EFFECT OF SPIRULINA (*Spirulina platensis)* AND RIBOFLAVIN AGAINST ARSENIC TOXICITY IN RAT

A THESIS

BY

SHEFALY AKTER

Registration No.: 1605514

Session: 2016-2017

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MASTER OF SCIENCE (MS)

IN

PHARMACOLOGY

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DEDICATED TO MY BELOVED PARENTS

ABSTRACT

This study was undertaken to observe the effects of spirulina (*Spirulina platensis*) and riboflavin in hematological and biochemical parameters against arsenic toxicity in Long Evans rat. Sixty male rats were taken to perform the study. Experimental rats were divided into five equal groups. Each group consists of 12 rats. Animals of T_0 group were given normal feed and water and kept as control. Rats of T_1, were given arsenic trioxide @ 100 mg/L drinking water orally. Rats of group T_2 were given arsenic trioxide @ 100 mg/L drinking water and with spirulina @ 1 gm/kg feed. Group T_3 were given arsenic trioxide @ 100 mg/L with riboflavin @ 10mg /kg body weight. Group T_4were given arsenic trioxide and spirulina and riboflavin with same dose up to 45 days respectively. Four rats from each group (T_0, T_1, T_2, T_3 and T_4) were sacrificed at 15 days interval to determine body weight, hematological and biochemical parameters. Result showed that in group T_1, body weight gain was minimum, whereas in groupT_2, T_3 and T_4 the body weight gain in rats were better. Reduction of TEC, Hb values were significant ($P<0.01$) in T_1 group. Whereas in rest groups reduction of TEC, Hb were less than arsenic treated groups. The values of SGOT and SGPT were significantly ($P<0.01$) decreased in all group but in it is more effective in combined group. In conclusion, spirulina and riboflavin has significant effect on hematological and biochemical parameters and increase body weight.

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LIST OF ABBREVIATIONS

µg	Microgram
As	Arsenic
BW	Body weight
CAT	Catalase
DMA	Dimethylarsinic acid
ESR	Erythrocyte sedimentation rate
et al.	Associates
FAD	Flavin adenine dinucleotide
FMN	Flavin adenine mononucleotide
g	Gram
GLA	γ- Linoleic acid
Hb	Haemoglobin
HDL	High density lipoprotein
ICDDRB	International centre for diarrhoeal disease research Bangladesh
IEH	Institute of environment and health
Kg	Kilogram
L	Litter
LDL	Low density lipoprotein
LH	Luteinizing hormone
MCH	Mean corpuscular haemoglobin
MCL	Maximum contaminant level
MCV	Mean corpuscular volume
MDA	Malondialdehyde
mg	Milligram
MMA	Monomethylarsinate
$NaAsO_2$	Sodium arsenite
NO	Nitricoxide
PCV	Packed cell volume
rpm	Rotation per minute
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxide dismutase
SPI	Spinal cord injured
TC	Total cholesterol
TEC	Total erythrocyte count
TLC	Total leukocyte count
WHO	World health organization

CHAPTER 1

INTRODUCTION

Arsenic is a shiny grayish non essential trace element that is widely distributed in nature. It appears in three allotropic forms in nature. The forms are yellow, black and gray; the stable form is a silver gray, brittle crystalline solid. Arsenic can be found in both organic and inorganic forms in water, food, air soil. The most important inorganic forms of arsenic compounds are arsenic trioxide, sodium arsenite, arsenic trichloride, arsenic acid and arsenites (trivalent forms) and lead and calcium arsenates (pentavalent forms). Common organic arsenic forms are arsanilic acid monomethylarsinate (MMA), dimethylarsinic acid (DMA) (David J. Thomas *et al.,* 1995) and arsenobetaine. The inorganic forms of arsenic exhibit the highest toxicity level (FAO, WHO, 1983).

The Earth's crust is an abundant natural source of arsenic. It also found in small quantities in rock soil, water and air. It also found due to industrial exposure. The safety limit of arsenic accepted by Bangladesh Government is 0.05 mg/liter for drinking water (WHO, 1999). The World Health Organization limit for drinking water 0.01 mg/liter and far foodstuffs is 2 mg/liter on a fresh weight basis (Robinson *et al.*, 2003).

Groundwater arsenic contamination in Bangladesh is reported to be the biggest arsenic calamity in the world in terms of the affected population (Talukder *et al.,* 1998). Chronic arsenic exposure is associated with many human health conditions, including skin lesions and cancers of the liver, lung, bludder and skin (Uddin and Huda, 2011). Arsenicosis presents with significant changes in the serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum creatinine, urea, uric acid levels and various hemato- logical parameters like TEC, TLC, Hb, blood sugar level in the Swiss albino rats (Yasmin *et al.,* 2011). The clinical feature initially invariably relate to the gastrointestinal system and are nausea, vomiting, colicky abdominal pain and profuse watery diarrhoea (Mueller PD *et al.,* 1989). Arsenic can enter into the blood chain (Ulman *et al.,* 2004). Recently arsenic intoxication in experimental animals has been found to be associated with hepatic tumors (Waalkers *et al.,* 2003), Spermatogenesis (Sukla and Pandey, 1984), Inhibition of testicular steroidogenic function (sarker *et al.,* 1991) and severe metabolic disorders such as diabetes in human (Longnecker and Daniels, 2001, Tseng *et al.,* 2002). The natural source of human exposure to arsenic occurs through consumption of drinking water sourced from groundwater that contains dissolved inorganic arsenic (Nandi *et al.,* 2006).

Significantly elevated standard mortality from cancer of the bladder, lung, liver, kidney, skin and colon were found in the population living in an area of Taiwan, China and some part of Africa where arsenic contamination of the water supply was endemic (Azcue and Nriagu, 1995; Meliker *et al.*, 2007 and Asaolu, 2010).

Still there is no specific curative treatment against arsenic toxicity. Immediate stoppage of drinking arsenic contaminated water and consumption of arsenic free drinking water are the mainstay of therapy (Dey, 2002).

Many studies have shown that vegetables possess valuable effects against oxidative stress related diseases through scavenging free radicals and by enhancing the activities of antioxidant enzymes (Orozco *et al.,* 2003; Gupta and Flora, 2006; Hord *et al.,* 2009). Chelation therapy is recommended through intravenous line but the use of chelators in patients exposed to arsine gas is controversial (Anderson *et al.,* 2016).

Spirulina is a microscopic filamentous aquatic non-toxic blue-green algae belongs to the group cyanobacterium (genus *Spirulina*, especially *S. platensis* synonym *Arthrospira platensis*) (Spolaore*et al.,* 2006) that is rich in proteins, lipids, carbohydrates, β-carotene, riboflavin, α-tocopherol and α- linoleic acid (El-Desoky *et al.,* 2013). It is not only a whole food, but it seems to be an ideal therapeutic supplement. Spirulina has been shown to prevent cataract (Haque *et al.,* 2005), acute allergic rhinitis and vascular reactivity (Mao *et al.,* 2005) and cerebral ischemia (Khan *et al.,* 2005) has also been shown to be effective against cadmium and arsenic induced-toxicities (Saha *et al.,* 2005). Spirulina alone or in combination with other vitamin and/or mineral was found to be effective in the removal of arsenic from arsenic-loaded tissues in various species including man (Fariduddin *et al.,* 2001; Misbahuddin *et al.,* 2006; Awal, 2007), in the treatment of chronic arsenic poisoning (Khan *et al.,* 2001), in reducing arsenic toxicity induced skin manifestations of patients in Bangladesh (Karim *et al.,* 1999). A recent study by the Bangabandhu Sheikh Mujib Medical University found improvement in skin manifestation of arsenic-stricken patients after they were given treatment of spirulina. Spirulina is known to strengthen the immune system and is used for treatment of HIV and AIDS (Teas *et al.,* 2004). It also exhibits antiviral (Herna´ndez-Corona *et al.,* 2002), anti-bacterial (Ozdemir *et al.,* 2004), anti-platelet, anti-cardiotoxic, hypocholesterolemic and anti-nephrotoxic effects (Khan *et al..,* 2006). Spirulina extract plus zinc was found to be advantageous in patients of chronic arsenic poisoning (Misbahuddin *et al.,* 2006). It was found in the laboratory that the natural carotene of

spirulina could inhibit, shrink and destroy oral cancer cells. Phycocyanin of spirulina also prevents cancer and its growth (Peto *et al.,* 1981; Shekelle *et al.,* 1981). The bioremediation potential of spirulina against heavy metal ions in industrial effluents was studied by various researchers (Balaji *et al.,* 2014). Spirulina supplementation is useful in adjuvant treatment of leukaemia and anaemia caused by lead (Pb) and Cadmium (Cd) toxicity (Simsek, *et al.,* 2009). Spirulina has been recommended as a chemoprotective against arsenic induced toxicity in humans (Rahman *et al.,* 2008). It is reported that administration of spirulina provide a protective mechanism against arsenic induced toxicity in goats (Ghosh A, *et al.,* 2014).It is found that combined treatment of using spirulina and vitamin A is effective against chronic arsenicosis in rat (Hossain *et al.,* 2013).It has also protective effect against galactosamine-induced hepatotoxicity in mice (Vedi, *et al.,* 2013). Spirulina is helpful on toxic signs, body weight and hematological parameters in arsenic induced toxicities in ducks (Islam *et al.,* 2009).

Riboflavin, also known as vitamin B_2 is one of the B vitamins, which is water soluble. It is an important micronutrient that plays a key role in maintaining health in humans and animals. It is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which act as electron carriers, and therefore, plays an important role in a range of redox reactions, mitochondrial energy production, and cellular function (Depeint *et al.,* 2006). Riboflavin which causes plants to produce reactive oxygen species (ROS) when exposed to light, is an excellent photosensitizer for biocidal reactions. Riboflavin is involved in antioxidation and peroxidation; both process affect the production of reactive oxygen intermediates (ROIs) in oxidative burst and consequent hypersensitive response (Packer *et al.,* 1996 and Upreti *et al.,* 1991). Riboflavin induces disease resistance in plants by activating a novel signal transduction pathway (H. Dong and S. V. Beer 2000). Riboflavin showed anti-inflammatory effects in various experimental models such as carrageenan-induced paw edema, lipopolysaccha- ride-induced fever, and implantation of cotton pellet-induced fibro- vascular tissues (Bertollo *et al.,* 2006 and Granados-Soto *et al.,* 2004). Riboflavin treatment reduces apoptosis and oxidative DNA damage in a rat spinal cord injury model (Sinem Sakarcan *et al.,* 2017).

In Bangladesh, elaborate data is available for arsenic toxicity only on tube-well water; however, data on the specific treatment for the prevention of arsenic toxicity in both human and animals is very little. Therefore, data on the effective prevention of arsenic toxicity with spirulina and riboflavin and their comparative efficacy will be the expected new findings

especially for Bangladesh as well as for the world. So in the context of above, the present study was undertaken with the following objectives:

I. To determine the efficacy of spirulina and Riboflavin on arsenic induced toxicity in rats.
II. To know the effect of arsenic, spirulina and riboflavin on biochemical parameters in arsenic fed rats.
III. To determine the effect of arsenic, spirulina and riboflavin on hematological parameters in arsenic fed rats.
IV. To know the effect of arsenic, spirulina and riboflavin on the body weight in arsenic fed rats.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Physical and Chemical Properties of Arsenic

Arsenic is a chemical element in the nitrogen group (Group 15 [Va] of the periodic table), with symbol As an atomic number 33 valences 3 and 5, andmolecular weight = 74.92 gm. It has a garlicky odour, and is chemically stable, yet fairly brittle. Arsenic can be found in nature in two forms, inorganic and organic. The most important inorganic arsenic compounds are arsenic trioxide, sodium arsenite, arsenic pentoxide, arsenic acid, arsenic trichloride (trivalent forms), and arsenates, such as, lead and calcium arsenates (pentavalent forms). Common organic arsenic compounds are arsanilic acid, methylarsonic acid, dimethylarsinic acid (cacodylic acid), and arsenobetaine.

Mazumder, (2008), chronic arsenic toxicity due to drinking of arsenic contaminated ground water is a major environmental health hazard throughout the world. Organic arsenic exposure can also occur by eating food. Organic arsenic is 500 times less harmful than inorganic arsenic. Inorganic arsenic trioxide is a component of geologic formations and can be washed out into the ground water. Arsenic poisoning can be related to human activities such as mining and ore smelting but is more often associated with dissolved solids naturally endemic in the aquifer environment.

ATSDR, (1997), the inorganic arsenic compounds are solids at normal temperatures and are unlikely to volatilize. The water solubility of these compounds varies from quite soluble (sodium arsenite and arsenic acid) to practically insoluble (arsenic trisulfide). Some organic arsenic compounds are gases or low-boiling liquids at ambient temperatures. Except for the organic arsenic acids, they are not readily water-soluble.

Lissi, (2000), studied that the main active ingredient of Spirulina are commonly seen as the Phycocyanobilin proteins, with C-phycocyanin being the most commonly touted meta-component and consists of smaller protein components such as Phycocyanibilin. These structures resemble the body's endogenous bilirubin molecule. The bilin groups are also the source of the anti-oxidative effects of Phycocyanobilin proteins.

Table 1. Physical and Chemical Properties of Arsenic and Selected Arsenic Compounds

Chemical Name	Molecular Weight	Oxidation State	Physical State	Water Solubility (g/100 mL)
Arsenic	74.92	0	Solid	Insoluble
Arsenic acid	141.95	+5	Solid	302
Arsenic trioxide	197.82	+3	Solid	2.1
Arsenic pentoxide	229.84	+5	Amorphous solid	Freely soluble
Sodium arsenate	185.91	+5	Solid	Very soluble
Sodium arsenite	130.92	+3	Solid	Freely soluble
Arsine	77.93	+3	Gas	20 mL/100g
Dimethylarsinic acid	138.01	+5	Solid	Soluble
Methanearsonic acid	139.98	+5	Solid	Freely soluble
Sodium dimethyl arsinate	159.98	+5	Solid	ND
Sodium methane arsonate	161.96	+5	Solid	57
Trimethylarsine	120.03	+3	Liquid	NA

Source: Arsenic in Drinking Water (California Public Health Goal

2.2 Sources of arsenic

Monique Bissen *et al.* (2003), Arsenic is widely distributed in the environment. It ranks twentieth in the abundance of elements in the earth's crust. The total amount of arsenic in the upper earth crust is estimated to be 4.01×10^{16} kg with an average of 6m g/kg. In the global arsenic cycle 3.7×10^{6} kt occur in the oceans, another 9.97×10^{5} kt on earth (land), 25×10^{9} kt in sediments, and 8.12 kt in the atmosphere. In sea water the concentration of arsenic varies between 0.09 µg/L and 24 µg/L (average: 1.5 µg/L), and in fresh water between 0.15 µg/L and 0.45 µg/L (maximum: 1 mg/L). In mineral and thermal waters arsenic was found in concentrations up to a factor of 300 of the mean concentration of arsenic in groundwater.

Léonard *et al.* (1991) and Ahmed *et al.* (1997), today, natural sources of arsenic in groundwater used for drinking water purposes are a significant problem particularly in Bangladesh. The WHO, which recommends a maximum contaminant level (MCL) for arsenic in drinking water of 10 µg/L, presumes that around 40 million people are acute at risk

in Bangladesh. Consequently, the chronic toxicity of arsenic will be a common cause of death in Bangladesh, if it is not possible to produce arsenic-free drinking water.

Williams (2001) Smedley *et al.* (2002), Sadler *et al.* (1994) and Rüde *et al.* (1996), published a study which gives an overview on arsenic concentrations in mine waters in seven countries of south-east Asia, Africa, and Latin America showing that arsenic concentrations in mine drainage vary between 5 µg/L and 72 mg/L. Arsenic poisoning of groundwater and soils related to mining activities are found in Thailand, Ghana, Zimbabwe, South Africa, England, Greece, Mexico, Canada, and the United States. The smelting of Cu, Ni, Pb to be emitted annually with 80% emitted by copper smelters. Arsenic amounts measured in soils near a lead smelter were 2 g/kg, near a copper smelter 0.55 g/kg, and a gold smelter 0.5 to 9.3 g/kg.

2.3 Metabolism of arsenic

Styblo *et al.* (2002), conclude that there is new compelling evidence that biomethylation is a process which activates arsenic as a toxin and carcinogen. The production of methylated trivalent arsenic in particular, has been associated with a variety of adverse effects that have a profound impact on cell viability or proliferation. Known effects include the inhibition of several key enzymes, damage to DNA structure and activation of AP-1-dependent gene transcription.

EPA, (1984) and Irgolic *et al.* (1983), arsenic is present in all sources of water. Water devoid of living organisms will very likely contain only inorganic arsenic in the form of arsenate and/or arsenite. Studies examining the form of arsenic in water supplies have largely reported only arsenate and arsenite in varying ratios**.**

Schoen *et al.* (2004), suggest that there is evidence that arsenic's trivalent methylated metabolites may induce comparable or greater toxicity than inorganic arsenic. However, there is limited evidence that these metabolites are present in sufficient quantities or for sufficient length of time to induce toxicity at target locations.

Villa-Bellosta and Sorribar (2010), studied that there are three Na/P co transporters that are expressed in the apical membrane of epithelial cells. While NaPi-IIa and-IIc are mainly expressed in the renal proximal tubules, NaPi-IIb is expressed in the intestine, liver, and lungs. Since the serum Pi concentration is approximately 1.1 mM, and the Ki values of iAsV for NaPi-IIa and -IIc are much higher than that for NaPi-IIb, the only Pi transporter out of the

three co-transporters capable of transporting iAsV in physiological conditions would appear to be NaPi-IIb.

2.4 Contamination of arsenic

Hossain (2006), stated that Bangladesh is currently facing a serious threat to public health, with 85 million people at risk from arsenic (As) in drinking water and in food crops. In Bangladesh, the groundwater As contamination problem is the worst in the world. Ninety-seven percent of the population in the country uses groundwater for drinking and domestic purposes as surface water is mismanaged. High levels of As in groundwater are causing widespread poisoning in Bangladesh. Different studies have addressed various aspects of the As issue in Bangladesh. This review is undertaken to give an overview of the latest findings and statistical data on the issue especially on soil, water and food cycle. The World Health Organization (WHO) recommends a safe limit for As in drinking water of 10μg/L. A recent survey looked at the As concentrations of drinking water from deep wells in 64 districts in the country and found that 59 had concentrations >10μg/L and 43 had concentrations >50μg/L. Contaminated groundwater is also used for irrigation of paddy rice, which is the main staple food for the population. This practice enhances the level of As in the soils rendering them unsuitable for agriculture. A few recent studies have reported that 85–95% of total As in rice and a vegetable was inorganic, which outlines the need for more studies for standardization. Arsenic concentration is higher in Bangladeshi soils, groundwater and plants (data based on 4% area of the country) than the permissible limits or normal range reported. This situation poses a serious threat on human and livestock health and highlights the need for scientific studies that would better describes the fate of As in the natural environment and identify all potential routes of exposure.

Azizullah *et al.* (2011), Bhowmik *et al.* (2015) and Rabbani, *et al.* (2017), numerous small-scale local studies, generally at the village level, have reported high arsenic concentrations in groundwater up to hundreds of micrograms per liter, primarily in the provinces of Punjab and Sindh. However, a lack of resources in the country has prevented the comprehensive evaluation of arsenic in groundwater (Nickson *et al.*, 2005, Farooqi *et al.*, 2007, Baig *et al.*, 2010). Considerable arsenic contamination has also been reported in other South and East Asian countries, for example, India, Bangladesh, Cambodia, and Vietnam (Brahman *et al.*, 2013). Shallow small-scale and family-based hand and motorized pumps have long been a major source of drinking water in the Indus Plain and are as widespread in Pakistan as in

those other arsenic affected regions of Asia. Higher-volume pumping with tube wells became popular throughout Pakistan in the 1960s and is used primarily not only for irrigation but also for municipal water supplies.

2.5 Health risk for arsenic

Hall (2002), arsenic can cause acute and chronic poisoning. Chronic arsenic poisoning involves non-specific symptoms such as chronic weakness, loss of reflexes, weariness, gastritis, colitis, anorexia, weight loss, and hair loss. Long-term exposure through food or air results in hyperkeratosis, hyper pigmentation, cardiovascular diseases, disturbance in the peripheral vascular and nervous systems, circulatory disorders, brittle loose nails with transverse white bands across the nails called Mees lines, eczema, suffering from liver and kidney disorder. Arsenic is deposited in hair, skin, nails, and bones.

Monique Bissena *et al.* (20003), acute arsenic poisoning may cause vomiting, dryness of the mouth and throat, muscle cramps, colicky abdominal pain, tingling of the hands and feet, circulatory disorders, and nervous weakness. Cold and clammy skin, hallucinations, delirium, and diarrhoea appear. Fatal shock can develop due to renal failure. If death does not occur within 24 h irreversible organ disorders occur. Death may result in the next days due to hepatic failure, renal failure, or heart attack.

Alam (2004), concluded the effects may include shortened life expectancy, decrease in reproduction, and behavioural changes. In arsenic toxicity, excitement, restlessness, ruffled hair coat, ataxia, in coordination, muscle tremor, paralysis and severe skin lesions were observed in rats. Significant ($P<0.01$) decrease in body weight, TEC, Hb and PCV and significant ($P<0.01$) increase in ESR, SGOT and SGPT were noticed in his findings. He also observed heavy congestion in liver, spleen, kidney and heart with severe haemorrhagic enteritis and rose-red inflammation in the stomach.

The IEH review (2003) suggested that several epidemiological studies found associations between increased spontaneous abortions, still births and foetal mortality, lowered birth weight and congenital malformations and arsenic in drinking water, airborne dust and smelter environments; but that there was no consistent evidence for any specific end-point (WHO, 2001, IEH, 2003).

Sarkar *et al.* (2003) and N. Pant *et al.* (2004), observed that arsenic exposure causes obvious damage in various organs, including the male reproductive function as manifested by

decrease of and rogenesis, suppression of spermatogenesis, and a reduction in the weight of testes and sex organs.

Navas-Acien *et al.* (2008); Kile and Christiani (2008), found that Arsenic toxicity has been linked to heart disease and hypertension, cancer stroke, cerebro-vascular diseases, chronic lower respiratory diseases and diabetes (Hendryx, 2009).

Tchounwou *et al.* (2004), concluded that in animal experiments, arsenic compounds have been found to be fetotoxic and teratogenic. The common developmental effects seen include malformations of the brain, urogenital organs, skeleton, ear and small or missing eye.

Defra and Environment Agency (2002), summarised that Lung cancer in particular is implicated in arsenic exposure by inhalation and is considered to be the critical effect. This conclusion is supported by various investigations involving smelter workers in the USA, Sweden, and Japan. There is also evidence for an increased risk of lung cancer in people living near industries where arsenic is emitted. It is also important to note that only inorganic arsenic is clearly implicated as a carcinogen; there are no studies concerning cancer in humans from the ingestion or inhalation of organic arsenic.

2.6 Composition of spirulina

2.6.1 Protein and amino acids

Vonshak *et al.* (1997) and Fujisawa *et al.* (2010), observed that platensis is the most useful microalgae for nutrition due to its components, especially protein. The nutritional level of protein is almost 70% of its dry weight and also has a high quantity and quality belonging to amino acids. *S. platensis* contains all of the essential amino acids. Researchers reported that although methionine and cysteine are found in a lower value, albumin and casein are found in a higher value, of animal proteins, respectively, ineggs and milk. *S. platensis* contains biliproteins, especially C-phycocyanin which is 20% of all protein fractions. C-Phycocyanin

molecule has an antioxidant feature, which regulates immunity and protects the organism against disease.

2.6.2 Vitamins

Kapoor *et al.* (1993), Watanabe (2007) and ADA (2003), *S. platensis* has the richest vitamin source of vitamin A (beta-carotene), vitamin E, thiamin (vitamin B1), biotin (vitamin B7), and inositol (vitamin B8) in food. Beta-carotene is in a biotransformed state which can be absorbed by humans, and is also important for antioxidant processes in organisms. On the other hand, there is a conflict of cobalamin (vitamin B12) content in S. platensis. Some researchers reported that *S. platensis* has no reliable vitamin B12. They explain that it is a pseudo vitamin B12 which is inactive and in a form that the human organism cannot uptake at a cellular level. However, other researchers claimed that *S. platensis* has a great amount of B12 compared to other sea algae and they indicated that vitamin B12 in this microalgae is important for vegetable nutrition, especially for humans who do not eat meat.

2.6.3 Carbohydrates

Walter (1997), Pugh (2001) and Nielsen (2010), summarised that *S. platensis* contains 13.6% carbohydrates, which are glucose, mannose, galactose, and xylose. *S. platensis* easily digestible and a safe nutrient for human consumption. Nevertheless, it does not contain cellulose, which cannot be absorbed by humans and thereby this feature makes. It is significant for people who have intestinal mal absorption and for the elderly. Likewise, there is also a polysaccharide molecule, isolated from *S. platensis*, which has a huge molecular weight. This polysaccharide has an immunomodulator effect called “immulina” by scientific authorities.

2.6.4 Lipids

Colla *et al.* (2004), Jubie*et al.* (2012) and Li*et al.* (2007), total fatty acids tend to fluctuate around 6% by dry weight. Fatty acids such as γ-Linolenic acid (GLA) at up to 20.8mg/g (up to 25% of total fatty acids), Stearic Acid, Alpha Linoleic Acid, Palmitic Acid, and Linoleic Acid; exact composition varies depending on production. These components are also mediators of immune and cardiovascular system due to their precursor effects of prostaglandins and leukotrienes.

2.6.5 Minerals

Viswanadha *et al.* (2011), *S. platensis* contains many minerals such as potassium, calcium(600–1,200mg/100g),chromium, copper, iron(50-150mg/100g), magnesium(200-600mg/100g), manganese, phosphorus, selenium(50-200mcg/100g), sodium, and zinc. This microalgae is a good component due to its iron, calcium, and phosphorus contents. The ferrous component in this microalgae can be easily digested and bioactive in an organism easily which is important for pregnant adult females. The utilization of calcium and phosphorus contents of *S. platensis* has an important impact on bone calcification and improves bone health.

2.6.6 Pigments

Babadzhanov *et al.* (2004), *S. platensis* has some natural pigments which color this microalgae, such as c-phycocyanin, chlorophyll, xanthophyle, beta-carotene, zeaxanthin, and allophycocyanin. The most important are phycocyanin, chlorophyll, and beta-carotene. C-Phycocyanin is the most important pigment, which includes iron, and contains 14% of its dry weight. Also, *S. platensis* is one of the best nutrients that contains the highest chlorophyll value (1%). Chlorophyll is known as a detoxifier and purifier phyto-nutrient. It improves the carbohydrate, protein, and lipid metabolism and affects reproduction positively. Carotenes constitute half of this microalgae, especially beta-carotene. The carotenes and xanthophyle in *S. platensis* are demonstrated in different metabolism pathways in the body, and also better influence the function of vitamins and minerals in an organism. Nowadays, diets rich in carotenes are found to be important for human health due to its effects in reducing the risk of diseases

2.7 Treatment with spirullia

Duncan *et al.* (2015), pointed out that As metabolism by microalgae may be affected by the composition of growth media, yet more work should be conducted to evaluate the risks of As accumulation in the algal products and to establish optimal culture conditions for eliminating As threats to the health of humans and animal.

Islam *et al.* (2009), observed that spirulina has protective effect against arsenic toxicity in rats. Thirty six female Long Evans rats were randomly divided into three equal groups (n=12) and marked as T_0, T_1 and T_2 groups. Rats of T_0 group were given normal feed and water and kept as control. Rats of T_1 and T_2 groups were given 5mg Sodium arsenite/kg body weight (BW) and 5mg Sodium arsenite/kg (BW) plus spinach extract 100 mg/kg body weight

respectively daily for 30 days orally. This indicates that As causes liver injury. Chronic exposure of experimental animals to inorganic arsenic has been shown to produce various liver lesions, including inflammation and oxidative damage, fatty accumulation, parenchymal cell degeneration, hepatic fibrosis and liver proliferative lesions (Mazumder, 2005). From this findings it may be stated that spinach somehow prevent liver injury caused by arsenic.

Selmi (2011) conducted one study in older persons with a history of anaemia taking 3g of Spirulina daily for 12 weeks failed to note an increase in red blood cell count yet increased mean corpuscular haemoglobin (MCH), MCV, and MCHC in men and increased in MCH in women. Platelets were unchanged over 12 weeks, and white blood cells increased significantly at 6 weeks in time; high variability noted in this study.

Vedi *et al.* (2013), conducted a study on to evaluate the protective properties of *Spirulina fusiformis* against galactosamine induced toxicity in swiss albino mice. Galactosamine injection significantly increased the levels of SGOT, SGPT, SBLN and TNF–α in the serum and caused depletion in the antioxidant status in the liver. Administration of *Spirulina fusiformis* (100mg/ kg body weight, i.p.) altered these parameters and brought them activity against galactosamine induced toxicity in mice.

Awal *et al.* (2013), summarised that spirulina has protective effect in reducing toxic signs, body weight and haematological parameters in arsenic induced toxicities in rats. He treated the arsenic induced rat with spirulina in three different doses i.e. 30, 60 and 120 mg/L in drinking water daily for 90 days starting from day 15. He found that in spirulina treated groups reduction of TEC, Hb and PCV were less than arsenic treated groups. He concluded that spirulina may be helpful for reducing the body burden of arsenic in ducks.

Naif Abdullah Al-Dhabi (2013), conducted a study to reveal the concentrations of six typical heavy metals/minerals (Ni, Zn, Hg, Pt, Mg, and Mn) in 25 spirulina products commercialized worldwide for direct human consumption. Samples were ground, digested and quantified by Coupled Plasma Mass Spectroscopy (ICP–MS). The concentrations (mg/kg d.w.) were range from 0.001 to 0.012 (Pt) followed by 0.002–0.028 (Hg), 0.002–0.042 (Mg), 0.005–2.248 (Mn), 0.211–4.672 (Ni) and 0.533–6.225 (Zn). The inorganic elements of the present study were significantly lower than the recommended daily intake (RDI) level of heavy metal elements (mg/daily) Ni (0.4), Zn (13), Hg (0.01), Pt (0.002), Mg (400) and Mn (4). Based on this study the concentration of inorganic elements was not found to exceed the present regulation levels, and they can be considered as safe food.

Samah M.M. Fathy, *et al.* (2015) conducted a study to investigate the effect of intra-peritoneal injection of purified exudates of arsenic *Spirulina platensis* on the mammalian endocrine and nervous systems. The intra-peritoneal injection of the cyanobacterial exudates in mice was applied line assay kit. A sharp disruption in the sex hormones levels of testosterone, progesterone, follicular stimulating hormone and luteinizing hormone was demonstrated in the serum of the treated mice. At the same time, a significant reduction in the endogenous antioxidant defence enzymes, superoxide dismutase, catalase and glutathione peroxidase was observed in the hippocampus region of the injected mice. Moreover, levels of dopamine, nor adrenaline, serotonin and acetyl choline neurotransmitter in the same region were significantly affected as a result of the treatment with spirulina filtrate. The gas chromatography–mass spectrometer and liquid chromatography mass spectrometry/mass spectrometry analysis showed the presence of some sterol-like compounds in the cyanobacterial filtrate. He demonstrated the capability of Spirulina to release detrimental bioactive metabolites into their surrounding that can disrupt the mammalian endocrine and nervous systems.

Samir A. E. Basshandy *et al.* (2016) conducted a study to examine the protective role of *Spirulina platensis* against arsenic-induced testicular oxidative damage in rats. Arsenic (in the form of $NaAsO_2$ at a dose of 6.3mg/kg body weight for 8 weeks) caused a significant accumulation of arsenic in testicular tissues as well as a decrease in the levels of testicular superoxide dismutase (SOD), catalase (CAT), reduced glutathione, and zinc. Moreover, it significantly decreased plasma testosterone, luteinizing hormone (LH), triiodothyronine (T_3), and thyroxine (T_4) levels and reduced sperm motility and sperm count. Arsenic (AS) led to a significant increase in testicular malondialdehyde (MDA), tumour necrosis factor alpha (TNF-α, nitricoxide (NO) and sperm abnormalities. *S. platensis* at a dose of 300mg/kg was found to attenuate As-induced oxidative stress, testicular damage, and sperm abnormalities by its potent antioxidant activity.

Rakhi Bajpai Dixit and M. R. Suseela (2013) found that pharmaceutical importance of bioactivities of cyanobacterium including anti proliferative, antitumor, antifungal, antibacterial, antimalarial, antiviral, antimycotics, cytotoxicity, multi-drug resistance

reversers and immunosuppressive agents. Blue green algae, *Spirulina* display antitumor activity against many cancers both in human and animal systems.

Besednova *et al.* (1979), shown that *Spirulina platensis* enhances functions of selected effector cells of immune system of chicken. The available data suggesting that the *Spirulina platensis* exposure improves chickens immune performance without adversely affecting other performance characteristic. The whole cells of blue green algae, *Spirulina platensis* and its lipopolysaccarides were shown to stimulate production of macro and microglobulin antibodies in rabbits.

Sung-Ho Oh *et al.* (2010) and Karkos *et al.* (2011), summarised that the ultrasonic extraction of Spirulina maxima exhibited potential anticancer activity. The extract is effective against different types of human cancer cell lines such as lung (A549), liver (Hep3B), stomach (AGS) and breast (MCF-7) cell lines.

Rawshon Jamil *et al.* (2015), conducted to evaluate the prebiotic effects of spirulina as a growth and immunity promoter for broiler chickens. Birds were randomly and equally distributed into four groups (T_0, T_1, T_2 and T_3) and fed on a diet containing 0, 2, 4 and 8 g Spirulina/kg feed respectively for 4 weeks. The body weight was increased in the treatment groups fed with spirulina diet from 7^{th} days to 28^{th} days old. FCR was also decreased among the treatment groups. Haematological parameters were increased except ESR which was decreased in the treatment group. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) level were decreased in all the treatment groups. The study suggests that, Spirulina is a good natural feed additive which has a tremendouseffect to improve the broiler production and thereby may reduce the production cost.

Norma Paniagua-Castro *et al.* (2011), studied the role of *Spirulina (Arthrospira*) in preventing cadmium (Cd) teratogenicity in ICR mice. He administered Cd intraperitoneally to female mice at 1.5 mg =kg on gestation day (GD)-7, and spirulina was given by peroral (intragastric) administration at 62.5, 125, 250, or 500 mg =kg from GD-0 through GD-17. Administration of Cd caused reproductive damage, oxidative stress and lipoperoxidation, embryonic hydroperoxides were also determined. Treatment with Spirulina at the three highest doses significantly decreased the frequency of foetuses with anencephaly, micrognathia, and skeletal abnormalities induced by Cd. Furthermore, spirulina treatment significantly and dose-dependently decreased lipid peroxidation, which was dramatically

increased by administration of the metal. He suggested that the therapeutic potential of spirulina in Cd-induced teratogenicity and probably through its antioxidant activity.

Sun Hee Cheong *et al.* (2010), investigated the anti-atherogenic effect of spirulina in New Zealand white rabbit model. The animal had hypercholesterolemia induced by being fed a high cholesterol diet containing 0.5% cholesterol for 4 weeks, then fed with 1% or 5% for an additional 8 weeks. Spirulina supplementation lowered intimal surface of the aorta by 32, 2 to 48.3% compared to high cholesterol diet. Serum triglyceride (TG) and total cholesterol (TC) were reduced in spirulina treated group. After 8 weeks serum low density lipoprotein cholesterol (LDL-C) remarkably decreased by 26.4% and 41.2% compared to high cholesterol diet. He suggested that spirulina intake can cause the reduction of hyper cholestorolemic atherosclerosis associated with a decreased level of serum TC, TG and LDL-C and an elevation of HDL-C level.

Leandro P Moura *et al.* (2011), conducted a study to analyse the effects of physical exercise and spirulina intake on the control of NAFLD in diabetic Wistar rats. Diabetes was induced in the animals through intravenous administration of alloxan. The rats were divided into four groups: Diabetic Control (DC) – diabetic rats fed with a control diet and no physical exercise; Diabetic Spirulina (DS) – diabetic rats fed with a diet that included spirulina; Diabetic spirulina and Exercise (DSE) – diabetic rats fed with a diet that included spirulina and that exercised; and Diabetic Exercise (DE) – diabetic rats fed with a control diet and that exercised. The groups DS, DSE, and DE presented lower plasma concentrations of LDL cholesterol than DC, as well as lower levels of total liver lipids in groups DS, DSE, and DE in comparison to DC. Spirulina appears to be effective in reducing total circulating levels of LDL-cholesterol and hepatic lipids, alone or in conjunction with physical exercise in diabetic rats.

2.8 Beneficial effect of Riboflavin

Sinem Sakarcan *et al.* (2017), conducted a study on Spinal Cord Injured (SPI) rats to investigate the putative protective effect of riboflavin against SCI-induced spinal cord and kidney damage. Injured animals were given either 25 mg/kg riboflavin or carboxymethyl cellulose 15 min after injury, and this regimen was repeated twice daily for 7 days. SCI caused tissue injury through oxidative stress and neutrophil infiltration into tissues. Riboflavin inhibited tissue injury through its neuro protective and antiapoptotic effects. He

demonstrated that riboflavin not only exerts antioxidant and antiapoptotic effects on the spinal cord but also has a significant impact on preventing kidney damage secondary to SCI.

Lydia B. Zablotska *et al.* (2008), conducted a study to clarify the effects of the vitamin B group (29 thiamine, riboflavin, niacin, pyridoxine, and cobalamin) and antioxidants (vitamins A, C, and E) on arsenic-related skin lesions. A total of 14,828 individuals meeting a set of eligibility criteria were identified among 65,876 users of all 5,996 tube wells in the 25-km^2 area of Araihazar, Bangladesh; 11,746 were recruited into the study. This analysis is based on 10, 628 subjects (90.5%) with non-missing dietary data. Skin lesions were identified according to a structured clinical protocol during screening and confirmed with further clinical review. Riboflavin, pyridoxine, folic acid, and vitamins A, C, and E significantly modified risk of arsenic-related skin lesions. The deleterious effect of ingested arsenic, at a given exposure level, was significantly reduced (ranging from 46% reduction for pyridoxine to 68% for vitamin C) for persons in the highest quintiles of vitamin intake. He concluded that intakes of B-vitamins and antioxidants, at doses greater than the current recommended daily amounts for the country, may reduce the risk of arsenic-related skin lesions in Bangladesh.

Sandor *et al.* (2000), summarised that Riboflavin was significantly better than placebo in reducing attack frequency and the number of headache days, though the beneficial effect was most pronounced during the third month of treatment. Another study by the same investigators found that treatment with either a medication called a β-blocker or high-dose riboflavin (400 mg/day) for four months resulted in clinical improvement.

Yuvaraj *et al.* (2008), studied the antioxidant effect of co-administering riboflavin (10 mg/day), niacin (50 mg/day), and coenzyme Q_{10} (100 mg/day) was evaluated in 78 postmenopausal breast cancer patients treated with Tamoxifen for 90 days. This supplementation effectively prevented the oxidative stress associated with tamoxifen treatment. Riboflavin can also act as a photosensitizer, and this property may have value in photodynamic therapy of cancer.

Agte *et al.* (1998) and Shi, Zumin (2015), summarised that iron is an important mineral for growth and development. Iron deficiency can lead to anaemia, particularly in women who are pregnant or nursing and young children. Riboflavin helps the body absorb both iron and zinc and also helps make them both more available to your body. This increase in absorption helps to prevent iron deficiency and other accompanying symptoms.

Sebrell and Butler (1938); Vilter and Spies (1939), studied that the use of riboflavin in the human has been in conditions other than those associated with neurological disease. They investigate peripheral neuritis become worse in persons with the healing lesions of riboflavin deficiency (roughening of the skin around the mouth and across the tip of the nose) during the administration of riboflavin. The amounts of riboflavin given to cure these lesions do not affect the peripheral neuritis

Hassan *et al.* (2013), summarised that Cisplatin (CP), though one of the most valued anticancer drugs against various forms of cancer has limitation because of many side effects. One study suggests that a moderate amount of riboflavin can persuade the extrinsic pathway of apoptosis while higher amounts can activate additional cell death mechanisms such as the intrinsic pathway of apoptosis by down-regulating many anti-apoptotic factors as well as up-regulating many other apoptosis inducing factors.

Alam *et al.* (2015), studied that Each B-group vitamin acts in synergy to maintain the body‘s homeostasis by playing major roles in metabolic processes such as energy production and red blood cell formation. One of such essential vitamins, riboflavin is an essential vitamin that generally acts as a co-factor; Flavin Adenine Mononucleotide (FMN) and Flavin Adenine Dinucleotide (FAD) in numerous enzymatic reactions in all forms of life and performs key metabolic functions by mediating the transfer of electrons in biological oxidation-reduction reaction. They also suggest that it helps to maintain the integrity of mucous membranes, skin, eyes and the nervous system. During periods of dietary deprivation or physiological and pathological stress, humans are vulnerable to developing riboflavin deficiency.

CHAPTER 3

MATERIALS AND METHODS

This experiment was conducted during the period between 12th September to 10th November 2017 at the animal shed under the Department of Physiology and Pharmacology, Faculty of Veterinary and Animal Science, in Hajee Mohammad Danesh Science and Technology University, Dinajpur.

3.1 Experimental site

The laboratory animal house at the Department of Physiology and Pharmacology was the experimental site.

3.2 Layout of experiment

The layout of the experiment is presented below:

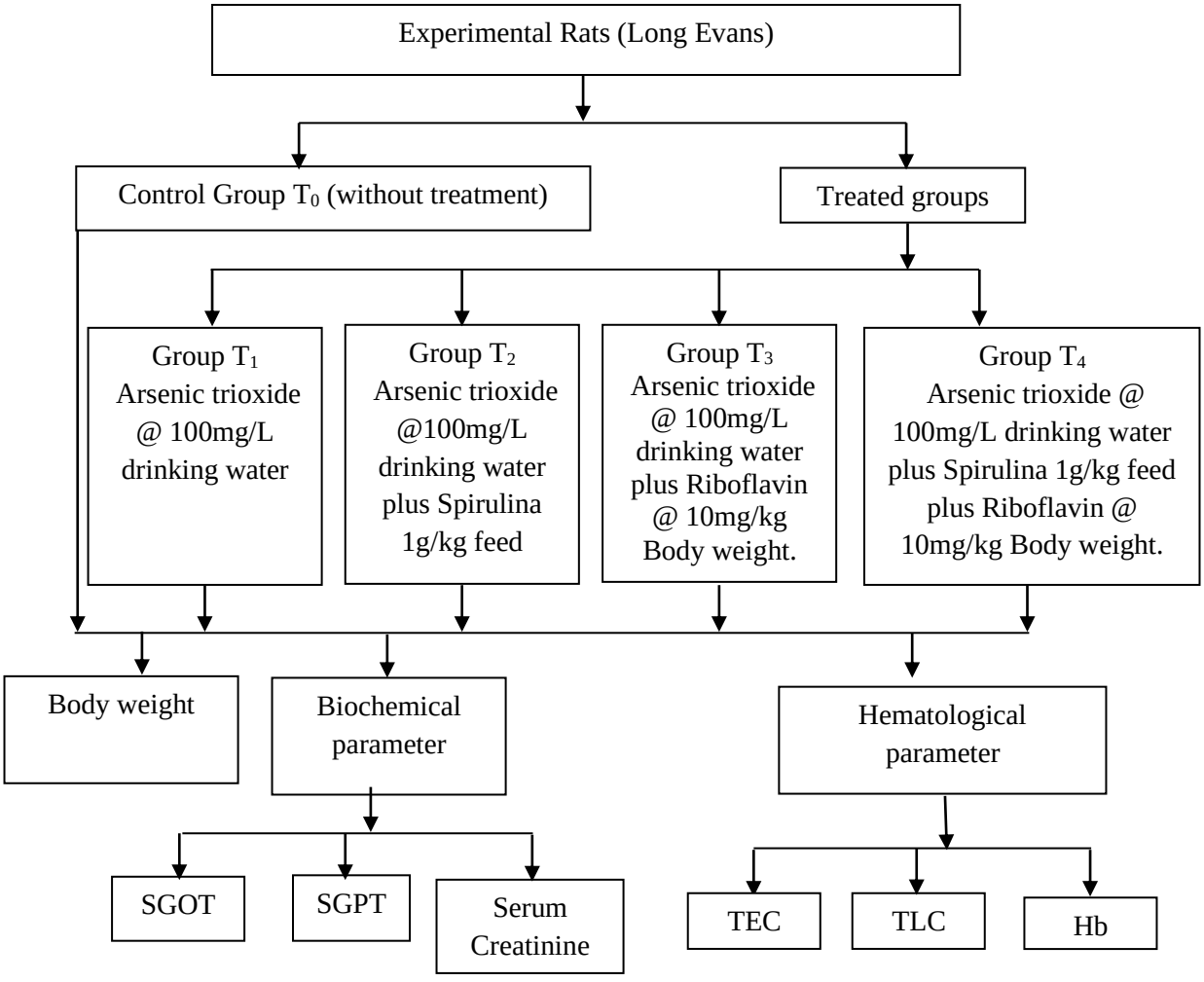

Figure 1. Layout of the experiment

3.3 Experimental animal

A total of 60 male Long Evan rats of 6 weeks age was purchased from International Centre for Diarrheal Disease Research Bangladesh (ICDDRB). The animals were housed in compartmented rectangular metallic cage under standard laboratory conditions (12 h light: 12 h dark, 25 ± 20C and humidity 60 ± 5%). Rats were acclimatized for 15 days in the laboratory before the experiment started.

3.4 Preparation of house

At first the room as well as wire cages were washed by sweeping and washing with tap water using hose pipe connected with tap. The room was disinfected with a phenolic disinfectant and allowed to dry the room leaving unused with the electric fan and the bulb switched on. Proper ventilation was provided.

3.5 Test Chemicals

Arsenic trioxide was purchased from a scientific laboratory. Spirulina capsule (Navit®) was collected from Square Pharmaceuticals Limited and Riboflavin (Riboson®) from Jayson Pharmaceuticals Limited.

3.6 Experimental animal grouping

Sixty rats were collected for this investigation. These rats were divided into five groups containing 12 rats in each group. Then they were individually marked using different color on their tail tips for identification. The groups were designed maintained as follows:

Group T_0: The rats were fed with pellet diet, as recommended by ICDDRB and given water *ad libitum* and their initial body weight were recorded after acclimatization. This group of rats served as negative control.

Group T_1: After acclimatization body weights were measured. The rats were fed with pellet diet and given water mixed with arsenic trioxide. This group served as positive control.

Group T_2: After acclimatization body weights were measured. The rats were fed with pellet diet mixed with spirulina and given water mixed with arsenic trioxide.

Group T_3: After acclimatization body weights were measured. The rats were fed with pellet diet plus given water mixed with arsenic trioxide, and in another pot water mixed with riboflavin.

Group T_4: After acclimatization body weights were measured. The rats were fed with pellet diet mixed with spirulina plus given water mixed with arsenic trioxide, and in another pot water mixed with riboflavin**.**

3.7 Body weight (BW)

The rats were individually weighed firstly on Day 0 (Day 0= immediate previous day of starting treatment) after grouping and marking, Day 15, Day 30 and finally on Day 45 and the results were recorded.

Plate 1: Weighing of body weight of rat

3.8 Clinical signs

Experimental rats were closely observed after feeding arsenic trioxide and spirulina daily for 3 times (morning, afternoon and evening) for the appearance of any toxic signs if in them, during the entire experimental period (from Day 1 to Day 45) and the findings were recorded.

3.9 Experimental trial

The experimental trial was conducted for 45 days. Rats of Group T_0 were maintained with only normal pellet feed and water *ad libitum* as control, that of Group T_1 were treated with arsenic trioxide at a dose of 100mg/L drinking water. The rats of Group T_2 were treated with arsenic trioxide at 100mg/L in drinking water daily and spirulina (*Spirulina platensis*) simultaneously at a dose of 1 gm/kg feed. The spirulina (Navit®) used in this experiment was

collected from Square Pharmaceuticals Limited; as a capsule form. The rats of Group T_3 were treated with arsenic trioxide at 100 mg/L in drinking water daily and riboflavin Tablet (Riboson®; Jayson Pharmaceuticals Limited; Bangladesh) simultaneously at a dose of 10mg/kg bodyweight. The animals of Group T_4 were treated with arsenic trioxide at 100mg/L in drinking water daily and riboflavin at a dose of 10mg/kg body weight and spirulina (*Spirulina platensis*) simultaneously at a dose of 1gm/kg feed. All treatments were given for 45 days.

3.10 Preparation of treatment materials

3.10.1 Arsenic trioxide solution

On the basis of the total body weight of the rats, the required amount of arsenic trioxide for a day (100mg/L drinking water) was weighted separately for each group of rats. The respective pre-weighed arsenic trioxide was mixed with the drinking water daily for that particular group. Generally, 10ml drinking water per rat was allotted for mixing arsenic trioxide to make sure that the full amount of arsenic trioxide was taken by the rats. After finishing the drinking of the arsenic trioxide mixed water, normal drinking water was supplemented *ad libitum.*

3.10.2 Spirulina mixed feed

Each capsule of Spirulina (Navit®; Square Pharmaceuticals Limited, Bangladesh) containing 500mg of *Spirulina platensis.* The powder of spirulina was kept in a cup after opening from the capsule. The required amount of spirulina (1gm/kg feed) was measured with the help of electric balance. The powdered spirulina was kept in desiccators to prevent water absorption and change in quality of the powder. For proper homogenous mixing, small amount of distilled water was added to the pre-weighed spirulina powder to make it a suspension and then the suspension was added drop by drop to the feed and simultaneously the feed was stirred by a glass rod for homogenous mixing. As the feed was dried pellet, the spirulina was adhered on the pellets. After finishing the spirulina mixing, feed was dried in an electric oven at 50°C overnight and kept in air-tied plastic container then supplied to rats *ad libitum*.

Plate 2: Feed

3.10.3 Riboflavin mixed Water

Each Tablet of Riboflavin (Reboson®; Jayson Pharmaceuticals Limited; Bangladesh) containing 5mg of riboflavin. The tablet was made to a homogeneous powder with the help of pestle and mortar. Then the powder was mixed with required amount of distilled water and simultaneously the water was stirred by a glass rod for homogenous mixing. After completion of proper mixing, the mixed water was provided to rat.

3.11 Sampling

After starting treatment of 15 days 4 rats from each group were anesthetized using chloroform anesthesia and they were sacrificed and about six milliliters (ml) of blood samples were collected directly from cardiac puncture of each rat by using disposable plastic syringe. The blood from each rat was then transferred into two tubes for determination of biochemical parameters, hematological test. . For the biochemical test 4ml of blood sample was taken into pre-marked centrifuge glass test tubes immediately after collection. Collected blood kept at was room temperature to allow it to clot properly then stored in a refrigerator overnight. Serum was separated following centrifugation of the blood in the next morning and the supernatant serum was taken into pre-marked eppendorf tubes. The harvested serum were kept at-20^{o}C until used. For the hematological test and detection of arsenic concentration in blood 1 ml of blood for each was taken separately into EDTA coated tube. The total lung, liver, and kidney were collected aseptically, washed with physiologic saline and were kept in the pre-marked zipper polythene bag. Bloods samples for hematological investigation were preserved at 4^{0}C temperature. All blood were taken 1st on Day 15, 2nd on Day 30, and 3rd on Day 45.

3.11.1 Preparation of samples for examinations

3.11.1.1 Serum collection

Properly clotted each blood sample was detached from the inner wall of the respective test tube with a separate long fine needle by moving it slowly between the clot and the inner wall of the tube after 1 hour and then kept 24 hours at 4^0C in a refrigerator. In the next day, tubes with blood clot were kept outside the refrigerator for thawing. Following thawing, the test tubes containing blood clot were centrifuged by centrifuge machine (EBA 20, Hettich, ZENTRIFUGEN, Germany) at 2000rpm (rotation per minute) for 10 minutes. The separated supernatant serum was collected from each test tube into the correspondingly marked screw capped sterile eppendorf tubes with separate sterile Pasteur pipette and kept in a deep freeze at -20^0C until analysis.

Plate 3: Collection of serum

3.12 Biochemical tests

Sera were thawed on the laboratory bench and the SGOT, SGPT activity and serum creatinine were determined through the use of Reflotron® Plus (Boehringer Mannheim, Germany) according to the method described by Deneke and Rittersdorf (1984) and Deneke *et al.* (1985).

3.12.1 Serum Glutamate Oxaloacetate Transaminase (SGOT)

Serum of the sample was 4-fold diluted in Phosphate Buffered Solution (PBS) with p^H 7.4. Twenty five micro liters of diluted serum was placed on the centre of the red application zone

(xx marked) of the Glutamic Oxaloacetic Transaminase (GOT) test strip with the help of micropipette after opening the sliding cover of the test strip. The strip was then placed on to the strip guide within 15 seconds from placing of serum on the strip and the slide was forwarded until it locks into place. The sliding cover was closed. The GOT level was displayed on the monitor in 75 seconds in Unit/Liter (U/L).

3.12.2 Serum Glutamate Pyruvate Transaminase (SGPT)

Serum of the sample was 4-fold diluted in Phosphate Buffer Solution (PBS) with p^H 7.4. Twenty five micro liters of diluted serum was placed on the centre of the red application zone (xx marked) of the Glutamic Pyruvate Transaminase (GPT) test strip with the help of micropipette after opening the sliding cover of the test strip. The strip was then placed on to the strip guide within 15 seconds from placing of serum on the strip and the slide was forwarded until it locks into place. The sliding cover was closed. The GPT level was displayed on the monitor in 75 seconds in Unit/L.

3.12.3 Serum creatinine

Serum of the sample was diluted in PBS. Twenty five micro liters of diluted serum was placed on the centre of the red application zone of the creatinine test strip with the help of micropipette after opening the sliding cover of the test strip. The strip was then placed on to the strip guide within 15 seconds from placing of serum on the strip and the slide was forwarded until it locks into place. The sliding cover was closed. The creatinine level was displayed on the monitor in 75 seconds in mg/dl.

3.13 Examination of blood for determination of hematological parameters

3.13.1 Total Erythrocyte Count (TEC)

RBC number was calculated as number of cells counted x 10,000 and the result was expressed in million/µl of blood. Total erythrocyte count was done following the method described by Lamberg and Rothstein (1977). Blood sample well-mixed was drawn with red blood cell diluting pipette exactly up to 0.5 marks of the pipette. Outside of the tip of the pipette was wiped with cotton. Then the pipette was immediately filled with the red cell diluting fluid (Hayem's solution) up to 101 marks. The free end of the pipette was wrapped around with the rubber tube stretching to both the ends and held with thumb and middle finger. The content of the pipette was mixed thoroughly by shaking with 8-knot motion for 3-

5 minutes. Then the counting chamber was placed with special cover glass under microscope using low power (10x) objectives. After discarding 2 or 3 drops of fluid from the pipette, a small drop was placed to the edge of the cover glass on the counting chamber as the entire area under the cover glass was filled by the fluid. One-minute time was spared to allow the cells to settle on the chamber under the cover glass. Taking 5 larger squares (4 in the 4 corners and the central one) of the central large square, the cells were counted from all the 80 small squares (16×5) under high power objectives (45x).

3.13.2 Total Leukocyte Count (TLC)

Well mixed blood sample was drawn up to the 0.5 mark of while blood cell pipette. The pipette was filled up to the 11 mark with the diluting fluid (N/10 HCl) by steady sucking and the content was thoroughly mixed for 2 minutes and then 2 or 3 drops of the content was discarded and counting chamber was then filled in the same way as done in the red blood cell count. The leukocytes in the 4 large corner squares (each 1 square mm) of the counting chamber were counted. The counting of leukocyte were performed as per methods described by Lamberg and Rothstein (1977). The result was expressed as thousand/µl.

Plate 4: Anaesthesia of rat

3.13.3 Determination of Hemoglobin Concentrations (Hb)

The N/10 hydrochloric acid was taken in a graduated tube up to 2 marks with the help of a dropper. Well-homogenized blood sample was then drawn into the Sahli pipette up to 20 cm. mark. The tip of the pipette was wiped with sterile cotton and the blood of the pipette was immediately transferred into the graduated tube containing hydrochloric acid. This blood and acid were thoroughly mixed by stirring with a glass stirrer. There was a formation of acid hematinic mixture in the tube by hemolysing red blood cells by the action of hydrochloric acid (HCL). The tube containing acid hematinic mixture was kept standing in the comparator

for 5 minutes. After that distilled water was added drop by drop. The solution was mixed well with a glass stirrer until the color of the mixture resembled to the standard color of the comparator. The result was read in daylight by observing the height of the liquid in the tube considering the lower meniscus of the liquid column. The result was then expressed in g %. The above procedure was matched by the Hellige hemometer method as described by Lamberg and Rothstein (1977).

Plate 5: Collection of blood sample

3.13.4 Determination of Packed Cell Volume (PCV)

The citrated well mixed blood sample was drawn into special loading pipette (Wintrobe pipette). The tip of the pipette was inserted up to the bottom of a clean, dry Wintrobe hematocrit tube. Then the Wintrobe tube was filled from the bottom by pressing the rubber bulb of the pipette. As blood came out, the pipette was slowly withdrawn but pressure was continued on the rubber bulb of the pipette so as to exclude air bubbles. The tip of the pipette was tried to keep under the rising column of blood to avoid foaming and the tube was filled exactly to the 10 cm mark. Then the Wintrobe hematocrit tube was placed in the centrifuge machine and was centrifuged for 30 minutes at 3000 rpm. Then, the hematocrit or PCV was recorded by reading the graduation mark; the percent volume occupied by the hematocrit was calculated by using the following formula as described by Lamberg and Rothstein (1977).

$$\text{PCV\%} = \frac{\text{Height of the red cell volume in cm}}{\text{Height of total blood in cm}} \times 100$$

3.13.5 Determination of Erythrocyte Sedimentation Rate (ESR)

The fresh anticoagulant blood was taken into the Wintrobe hematocrit tube by using special loading pipette exactly up to 0 marks. Excess blood above the mark was wiped away by sterile cotton. The filled tube was placed vertically undisturbed on the wooden rack for one hour. After one hour the ESR was recorded from the top of the pipette. The result was expressed in mm in 1st hour.

3.14 Statistical analysis

The collected data were statistically analyzed as per Steel and Torrie (1980) using Completely Randomized Design (CRD). Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were performed with the help of SPSS 20 software to find out the difference among the treatments.

CHAPTER 4

RESULTS AND DISCUSSION

The experiment was conducted to determine the efficacy of spirulina and Riboflavin on arsenic toxicity in rats. It was also undertaken to observe the effects of spirulina and Riboflavin on body weight, hematological and biochemical parameters in arsenic fed rats. Sixty rats were randomly divided into five equal groups to conduct the experiment. T_0 group served as negative control and fed with normal diet. Group T_1 were treated with arsenic trioxide at a dose of 100mg/L drinking water and this group were kept positive control. Group T_2 were treated with same dose of arsenic trioxide and Spirulina (*Spirulina platensis*) simultaneously at a dose of 1 gm/kg feed. Group T_3 were treated with same dose of arsenic trioxide and riboflavin tablet simultaneously at a dose of 10mg/kg bodyweight. Group T_4 were treated combine with arsenic, spirulina and riboflavin at a same dose. All the treatment were continued for 45 days and treated rats were closely observed through the entire period.

4.1 Clinical signs

There were no significant change in clinical signs of arsenic toxicity were observed in trial rats during the entire experimental period.

4.2 Body weight (BW) of the rats

Body weights (BWs) of experimental rats of all groups were taken fifteen days interval on day 0, day 15, day 30 and day 45. Table 2 showed that the body weight gain was highest (299.40 ± 3.70) in T_2 group rats at 45 days but the body weight gain was lowest (96.60 ± 2.62) in arsenic treated T_1 group at 45 days whereas body weight gain in T_0, T_3 and T_4 were 255.80±5.12, 284.80±7.15, and 275.80±2.85 which were better than arsenic treated T_1 group. The body weight of initial groups were not significant ($p > 0.05$) but in 15 days, 30 days and 45 days mean value of body weight were significant ($p<0.01$).

The body weight of treated group were increased with their age but in T_1 group it decreased compared to other groups. In the present study arsenic reduced the body weight with their increasing age. The highest body weight gain was found in T_2 group where spirulina were treated with arsenic. It recommends that spirulina act against arsenic in decreasing body weight. Sharma *et al.* (2007) reported that decreased body weight was observed in arsenic

treated group of Swiss albino mice. Jun *et al.* (2008) who reported As significantly ($p<0.01$) decreases the body weight of rats.

Table 2 Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus spirulina on the body weight of rats

Treatment	T_o	T_1	T_2	T_3	T_4	P. value
Initial	78.20 ± 3.28	82.60 ± 2.50	88.46 ± 3.32	84.20 ± 4.32	85.00 ± 2.78	NS
15 days	171.00^{b} ±5.56	87.40^{a} ±4.02	181.80^{bc}±5.21	188.40^{c} ±4.97	195.00^{c} ±5.17	**
30 days	220.20^{b} ±4.32	94.80^{a} ±2.63	261.20^{d} ±2.92	255.40^{d} ±2.04	239.40^{c} ±4.74	**
45 days	255.80^{b} ±5.12	96.60^{a} ±2.62	299.40^{d} ±3.70	284.80^{c} ±7.15	275.80^{c}±2.85	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.3 Hematological parameter

4.3.1 Total Erythrocyte Count (TEC)

In Table 3, Total Erythrocyte Count (TEC) values were highest (8.53 ± .14) found in T_4 group at 45 days where spirulina and riboflavin were treated against arsenic toxicity but lowest (6.35 ± .25) value was found in T_1 group where only arsenic were given. TEC value found at 15 days (7.10 ± .04) and 45 days were significant ($p<0.01$) and values found at 30 days (7.63 ± .28) were significant ($p<0.05$).

Table 3: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus spirulina on Total Erythrocyte Count (TEC) values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	$6.40^{a} \pm .13$	$6.20^{a} \pm .04$	$6.48^{a} \pm .13$	$6.43^{a} \pm .13$	$7.10^{b} \pm .04$	**
30 Days	$6.48^{ab} \pm .18$	$6.23^{a} \pm .04$	$6.98^{b} \pm .18$	$6.98^{b} \pm .13$	$7.63^{c} \pm .28$	*
45 Days	$6.71^{ab} \pm .25$	$6.35^{a} \pm .25$	$7.34^{c} \pm .26$	$7.55^{c} \pm 17$	$8.53^{d} \pm .14$	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.3.2 Total Leukocyte Count (TLC):

In Table 4, Total leukocyte counts on Day 30 was found highest (10.87 ± .005) in control group rats and lowest in T_4 group rats where sprulina and riboflavin were treated and the difference were statistically significant among all group of rats ($p<0.01$). So it can be recommended that sprulina and riboflavin decrease the TLC level.

Table 4: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus spirulina on Total Leukocyte Count (TLC) values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	$9.56^{d} \pm .003$	$9.09^{a} \pm .005$	$9.41^{b} \pm 005$	$9.37^{b} \pm .030$	$9.51^{c} \pm .006$	**
30 Days	$10.87^{e} \pm .005$	$10.24^{a} \pm .005$	$10.42^{c} \pm .005$	$10.27^{b} \pm .005$	$10.76^{d} \pm .006$	**
45 Days	$10.82^{c} \pm .003$	$9.79^{a} \pm .008$	$9.87^{ab} \pm .005$	$9.88^{b} \pm .010$	$10.80^{c} \pm .058$	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.3.3 Hemoglobin (Hb):

Highest (15.25 ± .78) Hb concentration was found in T_4 group at 30 days and lowest concentration was found in T_0 group (Table 5). Difference among values of 30 days of Hb concentration were statistically significant ($p<0.01$) and the difference among values of 15 and 45 days of Hb concentration were statistically significant ($p<0.05$). It might be concluded that Spirulina and riboflavin might slightly increase the values of Hb against arsenic toxicity in rats.

Table 5: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus spirulina on Hemoglobin concentration (Hb) (gm/dl) values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	13.50^{b} ± .65	11.13^{a} ±.43	14.00^{b} ± .41	13.13^{b} ± .31	12.88^{b} ± .43	*
30 Days	11.03^{b} ± .89	8.45^{a} ± .65	15.25^{c} ± .78	14.98^{c} ± .72	15.25^{c} ± .78	**
45 Days	15.13^{b} ± .97	7.75^{a} ± 1.78	15.28^{b} ± .83	15.05^{b} ± 1.05	17.20^{b} ± 1.54	*

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.3.4 Erythrocyte Sedimentation Rate (ESR):

The highest ESR value was observed in T4 group and lowest value was observed in control group (Table 6). The differences between the mean values of different groups were found significant ($p<0.05$). It was found that hemoglobin and hematocrit values were reduced in arsenic toxicities in rats as observed in the present study and in other groups it was increased. The cause of change in hematological values might be due to the toxic effect of arsenic on haematopoeitic system which is responsible for such alterations in hematological parameters. However, Islam *et al.* (2005) assumed that toxic effects of arsenic trioxide on bone marrow may be responsible for erythrocytopenia.

Table 6: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus spirulina on Erythrocyte Sedimentation Rate (ESR) (gm/dl) values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	$43.88^{b} \pm 2.78$	$39.75^{ab} \pm 1.03$	$38.38^{a} \pm .85$	$36.25^{a} \pm .85$	$36.50^{a} \pm .65$	*
30 Days	$42.00^{b} \pm 3.58$	$31.00^{a} \pm 1.47$	$45.50^{b} \pm 2.10$	$45.13^{b} \pm 2.56$	$41.50^{b} \pm 1.32$	*
45 Days	$42.25^{b} \pm 2.81$	$24.25^{a} \pm 2.17$	$47.00^{b} \pm 3.45$	$49.63^{b} \pm 3.90$	$50.25^{b} \pm 7.16$	*

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.3.5 Packed Cell Volume (PCV):

The PCV values were decreased in arsenic treated group. The highest values were found in T_4 group and lowest values were found in arsenic treated T_1 group (Table 7). The differences between the mean values of different groups were found not significant. The cause of change in hematological values might be due to the toxic effect of arsenic on haematotopoeitic system which is responsible for such alterations in hematological parameters. However, Islam

et al. (2005) assumed that toxic effects of arsenic trioxide on bone marrow may be responsible for erythrocytopenia.

Table 7: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus on Packed Cell Volume (PCV) (gm/dl) values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	13.67 ± 1.33	9.00 ± .58	12.00 ± 1.53	15.00 ± 2.52	16.00 ± 3.21	NS
30 Days	12.00 ± .58	11.00 ± .58	13.33 ± .88	15.67 ± .67	19.00 ± 2.08	NS
45 Days	18.33 ± 3.84	9.00 ± .58	15.33 ± .33	21.67 ± 6.01	20.00 ± 2.89	NS

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.4 Biochemical parameters

4.4.1 Serum Glutamate Oxaloacetate Transaminase activity (SGOT)

The highest values of SGOT were observed in the control group (T_0) while the lowest values were observed in theT_4 group (Table 8). There were significant differences within the groups during the two days (30 and 45) of measurement ($P<0.01$) and in 15 days significant differences within the groups of measurement ($P<0.05$). It appears that while Spirulina alone has some effect in lowering the SGOT values in response to prolonged administration of arsenic, the combination of spirulina and riboflavin produced a more significant reduction in SGOT level comparable to the control group ($P<0.01$)**.** Although this finding disagreed with the previous findings that SGOT was reduced by As alone (Mahaffey *et al.,* 1981). It is similar with the findings of Yasmin *et al.* (2011) who indicated similar results. In Spirulina treated (T_2), riboflavin treated (T_3) and Spirulina plus riboflavin treated (T_4) experimental arsenicosis groups, there were significantly decreased values of arsenic recorded ($P<0.01$).

Table 8: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus on SGOT values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. value
15 Days	$115.00^{c} \pm 2.89$	$110.00^{bc} \pm 289$	$100.00^{a} \pm 2.52$	$106.67^{abc} \pm 4.41$	$102.00^{ab} \pm .58$	*
30 Days	$109.00^{bc} \pm 3.79$	$117.00^{c} \pm 8.50$	$97.33^{ab} \pm 1.45$	$98.67^{ab} \pm 1.86$	$88.00^{a} \pm 1.53$	**
45 Days	$109.67^{b} \pm 3.18$	$141.67^{c} \pm 6.23$	$106.67^{b} \pm 1.67$	$105.00^{b} \pm 2.89$	$84.33^{a} \pm 2.33$	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.4.2 Serum Glutamate Pyruvate Transaminase activity (SGPT)

Continuous administration of arsenic to Long-Evans rats caused a significant increase in the blood SGPT level. The highest values of SGPT were observed in theT_1 group where the rats were treated with only arsenic. There were insignificant differences within the groups during days 15 and 30 but this difference became statistically significant ($p<0.01$) by day of 45.The lowest values of SGPT were observed in the T_4 group where combine administration of spirulina and riboflavin against arsenic toxicity. In 15 days and 30 days blood SGPT level were increased however it is not statistically significant ($p > 0.05$). In 45 days the level of blood SGPT were decreased and it is statistically significant ($p<0.01$) (Table 9). Overall SGPT values have decreasing trend with the progress of time in all groups which was agreed with the findings of (Islam, 2008). It may be concluded that prolonged treatment with spirulina and riboflavin may reduce the blood SGPT level.

Table 9: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus on SGPT values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P Value
15 days	71.67 ± .89	77.00 ± 1.53	64.33 ± 7.17	72.67 ± 1.45	79.00 ±1.52	NS
30 Days	73.00 ± .58	76.00 ± 3.51	68.67 ± 8.95	64.33 ± 2.33	70.67 ± .67	NS
45 Days	74.00^{b} ± .00	89.33^{c} ± 4.84	68.67^{b} ± 7.36	42.33^{a} ± .89	45.67^{a} ± 2.33	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

4.4.3 Serum creatinine

Serum creatinine value were highest found in T_3 group at 45 days and lowest values were observed in control groups. The differences were found significant ($P<0.05$) on day 15. The differences between the mean values of 30 and 45 days groups were found significant ($p<0.01$). On the day 30 lowest mean value were observed in control group and highest mean value were observed in T_3 group rats and the differences were statistically significant ($p<0.01$). On the day 45 lowest mean value were observed in T_2 group and highest mean value were observed in T_3 group rats and the differences were statistically significant ($p<0.01$). The differences of As content between T_2 and T_3 were statistically significant ($P<0.01$). However the As contents increased in T_1, T_3 and T_4 group but decreased in T_2, groups on day 30 compared to day 45. On day 45, the values of serum creatinine was the highest in T_3 group rats and lowest in T_2 group. The differences were observed significant ($P<0.01$) on day 45 (Table 10). There was significant difference in serum creatinine level observed between the control group and all other treatment group rats through the whole study period. Which disagree with the findings of Nabi *et al.* (2005) in human being who showed that the patients of arsenicosis had significantly lower level of serum creatinine compared to the control and Zhang *et al*., (1995) who observed that there is a relationship between arsenic level and degree of chronic renal insufficiency in men. Islam *et al.* (2009)

and Roger *et al.* (2000) which concluded that there were no significant rises in the serum creatinine levels of arsenic treated mice.

Table 10: Effects of arsenic, arsenic plus riboflavin, and arsenic plus riboflavin Plus on Serum creatinine values of rats

Treatment	T_0	T_1	T_2	T_3	T_4	P. Value
15 Days	$0.51^{a} \pm .005$	$0.62^{c} \pm .003$	$0.52^{a} \pm .005$	$0.59^{c} \pm .042$	$0.53^{a} \pm .003$	*
30 Days	$0.52^{a} \pm .003$	$0.65^{c} \pm .005$	$0.54^{b} \pm .008$	$0.64^{c} \pm .005$	$0.52^{a} \pm .006$	**
45 days	$0.52^{a} \pm .006$	$0.67^{c} \pm .003$	$0.51^{a} \pm .003$	$0.69^{c} \pm .012$	$0.57^{b} \pm .023$	**

In a row figurers with same or without superscripts do not differ significantly as per DMRT, data were calculated at 99% level of significance ($p<0.01$).

Figures indicate the Mean ± SE (standard error); NS means not significant

** = Significant at $p<0.01$ level of probability

*= Significant at $p<0.05$ level of probability

CHAPTER 5

CONCLUSION

The results of this study may be concluded as the following

- From this study it may be recommended that arsenic toxicity reduce the body weight of rats.
- Treatment with spirulina and riboflavin might increase the body weight. Spirulina and riboflavin alone can reduce the effects of arsenic toxicity but if both of these used in combination it may be more effective.
- Arsenic toxicity has adverse effect in hematological and biochemical parameters in rat.
- Spirulina and riboflavin have protective effect in improving these parameters.
- Combined spirulina and riboflavin treatment is more effective arsenic toxicity.
- This study suggested that spirulina and riboflavin has significantly reduced the arsenic concentration of inorganic arsenic toxicity in rats.
- Further investigation in this line may make more clear evidence to use spirulina as a therapeutic treatment for arsenic toxicity.

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