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Research Article

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ANTIOXIDANT ACTIVITIES OF *MORINGA OLEIFERA* LEAF EXTRACT AGAINST ARSENIC INDUCED TOXICITY IN *CIRRHINUS MRIGALA*

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ABSTRACT

This experimental study was conducted to evaluate the antioxidant activities of Moringa oleifera leaf extract against Arsenic (As) induced toxicity in Cirrhinus mrigala in Tawakkal Fish Hatchery at Muzaffargarh, Punjab, Pakistan. 288 fingerlings were collected from fish pond and kept in circular tank for acclimatization. 12 fish about 100-day old having similar size were selected randomly and kept in separate glass aquaria for each treatment groups T1, T2, T3 and control group T4. Fish in treatments groups T1, T2 and T3 were exposed with water born sublethal concentration of 1/10th LC50 of arsenic (As) for 7days (240 hours). On 8th and 16th days of the experiment three fish were collected from each aquarium, humanly dissected targeted organ was taken out and used for liver, muscle, and gills antioxidant enzyme activities and histopathological alteration. The findings indicate that in treatment group T2 which feed with 2% and 4% Moringa oleifera supplemented diet reduced significantly ($P<0.05$) arsenic induced oxidative stress in fish, enhance the superoxide dismutase and catalase activities but treatment group T2 is 2% Moringa oleifera supplemented diet is more effective near to control group T4 as compared to treatment group T3 with 4% Moringa oleifera supplemented. After 16 days exposure of 1/10th concentration of arsenic with 0% Moringa oleifera various degenerative alteration were seen in gills. In 2% and 4% Moringa oleifera with 1/10th arsenic, spiked secondary lamellae and lamellar epithelium lifting (EL) and rupture of epithelial layer (↑↑) and fusion of lamellae were observed at several points. Moringa oleifera is medicinal herb, which has various tremendous benefits.

Keywords: *Cirrhinus mrigala, Moringa oleifra, Arsenic, antioxidant, Catalase and Superoxide dismutase*.

INTRODUCTION

Aquaculture and fisheries are the world fastest emerging, food processing, manufacturing sectors and growing more rapidly as compared to other animal food manufacturing sector to overcome food shortage due to overpopulation (Bahri et al., 2021, Pauly & Zeller, 2017, Cai et al., 2021). The aquaculture industry is contributing almost 50% of the world food fish provision and is recognized as having the best potential to satisfy the increasing demand for aquatic products, and Asia accounts for the majority of aquatic products. (Gjedrem et al., 2012, Béné et al., 2016, Garlock et al., 2022). More items from aquaculture are currently consumed directly than from conventional fisheries (Gempesaw et al., 1995, Chopin & Tacon, 2021, Mobsby et al., 2020). These sectors are providing aquatic products, having nutritional values such as proteins, carbohydrates, omega-3 fatty acids, highly rich in micronutrients and vitamins (Balami et al., 2019, Khalili Tilami & Sampels, 2018, Mishra & Pradesh, 2020, Masi et al., 2022). Aquatic products are more efficient converter of protein and energy as compared to other farm animals (Crab et al., 2012, Gephart et al., 2020, Stankus, 2021). Development and advancement in the field of science and technology, industries and informal settlement are major factors causing water pollution that are continuously affecting the water quality parameters leads to decline in population of

freshwater species (Malik et al., 2020, Zeitoun & Mehana, 2014, Fernando et al., 2007). Due to mismanagement of human activities are introducing many undesirable hazardous substances into the freshwater and promoting many complications for aquatic organisms (Owa, 2013). Water pollutants promote the biochemical and physiological changes in fish that leads to growth and development inhibition (Shahjahan et al., 2022). Many non-degradable heavy metals are considered as lethal for aquatic organisms due to their environmental endurance and for bioaccumulation (Das et al., 2001, Garg et al., 2009, Humtsoe et al., 2007). Hence, the study of aquatic animal toxicology has become an important field in the field of water pollution. Toxicology is an offshoot of animal and plant sciences that explores the harmful effects of chemicals and other aligned agents on living organisms (Mance, 2012, Ali & Khan, 2018). It is valuable for protecting public health from the negative effects of toxic substances such as heavy metals in food, air, and water (Maity et al., 2021, Briffa et al., 2020, Baker et al., 2014, Wood, 2011). According to toxicological studies, enzymes are a common target of toxicants (Karaca et al., 2010). Enzymes are metabolic functional units (Zammit & Newsholme, 1979, Kumar et al., 2022). Oxidative enzymes are essential in respiratory metabolism (Coutinho & Gokhale, 2000, Natarajan, 1984, Barathinivas et al., 2022). Succinic dehydrogenase is a key oxidative enzyme in the (TCA) cycle (Kregiel, 2012, Moosavi et al., 2019, Bubber et al., 2011). Heavy metals discharged into waters from industrial effluents and other sources have a negative impact on fish growth and metabolism (Javed & Usmani, 2015, Ali & Khan, 2018). There are numerous heavy metals that are toxic to humans and aquatic organisms (Shahjahan et al., 2022). These metals enter into aquatic habitats through a variety of routes, causing negative effects on physiology and morphology and have a unique property of accumulating over time extremely high amounts accumulating in organisms along the food chain with very low concentrations in water and sediment (Sörme & Lagerkvist, 2002, Wang et al., 2011, Zhou et al., 2020). Over the past few decades, the amount of heavy metals in the aquatic environment has dramatically increased (Waheed et al., 2018). Mercury, cadmium, arsenic, and selenium are some of the heavy metals that are harmful. Among these heavy metals, arsenic is one of the most significant global environmental pollutants and is a chronic bio-accumulative carcinogen (Khayatzadeh & Abbasi, 2010, Baby et al., 2010, Atli et al., 2006). There are two main inorganic forms of this hazardous metalloid: arsenate and arsenite (Kaur et al., 2011, Jomova et al., 2011). Arsenite interferes with cellular enzymatic processes, whereas arsenate functions as a phosphate analogue and prevents the uptake and use of phosphate (Vahter, 2009, Gaim et al., 2015). Despite being poisonous, arsenic can be promptly sequestered in animal and plant tissues (Gunes et al., 2009). Water is major source of arsenic transporting under natural circumstances. Arsenic (As) is a metalloid element that is naturally occurring and pervasive throughout the environment and is particularly common in aquatic environments as a result of anthropogenic and natural processes (Wang & Mulligan, 2006, Ali et al., 2019, Chung et al., 2014). Two forms of arsenic are reported in water are arsenate and arsenate (Chouhan & Flora, 2010, Radfard et al., 2019). Arsenic primarily affects the different organs involved in excretion, absorption, and accumulation (Shahjahan et al., 2022). It shows negative effects on circulatory system, gastrointestinal tract, liver, skin, and kidney (Lavanya et al., 2011, Squadrone et al., 2013). It also has negative impact on the biochemical, hematological, and ion regulatory parameters of organisms and fish in aquatic media, and alters in these parameters are useful in environmental bio monitoring of arsenic contamination (Xu et al., 2021, Naz et al., 2021). Arsenic toxicity is caused by the inhibition and impairing of mitochondrial enzymes and the uncoupling of oxidative phosphorylation (Squibb & Fowler, 1983, Selvaraj et al., 2013, Sun et al., 2022). Arsenic's toxicity results from its capacity to bind with sulfahydryl groups of proteins and enzymes and act as a substitute for phosphorus in a number of metabolic processes (Rosen et al., 2011). The most frequent cause of increased arsenic concentrations in aquatic environments is anthropogenic activity (Bukola et al., 2015, Ochieng et al., 2007, Vareda et al., 2019). *Moringa oleifera* is a well-liked and medicinal plant accepted for protein sources and is used in fish diets (Puycha et al., 2017, Richter et al., 2003, Hardy, 2010). It is gaining popularity in aquaculture because all of its parts, including the flowers, leaves, and seeds, can be used (Nascimento et al., 2013). Antioxidants are a key component of nutritional biochemistry and are found in abundance in *Moringa oleifera*, the leaves and flowers have significant nutritional value to the populace (Hamed & El-Sayed, 2019, Santos et al., 2012, Charoensin, 2014, Verma et al., 2009). The current experimental study was designed to investigates the antioxidant activity of *Moringa oleifera* leaf extract against Arsenic induced toxicity in *Cirrhinus mrigala*.

MATERIALS AND METHODS

Ethical statement: This experimental study was conducted under the strict rules and regulation of Pakistan Animal Welfare Society. The fish fingerling collection and managing were done under the instructions and guidelines of Fisheries Development Board (FDB). All possible efforts were made to minimize the pain and suffering of fish during experiment.

Study site: The experimental investigation was carried out in Tawakkal Fish Hatchery Muzaffargarh, Punjab, Pakistan. The hatchery's entire size is 8435 km2, and it is located between latitudes 30.0736° North and 71.1805° East.

Experimental design: *Cirrhinus mrigala* fingerlings were collected from fish ponds and placed in cement tanks for 14 days. This experimental study was conducted in glass aquarium for two weeks, with each aquarium filled with 240 litter waters. Following acclimatization, 12 individuals of *Cirrhinus mrigala* of similar size were selected randomly and kept in separate glass aquaria for each treatment and control group. Three treatment groups or experimental groups were designated as T_1, T_2 and T_3. All treatments were exposed with water born sublethal concentration of arsenic (Sanaullah et al., 2021). A control group without arsenic was used in tandem. Fingerlings of *Cirrhinus mrigala* in treatment groups T1, T2 and T3 were fed with *Moringa oleifera* leaves extract supplemented diet 0 %, 2 % and 4 % and exposure of $1/10^{th}$ of LC50 of arsenic (As). Fish in the control group were fed commercially available feed without additional *Moringa oleifera*. A study was carried out with constant water temperature, pH, and hardness of 28 °C, 7.1 0.4, and 196 10 mg/L, respectively. During the experimental trial, three fish were selected at random and tested for histology and oxidative enzyme assays after each 8^{th} days.

Physico-chemical Parameters of Water: The physico-chemical parameters containing carbon dioxide (mg/L), pH, total ammonia (mg/L), total hardness (mg/L), water temperature (°C), electrical conductivity (µS/cm), dissolved oxygen (mg/L) and were noted and maintained (Table 1).

Table 1 Physico-chemical parameters of hatchery water.

Sr. No.	Parameters	Measurements
1	Temperature (^{o}C)	28.2±0.43
2	pH	7.5±0.085
3	Dissolved Oxygen (mgL^{-1})	7±0.61
4	Total alkalinity (mgL^{-1})	34.7±0.61
5	Calcium hardness (mgL^{-1})	34±1
6	Chlorides (mgL^{-1})	31±0.23
7	Electrical conductivity ($uScm^{-1}$)	192±0.69
8	Total dissolved solid (mgL^{-1})	117±0.5
9	Salinity (ppt)	0.15±0.02

Enzyme assays: Fish kidney, liver, gills, and muscles were homogenized in cold buffer (1:4 W/V) after being rinsed with phosphate buffer with a pH of 6.5 (0.2M). This was carried out in order to remove tissues red blood cells (liver, kidney, gills and muscles). After homogenization, the organ homogenate was centrifuged for 15 minutes at 4 °C at 10,000 rpm. The clear supernatant from centrifugation was kept at -4 °C for the enzyme assay (Sanaullah et al., 2021).

Catalase (CAT)

Preparation of 50mM phosphate buffer, pH 7.0: Prepared 1.2 ml of 50 mM phosphate buffer pH 7.0, 0.2 ml of tissue homogenate, and 1.0 ml of 30 mM H_2O_2 solution were added to initiate the reaction. Throughout the course of three minutes, the absorbance decreased at 30-second intervals. Instead of using hydrogen peroxide, 1.0 ml of pure water was run concurrently with the enzyme blank. In nanomoles of H_2O_2 degraded per minute per gramme of tissue, the enzyme activity was measured.

Superoxide dismutase (SOD): The tubes containing 0.75ml ethanol and 0.15ml chloroform were refrigerated in ice and then centrifuged with 0.1ml of tissue homogenate added. A 0.1 M carbonate and bicarbonate (pH 10.2) buffer and 0.6 mM EDTA solution were each added to 0.5 ml of supernatant. The reaction was initiated by the addition of 0.5ml of 1.8mM NBT (freshly prepared) and increase in absorbance at 480 nm by using a spectrophotometer.

Histology of Fish: The fish from each aquarium were collected and slaughtered to observe the modification in tissue of organs i.e., gills. Slides were organized. A fraction of 4-6 µm was formed by microtome (AEM450). The fraction was marked with hematoxylin and eosin to imagine the variations in tissue by a camera fixed microscope (S/N-EU1711056).

RESULTS AND DISCUSSION

The current experiment was designed to determine the antioxidant effect of *Moringa oleifera* leaf extract against arsenic-induced toxicity in *Cirrhinus mrigala*. It was carried out in a controlled laboratory setting with water at a constant pH of 7.5 and 28 °C. A total of 288 acclimatized individuals of *Cirrhinus Mrigala* were partioned into four groups with three replicates each. Treatment groups T_1, T_2 and T_3 were exposed with 1/10th concentrations of LC50 of arsenic. Groups T_1,

T_2 and T_3 were also feed with 0, 2 and 4% *Moringa oleifera* leaves extract supplemented diet respectively. Control group T_4 with no arsenic and no *Moringa oleifra* supplemented diet. On 8th and 16th days of the experiment three fish were collected from each aquarium; humanly dissected targeted organ was taken out and used for liver, muscle, and gills antioxidant enzyme activities and histopathological alteration. The result of Superoxide dismutase enzyme activity in different tissues like liver, muscle, and gills of fish after introduction of 1/10th concentration of LC50 of Arsenic and its ameliorative effect with 2% and 4% feed supplementation of *Moringa oleifera* leaf extract for 16 days are presented in tables (Table 2, Table 3 and Table 4). The findings of Catalase enzyme activity in different tissues like liver, gills and muscles of fish after exposed to 1/10th concentration of LC50 of arsenic and its ameliorative effect with 2% and 4% feed supplementation of *Moringa oleifera* leaf extract for 16 days are presented in tables (Table 5, Table 6 and Table 7).

Table 2. Change in Superoxide dismutase (SOD) (IU/g of tissue) enzyme activity in liver tissue of *Cirrhinus mrigala* after exposure of arsenic along with varying levels of *Moringa oleifera* leaves extract supplemented diet.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0% M+1/10th)	3.34±0.02[a]	3.54±0.01[a]
T2 (2% M+1/10th)	2.64±0.01[b]	2.72 ±0.03[b]
T3 (4% M+1/10th)	2.84±0.02[b]	2.98±0.01[b]
T4 Control	2.66±0.01[b]	2.74±0.02[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Table 3. Changes in Superoxide dismutase (SOD) (IU/g of tissue) enzyme activity in gill tissue of *Cirrhinus mrigala* after exposure of arsenic along with varying levels of *Moringa oleifera* leaves extract supplemented diet.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0% M+1/10th)	3.50±0.03a	3.56±0.02[a]
T2 (2% M+1/10th)	2.08±0.02[b]	2.10±0.01[b]
T3 (4% M+1/10th)	2.90±0.01[c]	3.08±0.06[c]
T4 Control	2.11±0.01[b]	2.20±0.01[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Table 4. Change in Superoxide dismutase (SOD) (IU/g of tissue) enzyme activity in in muscle tissue of*Cirrhinus mrigala* after exposure of arsenic along with varying levels of *Moringa oleifera* leaves extract supplemented diet.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0%M+1/10th)	2.13±0.02[a]	2.25±0.02[a]
T2 (2% M+1/10th)	1.34±0.02[b]	1.68±0.01[b]
T3 (4%M+1/10th)	1.68±0.01[b]	1.76±0.01[b]
T4 Control	1.41±0.02[b]	1.69±0.01[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Table 5. Changes in the Catalase (CAT) enzyme activity (IU/g of tissue) in liver tissue of *Cirrhinus mrigala* after exposure arsenic with varying levels of *Moringa oleifera* leaves extract.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0% M+1/10th)	24.1±0.21[a]	24.60±0.18[a]
T2 (2% M+1/10th)	18.89±0.02[b]	19.89±0.27[b]
T3 (4% M+1/10th)	21.67±0.14[c]	22.73±0.44[c]
T4 Control	19.10±0.11[b]	20.34±0.16[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Moringa oleifera is a familiar and well-known medicinal plant with numerous therapeutic properties. (Kumar, 2017). It is a significant source of antioxidants, which are nutrients used in nutritional biochemistry and may be advantageous for human health (Gopalakrishnan et al., 2016, Patel et al., 2010). Minerals, vitamins, and other vital phytochemicals are abundant in the leaf extracts of this plant. It is employed as a potential anti-inflammatory, anti-cancer, and antioxidant agent (Gupta et al., 2018).

Table 6. Changes in the Catalase (CAT) enzyme activity (IU/g of tissue) in gills *Cirrhinus mrigala* afterexposure arsenic with varying levels of *Moringa oleifera* leaves extract.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0% M+1/10th)	20.10±0.10[a]	20.68±0.12[a]
T2 (2% M+1/10th)	16.86±0.23[b]	16.96±0.17[b]
T3 (4% M+1/10th)	18.87±0.05[c]	18.48±0.66[b]
T4 Control	17.05±0.13[b]	17.12±0.03[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Table 7. Changes in the Catalase (CAT) enzyme activity (IU/g of tissue) in muscles of *Cirrhinus mrigala* after exposure arsenic with varying levels of *Moringa oleifera* leaves extract.

Treatments	8th Day (Mean±SD)	16th Day (Mean±SD)
T1 (0% M+1/10th)	9.87±0.01[a]	9.98±0.08[a]
T2 (2% M+1/10th)	7.82±0.02[b]	7.94±0.06[b]
T3 (4% M+1/10th)	8.02±0.14[b]	8.21±0.28[b]
T4 Control	7.28±0.01[b]	8.41±0.03[b]

Mean values labeled with same alphabet are non-significantly different at α=0.05

Effect on Superoxide dismutase (SOD) enzyme activity

The results indicated that activity of superoxide dismutase decreased in tissues of liver, gills and muscles of fish when exposed to 1/10th concentration of arsenic (T_1) as compared to the control group of fish and similar concept was reported by (Farombi et al., 2007). While, in treatment groups T_2 and T_3 which are feed with 2% and 4% of *Moringa oleifera* extract supplemented diet along with 1/10th concentration of LC50 of arsenic, superoxide dismutase enzyme recovers and increased in tissues of liver, gills and muscles of fish and oxidative stress is reduced. Treatment group T_2 which feed with 2% *Moringa oleifera* supplemented diet reduced arsenic induced oxidative stress more effectively as compared to the 4% *Moringa oleifera* supplemented. But 4 % *Moringa oleifera* supplemented diet also significantly reduced arsenic induced oxidative stress, these findings are strongly supported by the (Hamed & El-Sayed, 2019). Changes occurred in enzyme activity that depends on concentration of arsenic and *Moringa oleifera*. The primary superoxide scavenger SOD also acts as the first line of defense against cellular damage brought on by environmental stress (Gratão et al., 2005). SOD transforms the extremely reactive oxide ion into H_2O_2. (Bhattacharya & Bhattacharya, 2007) reported that the arsenic-treated Indian catfish *Clarias batrachus* had significantly lower SOD activity. Similar to earlier research, liver and gill SOD activity in rockfish tissues significantly decreased after exposure to waterborne arsenic (Kim & Kang, 2017, Diaconescu et al., 2008).

Effect on Catalase (CAT) enzyme activity: The findings indicated that when fish exposed to arsenic. Catalase activity decreased in the muscles, gills, and liver tissue. CAT detoxifies excess H_2O_2 created by SOD action. CAT activity was found to be lower in living tissues exposed to arsenic in other studies (Altikat et al., 2015). In my research work, the CAT activity was lower in T_1 as compared to in the control group T_4. The CAT activity was less decrease in T_2 and T_3 was near to control group, when exposed to 1/10th concentration of arsenic treatment group T_1 as compared to the control group T_4 of fish. While, in treatment groups T_2 and T_3 which are feed with 2 and 4% *Moringa oleifera* extract supplemented diet along with 1/10th concentration of LC50 of arsenic, Catalase enzyme recover and increased in tissues of liver, gills and muscles of fish and oxidative stress is reduced. Group 2 which feed with 2% *Moringa oleifera* supplemented diet reduced arsenic induced oxidative stress more effectively as compared to the 4% *Moringa oleifera* supplemented. But 4 % *Moringa oleifera* supplemented diet also significantly reduced arsenic induced oxidative stress. Similar outcomes were reported that *Moringa oleifera* reduced the significantly reduced the effect of Arsenic in animals tissues and blood which supports the current finding this study (Gupta et al., 2005, Gupta et al., 2007, Mishra et al., 2009).

Effects on histology of *Cirrhinus mrigala* gills: Four gill arches supported two hemibranchs with numerous filaments bearing lamellae, which were separated by substantial interlaminar spaces and well-differentiated primary lamellae (PL), secondary lamellae (SL), and inter filamental lamella spaces in the fish's gill structure shown in (Figure 1). After 16 days exposure of 1/10th concentration of arsenic with 0% *Moringa oleifera* various degenerative alteration were seen in gills. There was the filament epithelial proliferation (FP), necrosis (N) and desquamation (D) in the inter filament region (Ahmed et al., 2013, Zeitoun & Mehana, 2014). With the collapse of the pillar cell system, secondary lamellae fused, the epithelial layer ruptured, and numerous secondary lamellae developed aneurisms (A) as shown

in (Figure 1). In In group 2 which feed with 2% *Moringa oleifera* supplemented diet along with 1/10th concentration of LC50 of arsenic, cellular necrosis (CN) in secondary lamella, epithelial hyperplasia (EH) was observed as shown in (Figure 2). In 4% *Moringa oleifera* with 1/10th arsenic, spiked secondary lamellae and lamellar epithelium lifting and multiple instances of lamellae fusion and epithelial layer rupture were seen as shown in Figure 2.

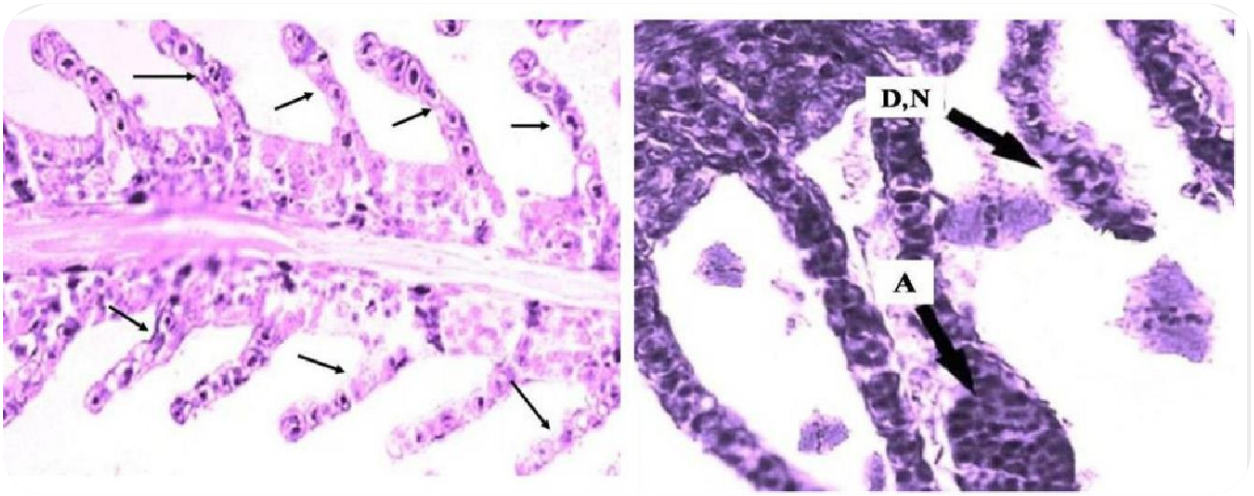

Figure 1. Degeneration of gills structure aneurisms (A) necrosis (N) and desquamation (D) in the inter filament region after arsenic exposure.

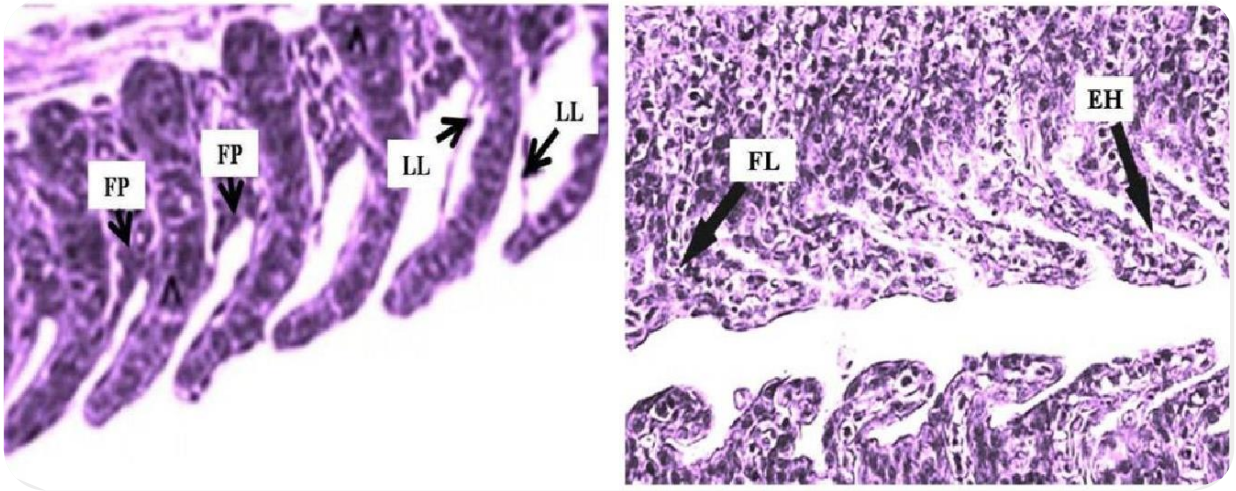

Figure 2. Fusion of secondary lamellae, lamellar epithelium lifting (EL) and multiple instances of lamellae epithelial layer, filament epithelial proliferation (FP) after antioxidant activity of 2% and 4% *Moringa oleifera.*

The finding of current study indicated that Catalase (CAT) and Superoxide dismutase (SOD) activities decreased in the experimental groups T_1, $T_2$and T_3 of fish as compare to control groups T_4 because of arsenic exposure. In Treatment group T_1 which was supplemented with 0% diet of *Moringa oleifera* and 1/10th of LC50 of arsenic, Catalase (CAT) and Superoxide dismutase (SOD) activities significantly decreased because of oxidative stress of arsenic. But in Treatment groups T_2 and T_4 activity of Catalase (CAT) and Superoxide dismutase (SOD) was recovered close to control group T_4. In these groups, *Moringa oleifera* leaves extract supplemented diet used and its antioxidant potential reduced the oxidative stress of arsenic. But 2% *Moringa oleifera* dose cure best in experimental groups of fish. According to this study, the extract of *Moringa oleifera* leaves has strong antioxidant activity against arsenic free radicals, protects important biomolecules from oxidative damage, and offers significant protection against oxidative damage in fish. *Moringa oleifera* leaf extracts are an inexpensive source of high-quality plant protein that has no negative impact on the body

composition of fish.

CONCLUSION

In this experimental research, it is concluded that *Moringa oleifera* has strong, anti-oxidant ability. It is suggested that *M. oleifera* is medicinal herb, which have various tremendous benefits like it possess anti-oxidant ability thus used as antioxidant in diet to reduced physiological alteration caused by pollutants and improve the growth of fish at low cost. Histopathology has shown that *M. oleifera* has reduced hepatic inflammation, hepatic degeneration, cytoplasmic necrosis, induced by arsenic intoxication. Hence, it is concluded that *M. oleifera* leaf extract has reduced Arsenic induced oxidative stress in liver enhanced growth of fish and protected from diseases.

Credit authorship contribution statement

Muhammad Shahbaz Azhar: Execute the experiment, Investigation, Data curation, Visualization, Writing – original draft, Writing – original draft. **Muhammad Zubair Anjum:** Supervision, Review and editing. **Tahira Sarwar, Basharat Mahmood and Shameen Arif:** Conceptualization, Resources. **Muhammad Niaz Asghar and Asim Shamim:** Writing- review and approved the final manuscript. **Muhmmad Mujahid Anwar:** Statistical analysis.

Declaration of competing interest

The authors declare that there no conflicts of interest to report.

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