. A feasible explanation for this would be the changes of geochemical environment due to high withdrawal of groundwater resulting decomposition of pyrites to ferrous sulfate, ferric sulfate, and sulfuric acid, thus arsenic in pyrites becomes available (Bridge and Husain, 1999; Das et al., 1996; Welch et al., 1988). Due to massive withdrawal of underground water in the last three decades, it may cause the decomposition of pyrites to oxides of iron, arsenic and sulphuric acid which are soluble in water containing sulphuric acid. Under reducing condition below the water table and in the presence of organic matter, non toxic oxides of arsenic are reduced to toxic oxide forms. In 1999, British Geological survey reported that the 'pyrite oxidation' hypothesis proposed by scientists from West Bengal is not a major process for arsenic recruitment in ground water and also stated that 'oxyhydroxide reduction' hypothesis proposed by Nickson et al. (1998) is probably the main cause of arsenic mobilization in groundwater. According to this hypothesis, the origin of arsenic rich ground water is due to a natural process, and it seems that the arsenic in ground water has remained for thousands of years without

being flushed from the delta. Arsenic is assumed to be present in alluvial sediments with high concentrations in sand grains as a coating of iron hydroxide. The sediments deposited in valleys eroded in the delta when the stream base level was lowered due to the drop in sea level during the last glacial advance. The organic matter deposited with the sediments reduces the arsenic bearing iron hydroxide and releases arsenic into groundwater. Organic matter deposited in the sediments reduce the arsenic adsorbed on the oxy-hydroxides and releases arsenic into the ground water and dissolution occurs during recharge, caused by microbial oxidation of the organic matter as bacteria dissolves surrounding oxygen.

1.10 Social stigma of arsenic toxicity in Bangladesh

In Bangladesh, people with arsenic poisoning suffer enormous social stigma. Many people believe, arsenic poisoning is infectious or a curse. Usually parents are reluctant to let their children play with other children suffering from arsenic poisoning and patients of arsenic poisoning can be shunned within their villages. In case of women, the situation is more serious. Usually in Bangladesh, a women's magnetism lies in her beauty which is judged by her pale complexion. Unfortunately, this makes it harder and in some cases impossible to get marry, for single woman who is suffering from arsenic-induced melanosis and keratosis of the skin. Even once married, after then women have to face the risk of divorce if they develop arsenic exposure-related skin diseases. In fact, this is a big social problem in male-dominated society in Bangladesh (UNICEF, 2009).

1.11 Standards and regulations for arsenic exposure through drinking water

According to the World Health Organization (WHO), arsenic is one of 10 chemicals of major public health concern. The toxic effects of arsenic depend on the nature and extent of exposure, particularly the frequency of exposure, duration of exposure and type of arsenic present. Efforts of WHO to reduce arsenic exposure includes setting guideline values, reviewing evidence and providing risk management recommendations. WHO publishes a guideline value for arsenic in its Guidelines for Drinking-Water Quality (GDWQ). The intention of the guidelines is to be used as the basis for regulation and standard setting worldwide, for the development of national

standards that, if properly implemented, will ensure the safety of drinking water supplies through the elimination, or reduction to a minimum concentration, of constituents in drinking water that are known to be hazardous to public health. The current recommended limit of arsenic in drinking water is 10 µg/L although this guideline value is designated as provisional due to the measurement difficulties and practical difficulties in removing arsenic from drinking-water. This is based on a 6×10^{-4} excess skin cancer risk, which is 60 times higher than the factor normally used to protect human health. However, the WHO states that the health-based drinking water guideline for arsenic should in reality be 0.17 µg/L (Kapaj et al., 2006).

The US EPA drinking water standard for arsenic was set at 50 µg/L in 1975, based on a Public Health Service standard originally established in 1942 (US EPA) Recently, the US EPA has established a health based, non-enforceable Maximum Contaminant Level Goal (MCLG) of zero arsenic and an enforceable Maximum Contaminant Level (MCL) of 10 µg As/L in drinking water (US EPA). However, the current drinking water guideline for arsenic adopted by both the WHO and the US EPA is 10 µg/L. This is higher than the proposed Canadian and Australian maximum permissible concentrations of 5 and 7 µg As/L, respectively (Kapaj et al., 2006).

1.12 Minimal Risk Levels (MRLs) for arsenic

An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse non-cancer health effects over a specified duration of exposure. They are below levels that might cause adverse health effects in the people most sensitive to such chemical-induced effects. MRLs are derived for acute (1-14 days), intermediate (15-364 days), and chronic (365 days and longer) durations and for the oral and inhalation routes of exposure (<http://www.opentoxipedia.org/>). Health effects of arsenic are determined by the dose, the duration (how long), and the route of exposure. According to Agency for Toxic Substances and Disease Registry (ATSDR) acute-, intermediate- or chronic-duration inhalation MRLs were not derived for any inorganic arsenic or organic arsenic compounds. For inorganic arsenic an MRL of 0.005 mg arsenic/kg/day has been derived for acute-duration oral exposure (≤ 14 days) but there is no intermediate-duration oral MRL was derived for inorganic arsenic. For chronic-duration oral

exposure (≥ 1 year) to inorganic arsenic an MRL of 0.0003 mg arsenic/kg/day has been derived. There is no acute-duration oral MRL derived for monomethylarsonic acid (MMA). An MRL of 0.1 mg MMA/kg/day has been derived for intermediate-duration oral exposure (15-364 days) to MMA and for chronic-duration oral exposure (≥ 1 year) the MRL is 0.01 mg MMA/kg/day. For DMA there is no acute- or intermediate duration oral MRLs but an MRL of 0.02 mg DMA/kg/day has been derived for chronic-duration oral exposure (≥ 1 year) to DMA. The normal human levels of arsenic in unexposed individuals are < 1 $\mu\text{g/L}$ in blood, < 100 $\mu\text{g/L}$ in urine, ≤ 1 ppm in nails and ≤ 1 ppm in hair (ATSDR, 2007).

1.13 Health effects of arsenic

Worldwide chronic arsenic toxicity has become a major threat to human health. Arsenic exposure to humans mainly occurs from the ingestion of arsenic contaminated water and food. Chronic exposure to arsenic has more effects on health than any other toxicant, and the list continues to grow. Arsenic poisoning takes between 8 and 14 years to its effect on health, depending on the amount of arsenic ingested, nutritional status, and immune response of the affected individual (Ahsan et al., 2006; Maharjan et al., 2007; Milton et al., 2004; Watanabe et al., 2001). Several studies have clearly indicated that the toxicity of arsenic depends on the exposure dose, frequency and duration, age, and sex, as well as on individual susceptibilities, genetic and nutritional factors (Ahsan et al., 2007). Toxicity of arsenic has been heightened by recent reports of large populations in Bangladesh, West Bengal, Inner Mongolia, Taiwan, China, Mexico, Argentina, Chile and Hungary that have been exposed to high concentrations of arsenic in their drinking water (Argos et al., 2010; Chen et al., 1988a, 1988b; Mandal and Suzuki, 2002; Meliker et al., 2007; Mukherjee et al., 2006).

Human health effects of chronic arsenic toxicity (CAT) are designated by the term arsenicosis which was first coined by Mazumder et al. (1988) group and later used by WHO to imply a chronic disease caused by prolonged exposure in humans to arsenic. Previously the condition were described as arseniasis, arsenism, arsenicism, etc (Mazumder, 2008). Many different systems or organs within the body are affected by chronic exposure to inorganic arsenic, particularly because of its potential to be a

human carcinogen. The clinical manifestations of arsenic toxicity are many, but the most commonly observed symptoms in people who suffer from chronic arsenic poisoning are the characteristic skin lesions. The main dermatological symptoms observed in arsenic affected people are melanosis (change of pigmentation) and keratosis (rough, dry, papular skinlesions). Chronic arsenic exposure may also cause reproductive, neurological, cardiovascular, respiratory, hepatic, hematological, and diabetic effects in humans (NRC, 1999). Intake of inorganic arsenic was recognized as a cause of skin, bladder, and lung cancer (Cantor, 2001; IARC, 2004). A good number of articles have been published on chronic arsenic exposure and its associated health effects (Chen et al., 2007; Hossain et al., 2012; Huda et al., 2014; Islam et al., 2011; Kapaj et al., 2006; Mandal and Suzuki, 2002; Morton and Dunnette, 1994; Naqvi et al., 1994; Ng et al., 2003; Wang et al., 2007; Yoshida et al., 2004).

1.13.1 Skin manifestations

Skin lesions are a classical sign of chronic arsenic poisoning. Chronic exposure to arsenic by either ingestion or inhalation produce a variety of skin symptoms including diffused and spotted melanosis, leucomelanosis, keratosis, hyperkeratosis, dorsum, Bowen's disease, and cancer. Skin disorders are well documented in several epidemiological studies conducted in different parts of the world in which the population are exposed to arsenic through drinking water (Ahsan et al., 2006; Chakraborti et al., 2003; Khan et al., 2003; Mazumder et al., 1998; Rahman et al., 2005). Recently, Yoshida et al. (2004) reported dose-response relationship between arsenic levels in drinking water and risk of skin lesions. Melanosis and keratosis are found at the primary stage of arsenic-induced all dermatological manifestations, leuko-melanosis and hyperkeratosis in the second stage and ultimately may turn to skin cancer such as Bowen's disease, basal cell and squamous cell carcinoma (Khan et al., 2003; Milton et al., 2003; Yoshida et al., 2004). Hyperpigmentation may occur, particularly in body areas where the skin tends to be a little darker (Shannon and Strayer, 1989). Photograph of some skin lesions are given below (Figure 1.6)



Figure 1.6: Some phenomenon caused by chronic exposure to arsenic

Source: [Environmental Health Sciences group, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh]

1.13.2 Carcinogenic effects

The evidence of carcinogenicity in humans from exposure to arsenic is based on epidemiological studies of cancer in relation to arsenic in drinking water. The Working group of International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency (EPA) evaluated data from ecological studies, cohort studies and case-control studies from many countries and observed that arsenic was potentially carcinogenic for skin cancer (IARC, 2004; Smith et al., 1992). The carcinogenic potential of arsenic was recognized over 110 years ago by Hutchison, who observed an unusual number of skin cancer occurring in patients treated for various diseases with medical arsenicals (Klassen, 2008).

Different epidemiological studies demonstrated an evident causal relationship between environmental, occupational, and medical exposure of millions of people worldwide to inorganic arsenic and increased risks of cancer of the skin, lungs, urinary bladder, kidney, prostate, liver and other sites (Chen et al., 1992; IARC, 2004; Smith et al., 1998; Wu et al., 1989; Yu et al., 2000). It is thought that the mechanism by which these cancers originate may involve the promotion of oxidative stress by arsenic compounds, in which the antioxidant capacity of the living organism is overwhelmed by ROS (reactive oxygen species), resulting in molecular damage to proteins, lipids and most significantly DNA (Cohen et al., 2006; Lynn et al., 2000; Shi et al., 2004; Valko et al., 2005).

In exposed human populations, arsenic has been primarily associated with tumors of the skin and lungs, but also can be associated with tumors of the bladder, kidney, and liver. A large number of epidemiological studies have reported that inhalation exposure to inorganic arsenic increases the risk of lung cancer (Enterline et al., 1995; Järup et al., 1989; Rossman et al., 2004; Smith et al., 1998) pointed out that arsenic can play a role in the enhancement of UV-induced skin cancers. The mechanism of action may involve effects on DNA methylation and DNA repair. Malignant arsenical skin lesions may be Bowen's disease (intraepithelial carcinoma, or carcinoma *in situ*), and multiple basal cell carcinomas, arising from cells not associated with hyperkeratinization or squamous cell carcinomas, which develop from some of the hyperkeratotic arts or corns. Skin cancer may arise in the hyperkeratotic areas or may appear on non-keratotic areas of the trunk, extremities, or hand. Arsenic in drinking water is associated with kidney cancer. Ecological studies in Taiwan, Chile, Argentina and Australia, and cohort studies from Taiwan and the USA demonstrated that long-term exposure to arsenic increased the risks for kidney cancer (Chen et al., 1985, 1988a, 1992; Hopenhayn-Rich et al., 1998; Kurttio et al., 1999). There is general agreement that inhalation of inorganic arsenic has been documented as a lung carcinogen in humans. Lung cancer is the leading cause of cancer-related mortality in the United States and worldwide. An association between lung cancer and exposure to inorganic arsenic through different sources has been confirmed in several epidemiologic studies (Boyle and Maisonneuve, 1995; Hopenhayn-Rich et al., 1998).

Liver cancers can develop from specific chronic liver diseases. Liver cirrhosis appears to be a primary cause of arsenic-related mortality in Guizhou, China, and is potentially associated with hepatocellular carcinoma (Liu et al., 1992, 2002). International Agency for Research on Cancer listed the liver as a potential organ for arsenic carcinogenesis (IARC, 2004). The association between environmental arsenic exposure and human liver cancers has been repeatedly reported (Centeno et al., 2002; Chiu et al., 2004; Liaw et al., 2008). Although it is clear that arsenic is a human carcinogen, the precise cellular mechanism by which arsenic induces cancer is still largely unknown.

1.13.3 Cardiovascular effects

Cardiovascular disease is the most common cause of death worldwide. Atherosclerosis is the central event of cardiovascular disease and proatherogenic role of arsenic have been well established (Chen et al., 1988b; Chiou et al., 1997; Hossain et al., 2012; Mumford et al., 2007; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, Tseng, 2005; Wang et al., 2002). The cardiovascular system is a very sensitive target of arsenic toxicity. Arsenic-induced cardiovascular diseases in human population may result from the interaction among genetic, environment and nutritional factors. Epidemiological studies have shown that arsenic ingestion through food or water may have serious effects on the human cardiovascular system including heart damage (myocardial depolarization, hypertrophy of the ventricular wall, cardiac arrhythmias), vascular damage (Raynaud's disease, Blackfoot disease, arterial thickening), ischemic heart disease, cerebrovascular diseases, and hypertension (Chen et al., 1988b; Chiou et al., 1997; Hossain et al., 2012; Huda et al., 2014; Karim et al., 2013; Mumford et al., 2007; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, 2005; Wang et al., 2002). The first evidence of a link between cardiovascular disease and arsenic in drinking water came in 1980 from Antofagasta, Chile, with a report of 17 deaths from myocardial infarction in people under the age of 40 (Yuan et al., 2007). Increased risk of cardiovascular disease was reported in smelter workers due to arsenic exposure (Axelson et al., 1978; Lee-Feldstein, 1989). Recently we found that arsenic exposure causes endothelial damage or dysfunction, an early event of atherosclerosis (Hossain et al., 2012).

It is believed that vascular endothelial cells play a pivotal role in arsenic-induced cardiovascular diseases. Arsenic causes endothelial damage through reactive oxygen species (ROS) production. Endothelial cell activation/dysfunction by arsenic increases the production of several inflammatory and adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), monocyte chemoattractant protein-1 (MCP-1) related to the atherosclerotic lesions (Blankenberg et al., 2001; Chen et al., 2007; Hsieh et al., 2008; Hwang et al., 1997; Lee et al., 2005; Ridker et al., 1998).

Several epidemiological studies reported that chronic arsenic poisoning through ingestion of arsenic-contaminated water can affect the platelets which increase the risk of death in humans from various cardiovascular diseases (Axelson et al., 1978; Lee et al., 2002; Lee-Feldstein, 1983; Wu et al., 1989). A dose-response relationship between prevalence of CVD and ingested arsenic was reported in northeastern Taiwan (Chiou et al., 1997). Ischemia is localized tissue anemia due to obstruction of the inflow of arterial blood. Mounting evidence indicated that arsenic increases mortality from ischemic heart disease (Chang et al., 2004; Chen et al., 2011; Tsai et al., 1999). Black foot disease, (BFD) a unique form of peripheral vascular disease, has been reported to be one of the important complications of chronic arsenic toxicity in southwestern Taiwan (Tseng, 1977). It is characterized by the severe systemic arteriosclerosis as well as dry gangrene and spontaneous amputations of affected extremities at end stages. Increased prevalence of peripheral vascular disease has also been reported among residents with long-term arsenic exposure from arsenic present in drinking water in Taiwan, Chile, the USA, and Mexico (Chen et al., 2007; NRC 1999, 2001; Tseng et al., 1996; Wang et al., 2007).

1.13.4 Hepatotoxic effects

Liver is the major site of arsenic detoxification. Abnormal liver function, manifested by gastrointestinal symptoms such as abdominal pain, indigestion, loss of appetite and by clinical elevations of serum enzymes, frequently occurs from exposure to arsenic in the drinking water (Islam et al., 2011; Mazumder, 2005), or from environmental exposure to arsenic through burning high-arsenic coal in interior stoves (Liu et al., 1992). Histological examination of the livers has revealed a consistent finding of

portal tract fibrosis (Mazumder, 2005). Individuals exposed more frequently to arsenic suffer from cirrhosis, which is considered to be a secondary effect of damage to the hepatic blood vessels. Hospitalized Indian arsenicosis patients have very high rates of hepatportal sclerosis developed from drinking water highly contaminated with arsenic (Dhawan et al., 1983; Santra et al., 1999). Chronic arsenic exposure in animals can also produce liver endothelial cell damage, which subsequently damages parenchymal cells (Straub et al., 2007). All these studies clearly revealed that prolonged drinking of arsenic-contaminated water is associated with hepatomegaly, hepatic fibrosis and cirrhosis.

1.13.5 Renal effects

The kidneys are the major route of arsenic excretion, as well as major site of conversion of pentavalent arsenic into the more toxic and less soluble trivalent arsenic. Sites of arsenic damage in the kidney include capillaries, tubules, and glomeruli (Winship, 1984). Damaged proximal tubular cells lead to proteinuria and casts in the urine. In some cases elevated levels of creatinine or bilirubin have been reported (Moore et al., 1994). Mitochondrial damage is also prominent in tubular cells. Several animal studies have reported renal effects following intermediate- or chronic-duration of oral arsenic exposure (Brown et al., 1976). The effects include increased kidney weight, swollen mitochondria and increased numbers of dense autophagic lysosome-like bodies in the proximal tubules, increased pigmentation in the proximal tubules, and cysts.

1.13.6 Neurological effects

The most typical neurological feature of arsenic neurotoxicity is peripheral neuropathy (Mathew et al., 2010). A large number of epidemiological studies and case reports in arsenic affected areas revealed that chronic arsenic exposures are associated with various neurologic problems such as mental retardation, and developmental disabilities such as physical, cognitive, psychological, sensory and speech impairments (Saha, 1995; Winship, 1984; Zierold et al., 2004). Studies on patients with arsenic neuropathy have shown a reduced nerve conducting velocity in their peripheral nerves, and this has become a hallmark of arsenic-induced neurotoxicity. Studies in China and Bangladesh have shown that mental health

problems (e.g. depression) are more common among the people affected by arsenic contamination (Brinkel et al., 2009). Additionally, a significant association between decreased reading and spelling performance and hair arsenic levels was found in a group of elementary school children (Moon et al., 1985), suggesting that arsenic may also cause neurobehavioral effects. Like the cardiovascular system, both the peripheral and central components of the nervous system can be damaged by arsenic (Saha, 1995; Winship, 1984). Symptoms of chronic encephalopathy include persistent headache, diminished recent memory, distractibility, abnormal irritability, restless sleep, loss of libido, increased urinary urgency, and increased effects of small amount of ethanol (Morton and Caron, 1989). Secondary depression, anxiety, panic attacks and somatizations are common. In addition, experiences with animals have pointed out that prenatal arsenic exposure was associated with depressive-like behaviors in the affected offspring mouse (Martinez et al., 2008).

1.13.7 Reproductive effects

Arsenic exposure has been associated with a number of adverse health outcomes, but relatively little attention has been directed toward the potential impact of arsenic on human reproductive system. Both animals and human experiments have demonstrated that arsenic and its methylated metabolites cross the placenta (Concha et al., 1998; He et al., 2007), and thus fetuses may be exposed to arsenic. Several studies suggest the association between arsenic exposure and adverse pregnancy outcomes, such as spontaneous abortion and stillbirth, and infant death (Ahmad et al., 2001; Milton et al., 2005; Rahman et al., 2007; von Ehrenstein et al., 2006). In a study with mice, a significant decrease in sperm count and motility along with increase in abnormal sperm were observed at high concentration (Pant et al., 2001).

1.13.8 Genotoxic effects

Inorganic arsenic is generally recognized as a mutagenic agent. Several studies have been carried out exploring the genotoxic effect of inorganic arsenicals (Cohen et al., 2006; Yamanaka et al., 2004). Arsenic causes DNA damages, chromosomal abnormalities; epigenetic changes that alter DNA methylation status (Chanda et al., 2006; Kitchin, 2001; Rossman et al., 2004; Zhao et al., 1997). Chromosomal aberrations, DNA-protein cross-links, and sister chromatid exchanges were observed

in hamster embryo cells, human lymphocytes and fibroblasts after exposure to inorganic arsenic (Dong and Luo, 1993; Jha et al., 1992; Kochhar et al., 1996; Lee et al., 1985; Okui and Fujiwara, 1986; Rasmussen and Menzel, 1997; Wiencke and Yager, 1991). Arsenic-induced chromosomal aberrations are characterized by chromatid gaps, and fragmentation, endoreduplication, and chromosomal breaks. It has already been reported that both arsenic and its metabolites can have a variety of genotoxic effects, which may be mediated by oxidants or free radical species. All of these species also have effects on signaling pathways leading to proliferative responses. There are interesting differences in the activities of inorganic and organic species both in terms of target organ carcinogenicity, toxic and genotoxic mechanisms. Mass et al. (2001) indicated that exposure of human lymphocytes to methylated trivalent arsenic causes direct DNA damage. A study using an earlier version of the alkaline elution method has indicated that arsenic induces DNA strand breaks in human fetal lung fibroblasts (Dong and Luo, 1993). Vuyyuri et al. (2006) reported that occupational exposure to arsenic among workers in a glass plant in India whose levels of blood arsenic were five times higher than in the control group had increased DNA damage in leukocytes. Li et al. (2001) reported that arsenic induced typical and various extents of DNA strand breaks in human cells via reactive oxygen species (ROS) in a dose-dependent manner. The most extensively studied DNA lesion is the formation of 8-hydroxyguanine (8-OH-G), one of the major products of DNA oxidation, which originates from the reaction of hydroxyl radical with guanine (Valko et al., 2006). 8-OH-G is a sensitive genotoxic marker of oxidative damaged DNA. Associations of arsenic exposure with increased urinary 8-OH-G concentrations have also been observed (Hu et al., 2006). Several studies showed that arsenic exposure causes epigenetic changes (Bailey and Fry, 2014; Hou et al., 2012; Reichard and Puga, 2010; Smeester et al., 2011). Epigenetic changes are the external modification of DNA without changing the sequences of bases. Hypo and hyper methylation of bases present in DNA are the main event in epigenetic changes. Epigenetic changes can be inherited to child from mother. Epigenetic alterations not only cause adverse effect on embryonic or neonatal growth but also can induce cancer or other deadly diseases in later life (Heindel, 2007; Vahter et al., 2008).

1.13.9 Respiratory effects

Effects of arsenic on the human respiratory system have been reported from both occupational exposures as well as from tube-well water arsenic toxicity. Human exposed to arsenic dust or fume inhalation are more apt to be encountered in mining and milling of ores, in industrial processing, such as smelting industry which often produces irritation of the mucous membrane, resulting in laryngitis, bronchitis, rhinitis and tracheobronchitis, causing stuffy nose, sore throat, hoarseness and chronic cough etc. (Dekundt et al., 1986; Saha et al., 1999). A fatal case of arsenic trioxide inhalation manifested widespread as tracheobronchial mucosal and sub mucosal hemorrhages with mucosal sloughing, alveolar hemorrhages, and pulmonary edema (Gerhardsson et al., 1988).

Noncancerous respiratory effects of arsenic on the human have been reported both from occupational exposure as well as from tube well water arsenic toxicity. Mazumder et al. (2000) reported an association between arsenic ingestion in drinking water and the prevalence of respiratory disorders. The relationship between ingested arsenic and nonmalignant respiratory effects has so far only been reported from Chile, India, and Bangladesh (Smith, 1998). Ingestion of inorganic arsenic for a prolonged period causes respiratory problems, including cough, chest sound, bronchitis, and shortness of breath (Islam et al., 2007; Mazumder et al., 2000; Milton and Rahman, 2002; Milton et al., 2001, 2003). In a small cross-sectional study, Milton and Rahman, (2002) investigated the link between arsenic exposure and the rate of chronic bronchitis in Bangladesh and they concluded that ingestion of inorganic arsenic present in drinking water may lead to increased risk of chronic cough and bronchitis. Saha (1995) conducted a study in West Bengal of India, and found a good number of patients with asthmatic symptoms in arsenic-endemic areas. They concluded that bronchitis and asthma were the common complications of ground water arsenic toxicity. Recently Islam et al. (2007) studied the link between arsenic exposure via drinking water and respiratory complications. They found high prevalence of respiratory complications such as breathing problems including chest sound, asthma, bronchitis, and cough in arsenicosis patients in Bangladesh. Asthma is a chronic disease worldwide. According to Global Initiative for Asthma (GINA) estimates, 300 million people worldwide currently suffer from asthma (Masoli et al., 2004). Asthma

is the most common chronic disease among children and adults. Asthma is a major public health concern in both developing and developed countries (Braman, 2006; Masoli et al., 2004). Mortality rate caused by asthma is still high. Although associations of arsenic exposure with other diseases have been well documented, a very little attention has been given on arsenic exposure-related asthma.

1.14 Biomarkers of the effect caused by arsenic

Arsenic is known to influence the activity of a number of enzymes, in particular the group of enzymes responsible for heme synthesis and degradation (Woods and Fowler, 1978) and activation of heme oxygenase (Sardana et al., 1981). Menzel and coworkers (Menzel et al., 1998) have also examined the *in vitro* induction of human lymphocyte heme oxygenase 1 (HO1) as a biomarker of arsenite exposure. Arsenite was observed to induce *de novo* synthesis of HO1 in human lymphoblastoid cells, but it has not been determined whether the same response is induced *in vivo*. Animal tests have shown that arsenic poisoning increased urinary levels of uroporphyrin, coproporphyrin and bilirubin (Albores et al., 1989). These tests have also been shown to be applicable to human subjects (García-Vargas and Hernández-Zavala, 1996). Hence, altered urinary levels of these heme-related compounds could serve as a sensitive biomarker of the effect of arsenic. Arsenic induces oxidative stress that causes the DNA damage. 8-hydroxy deoxy guanosine (8-OH-G) has been recognized as genotoxic marker of the oxidative damage of DNA caused by arsenic (Hu et al., 2006). In attempt to establish the serum biomarkers and the health effects for long-term arsenic exposure, very recently Ali et al. (2010) demonstrated the dose-response relationship between arsenic exposure and plasma cholinesterase activity in the population of arsenic-endemic areas in Bangladesh. Plasma cholinesterase activity has been reported to be involved in liver and neuro toxicity (Ali et al., 2010). The same group (Islam et al., 2011) in their recent study has also demonstrated the exposure- and dose- response relationship between serum hepatic enzymes and arsenic in drinking water, hair and nail arsenic concentrations. Serum hepatic enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) act as biomarkers for liver injury. Same group has also reported arsenic exposure increased several circulating markers of cardiovascular diseases that include big endothelin-1(big ET-1), oxidized-low density lipoprotein

(Ox-LDL), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), C-reactive protein (CRP), vascular endothelial growth factor (VEGF) and plasma uric acid (Hossain et al., 2012; Huda et al., 2014; Karim et al., 2013). We also found that arsenic exposure dose-dependently decreased high density lipoprotein cholesterol (HDL-C) (Karim et al., 2013). HDL-C is a good lipoprotein that has protective effects against cardiovascular diseases because of its anti-oxidant and anti-inflammatory properties.

1.15 Mitigation of arsenic problem

In Bangladesh, a good number of institutions, public universities, non-government organizations and donor agencies are working on various aspects of ground water arsenic contamination. Several projects have been undertaken to understand the magnitude of the problem and adopt mitigation measures but it appears to be the beginning of addressing the problem nation-wide. To mitigate arsenic problem, the following emergency steps should be taken.

- i) Raising public awareness should be the starting point for any approach to deal with the arsenic problem.
- ii) Training community members to test the tube-wells using field kits, and marking all the tested tube-wells (red if arsenic detected above 50 ppb, otherwise green if safe for drinking).
- iii) Monitoring operation, maintenance and water quality parameters of the alternative drinking water systems and continued promotion of safe water use in the community
- iv) Ground water treatment technologies that are cheap, efficient and easy to use should be applied at a large scale as an interim or mid term solution. Immediate measures must be taken to protect the health of those living in areas where water is contaminated by arsenic.
- v) Improving nutrition and fighting under-nourishment has to be a central element of the fight against the arsenic crisis.
- vi) Participation of the civil society has to be a key element of designing, planning and implementing remedial strategies.

vii) Coordination of the various government and non government organization are critically important to develop confidence of the people especially who are in danger of arsenic toxicity.

1.16 Further actions needed for arsenic mitigation

To save the people from arsenic contamination, an integrated and extensive program of action is needed.

1.16.1 National survey

The extent of arsenic problem is yet to be assessed. A national survey is required to understand the magnitude of the problem. The survey should be conducted to achieve the following goals:

1. Examination of the quality of water of all tube wells.
2. Delineation of the population exposed to arsenic contamination.
3. Identification of all arsenic affected people in the high risk areas.
4. Increasing the number of qualified doctors and health workers to treat the people who have already developed adverse health effect due to the arsenic exposure.

1.16.2 Provision of safe drinking water

Supply of safe drinking water in the arsenic affected areas is urgently required to avoid further ingestion of arsenic and arsenic related diseases. The provision of safe drinking water includes:

- i) Installation of tube-well is alternative aquifer producing water without arsenic content. Sinking of deep tube-well is a promising option for supply from uncontaminated deep aquifers having protective over impermeable clay layer which is common in stratified aquifers in Bangladesh.
- ii) Installation of community type treatment plants for the treatment of surface water for water supply in the absence of good quality ground water. Protected ponds may provide safe water with minimal treatment.

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- iii) Rainwater harvesting should be greatly encouraged as an alternative as well as supplementary water supply system in arsenic affected areas.
 - iv) Dug wells with adequate sanitary protection may be constructed for domestic water supply where aquifer and ground water conditions permit such constructions.
 - v) Instillation of community type arsenic removal plants attached or close to the tube-well to produce good quality water.
 - vi) Development and instillation of household level arsenic removal units are encouraged.

1.16.3 Awareness building

Awareness of people about ground water arsenic contamination and arsenic exposure-related diseases are essential to combat arsenic problem in Bangladesh. People are to be aware of:

- i) The possible health effects of drinking arsenic contaminated water as well as unsafe water from unprotected sources.
- ii) Necessity of having the sources of drinking water tested for arsenic and pathogens from a laboratory.
- iii) Alternative sources of safe water and good hygienic practices to preserve quality of drinking water.

1.16.4 Capacity building through training

Appropriate and comprehensive training programs to be developed with following targets:

- i) Development of skills of the doctors and health workers.
- ii) Enhancement of the knowledge and skills of engineers, hydrologist, NGO workers etc.
- iii) Strengthening the implementation capacity of the organizations involved in the planning and implementation of arsenic contamination free water supply system.

1.17 Phytoremediation of arsenic toxicity

Phytoremediation is an excellent way to solve the environmental problem especially environmental pollution by plant. Over the past 20 years, this technology has become increasingly popular and has been employed at sites with soils contaminated with lead, uranium, and arsenic (Comis et al., 1997; Prasad and Freitas, 1999, 2000; Prasad, 2004; Salt et al., 1998). Among phyto-remediation technologies, phytoextraction is one of the most recognized and acceptable technique for the scientific communities. Several researches have been conducted to test the efficacies of plant materials to remove or reduce arsenic toxicity. Mahmud et al. (2008) has tested 49 indigenous plant species and found that only one species of fern (*Dryopteris filix-mas*), three herbs (*Blumea lacera*, *Mikania cordata*, and *Ageratum conyzoides*), and two shrubs (*Clerodendrum trichotomum* and *Ricinus communis*) were found to be suitable for phytoremediation. Al Rmali et al. (2005) showed the good efficacies of water hyacinth plant to remove arsenic from water. One of the big limitations for phytoremediation of arsenic contamination is that it is rather difficult to remove arsenic from large amount of water or soil. Arsenic has entered the food chain because of the use of contaminated ground water for the irrigation of agricultural production. It is really a big challenge to remove arsenic from the bulk amount of underground water used for agricultural production. Therefore, another exciting approach is phytobioremediation by which arsenic toxicity can be reduced or prevented in human. This approach has opened a new window to reduce arsenic toxicity in humans. A good number of studies have been conducted to check the efficacies of plant materials against arsenic toxicity through animal model experiment. Flora et al., (2009) reported that garlic extract can prevent arsenic-induced hepatic apoptosis. Curcumin, an active ingredient of turmeric has been reported to inhibit arsenic-induced several intracellular signals (Gao et al., 2013; Hossain et al., 2000). Further, in animal experiments, it has been observed that curcumin attenuates the arsenic-induced biochemical perturbation in rat (Yousef et al., 2008). Curcumin also inhibits the arsenic-induced genotoxicity in mice (Biswas et al., 2010). Karim et al. (2010) has reported the ameliorating effects of turmeric on arsenite-induced biochemical perturbation of blood indices. A study conducted by Sarkar et al. (2012) has shown the reduction of arsenite-mediated adverse effects by

water hyacinth root powder in mice. Very recently Sheikh et al., (2014) has reported the protective effects of *Moringa oleifera* Lam. leaves against arsenic toxicity in mice. Same group (Rahman et al., 2012) in a separate study has showed the ameliorating effect of *Zingiber zerumbet* Linn. on arsenite-induced changes of blood indices in experimental mice.

1.18 Objective of the present study

Arsenic is a ubiquitous element present in food, soil, water and airborne particles. The general population is exposed to inorganic and organic arsenic through water, food, occupation and other environmental sources. Arsenic toxicity is a global health problem affecting millions of people in the many countries including Bangladesh and India. Arsenic toxicity has caused an environmental tragedy in Bangladesh and West Bengal of India where millions of peoples have been affected due to the drinking of water contaminated by arsenic (Chowdhury et al., 2001; Golub et al., 1998). A huge numbers of toxicity cases have already been reported in the north-west region of Bangladesh and it is becoming alarming day by day as the new cases of toxicity are being reported. Around 80-100 millions of peoples are at risk of arsenic toxicity in the country (Chowdhury, 2004). In Bangladesh the number of toxicity cases exceeded over the number of Chernobyl catastrophe (Sambu and Wilson, S2008). Although arsenic is a well established human carcinogen, paradoxically, arsenic is also used to treat acute promyelocytic leukemia (PML) for its potentiality to induce apoptosis in cancer cells. Due to its dual roles in therapeutic application and in acute toxicity, arsenic has created a renewed attention.

Arsenic toxicity induces dermatitis, multi site cancers, cardiovascular diseases, diabetes mellitus, peripheral neuropathy, liver damage, renal failure, and many other pathogenesis (Akila et al., 1998; Chen et al., 2007; Hossain et al., 2012; Islam et al., 2011; Karim et al., 2013; Meliker et al., 2007; Mumford et al., 2007; Tapio et al., 2006; Vahidnia et al., 2008; Wang et al., 2002). The major metabolic pathway of inorganic arsenic in humans is its methylation in liver. This methylation of arsenic is proved by the presence of monomethylarsonic acid (MMA) and dimethylarsinic acid in urine and bile (Cui et al., 2004; Li et al., 2008). Generally toxicity of arsenic is thought to cause largely by its reaction with free sulfhydryl groups of enzymes and

proteins followed by their cross linking (Hossain et al., 2000). The cross linking of enzymes or proteins activate the multiple intracellular signaling pathways inside the cells that may be responsible for arsenic-mediated pathogenesis. Moreover, arsenic-induced intracellular signals are largely mediated through redox-linked mechanism since reactive oxygen species (ROS) produced by arsenic act as second messengers for many signaling events (Hossain et al., 2000; Hossain et al., 2003).

Attempt to apply nutritional antioxidant to prevent or to treat the diseases caused by oxidative stress have been getting attention in recent years. Many plant products exert their protective effects against oxidative stress-mediated diseases by scavenging free radicals. Although arsenic as an oxidative stress causes serious human sufferings, few reports on the beneficial effects of plant products against arsenic toxicity are available. *Raphanus sativus*, an edible root vegetable, belonging to a Brassicaceae family, is commonly consumed as important components in traditional foods in many countries in Asia including Bangladesh, India, Bangladesh, China and Japan. It has been used as a traditional medicine for the treatment of whooping cough, cancer, gastric discomfort, liver problems, constipation, dyspepsia, gallbladder problems, arthritis, gallstones, kidney stones etc. (Singh and Singh, 2013). Methanolic and acetone extracts of *Raphanus sativus* leaves had total polyphenolic content of 86.16 and 78.77 mg/g dry extract, respectively (Beevi et al., 2010). In this paper, they reported the presence of catechin, protocatechuic acid, syringic acid, vanillic acid, ferulic acid, sinapic acid, o-coumaric acid, myricetin, and quercetin in leaves. Leaves showed potent reductive capacity, significantly inhibited linoleic acid peroxidation and displayed metal chelating activity and most potent antioxidant and radical scavenging activity, which may be accounted for the high polyphenolic content. From the ancient time it has been used in folk medicine as a natural drug against many toxicants (Lee et al., 2012). *Momordica charantia* fruits (bitter guard) are an indigenous medicinal and vegetable plant found in the tropical and subtropical regions of the world and is also known as bitter melon (Lee et al., 2009). Fruits of bitter gourd plant contained highest antioxidant activity compared to the leaf and stem (Kubola and Siriamornpun, 2008; Wu and Ng, 2008). Bitter gourd believed to possess enzymatic and non-enzymatic antioxidant activities against the production of ROS in the cells (Dasgupta and De, 2006; Dhiman et al., 2012; Wu and Ng, 2008; Xanthopoulou et al., 2009). Several

biological effects of bitter gourd have been reported such as hypoglycaemic effects, anti-rheumatic, anti-inflammatory, antiseptic and anti-diabetic remedies (Anila and Vijayalakshmi, 2000; Leatherdale et al., 1981). In addition, bitter gourd also has been reported to have other medicinal properties such as anti-carcinogenic, hypocholesterolemic, anti-viral, anti-cytotoxic, hypoglycemic and anti-mutagenic properties and dissipate melancholia (Lotikar and Rajarama, 1966). The main constituents of bitter gourd which are responsible for these biological and medicinal effects are triterpenes, proteins, steroids, alkaloids, inorganic compounds, lipids, and phenolic compounds (Grover and Yadav, 2004). *Brassica nigra* leaves (black mustard green) are also nutritious green-leafy vegetables available in many parts of the world, belonging to the Brassicaceae family. It is an excellent source of vitamin C, vitamin E, vitamin A (in the form of carotenoids), and manganese. Beside these conventional antioxidants it also contains phytonutrients that include Hydroxycinnamic acid, quercetin, isorhamnetin, and kaempferol are among the key antioxidant phytonutrients provided by mustard greens. Mustard green has been shown to have benefits against cancer and inflammatory diseases. Because of their anti-oxidant and free radical scavenging activities raddish leaves, bitter guards and mustard greens may be effective to reduce arsenic toxicity. However, information regarding the effects of these three plant materials against arsenic toxicity is scarce. Therefore, this study has been designed to investigate protective effects of these plant materials against sodium arsenite-induced adverse effects in mice through biochemical and molecular approaches.

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CHAPTER II

Investigation of the ameliorating effects of RSL, MCF and BNL on Sa-induced perturbation of blood indices through biochemical approaches

Chapter 2: Investigation of the ameliorating effects of RSL, MCF and BNL on Sa-induced perturbation of blood indices through biochemical approaches

2. Abstract

Major cause of arsenic poisoning is the drinking of water contaminated by arsenic. High levels of arsenic have been found in the several food items in the arsenic-endemic areas in Bangladesh and other countries suggesting that exposure to arsenic is unavoidable. In this situation, phytoremediation may be a plausible way to reduce arsenic toxicity. This study was designed to check the efficacies of three plant materials having antioxidant and free radical scavenging activity against Sodium arsenite (Sa)-induced adverse effects through mice experiments. Mice were divided into eight equal groups: control, *Raphanus sativus* leaves (RSL), *Momordica charantia* fruit (MCF), *Brassica nigra* leaves (BNL), Sa, RSL plus Sa, MCF plus Sa and BNL plus Sa. Sa (10 mg/Kg body weight/day) was given orally and powder form of plant materials (50 mg/Kg body weight/day) were given as food supplement. In this study, it was observed that lactate dehydrogenase (LDH) activity was significantly ($p < 0.05$) higher in Sa-treated mice than that in the control group. RSL and MCF supplementation abrogated Sa-induced serum LDH activity significantly ($p < 0.05$). BNL could not abrogate Sa-induced serum LDH activity. Serum butyryl cholinesterase activity (BChE) in Sa-treated mice was significantly ($p < 0.05$) lower than the control group, and the food supplementation of RSL but not MCF and BNL could significantly ($p < 0.05$) prevent the reduction of Sa-mediated serum BChE activity. Sa administration increased the serum biomarkers used for liver function test that included alkaline phosphatase (ALP), Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST) activities. Among the plant material tested, only RSL could reduce Sa-induced elevation of the activities of these three enzymes in serum significantly ($p < 0.05$). MCF showed significant ameliorating effect ($p < 0.05$) only on Sa-induced elevation of ALP. BNL did not show any significant protective effect on Sa-induced elevation of ALP, ALT and AST activities. High density lipoproteins cholesterol (HDL-C), a serum biomarker of cardiovascular risk was found to be decreased in Sa-treated mice, and RSL but not MCF and BNL could ameliorate Sa-induced perturbation of HDL-C significantly ($p < 0.05$). Finally, RSL

could reduce the Sa-induced elevation of serum urea level significantly ($p < 0.05$); however, MCF and BNL could not show any significant protective effect on Sa-induced elevation of serum urea level. All these results explicitly stated that Sa treatment caused the perturbation of blood indices in mice associated with hepatic, cardiovascular and renal dysfunctions, and RSL showed protection against Sa-induced perturbation of those blood indices more effectively than the two other plant materials (MCF and BNL). Thus, the results indicated that RSL could be useful to reduce or prevent arsenic toxicity in human in future.

2.1 Introduction

Since decades, chronic arsenic toxicity is a widespread global problem affecting millions of people in many countries. In Bangladesh, it has become a great public health concern. This problem has not only created human sufferings and death but also made a huge socio-economic problem of the country. It is reported that 61 out of 64 districts of Bangladesh are affected by arsenic contamination. Recent surveys showed that approximately 80-100 million people of the country are living under the risk of arsenic poisoning (Chowdhury, 2004). Arsenic contamination of ground water poses a serious threat to man and agricultural sustainability in this country. Besides domestic use, significant quantities of water from shallow aquifers are being used in the dry season particularly for irrigating rice and others crops in the country. The dependency of ground water for drinking, cooking and irrigation resulting in a large quantity of arsenic is being cycled through environment each year with a major implication in public health and environment. Because of the uses of arsenic contaminated ground for the irrigation purposes, arsenic has entered the food chain (Ahsan and Del Valls, 2011). Therefore, exposure to arsenic is unavoidable. Several approaches including the filtering machine that can remove arsenic from the drinking water have been developed. But filtering machine is not sufficient for huge daily requirement of water for house hold purposes and for the irrigation of agricultural production. Therefore, alternative approaches are required to reduce the arsenic toxicity in human. Some synthetic drugs that include Meso-2, 3 dimercaptosuccinic acid, Succimer, Chemet, Sodium 2, 3-dimercapto-1-propane sulfonic acid, Dimaval, D-Penicillamine have tried for therapeutic purposes but they are not effectively useful for their adverse side effects on human health (Mazumder, 2000). Arsenic breaks the anti-oxidant system and exhibits its adverse effect through reactive oxygen species (ROS)-sensitive pathways (Hossain et al., 2000, 2003). Plant materials having antioxidant and free radical scavenging activity may be plausible target to remediate or prevent arsenic toxicity. Trends on applying nutritional antioxidants in diseases related to oxidative stress have gained immense interest in recent years. Plant products are known to exert their protective effects by scavenging free radicals and modulating antioxidant defense system. Recently some plant materials have shown their potential to reduce arsenic toxicity *in vitro* and *in vivo* models. In an attempt for the phytoremediation of arsenic toxicity, Karim et al. (2010) demonstrated the

ameliorating effects of turmeric powder against the arsenite-induced perturbation of blood indices. Curcumin, an active ingredient of turmeric has been reported to reduce arsenic toxicity (Yousef et al., 2008). Verma et al. (2007) showed that *P. fraternus* (commonly known as Bhumyamalaki), *Terminalia arjuna* (commonly known as Arjuna) and *Moringa oleifera* (commonly known as Sajina) seed had ameliorating effects on arsenic toxicity. Chowdhury et al. (2008) demonstrated that garlic (*Allium sativum*) could have potential to prevent arsenic toxicity. More recently protective effects of *Moringa oleifera* Lam. leaves and rhizomes of *Zingiber Zerumbet* Linn on the sodium arsenite-induced changes of several blood indices were reported (Rahman et al., 2012; Sheikh et al., 2014). All these studies suggested the potential application of plant materials against arsenic toxicity. In an attempt to check the efficacies of plant materials which have antioxidant and free radical scavenging activity, three edible plants materials were selected for this study: radish (*Raphanus sativus*) leaves (RSL), bitter gourd (*Momordica charantia*) fruits (MCF), mustard (*Brassica nigra*) leaves (BNL). RSL is a good source of polyphenols and other natural compounds that have antioxidant and free radical scavenging activity (Beevi et al., 2010). From the ancient time it has been used as a natural drug against many toxicants (Lee et al., 2012). Bitter gourd is an important nutritive food that contains highly biologically active compounds. Fruits of bitter gourd (MCF) plant contained highest antioxidant activity compared to the leaf and stem (Kubola and Siriamornpun, 2008; Wu and Ng, 2008). Bitter gourd is believed to possess antioxidant activities against the production of ROS in the cells (Dasgupta and De, 2006; Dhiman et al., 2012; Wu and Ng, 2008; Xanthopoulou et al., 2009). It is used as a folk medicine for the treatment for various chronic and degenerative diseases including diabetes mellitus, coronary heart disease and cancer (Aboa et al., 2008; Cheng et al., 2003; Fang et al., 2012; Nerurkar et al., 2006, 2010; Slater, 1984). *Brassica nigra* leaves (BNL) is a rich source of antioxidants flavonoids, indoles, sulforaphane, carotenes, lutein and zeaxanthin. Because of the potential antioxidant and free radical scavenging activities, it was hypothesized that RSL, MCF and BNL might have protective activity against arsenic toxicity. Therefore, this study was undertaken to check whether these plant materials could show protective effects against arsenic toxicity in mice.

2.2 Materials and methods

2.2.1 Chemicals and equipments

2.2.1.1 List of chemicals and test kits

1. Sodium arsenite
BDH Chemicals Ltd. England.
2. Lactate dehydrogenase (LDH) test Kit
dds Diagnostic systems, Turkey.
3. Butyryl cholinesterase (CHE) test kit
RANDOX, UK.
4. Alkaline phosphatase detection kit
Human, Germany.
5. Alanine aminotransferase detection kit
Human, Germany.
6. Aspartate aminotransferase detection kit
Human, Germany.
7. HDL-Cholesterol kit
Human, Germany.
8. Urea detection kit
Human, Germany.
9. Diethyl ether
Merck, Mumbai.
10. Ethanol
Merck, Germany.
11. RNA Stabilization Solution (*RNAlater*)
Ambion Company.

2.2.1.2 List of equipments

The equipments for animal maintenance and laboratory works are given below:

1. Plastic cages with iron lids (for housing mice).
2. Water bottles (with drinking nozzle).

3. Eppendorf tubes.
4. Scissors and forceps.
5. Injection syringe with needle.
6. Micropipettes (0.2-5 μ l, 0.5-10 μ l, 2-20 μ l, 20-200 μ l, and 100-1000 μ l) and micropipette tips.
7. Glass wares: Volumetric flux (50 ml, 100 ml), Conical flux (50ml, 100 ml), Beaker (250ml, 500ml and 1000ml), pipette (5ml, 10ml).
8. Different plastic wares (pipette filler, micro pipette tips holder, distilled water bottle).
9. Autoclave (ALP Co. Ltd. Tokyo, Japan).
10. -80° C Freezer. (Sanyo Electric Co. Ltd, Japan).
11. -20° C Freezer (Walton, Bangladesh).
12. Centrifuge machine (Eppendorf Model-5415).
13. Semi-automated bioanalyzer (Humalyzer 3000, Human, Germany).
14. Water bath (Digisystem Laboratories, Taiwan).
15. Heating and drying oven (Gallenkamp, UK).
16. Digital measuring balance (Unilab, USA).
17. Measuring cylinder.

2.2.2 Selection of plant materials

Laboratory where this research has been performed is continuously searching the efficacies of plant materials having antioxidant and free radical scavenging activity. In previous studies Turmeric, *Moringa oleifera* leaves, rhizomes of *Zingiber Zerumbet* (Karim et al., 2010; Rahman et al., 2012, Sheikh et al., 2014) were found to have protective effects against arsenic (Sa)-induced changes of blood indices (Karim et al., 2010; Rahman et al., 2012, Sheikh et al., 2014). In an attempt to investigate the efficacies of other plants, in this study, three plant materials were selected based on their availability and their anti-oxidant and free radical scavenging activities. During

the selection process emphasis was also laid on the cheapness and edibility of the plant materials. *Raphanus sativus* leaves (RSL), *Momordica charantia* fruits (MCF), *Brassica nigra* leaves (BNL) were selected for this study.

2.2.3 Ethical permission

Ethical permission for this study was taken from the Institute of Biological Sciences, Rajshahi University (No. 21/320-IAMEBBC/IBSc).

2.2.4 Collection of the vegetables

RSL, MCF and BNL were collected from the local farmers near the University of Rajshahi. Before collection from the local farmers, it was confirmed that no insecticides or pesticides were used. 3-4 weeks of ages of fresh RSL and BNL leaves were collected.

2.2.5 Animal maintenance

Adult healthy (four weeks of age) Swiss albino male mice with average body weight (BW) of 20-22 grams were purchased from ICDDR, B (International Centre for Diarrhoeal Disease Research, Bangladesh). The animals were randomly selected and housed in polycarbonate cages (Figure 2.1) with steel wire tops and wood-cube bedding (six mice per cage). After one week of acclimation, animals were divided into eight equal groups named control, RSL, MCF, BNL, Sa, RSL plus Sa, MCF plus Sa and BNL plus Sa. They were maintained with 12h: 12h dark-light cycle with available supply of distilled water and feed. Sa was given to the mice with distilled water (10 mg/kg body weight/day) and RSL/MCF/BNL powder (50 mg/ kg body weight/day) was added (as a supplement) to the normal diet. The mice experiments were conducted for 16 weeks after one week of acclimation.



Figure 2.1: Photograph of polycarbonate mice cage with steel wire tops, wood-cube and water supply bottle.

2.2.6 Mice food

The list of food ingredients (Table 2.1) was taken from ICDDR,B. Commercially available food ingredients were purchased and mixed homogeneously and this homogeneous mixture was then used as normal food for experimental mice (control and Sa group). For RSL/MCF/BNL, and RSL+Sa / MCF+Sa/ BNL+Sa group of mice, 50 mg of RSL/MCF/BNL powder was mixed with 5 gm (per day for each mouse) of normal food ingredients.

Table 2.1 Ratio formulation of different food ingredients for rat/mice

SL No.	Ingredients	% of Ingredients
1.	Maize	30
2.	Wheat	10
3.	Auto Rice Polish	20
4.	Til meal	10
5.	Soyabean meal	15
6.	Full fat Soya	10
7.	Fish meal	2.0
8.	Skimmed Milk Powder	0.50
9.	Soyabean Oil	2.0
10.	Salt	0.25
11.	Vitamin-Mineral Premix	0.25

2.2.7 Preparation of RSL, MCF and BNL powder

After collection from the local farmers, RSL, MCF and BNL were cleaned and washed repeatedly with distilled water. The MCF fruits were sliced. RSL, BNL and sliced MCF were then shade-dried. Finally, powder forms (Figure 2.2) were obtained by grinding the dried RSL, MCF and BNL. The powdered were kept at 4°C with sealed plastic packet to avoid the microbial contamination.

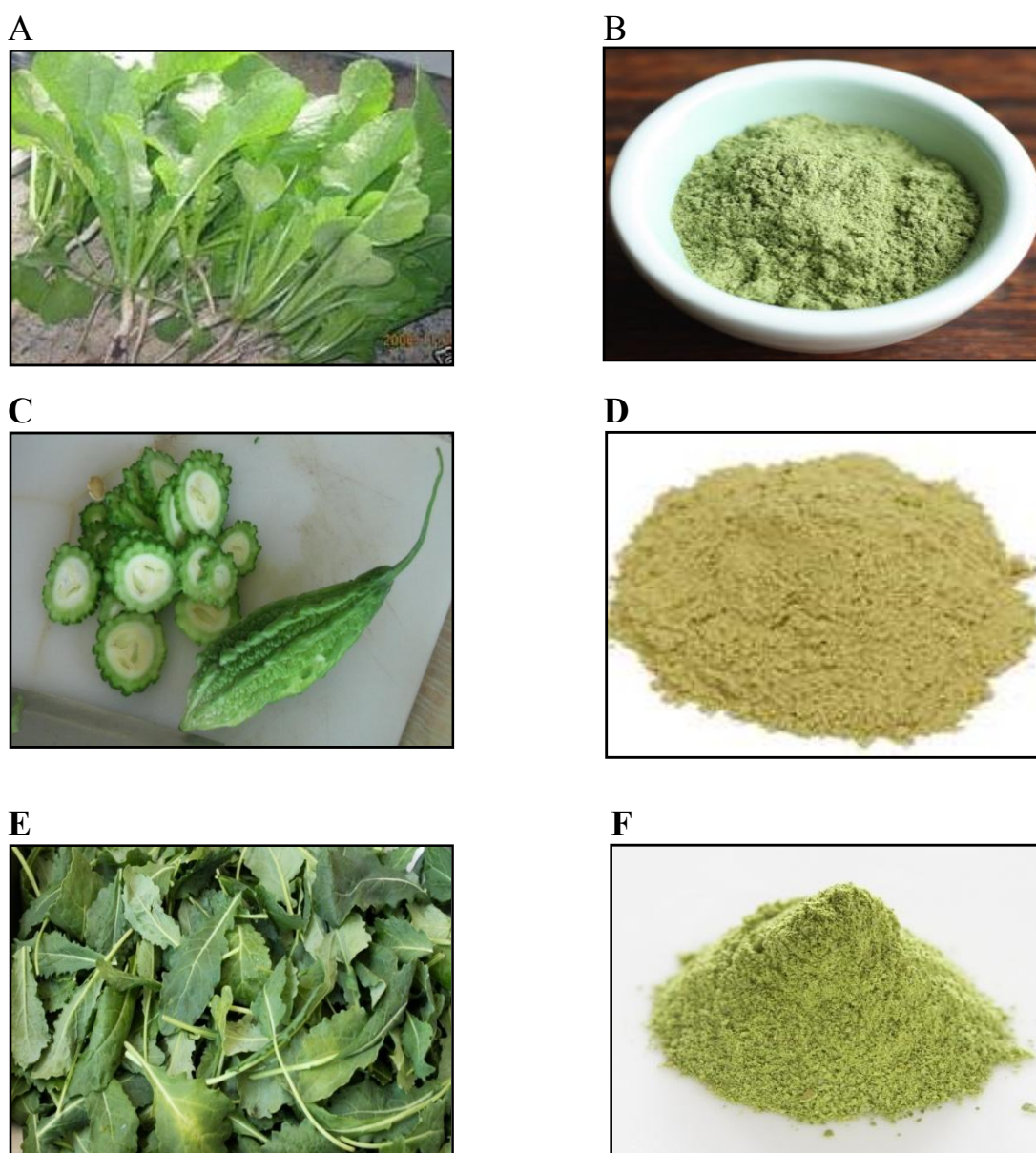


Figure 2.2: Photographs of plant materials used for the study: (A) *Raphanus sativus* Leaves (RSL), (B) powder of RSL, (C) *Momordica charantia* fruit (MCF), (D) powder of MCF, (E) *Brassica nigra* Leaves (BNL) and (F) powder of BNL.

2.2.8 Collection of serum from experimental mice

Eight groups of experimental mice were maintained for 16 weeks from starting day of the experiment. Then blood specimens were collected from the thoracic arteries of the mice after anaesthetization with diethyl ether. For coagulation, blood was kept about 30 minutes at room temperature. After centrifugation at 6000 rpm for 15 minutes at 4°C serum were drawn off and stored at -80°C until the experiments were performed.

2.2.9 Collection of liver and kidney tissue

Kidney and liver tissues were collected from the experimental mice from the eight groups (control, RSL, MCF, BNL, Sa, RSL plus Sa, MCF plus Sa, BNL plus Sa) of experimental mice after taking blood from thoracic arteries. A small section of liver and kidney were taken and kept in RNA stabilization solution (RNAlater® RNA Stabilization Solution, Ambion Company). Tissues in RNA stabilization solution were kept at -20°C until the extraction of total RNA from the collected specimens.

2.2.10 Laboratory examination

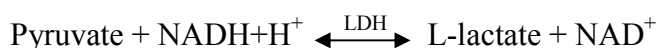
2.2.10.1 Determination of serum lactate dehydrogenase (LDH) activity

Plasma LDH activity was measured by using LDH kit according to the manufacture's protocol (dds Diagnostic systems, Turkey) through bioanalyzer (Humalyzer 3000, Human, Germany).

Reaction principle:

Lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. At high concentrations of lactate, the enzyme exhibits feedback inhibition and the rate of conversion of pyruvate to lactate is decreased.

LDH catalyzes the following reaction:



Type of specimen: Serum

Reagent composition:

Contents	P ^H	Concentration
Reagent-1(R1) :	7.5	
Phosphate buffer		50 mmol/L
Pyruvate		0.6 mmol/L
Reagent-2(R2) :	9.6	
Good's buffer		
NADH		0.18 mmol/L

Procedure:

The working reagent was prepared by mixing R1 (4ml) and R2 (1ml). Then working reagent was incubated for 5 minutes at 37°C. Plasma sample (10 µl) was added to pre-incubated working reagent. Finally, LDH activity was measured at 37°C spectrophotometrically (absorbance at 340 nm).

Calculation:

To calculate the LDH activity, the following formula was used according to the protocol:

$$\Delta A/\text{min} \times 16030 \text{ (Factor)} = \text{LDH activity [U/L]}$$

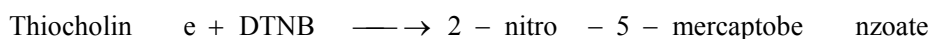
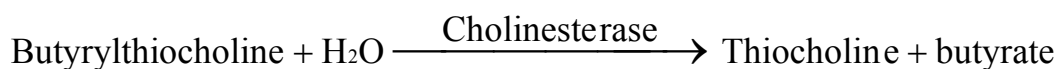
Where, ΔA = Changes of absorbance per minute at 37°C

Decrease of the absorbance value at 340 nm, due to the NADH oxidation in NAD^+ , is directly proportional to the enzyme activity. All serum samples were analyzed in duplicate, and then mean values were taken.

2.2.10.2 Determination of serum butyryl cholinesterase (BChE) activity

Serum cholinesterase activity was measured using butyryl cholinesterase (ChE) test kit according to the manufacture's protocol (RANDOX, UK).

Butyryl cholinesterase hydrolyses butyrylthiocholine to give thiocholine and butyrate. The reaction between thiocholine and DTNB gives 2-nitro-5-mercaptobenzoate, a yellow compound which can be measured by analyzer (Humalyzer 3000, Human, Germany) at 405 nm.



Here, DTNB = Dithiobis Nitrobenzoate

Type of specimen: Serum

Reagent composition:

Contents	Concentrations in the Test
R1.Buffer/Chromogen	
Phosphate buffer	50 mmol/L, pH 7.7
DTNB	0.25 mmol/L
R2. Substrate	
Butyrylthiocholine iodide	6 mmol/L
Procedure	
Wave length	Hg 405 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against air
Pipette into cuvette	37°C
R1	1.50 ml
Sample (dilute 1+1 with 0.9% NaCl solution)	0.01 ml
R2	0.05 ml

Sample was mixed with reagent and initial absorbance of the sample was read and again after 30, 60, and 90 sec.

Calculation:

To calculate the activity of serum cholinesterase, the following formula was used according to the protocol:

$$U/I (37^{\circ}\text{C}) = 23460 \times \Delta A \text{ 405 nm/min}$$

Where Δ = Changes of absorbance per minute at 405 nm.

All serum samples were analyzed in duplicate and then mean values were taken.

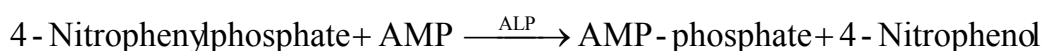
2.2.10.3 Determination of serum alkaline phosphatase (ALP) activity

ALP activity was measured using alkaline phosphatase (ALP) kit according to the manufacture's protocol (Human, Germany) through analyzer (Humalyzer 3000, Human, Germany).

Reaction principle:

Serum alkaline phosphatase (ALP) catalyzes in alkaline medium the transfer of the phosphate group from 4-nitrophenylphosphate to 2-amino-2-methyl-1 propanol (AMP), liberating 4-nitrophenol. The catalytic concentration is determined from the

rate of 4-nitrophenol formation, measured at 405 nm according to the manufacture's protocol (Human, Germany).



Type of specimen: Serum

Reagent composition:

Contents	Concentration
A. Reagent: 2-amino-2-methyl-1-propanol	0.4 mol/L
Zinc sulfate	1.2 mmol/L
N-hydroxyethylethylenediaminetriacetic acid	2.5 mmol/L
Magnesium acetate	2.5 mmol/L
B. Reagent: 4-Nitrophenylphosphate	60 mmol/L

Procedure:

The working reagent was prepared by mixing 4 ml reagent **A** + 1 ml reagent **B**. 20 μ l serum sample (which was previously centrifuged at 1600 g for 15 minutes) was mixed with 1000 μ l working reagent and the absorbance (405 nm) was measured after 1 minute and again exactly after 1, 2, 3 minutes against the air at 37 $^{\circ}$ C.

Calculation

To calculate the ALP activity, the following formula was used according to the protocol:

$$\Delta A/\text{min} \times \frac{V_t \times 10^6}{\epsilon \times l \times v_s} = \text{U/L}$$

Where, Δ = Changes of absorbance per minute at 405 nm.

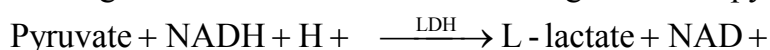
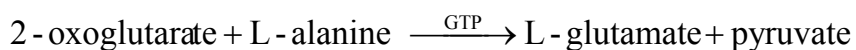
All serum samples were analyzed in duplicate and then mean values were taken.

2.2.10.4 Estimation of serum alanine aminotransferase (ALT) activity

Alanine aminotransferase activity was measured using ALT kit according to the manufacture's protocol (Human, Germany) through analyzer (Humalyzer 3000, Human, Germany).

Reaction Principle:

2-oxoglutarate reacts with L-alanine by the action of alanine aminotransferase to give L-glutamate and pyruvate. Pyruvate then converted to L-lactate by the action of LDH.



Type of specimen: Serum

Reagent composition:

Contents	Concentration
Buffer/Enzyme reagent	
TRIS buffer	150 mmol/l
L-alanine	750 mmol/l
LDH	>1.2kU/l
Substrate	
2-oxoglutarate	90 mmol/l
NADH	0.9 mmol/l

Working reagent:

The working reagent was prepared by mixing 2 ml of substrate into 8 ml buffer. The working reagent is stable for 4 weeks at 2-8° C and 5 days at 15-25° C.

Procedure:

Wave length	340 nm
Optical path	1 cm
Temperature	37° C
Measurement	Against air (Decreasing absorbance)
Pipette into cuvette	37° C
Sample	100µl
Working reagent	1000µl

Sample was mixed with working reagent and the absorbance (340 nm) was measured after 1 minute and again exactly after 1, 2, 3 minutes against the air at 37° C.

Calculation:

For $\Delta A/\text{min}$ within 0.12-0.16 (340nm) use only measurements from the first 2 minutes for calculation (1 min. incubation, 2 min. measurements).

Conversion factor from traditional units (U/L) in SI-units (kat/L):

$$1 \text{ U/L} = 16.67 \times 10^{-3} \mu\text{kat/L}$$

$$1 \mu\text{kat/L} = 60 \text{ U/L}$$

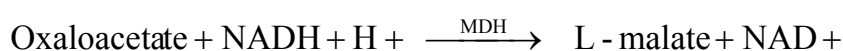
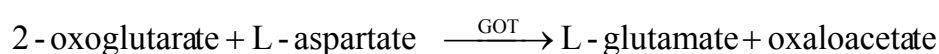
All serum samples were analyzed in duplicate and then mean values were taken.

2.2.10.5 Estimation of serum aspartate aminotransferase (AST) enzyme activity

Aspartate aminotransferase activity was measured using AST kit according to the manufacture's protocol (Human, Germany) through analyzer (Humalyzer 3000, Human, Germany).

Reaction Principle:

2-oxoglutarate reacts with L-aspartate by the action of aspartate aminotransferase to give L-glutamate and oxaloacetate. Oxaloacetate then converted to L-Malate by the action of malate dehydrogenase (MDH).



Type of specimen: Serum

Reagent composition:

Contents	Concentration
Buffer/Enzyme reagent	
TRIS buffer	100 mmol/l
L-aspartate	300 mmol/l
MDH	>0.9kU/l
Substrate	>0.6
2-oxoglutarate	60 mmol/l
NADH	0.9 mmol/l

Working reagent:

The working reagent was prepared by mixing 2 ml of substrate into 8 ml buffer. The working reagent was stable for 4 weeks at 2-8°C and 5 days at 15-25°C.

Procedure:

Wave length	340 nm
Optical path	1 cm
Temperature	37° C
Measurement	Against air (Decreasing absorbance)
Pipette into cuvette	37°C
Sample	100
Working reagent	1000

Sample was mixed with working reagent and absorbance of the sample was measured against the air.

Calculation:

For $\Delta A/\text{min}$ within 0.12-0.16 (340nm) use only measurements from the first 2 minutes for calculation (1 min. incubation, 2 min. measurements).

Conversion factor from traditional units (U/L) in SI-units (kat/L):

$$1 \text{ U/L} = 16.67 \times 10^{-3} \mu\text{kat/L}$$

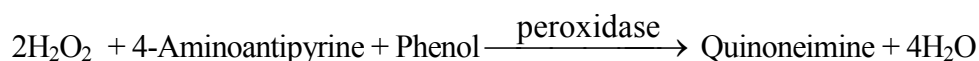
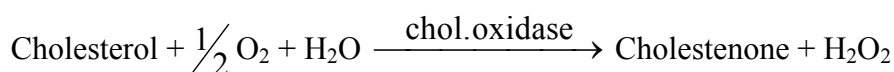
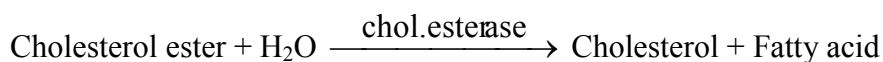
$$1 \mu\text{kat/L} = 60 \text{ U/L}$$

All serum samples were analyzed in duplicate and then mean values were taken.

2.2.10.6 Estimation of serum high density lipoprotein cholesterol (HDL-C)

Serum high density lipoprotein cholesterol (HDL-C) was measured using an assay kit according to manufacture's protocol (Human, Germany).

Very low density lipoproteins (VLDL) and low density lipoprotein (LDL-C) in the sample precipitate with phosphotungstate and magnesium ions. The supernatant contains high density lipoproteins (HDL-C). The HDL-C is then spectrophotometrically measured at 500nm.

Reaction principle:

Type of specimen: Serum

Reagent Composition:

Contents	Concentration
(PREC) :	
Phosphotungstic acid	0.55 mmol/L
Magnesium chloride	25.00 mmol/L
(STD) :	
Cholesterol	1.29 mmol/L

Procedure:

Precipitant reagent was diluted with distilled water as a ratio 4:1 (precipitant: distilled water). 20 μL samples were added to 50 μL diluted precipitant reagent. The mixture was then incubated for 10 min at room temperature and after incubation, centrifuged for 2 min at 4000rpm. 50 μL supernatant was added to 500 μL cholesterol reagents in

a test tube and incubated for 5 min at 37°C. Finally, HDL-C levels were measured at 37°C spectrophotometrically (absorbance at 500 nm).

Calculation:

The HDL-C concentration in the sample was calculated using the following general formula:

$$\frac{A \text{ sample}}{A \text{ standard}} \times C \text{ standard} \times \text{Sample dilution factor} = C \text{ sample (mg/dl)}$$

All serum samples were analyzed in duplicate and then mean values were taken.

2.2.10.7 Estimation of serum urea

Serum urea was measured using commercially available assay kit manufactured by Human Diagnostic, Germany through analyzer at 570-600 nm according to the manufacture's protocol.

Reaction Principle:

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. In a modified Berthelot reaction, the ammonium ions react with hypochlorite and salicylate to form a green dye. The absorbance increase at 580 nm is proportional to the urea concentration in sample.

Type of specimen: Serum

Reagent Composition:

Contents	Concentration
Reagent 1	
Phosphate buffer (pH 7.0)	120 mmol/l
Sodium salicylate	60 mmol/l
Sodium nitroprusside	5 mmol/l
EDTA	1 mmol/l
Reagent 2	
Phosphate buffer (pH < 13)	120 mmol/l
Hypochlorite	0.6 g/l Cl
Enzyme	
Urease	> 500KU/l
Standard	
Urea	13.3 mmol/l
Equivalent to BUN	6.2 mmol/l
Sodium azide	0.095 %

Reagent Preparation:

RG2 and STD were ready for use. The enzyme reagent 1a was prepared by mixing the contents of bottle ENZ with bottle RGT1 as a ratio 1:100.

Assay:

Wave length	570-600 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against reagent blank

Pipetting Scheme:

Pipette into cuvettes	Reagent blank	Sample or STD
Sample/STD		10 µl
Enzyme reagent 1a	1000 µl	1000 µl
Mix, incubate for 3 min at 37° C.		
RGT2	1000 µl	1000 µl

Sample was mixed with reagents and incubates for 5 min at 37° C. Absorbance of the sample was measured against reagent blank.

Calculation:

To obtain urea concentration from serum following formula was used according to the protocol:

$$\text{Conc.} = \frac{(\text{A}) \text{ Sample}}{(\text{A}) \text{ Standard}} \times 80(\text{factor}) = \text{mg/dL urea in the sample}$$

For, mmol/L factor = 13.3

All serum samples were analyzed in duplicate and then mean values were taken.

2.2.11 Statistical analysis

Statistical analysis for this study was performed using software of Statistical Packages for Social Sciences (SPSS). Blood parameters among the different groups of mice were analyzed by independent sample *t*-test.

2.3 Results

2.3.1 Analysis of the protective effects of *Raphanus sativus* leaves (RSL), *Momordica charantia* fruits (MCF) and *Brassica nigra* leaves (BNL) on sodium arsenite (Sa)-induced alteration of serum indices

2.3.1.1 Effect of RSL, MCF and BNL on Sa-induced alteration of serum lactate dehydrogenase (LDH) activity

Lactate dehydrogenase (LDH) is found extensively in body tissues. Elevated serum LDH activity has been recognized as a non specific marker for organ damage. It is reported that LDH activity is increased significantly in arsenic-treated mice and the human individuals exposed to arsenic chronically (Bhattacharjee and Pal, 2014; Karim et al., 2010). Serum LDH activities were evaluated in the all groups of experimental mice (Table 2.2). The levels of LDH activities in control, Sa, RSL, MCF, BNL, and RSL plus Sa, MCF plus Sa and BNL plus Sa were 119.56±13.20, 231.46±32.03, 106.87±28.43, 119.00±35.80, 177.63±21.32, 135.56±25.96, 134.30±33.26, 286.35±28.07, respectively. Serum LDH activities were found to be increased significantly ($p < 0.05$) after Sa treatment. Food supplementation of RSL and MC significantly inhibited the Sa-induced elevation of serum LDH activity. On the other hand, BNL alone increased the serum LDH activity. BNL further accelerated the Sa-induced LDH activity probably because of the synergistic action of BNL with Sa on serum LDH levels. These results suggested that RSL and MCF but not BNL could have protective effects on the Sa-induced elevation of serum LDH activity.

Table 2.2: Serum LDH activities in different groups of experimental mice

Serum index	Experimental groups				
	Control	Sa	RSL	RSL+Sa	
LDH (U/L)			106.87±28.43	135.56±25.96 ^b	
			MCF	MCF+Sa	
		119.56±13.20	231.46±32.03 ^a	119±35.80	134.30±33.26 ^b
			BNL	BNL+Sa	
			177.6±21.32	286.35±28.07	

Data were expressed as mean ± SE, n=6 for each group of mice. ^aSignificantly different from control at $p < 0.05$ and ^bsignificantly different from Sa treated group at $p < 0.05$. p -values were from independent sample t -test.

2.3.1.2 Effect of RSL, MCF and BNL on Sa-induced alteration of serum butyryl cholinesterase (BChE) activity

Previously Ali et al. (2010) showed that arsenic exposure decreased the BChE activity in human. Decreased BChE activity has been reported to be observed in hepatic dysfunction and neurotoxicity (Ali et al., 2010; Duysen et al., 2008; Uete et al., 1985). In this study, we investigated whether RSL, MCF and BNL could prevent the Sa-mediated decreased serum BChE activity (Table 2.3). Serum BChE activity (Mean \pm SE) measured in the control, Sa, RSL, MCF, BNL, and RSL plus Sa, MCF plus SA and BNL plus Sa were 11743.60 \pm 959.19, 8318.40 \pm 564.86, 11390 \pm 1370.45, 12391 \pm 1730.45, 9045 \pm 666.81, 10930 \pm 270.94, 10399.00 \pm 326.94 and 7028.33 \pm 1190.93 U/L, respectively. The results indicated that BChE activity was significantly ($p < 0.05$) lower in Sa group compared to the control group. Intriguingly, we observed that supplementation of RSL but not MCF and BNL significantly ($p < 0.05$) abrogated the Sa-induced perturbation of BChE activity. These results indicated that RSL could have protective effect against Sa-induced liver dysfunction and neurotoxicity.

Table 2. 3: Serum BChE activities in different groups of experimental mice

Serum index	Experimental groups				
	Control	Sa	RSL	RSL+Sa	
BChE (U/L)			11390 \pm 1370.45	10930 \pm 270.94 ^b	
			MCF	MCF+Sa	
		11743.60 \pm 959.19	8318.40 \pm 564.86 ^a	12391 \pm 1730.45	10399 \pm 326.94
			BNL	BNL+Sa	
			9045 \pm 666.81	7028.33 \pm 1190.93	

Data were expressed as mean \pm SE, n=6 for each group of mice. ^aSignificantly different from control at $p < 0.05$, and ^bsignificantly different from Sa treated group at $p < 0.05$. p -values were from independent sample t -test.

2.3.1.3 Effect of RSL, MCF and BNL on Sa-induced alteration of serum hepatic enzymes used for liver function test

Liver is the primary target organ for arsenic metabolism (Lu et al., 2001; Waalkes et al., 2000). Elevated levels of liver enzymes in serum represent liver dysfunction. Major serum enzymes used for liver function test are ALP, ALT and AST. In this

study, ALP, ALT and AST activities in the 8 groups (control, Sa, RSL, MCF, BNL, RSL plus Sa, MCF plus Sa and BNL plus Sa) of experimental mice were assessed. It was observed that Sa treatment significantly ($p < 0.05$) increased the activities of the all three enzymes in serum (Table 2.4). Intriguingly, supplementation of RSL significantly ($p < 0.05$) afforded the protection against Sa-induced perturbation of serum ALP, ALT and AST activities. MCF showed significant ($p < 0.05$) ameliorating effect on Sa-induced ALP activities. BNL could not show significant protective effects on Sa-induced perturbation of the activities of those three enzymes.

Table 2.4: Serum ALP, ALT and AST activities in the different groups of experimental mice

Serum indices	Experimental groups			
	Control	Sa	RSL	RSL+Sa
ALP (U/L)			107.36±12.35	245.62±6.01 ^b
			MCF	MCF+Sa
	121.73±4.73	330.23±19.44 ^a	100.37±7.70	260.40±26.35 ^b
			BNL	BNL+Sa
			114.60±7.70	178.50±15.73
ALT (U/L)			27.99±1.31	40.00±7.11 ^b
			MCF	MCF+Sa
	36.23±1.72	48.58±6.45 ^a	36.77±0.86	43.83±3.56
			BNL	BNL+Sa
			30.41±3.82	45.93±8.26
AST (U/L)			36.37±4.19	46.95±8.61 ^b
			MCF	MCF+Sa
	38.70±4.89	61.38±6.61 ^a	37.16±4.36	55.67±6.00
			BNL	BNL+Sa
			46.54±3.28	84.56±8.41

Data were expressed as mean ± SE, n=6 for each group of mice. ^aSignificantly different from control at $p < 0.05$ and ^bsignificantly different from Sa treated group at $p < 0.05$. p -values were from independent sample t -test.

2.3.1.4 Effect of RSL, MCF and BNL on Sa-induced alteration of high density lipoprotein cholesterol (HDL-C) levels

HDL-C has been reported to prevent atherosclerosis through its anti-inflammatory activity (Gordon et al., 1977; Kontush and Chapman, 2006). Proatherogenic roles of arsenic have been well documented (Karim et al., 2013; Simeonova and Luste, 2004; Wang et al., 2002). Karim et al. (2013) in their population based study conducted in the arsenic-endemic and non-endemic areas in Bangladesh showed that arsenic exposure decreased the circulating HDL-C levels. In this study, serum HDL-C levels in the all groups of experimental mice were evaluated (Table 2.5). The levels of HDL-C in the control, Sa, RSL, MCF, BNL, RSL plus Sa, MCF plus Sa and BNL plus Sa were 24.54±2.29, 18.91±3.75, 33.47±1.46, 30.85±4.03, 30.92±2.85, 30.75±1.05, 20.22±1.52 and 23.74±2.36 mg/dL, respectively. Results demonstrated that Sa treatment decreased significantly ($p < 0.05$) serum HDL-C levels. Intriguingly, RSL showed significant ($p < 0.05$) protection against Sa-induced perturbation of serum HDL-C levels. However, the protective effects of MCF and BNL on Sa-induced decreased level of HDL were not statistically significant.

Table 2.5: Serum HDL-C levels in different groups of experimental mice

Serum index	Experimental groups				
	Control	Sa	RSL	RSL+Sa	
HDL-C (mg/dL)			33.47±1.46	30.75±1.05 ^b	
			MCF	MCF+Sa	
		24.54±2.29	18.91±3.75 ^a	30.85±4.03	20.22±1.52
			BNL	BNL+Sa	
			30.92±2.85	23.74±2.36	

Data were expressed as mean ± SE, n=6 for each group of mice. ^aSignificantly different from control at $p < 0.05$ and ^bsignificantly different from Sa treated group at $p < 0.05$. p -values were from independent sample t -test.

2.3.1.5 Effect of RSL, MCF and BNL on Sa-induced serum urea level

Elevated level of serum urea is a marker of renal dysfunction (Carvounis et al., 2002; Finco and Duncan, 1976; Miller et al., 1978; Pedrini et al., 1988). In this study, serum urea levels in all groups of experimental mice were evaluated. As shown in Table 2.6, the serum urea levels (mean ± SE) of control, Sa, RSL, MCF, BNL, and RSL plus Sa,

MCF plus Sa, BNL plus Sa were 21.67±0.63, 31.67±1.97, 20.56±1.43, 24.01±1.63, 25.63±3.70, and 22.40±1.27, 29.65±2.76 and 28±8.70 mg/dL, respectively. Serum urea levels were significantly ($p < 0.05$) increased in Sa-treated mice compared to the control group. RSL supplementation could significantly ($p < 0.05$) abrogated the Sa-induced elevation of serum urea level. MCF and BNL could not show significant protective effects on Sa-induced serum urea levels.

Table 2. 6: Serum Urea levels in different groups of experimental mice

Serum index	Experimental groups				
	Control	Sa	RSL	RSL+Sa	
Urea (mg/dL)			20.56±1.43	22.40±1.27 ^b	
			MCF	MCF+Sa	
		21.67±0.63	31.67±1.97 ^a	24.01±1.63	29.65±2.76
			BNL	BNL+Sa	
			25.63±3.70	28±8.70	

Data were expressed as mean ± SE, n=6 for each group of mice. ^aSignificantly different from control at $p < 0.05$ and ^bsignificantly different from Sa treated group at $p < 0.05$. p -values were from independent sample t -test

2.4 Discussion

Arsenic exposure has been reported to be associated with dermatitis, variety of cancers, cardiovascular diseases, diabetes, peripheral neuropathy, and hepatic and renal dysfunction (Chen et al., 2007; Islam et al., 2011; Karim et al., 2013; Lai et al., 1994; Mazumder et al., 1998; Meliker et al., 2007; Murphy and Willenbrock, 1995; Okada et al., 1992; Tapio and Grosche, 2006; Vahidnia et al., 2008). Due to the notorious effects on human health, arsenic has become a major threat to the public health in Bangladesh and some other countries in the world. Widespread contamination of arsenic including foods and drinking water indicates that exposure to arsenic is unavoidable. Till today, no effective drugs have been developed that can reduce arsenic toxicity. In these circumstances, phytoremediation may be a plausible approaches for the reduction of arsenic toxicity in human. Therefore, this study was designed to check the efficacies of three edible plant materials: *Raphanus sativus* leaves (RSL), *Momordica charantia* fruits (MCF), *Brassica nigra* leaves (BNL)

against sodium arsenite (Sa)-induced perturbation of blood indices associated with organ damages and diseases.

Several soluble enzymes, proteins or other metabolites of serum have been considered as indicators of the organ damage, cardiovascular diseases, diabetics, and hepatic and renal dysfunctions. Pathogenic condition as well as organ dysfunction can be diagnosed by the alteration of serum indices. LDH in blood is often used as a marker for organ damage. Generally high concentrations of LDH are found in liver, heart, kidney, erythrocyte and skeletal muscle (Calbreath, 1992). Consequently, diseases affecting those organs such as renal dysfunction, hepatic disorders and myocardial infarction have been reported to be associated with significant elevation in total serum LDH activity. Usually, if tissue damage occurs, LDH is leaked from the damaged tissue or organ to blood where it is measured. In this study, it was observed that oral administration of Sa increased the activity of serum LDH levels in mice (Table 2.2). This result was consistent with the previous study that was conducted on human population (Karim et al., 2010). RSL and MCF showed significant ($p < 0.05$) protection against Sa-induced elevation of LDH activity. These results suggested that both RSL and MCF could have protective effects against Sa-induced organ damage. Serum BChE activity were decreased in Sa-treated mice significantly (Table 2.3). Effect of Sa exposure on serum BChE activity observed in this study were in good agreement with the results reported by Ali et al. (2010) in arsenic-endemic human subjects. BChE is considered to be a clinically important enzyme because it is involved in both liver function abnormalities and neurotoxicity by several toxic chemicals (Eisenbach et al., 2007; Montenegro et al., 2006; Rusyniak and Nañagas, 2004; Sugimura et al., 1995). Intriguingly, among the plant materials tested for this study, only RSL showed protective effect on Sa-induced serum BChE activity. The protective effect of RSL on Sa-induced serum BChE activity was noteworthy since liver dysfunction and neurological disorders are the major adverse health effects of chronic arsenic exposure (Ali et al., 2010; Islam et al., 2011; Le Quesne and Mcleod, 1977). Protective effect of RSL on Sa-induced liver dysfunction was further confirmed by the evaluation of the activities of other serum enzymes (ALP, ALT and AST) used for liver function test in the experimental mice (Table 2.4). Results showed that Sa treatment significantly increased the activities of serum ALP, ALT

and AST. Intriguingly, RSL exhibited significant protection against Sa-induced perturbation of the activities of those enzymes. MCF afforded limited protection on Sa-induced elevation of serum enzyme activities and significant ($p < 0.05$) protection was found only in the case of ALP. BNL did not show any significant effect on Sa-induced changes of these three enzymes.

In this study, it was observed that serum HDL-C levels were significantly lower in the mice treated with Sa than the non-treated control mice (Table 2.5). Decreased serum HDL-C levels observed in this study were in good agreement with the previous results in arsenic-exposed human subjects demonstrated by Karim et al. (2013) and Nabi et al. (2005). HDL-C removes deposits of LDL-C from the artery walls and returns it to the liver where they are broken down and eliminated from the body. For this reason HDL-C is considered to be protective against cardiovascular disease and is often referred to as "good" cholesterol (Gordon et al., 1977; Kontush et al., 2006). HDL-C transport from blood to liver where it is broken down (Barter et al., 2004, Zhang et al., 2005). Further, HDL-C is an anti-atherogenic factor with an antioxidant and anti-inflammatory properties. Through its antioxidant properties, HDL-C inhibits the conversion of LDL-C to oxidized LDL-C (Navab et al., 2007). The conversion of LDL-C to oxidized LDL by reactive oxygen species is now recognized as a predictive biomarker for the sub-clinical development of atherosclerosis and accepted as a key biochemical reaction in the initiation, progression and development of the atherosclerotic disease process (Albertini et al., 2002; Kopprasch et al., 2002; Kusuhara et al., 1997). Through anti-inflammatory properties, HDL-C also inhibits the secretion of different kinds of pro-inflammatory cytokines and molecules responsible for the development of atherosclerotic lesions by macrophage and endothelial cells (Barter et al., 2004). Interestingly, in this study, it was observed that food supplementation of RSL provided significant protection against Sa-mediated perturbation of serum HDL-C (Table 2.5). However, MCF and BNL could not show significant protective effects on Sa-mediated perturbation of HDL-C. Therefore, ameliorating effect of RSL on Sa-induced perturbation of HDL-C might be important for human in order to reduce the risk of arsenic-exposure related cardiovascular diseases.

Elevated serum urea level is correlated with an increased protein catabolism in mammalian body or from more efficient conversion of ammonia to urea as a result of

increased synthesis of enzyme involved in urea production in liver. Urea is excreted by kidney. Elevated level of serum urea is an indicator of renal dysfunction. In this study, it was found that Sa treatment increased the serum urea level in mice indicating the excessive catabolism of protein and renal dysfunction (Table 2.6). RSL but not MCF and BNL significantly inhibited the Sa-induced elevation of serum urea levels (Table 2.6), suggesting that RSL could have protective effect against Sa-induced renal dysfunctions.

This study tested the protective effects of three plant materials (RSL, MCF and BNL) against Sa-induced perturbation of blood indices. Results of this study stated that RSL among the plant material tested showed the highest protective effects against Sa-induced perturbation of blood indices. In this study, we could not show how RSL afford the protection against Sa-induced changes of blood indices. One possible mechanism is that RSL contains major active polyphenolics, (catechin, ferulic acid, protochatechuic acid, vanilic acid, sinapic acid) which have antioxidant and free radical scavenging activity (Beevi et al., 2010). Previous report suggested that arsenic-induced cellular dysfunction was mediated through the production of reactive oxygen species (ROS) (Hossain et al., 2000, 2003). Polyphenolic compounds present in RSL might inhibit Sa action through their antioxidant and free radical scavenging activities. More detail study, however, is needed in future to investigate what component(s) and how RSL reduces Sa-induced adverse effects. RSL is very cheap edible vegetable and nontoxic to human. Therefore, ameliorating effects of RSL against the adverse effects of Sa observed in this animal study could pave the ways for the future application of RSL against arsenic toxicity in human.

2.5 Conclusion

This study tested the efficacies of RSL, MCF and BNL against the Sa-induced perturbation of serum indices in mice. RSL and MCF but not BNL significantly could reduce the Sa-induced serum LDH activity. RSL also afforded protection against Sa-induced changes of BChE, ALP, ALT and AST activities and serum HDL-C levels, whereas MCF showed protection only on Sa-induced elevation of ALP activity, and BNL did not show any significant effect on Sa-induced perturbation of these blood parameters. Finally, RSL but not MCF and BNL significantly reduced Sa-induced serum urea level. Therefore, among three plant materials tested RSL showed highest efficacies against Sa-induced perturbation of blood indices related to hepatic, cardiovascular and renal dysfunction suggesting the future application of RSL against arsenic toxicity in human.

2.6 References

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CHAPTER III

Investigation of the protective effect of RSL on Sa-induced hepatic and renal expression of heat shock protein genes through molecular approaches

Chapter 3: Investigation of the protective effect of RSL on Sa-induced hepatic and renal expression of heat shock protein genes through molecular approaches

3. Abstract

Since *Raphanus sativus* leaves (RSL) showed the highest ameliorating effect against sodium arsenite (Sa)-induced perturbation of blood indices among the three plant materials tested in chapter 2, protective effect of RSL against Sa action were investigated in this section through molecular approaches. Molecular part of this study targeted the hepatic and renal expression of heat shock protein (HSP) genes that included HSP90 α , HSP90 β and HSP70. HSPs are stress-sensitive molecular chaperon that can be expressed by heat, oxidative stress, heavy metals etc. Liver and kidney samples from the experimental mice were collected as it was described in the Chapter 2. Total RNA was extracted from the livers and kidneys of control, Sa, RSL and RSL plus Sa groups of mice, and mRNA expression was analyzed by regular reverse transcription polymerase chain reaction (RT-PCR) and real time polymerase chain reaction (qRT PCR). In regular RT-PCR analysis, it was observed that Sa treatment increased the expression of hepatic and renal expression of HSP90 α , HSP90 β and HSP70. Intriguingly, food supplementation of RSL abrogated the Sa-induced hepatic expression of HSP90 α , HSP90 β and HSP70 genes. On the other hand, RSL was found to reduce renal expression of Sa-induced HSP90 β . Regular RT-PCR data also indicated that RSL had minimum or almost no protective effect on Sa-induced expression of HSP90 α and HSP70 genes in kidney. In real time PCR analysis, Sa treatment significantly ($p < 0.05$) increased the hepatic expression of HSP90 α , HSP90 β and HSP70 genes as compared to the control group of mice, and food supplementation of RSL could significantly ($p < 0.05$) inhibit the Sa-induced expression of all three HSP genes in liver. Real time PCR data indicated that relative expression of renal HSP90 α , HSP90 β and HSP70 were generally higher in Sa-treated mice compared to the control mice. However, elevations of the expression of hepatic HSP90 α and HSP90 β but not HSP70 were statistically significant ($p < 0.05$). In real time PCR analysis, although food supplementation of RSL showed a general trend in the inhibition of Sa-induced expression of HSP genes but only the inhibition of HSP90 α expression was statistically significant ($p < 0.05$). Thus the protective effects of RSL against Sa-induced molecular perturbation of hepatic and renal HSP gene transcriptions suggested the future application of RSL against arsenic toxicity in human.

3.1 Introduction

Under normal physiological conditions, a complete set of functionally competent proteins are maintained in the cells. When cell exposed to stress, disturbance of the intracellular milieu induces a stress response in the cell by inhibiting the expression of many housekeeping genes and by up regulating the expression of stressor genes to maintain protein homeostasis (Soo et al., 2008; Gao et al., 2004). Among the stressor proteins heat shock proteins (HSPs) are the prominent that can be over expressed in cells and tissues in response to the wide variety of stresses.

HSPs are an evolutionary family of proteins. They were first described in relation to heat shock (Ritossa et al., 1962). Later heat shock proteins are found to be associated with the response to other stress including exposure to cold, UV light and toxic chemicals (Bierkens et al., 1998; Cao et al., 1999; Matz et al., 1995). The functions of HSPs include protection and tolerance against cytotoxic conditions through their molecular chaperoning activity, maintaining cytoskeleton stability, and assisting in cell signaling. According to their size, they have been classified into the following families; HSP90, HSP70, HSP60, HSP40 and HSP27. Each HSP family may have several subtypes such as, α and β , the two sub types of HSP90 are found in human and mice. HSPs prevent the formation of nonspecific protein aggregates and they assist proteins in the acquisition of their normal architecture. Moreover, HSPs are likely to have anti-apoptotic properties and are actively involved in various processes as tumor cell proliferation, invasion, metastases and death. Notably, these proteins have been reported to be significantly elevated in a plethora of human cancers. Their over-expression has been robustly associated with therapeutic resistance and poor survival in cancer (Lianos et al., 2015). In this way, HSPs may have important therapeutic implications and they can be targeted by specific drugs in cancer treatment. Not only cancer, HSPs have been reported to be implicated in the development of atherosclerosis, a main biochemical event for cardiovascular diseases. Studies have reported that several HSPs may be potential risk markers of atherosclerosis and related cardiovascular diseases, or may be directly involved in the atherogenic process (Benjamin and McMillan, 1998; Snoeckx et al., 2001; Xu, 2002). HSPs especially HSP70 are expressed by cells in atherosclerotic plaque (Berbarian et al., 1990). Further several forms of HSPs are involved in hepatic injury, renal dysfunction and

neurological disorders (O'Neill et al., 2014; Sharp et al., 2013; Walsh et al., 2009). Previous reports have suggested that heavy metal exposure can induce several forms of HSPs (Bernstam and Nriagu, 2000; Wagner et al., 1999). Because of their responses to environmental chemicals, HSP genes have been suggested to be sensitive molecular biomarker for aquatic monitoring of heavy metal pollution. Arsenic has been reported to induce several forms of HSPs (Bernstam and Nriagu, 2000; Han et al., 2005; Xu et al., 2013). Previous chapter (Chapter 2) of the thesis mainly focused on the investigation of the efficacies of plant materials against the changes of blood biomarkers related to organ dysfunction caused by Sa exposure through biochemical approaches. Results in our previous chapter clearly indicated that among the plant materials tested, *Raphanus sativus* leaves (RSL) showed the most excellent efficacies against Sa-induced perturbation of blood biomarkers. Since HSPs have been reported to be sensitive molecular markers for environmental pollutant especially arsenic, in this section of the thesis, effects of RSL on the Sa-induced changes of the expression of several forms of HSP genes in hepatic and renal tissues were investigated through molecular approaches.

3.2 Materials and Methods

3.2.1 Chemicals and equipments

3.2.1.1 List of chemicals and test kits

1. TRIzol reagent
Life Technologies, Carlsbad, CA, USA.
2. ReverTra Ace- α -R
Toyobo, Osaka, Japan.
3. Primers
Invitrogen, Carlsbad, CA, USA.
4. DNase and RNase free water.
5. PCR master mix
Promega, Madison, WI, USA.
6. 100 bp DNA ladder
Life Technologies, Carlsbad, CA, USA.
7. DNA gel stain
Life Technologies, Carlsbad, CA, USA.
8. Agarose gel
Sigma-Aldrich.
9. CYBR safe DNA gel stain
Life Technologies, Carlsbad, CA, USA.

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10. TAE (Tris-acetate EDTA) buffer,
Life Technologies, Carlsbad, CA, USA.

3.2.1.2 List of equipments

The major equipments:

1. Autoclave, (ALP Co. Ltd. Tokyo, Japan).
2. -80° C freezer, (Sanyo Electric Co. Ltd, Japan).
3. -20° C freezer, (Walton, Bangladesh).
4. Centrifuge machine, (Eppendorf, Germany).
5. Nanodrop spectrophotometer, (Thermo Scientific, USA).
6. Regular PCR (thermal Cycler), (Applied Biosystems, USA).
7. Gel Electrophoresis, (UVP, USA).
8. Digital measuring balance,(Unilab Instruments, USA)
9. Gel documentation (Syngene, UK)
10. Real Time PCR (Winco International L.L.C, USA)

3.2.2 Extraction of RNA

Tissues were collected from the experimental mice as described in the previous chapter (Materials and Methods in Chapter 2). Total RNA was purified from hepatic and renal tissues of Control, Sa, RSL, RSL+Sa group of mice via TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was quantified and stored at -80°C. UV spectrophotometric quantification by nanodrop spectrophotometer (ND 2000) provided by Thermo Scientific (USA) was used to determine the concentration of RNA (ng/μl).

3.2.3 Preparation of cDNA

The RNA samples extracted from kidney and liver from the experimental mice were used for the preparation of cDNA. ReverTra Ace-α-R (Toyobo, Osaka, Japan) was

used to synthesize high quality cDNA from RNA (Hatta et. al., 2002). RNA samples were normalized with a volume of DNase and RNase free water by mixing a volume of total RNA for 200 ng up to 10 μ l total volume and then 5xRT buffer, dNTP mix, OligodT, RNase inhibitor into PCR tubes were added and mixed the preparation very well. After placing those PCR tubes in the thermal cycler, at first the preparation was incubated at 42°C for 20 min, then heated at 99°C for 5 min and finally stored the reacted solution at -20°C.

3.2.4 Reverse transcription (RT)-polymerase chain reaction (PCR) analysis

RT-PCR was performed as described previously (Meary et al., 2007). Semiquantitative RT-PCR was carried out by using PCR master mix (Promega, Madison, WI, USA; Catalog- M7501). Primer sequence for HSP90 α , forward AAACACCTGGAGATAATCCTGA and reverse TCAAAAATATAAAGCTTGAAT, HSP90 β , TCTTAAAGAAGATCAGACAGAG and reverse ATTCCCTCTCTACTCTTGA CAG, HSP70, forward, TGGTGCTGACGAAGATGAAG and reverse, CGACAAGGCCGCCTGAGCAA and β -actin, forward CTTCGAGCAAGAGATGGC, and reverse, GGTAGTTTCGTGGATGCC were obtained from Invitrogen (Carlsbad, CA, USA). β -actin were used as an equal loading control. PCR products were analyzed by electrophoresis on 1.5% agarose gel. The gels were stained with CYBR safe DNA gel stain (Life Technologies, Cat. No.: S33102). The 100 bp DNA ladder (Life Technologies, Carlsbad, CA, USA) was used for the size marker.

3.2.5 Quantitative reverse transcription–polymerase chain reaction (qRT PCR) analysis

Expression level of HSP 90 α , HSP 90 β and HSP70 were checked by quantitative real time PCR (qRT PCR) essentially as described elsewhere (Wagatsuma et. al., 2005). Forward and reverse primers for HSP90 α , HSP90 β and HSP70 and β -actin as shown in RT PCR section were used for the amplification of first stand cDNA by SYBR Green qPCR master mix (Wittwer, 1997). Thermal cycling and continuous monitoring of fluorescence detection system was maintained by Real Time PCR (Winc International L.L.C, USA) using universal temperature cycles: 10 min at 94°C followed by 35-45 two temperature cycles (15 sec at 94°C and 1 min at 60°C). The

relative expression of genes with respect to internal control; β -actin were analyzed with the corresponding software, and the number of PCR cycles to reach the threshold of detection (CT) was calculated. Samples were run in triplicates. The expression levels of respective genes in each sample were presented as fold increase to the mean value of the control.

3.3 Statistical Analysis

Statistical analysis for this study was performed by using software of Statistical Packages for Social Sciences (SPSS). Expression HSP genes among the different groups of mice was analyzed by independent sample *t*-test.

3.4 Results

3.4.1 Analysis of the effects of RSL on Sa-mediated expression of hepatic and renal HSP genes by regular RT-PCR

Hepatic and renal gene expression patterns of HSP90 α , HSP90 β and HSP70 in control, Sa, RSL and RSL plus Sa groups of mice were shown in Figure 3.1 and 3.2, respectively. It was observed that Sa treatment increased the hepatic expression of HSP90 α (upper panel), 90 β (second from the upper panel) and HSP70 transcripts (third from the upper panel) (Figure 3.1). Interestingly, RSL could reduce the Sa-induced expression of those transcripts. Like the hepatic expression of HSP genes, Sa treatment increased the renal expression of HSP90 α , HSP90 β and HSP70. RSL supplementation gave protection against Sa-induced hepatic expression of all three forms of HSP genes (Figure 3.2). On the other hand, RSL was found to reduce renal expression of Sa-induced HSP90 β . In regular RT-PCR data it was observed RSL had almost no protective effects on Sa-induced expression of HSP90 α and HSP70 genes. Lower panel in both Figure 3.1 and 3.2 indicated the expression of β actin as an internal control.

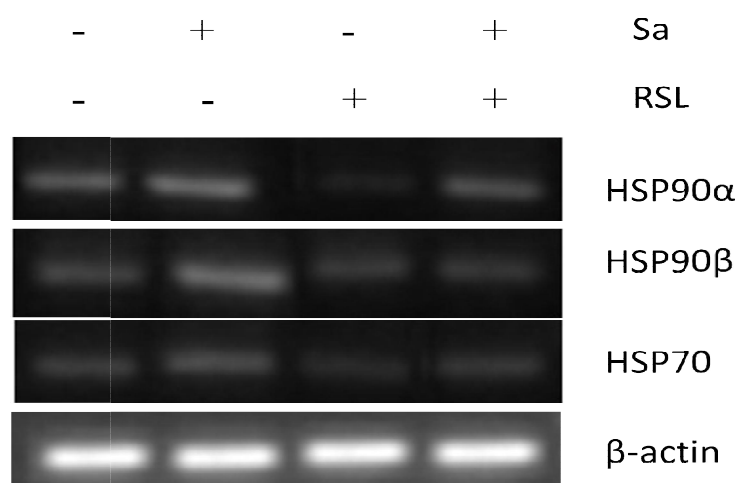


Figure 3.1: Analysis of the protective effects of RSL on Sa-induced hepatic expression of HSP genes through RT-PCR. Hepatic expression levels of HSP90α (upper panel), HSP90β (second from the upper panel) and HSP70 (third from the upper panel) of four different experimental groups of mice (from left: lane 1, control; lane 2, Sa; lane 3, RSL; lane 4, RSL plus Sa). β-actin were used as an internal control (lower panel). A representative results of one mouse from each group (n = 6) were shown.

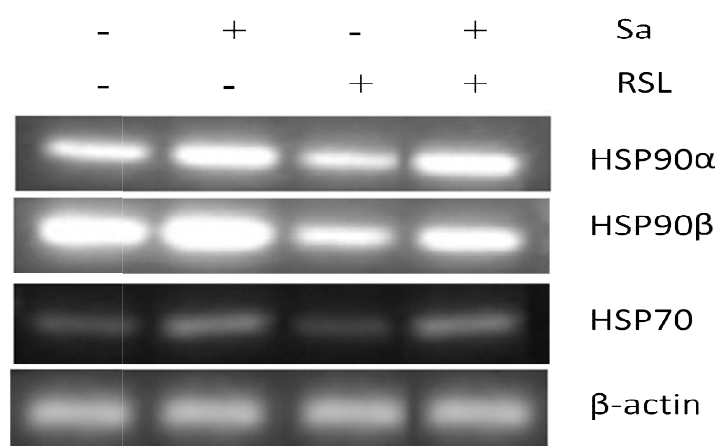


Figure 3.2: Analysis of the protective effects of RSL on Sa-induced renal expression of HSP genes through RT PCR. Renal expression levels of HSP90α (upper panel), HSP90β (second from the upper panel) and HSP70 (third from the upper panel) of different experimental groups of mice (from left: lane 1, control; lane 2, Sa; lane 3, RSL; lane 4, RSL plus Sa). β-actin were used as an internal control (lower panel). A representative results of one mouse from each group (n = 6) were shown.

3.4.2 Analysis of the effects of RSL on Sa-induced expression of HSP genes by quantitative real-time PCR (qRT-PCR)

Next, effects of RSL on Sa-induced expression of hepatic (Figure 3.3) and renal (Figure 3.4) HSP genes were examined by qRT-PCR. It was observed that relative expression of hepatic HSP90 α (Figure 3.3A), HSP90 β (Figure 3.3B) and HSP70 (Figure 3.3C) were significantly higher in Sa-treated mice than those in control mice. Intriguingly, RSL supplementation significantly ($p < 0.05$) inhibited Sa-induced expression of all these three hepatic HSP genes. Real time PCR data indicated that relative expression of renal HSP90 α (Figure 3.4A), HSP90 β (Figure 3.4B) and HSP70 (Figure 3.4C) were generally higher in Sa-treated mice compared to the control mice. However, elevations of the expression of HSP90 α and HSP90 β but not HSP70 were statistically significant ($p < 0.05$). Although food supplementation of RSL showed a general trend in the inhibition of Sa-induced expression of HSP genes in kidney but only the inhibition of HSP90 α expression was statistically significant ($p < 0.05$).

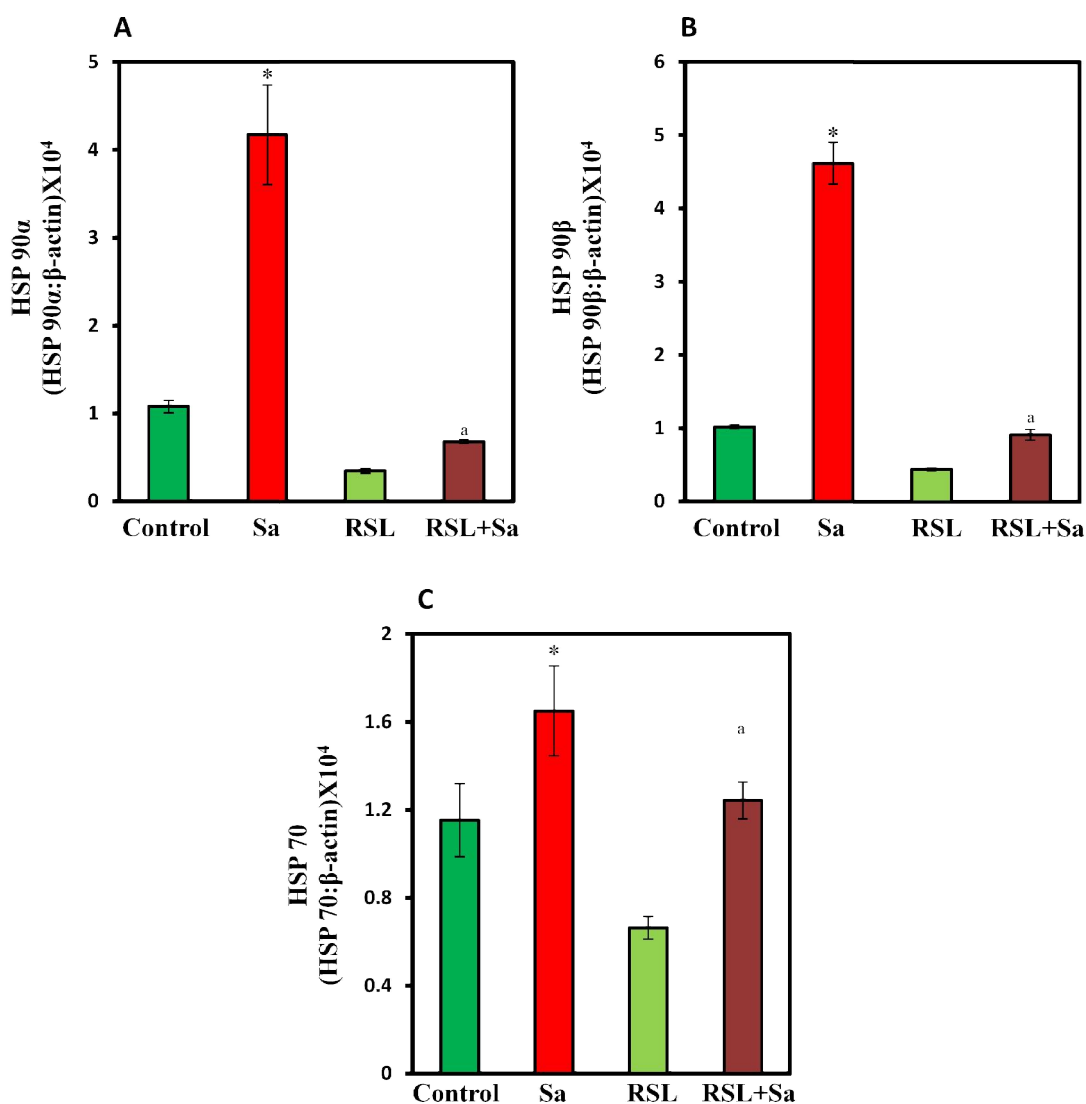


Figure 3.3: Analysis of the protective effects of RSL on Sa-induced expression of hepatic HSP genes through real time PCR (qRT-PCR). Hepatic HSP90 α , HSP90 β and HSP70 mRNA expression levels of four groups (n=6 mice in each group) of mice [control (C), Sa, RSL, RSL plus Sa] were quantified by real-time PCR, normalized to the mRNA expression levels of β actin. Bars depicted the expression levels (mean \pm SD) of respective gene which was presented as fold increase to the mean value of control of each group of mice (n=6 mice/group). *Significantly different from control at $p < 0.05$; ^asignificantly different from Sa at $p < 0.05$. p -values were from independent sample t -test.

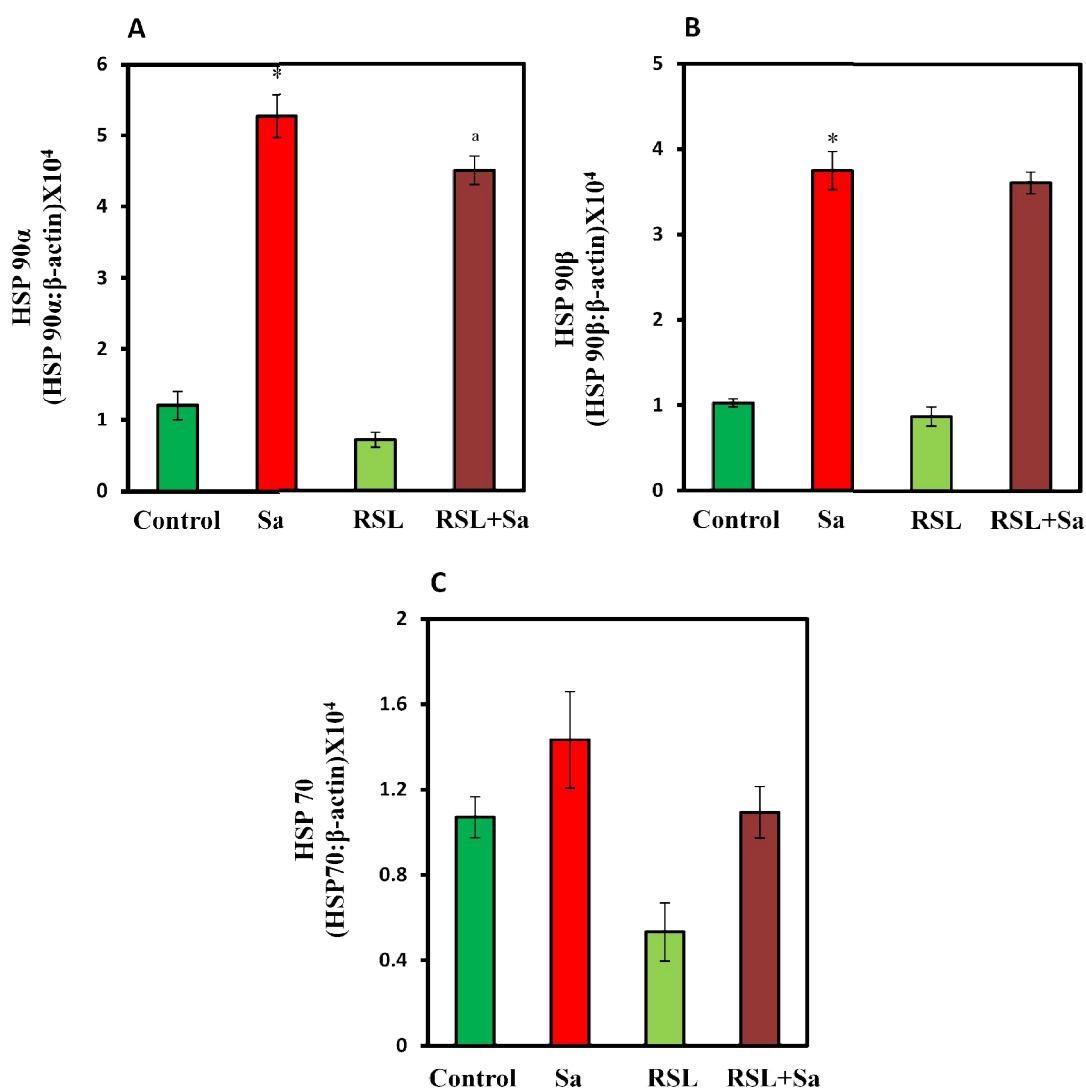


Figure 3.4: Analysis of the protective effects of RSL on Sa-induced expression of renal HSP genes through real time PCR (qRT-PCR). Renal HSP90 α , HSP90 β and HSP70 mRNA expression levels of four groups (n=6 mice in each group) of mice [control (C), Sa, RSL, RSL plus Sa] were quantified by real-time PCR, normalized to the mRNA expression levels of β actin. Bars depicted the expression levels (mean \pm SD) of respective gene which was presented as fold increase to the mean value of control of each group of mice (n=6 mice/group). *Significantly different from control at $p < 0.05$; ^asignificantly different from Sa at $p < 0.05$. p -values were from independent sample t -test.

3.5 Discussion

Regular RT-PCR and real time PCR data indicated that Sa treatment increased the hepatic and renal expression of HSP90 α , HSP90 β and HSP70 genes (Figure 3.1, 3.2, 3.3 and 3.4). Food supplementation of RSL to the mice inhibited the Sa-induced elevation of these genes in liver. Further real time PCR data indicated the general trend in the inhibition of Sa-induced expression of HSP genes in kidney by food supplementation of RSL.

Enhanced expression of HSPs is observed in response to the stressors such as temperature, toxic pollutant, chemicals, and irradiation. However, these proteins are also expressed in normal physiological conditions ubiquitously. HSPs function as molecular chaperones. HSPs are known to afford protection against protein aggregation, induce solubilisation of loose protein aggregates, facilitate folding of nascent polypeptides, participate in refolding of denatured proteins, and sequester damaged proteins and target them for degradation (Csermely et al., 1998; Hartl, 1996; Soti and Csermely, 2002). Further as chaperone HSPs have been reported to be played role in the transport of newly synthesized proteins within cells (Kiang et al., 1998). HSPs have been shown to protect cells against apoptosis induced by oxidative or other stresses (Buzzard et al., 1998; Garrido et al., 2001; Jaattela et al., 1998; Kirchhoff et al., 2002; Wang et al. 1995; Yaglom et al., 1999). Thus HSPs appear to be important in preventing damage and in cellular repair process after injury (Xu, 2002). Arsenic induces oxidative stress and arsenic-induced intracellular signals are largely mediated through the production of reactive oxygen species (ROS) (Hossain et al., 2000 and 2003). Arsenic-induced expression of HSPs observed (Figure 3.1, 3.2, 3.3 and 3.4) in this study was consistent with the previous studies reported by several other groups (Bernstam and Nriagu, 1998; Darach et al., 2005; Kim et al., 2005; Miura et al., 2014; Song et al., 2010).

It has been reported that over expression of HSPs are associated with several diseases that included a wide range of human carcinoma and cardiovascular diseases. HSPs are over expressed in solid tumors and haematological malignancies. (Chant et al., 1995; Ciocca et al., 1993; Kimura et al., 1993; Neckers, 2007; Ralhan et al., 1995; Takayama et al. 2003; Whitesell and Lindquist, 2005). Over expressed HSPs in cancer cells maintain protein homeostasis, and promote cell survival and proliferation

by inhibiting cell death (Soo et al., 2008; Whitesell and Lindquist, 2005). Over expressed HSPs allow cancer cells to tolerate changes from potentially lethal mutations that have role in carcinogenesis. HSPs act as biological buffers for genetic lesions found in cancer, allowing mutated proteins to perform their malignant functions (Soo et al., 2008; Whitesell and Lindquist, 2005). Because of their important roles in tumorigenesis and cancer progression, HSPs have been recognized as therapeutic target for cancer treatment. Since HSPs function through abrogating the apoptotic signals, inhibitors of HSPs can contribute to apoptosis of cancer cells. Based on this fact several inhibitors have been developed against different forms of HSPs (Itoh et al., 1999; Nadler et al., 1992; Wadhwa et al., 2000), and inhibitors of HSP90 have shown promising results in clinical trials against cancer. On the other hand, in cardiovascular pathology, several forms of HSPs were found to be implicated in atherosclerosis. Elevated levels of HSP70 were found in human and rabbit arteries and its distribution relation to necrosis and lipid accumulation (Berberian et al., 1990). Another study reported that HSP70 were higher in hypertensive patients than those of normotensive controls (Pockley et al., 2002). The same group also showed that antibodies against HSP70 had a protective role against the progression of atherosclerosis in hypertensive patients (Pockley et al., 2003). Further Hoppichler et al. (2000) reported the prognostic significance of HSPs in predicting morbidity and mortality due to atherosclerosis. All these reports indicated the involvement of HSPs in cardiovascular pathology. Major causes of arsenic exposure-related mortality are cancer and cardiovascular diseases. Therefore, inhibition of Sa-induced expression of HSP90 α , HSP90 β and HSP70 by food supplementation of RSL (Figure 3.3 and 3.4) observed in this study was noteworthy. In this study, however, we could not show how RSL could inhibit Sa-induced up-regulation of HSPs. Further study is needed to elucidate the mechanism of the effect of RSL on Sa-induced up-regulation of HSP genes in liver and kidney. RSL has antioxidant and free radical scavenging activity. From this point of view, it was hypothesized that RSL could inhibit Sa-induced expression of HSP genes through its potent antioxidant and free radical scavenging activity.

Advantage of phytobioremediation of toxic element is that most of the plant materials are non toxic to human. RSL is an edible vegetable and it is popular among the people in many countries including Bangladesh and India. Protective effects of RSL against

arsenic-induced up-regulation of HSP genes observed in this study has paved the way for the future investigation of the effect of RSL on other arsenic-mediated signals implicated in toxicity. Liver and kidney are the vital target organs for the metabolism and excretion of arsenic, respectively. Therefore, protective effects of RSL against arsenic-induced molecular perturbation in liver and kidney indicate the usefulness of RSL for the people who are now under the threat of arsenic poisoning.

3.6 Conclusion

Regular RT-PCR and real time PCR analysis showed that oral administration of Sa to the experimental mice increased expression of HSP90 α , HSP90 β and HSP70 genes in liver. Food supplementation of RSL could abrogate the expression of Sa-induced all three forms of HSP genes in liver. Regular RT-PCR and real time PCR data indicate that arsenic exposure increased the expression of HSP90 α , HSP90 β and HSP70 genes in kidney. Although food supplementation of RSL showed a general trend in the inhibition of Sa-induced expression of HSP genes in real time PCR analysis but only the inhibition of Sa-induced HSP90 α expression was statistically significant. Thus the protective effects of RSL against Sa-induced molecular perturbation of hepatic and renal HSP gene transcriptions suggest the future application of RSL against arsenic toxicity in human.

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Limitations

This study investigated the efficacies of three plant materials [*Raphanus sativus* leaves (RSL), *Momordica charantia* fruits (MCF), and *Brassica nigra* leaves (BNL)], against sodium arsenite (Sa)-induced adverse effects through animal experiment. Among the three plant materials, RSL was found to be the most effective against Sa-induced changes of serum biomarkers of hepatic, cardiovascular and renal dysfunction. RSL was also found to have protective effects against Sa-induced expression of hepatic and renal expression of heat shock protein (HSP) genes. Although this study depicted the ameliorating effects of RSL on Sa-induced adverse effects through biochemical and molecular approaches, there were several limitations of this study that warranted further discussion.

1. Plant materials for this study were selected based on the available information regarding the antioxidant and free radical scavenging activity. This study did not measure the antioxidant and free radical scavenging activity of plant materials. Several plant materials should have been screened first through checking their antioxidant activities. Then plant materials should have been selected for this study based on their levels of antioxidant activities. This way of selection of plant materials would be more scientific.
2. Based on the previous papers (Karim et al., 2010; Sheikh et al., 2014; Rahman et al., 2012) arsenite (Sa) dose (10 mg/kg body weight/day) for this study were selected. Before selecting the dose of Sa, a dose-dependent experiment should have been conducted to select minimum dose of Sa that could significantly change the blood indices and gene expression. This minimum dose of Sa could be more relevant to the human exposure to environmental arsenic.
3. Chapter 3 of this study only focused on the protective effects of RSL on the Sa-induced expression of HSP genes. There are other forms of HSPs that were not investigated in this study. Further arsenic has been reported to be associated with the expression of many other genes. Therefore, more extensive molecular study is needed in future to check efficacies of RSL on Sa-mediated expression of other genes. Further, gene expression does not always well correspond to the protein expression. Therefore, it is needed to investigate the effects of RSL on Sa-induced HSPs through western blotting.

4. Five times higher amount of plant materials than the dose of Sa were used in this study that showed the ameliorating effects against Sa-induced changes of blood indices. However, it was not known whether low dose of RSL could reduce arsenic toxicity. Therefore, dose-dependent experiment of RSL against arsenic-toxicity is required in future.

5. This study could not demonstrate how RSL ameliorated Sa-induced perturbation of hepatic, cardiovascular and renal biomarkers in blood, and expression of hepatic and renal HSP genes. Extracting several components of RSL and then checking the efficacies of those components against arsenic toxicity should be future expansion of this study.

6. RSL was the most effective against Sa-induced perturbation of blood indices related to the hepatic and renal dysfunction compared to the two other plant materials (MCF and BNL). Based on this fact, protective effect of RSL against Sa-induced expression of hepatic and renal HSP genes were analyzed. However, we could not rule out the protective effects of MCF and BNL against Sa-induced expression of HSP genes in liver and kidney.

In spite of those limitations, protective effects of RSL on Sa-induced perturbation of blood indices, and hepatic and renal expression of HSP genes indicate the future application RSL to reduce the arsenic toxicity in human.