

The Histo-protective Properties of *Celtis occidentalis* Methanolic Extract in Glyphosate-based herbicide intoxicated Renal and Gills Toxicity in *Cyprinus Carpio*

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Abstract

Pesticides are widely used to enhance crop yield, but their release can contaminate the environment. Glyphosate (N-phosphonomethyl glycine), a broad-spectrum organophosphorus herbicide, is extensively applied to control unwanted weeds. The present study evaluated the histo-protective efficacy of *Celtis occidentalis* leaf extract in *Cyprinus carpio* exposed to glyphosate-based herbicide (GBH). Twenty one fish were divided into three groups (n=7): Group I (control), Group II (GBH, 0.71 ml), and Group III (GBH + extract 300 mg/kg; EGBH) for 28 days. Histopathological analysis of kidneys and gills revealed that glyphosate exposure caused severe tissue damage, including swelling of Bowman's capsule, glomerular contraction, vacuolar degeneration of renal epithelial cells, tubular hypertrophy, necrosis, and interstitial hemorrhage with inflammatory infiltration. In gills, congestion of blood vessels, hyperplasia and hypertrophy of epithelial and pillar cells, hemorrhage, fusion and curling of secondary lamellae, mucous accumulation, and lamellar disorganization were evident. Co-administration of *C. occidentalis* extract markedly ameliorated these alterations, restoring normal tissue architecture in a dose-dependent manner. The findings demonstrate that glyphosate induces significant renal and gill histopathological alterations in *C. carpio*, while methanolic extract of *C. occidentalis* exhibits strong protective effects against herbicide-induced toxicity.

KEYWORDS: Pesticides, Glyphosate, *Celtis occidentalis*, Common carp, Histo-physiology of kidney and gills

1.0 Introduction

Pesticides are among the most potent and dangerous chemicals released into the environment. They adversely affect non-target organisms, which are of great importance [1]. Herbicides are the most commonly used

pesticides today, not only in agriculture but also in forests, horticulture areas and even homes. Safety data sheets indicate that glyphosate and glyphosate-based herbicides are dangerous to aquatic organisms [2]. Glyphosate (N-phosphonomethyl glycine) is a broad-range

organophosphorus herbicide widely used in agricultural and non-agricultural areas to eliminate various types of unnecessary plants and weeds, including terrestrial and aquatic plants. Its salt is known as isopropyl amine salt [3]. Recent studies have revealed that glyphosate increases the risks of toxicity in aquatic environments due to its high solubility in water and widespread use [4, 5]. Health research and experimental studies have found a positive link between glyphosate and carcinogens. IARC classified glyphosate as a Group 2A carcinogen for humans [6]. According to WHO and the Canadian Council of Ministers of the Environment (CCME) the upper limit of glyphosate in drinking water is 0.7 mg/L or 1 µg/L [7, 8].

Experimental studies show that glyphosate level has been exceeded from normal range in natural water in many countries and has harmful effects on aquatic animals, particularly on fish like common carp *Cyprinus carpio* [9]. *Round Up* was the first commercial glyphosate-based herbicide, introduced in 1974 by Monsanto Company. Then other types like Touchdown, Glifloglex, and Glyphogan have been developed, combining glyphosate with other agents to enhance their activity by increasing penetration power [10]. These agents are mostly surface-active, such as poly-ethoxylated tallow amine (POEA) [11].

Glyphosate is metabolized into methyl-phosphonic acid (MPA) and amino methylphosphonic acid (AMPA), the latter being the most active metabolite. Temperature plays a significant role in the growth, reproduction, and other metabolic functions of living organisms in both terrestrial and aquatic environments. *Cyprinus carpio*, found in freshwater aquaculture, especially in central

Europe, thrives best at temperatures between 16°C and 24°C, with optimal feeding rates reported within this range [12-14].

Glyphosate and glyphosate-based herbicides (GBH) have toxicological effects on various living organisms in different environments, affecting different body parts. These herbicides strongly affect aquatic organisms due to their continuous flow into water bodies and their different concentrations affect various aquatic organisms [15, 16]. The effects of glyphosate and GBH on *Cyprinus carpio* are more reported than any other organism [17]. Exposure to glyphosate behavioral changes, including quick and then slow swimming and also causes potential damages to the kidney, liver, gills, brain, and other organs of common carp, leading to various histopathological effects, including pyknosis, necrosis, edema, and hyperplasia [18-20].

As herbicides and pesticides are chemical species used for better crops production but on the other hand it is known that these pesticides and herbicides has harmful effect on aquatic environment where it accumulates in various organs of fish and produces harmful effect on the histopathology of organs. Therefore, we aimed this study to investigate the toxic effects of glyphosate-based herbicide on kidney and gills and its amelioration by the methanolic extract of *Celtis occidentalis*.

2.0 MATERIALS AND METHODS

2.1 Collection and acclimatization of Fish

Twenty one individuals of *Cyprinus carpio* (common carp) having average mass 105-110g and length 10-12cm were used in experiment. The fish were acclimatized in aquaria for one week having 40 L of water. The conditions of the aquaria were set to have 10h light and 14 h dark cycle and

provided with standard diet containing 30% crude protein, 3% crude fat, and 4% crude fiber. Fish were fasted for 24 h prior to experiment in order to prevent water contamination [21]. All of the experimental procedures were carried out according to the ethical committee the Departmental of Zoology, University of Malakand Animal Bye-Laws (Ref. no. E-SA-11-2009).

2.2 Glyphosate based herbicide Formulation and Dilution

Glyphosate based herbicide, a commercial herbicide having 480 g/L glyphosate (41% W/W), was diluted and added to aquarium and final concentration of 0.71 ml was achieved [22].

2.3 Preparation of *Celtis occidentalis* methanolic

The methanolic extract of *Celtis occidentalis* was used that was already prepared for another in-vivo activity as in our other article [23, 24].

2.4 Experimental Design and Treatment

After acclimatization period 21 fish were randomly categorized into three groups with n=7. Group-I was considered as a normal control group and was kept in water at a temperature of $23.25 \pm 2.30^{\circ}\text{C}$. Group-II was treated with glyphosate-based herbicide (0.71 ml) for 28 days. Group-III was treated to extract and glyphosate-based herbicide (300 mg/kg+0.71 ml) at constant temperature (25°C) for 28 days.

- **Group-I:** Control group
- **Group-II:** Glyphosate-bade herbicide treated group (GBH-0.71 mL)
- **Group-III:** Extract and Glyphosate-based herbicide treated group (EGBH-300 mg/kg+0.71 mL)

2.5 Anesthesia and Dissection:

On 28th day of the study fish were anaesthetized by clove oil and dissected. Kidneys and gills were collected,

washed with distilled water, and preserved in 10% formalin for histopathological examination.

2.6 Histopathological Examinations

The collected organ tissues were fixed in 10% formalin. Samples were dehydrated in graded ethyl alcohol (70%, 80%, 90%, and 100%) followed by Dealcoholization using Xylol. The tissues were embedded in paraffin wax at $57 \pm 1^{\circ}\text{C}$. Then Paraffin blocks were sectioned to $5 \pm 1 \mu\text{m}$ thickness with a rotary microtome, finally these sections were stained with Harris' hematoxylin and alcoholic eosin [23].

3.0. RESULTS

3.1. Renal Histo-physiology

Different histopathological changes were observed in the fish kidneys after exposure to 0.71ml of glyphosate-based herbicide. Renal tissues of the fish in control group shows normal histological architecture (Fig.1. A & B). Kidney sections of fish in the Group-II (GBH-0.71 mL) were found to have changes in the histopathology of their kidneys. The histological architecture of this group shows degeneration and contraction of glomerulus (G). The Bowman capsules were found expanded and swelling was also noted. The epithelial cells in the lining of the renal tubules (RT) were degenerated and swelling was also observed. Beside these changes, renal tubules were found elongated and a mild hypertrophy was also observed. Necrosis was also observed in renal tubules. Hematopoietic tissues (HT) were found infiltrated with mononuclear inflammatory cells. Interstitial hemorrhages were also seen (Fig.1. C-F). Histology of fish Group-III (EGBH) treated with extract and GBH combined has slight histopathological changes in their kidney. The histological architecture shows rare degeneration of the glomerulus (G) throughout the group. No glomerular constriction (G) and expansion of Bowman's capsule (BC) were noted. No

vacuolar degeneration in epithelial cells lining of the renal tubules (RT) was very evident. Rarely cloudy swelling and inflammatory cells infiltration were recorded in the renal tubules (RT). The increased size of the renal tubules due to hypertrophy was recovered immediately recovered by the extract. No hematopoietic tissues (HT) infiltrated with mononuclear inflammatory cells were recorded (Fig.1. G-J).

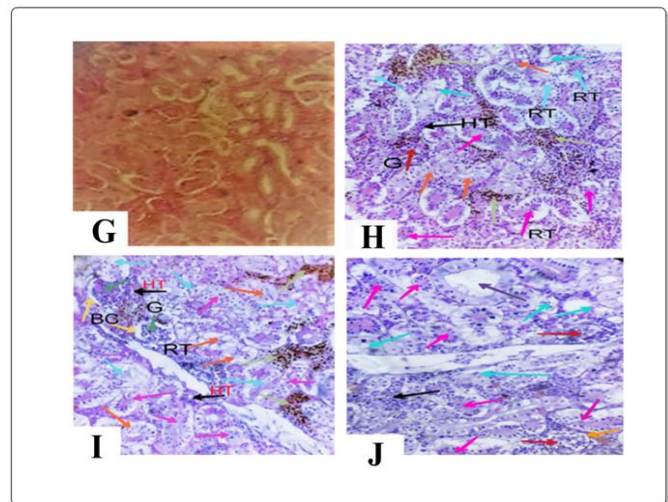
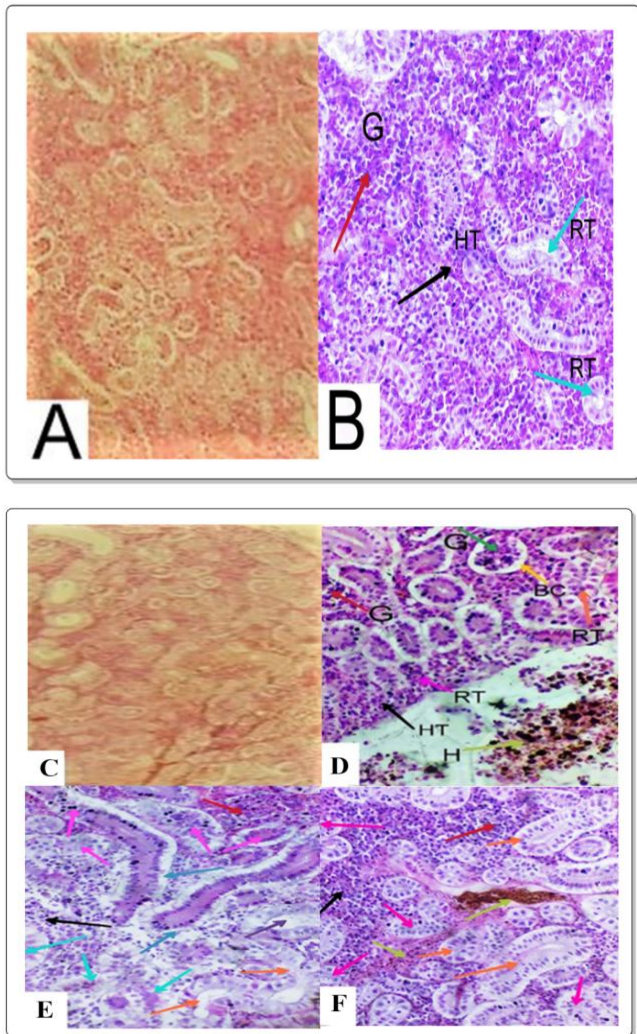


Fig. 1: Photomicrographs of kidney of the control, glyphosate-based herbicide and extract + glyphosate-based herbicide (Groups-I, II & III). (A & B) kidney sections of control group; the red arrow indicates normal histological structure of glomeruli shown by G. Renal tubules are indicated by blue arrow and are normal. The black arrow indicates normal hematopoietic tissues (HT). (C-F) kidney histology of the GBH-group; expansion and swelling of Bowmen’s capsule (BC) are indicated by yellow arrow. The red arrows in sections D, E and F indicate contraction of Glomerulus (G). In section E the sky blue arrow, deep blue arrow and purple arrow indicates vacuolar degeneration, elongation and mild hypertrophy of renal tubules respectively. Renal swelling in the sections (D-F) is indicated by orange arrow while necrosis of the renal tubules in these sections is indicated by pink arrows. The black arrows in sections (D-F) indicate the infiltration of mononuclear inflammatory cells in hematopoietic tissues. Interstitial hemorrhages are indicated by light Green arrows in section D and F. (G-J) Kidney sections of Group-III (EGBH); yellow arrows in sections I and J indicates the recovery of swelled and expanded Bowman’s capsule. The red arrows in sections (H & J) indicate the

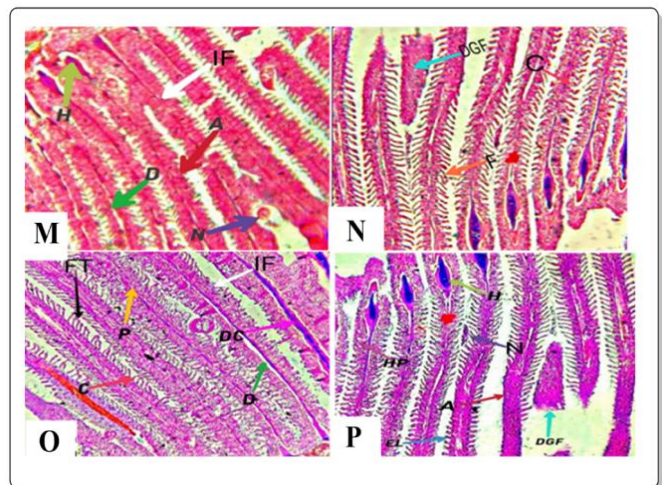
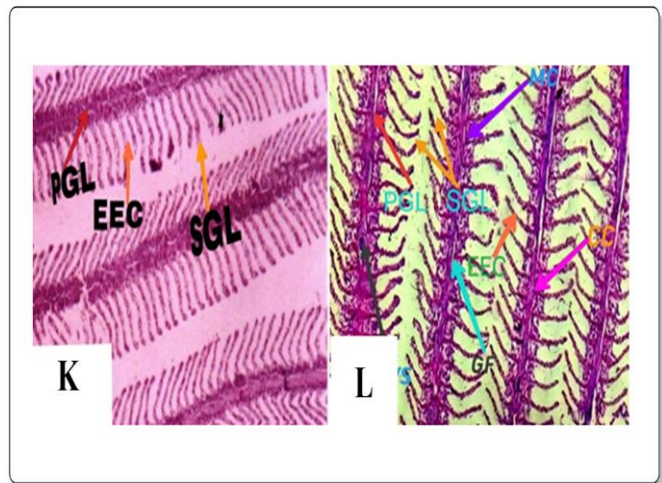
amelioration of glomerular degeneration. Swelling and necrosis in the renal tubules caused by GBH and attenuated by extract are indicated by orange and pink arrows, in sections (H-J) respectively, the recovery of vacuolar degeneration of the epithelial cells in lining of the renal tubules is indicated by sky blue arrows.

3.2. Gills histological assessment

The gills physiology of fish in Group-I treated as control group has recorded without any alterations. A normal thick lining of stratified epithelium was observed in the middle of primary and secondary gill lamellae consisting of mucous cells. No hemorrhage, atrophy, congestion of blood vessels, proliferation of the gill epithelium, hyperplasia of epithelial cells, hypertrophy of pillar cells, lamellar lifting, cellular atrophy, fusion of secondary lamellae at the base and terminal portion, lamellar curling and shortening of secondary gill lamella, lamellar disorganization and no vasodilation of central venous sinus were observed. Primary and secondary gill lamellae have a normal central cartilaginous core and the gill filament has a healthy structure (Fig. 2.K & L).

Fish in Group-II that was exposed to glyphosate-based herbicide (0.71ml), several histopathological changes were observed in their gills upon contrasting to control group. The histopathological architecture of this group revealed disruption of the cartilaginous core of gills, lamellar lifting, division of the cartilaginous core, proliferation of the gill epithelium, hemorrhage, vasodilation of the central venous sinus with blood congestion, detachment of gill filament, fusion of the secondary lamellae at the base and terminal portion, cellular atrophy, curling, lamellar curling and shortening of secondary gills lamellae and necrosis were observed (Fig. 2.M-P).

Gills histo-physiology of fish in Group-III exposed to extract and glyphosate-based herbicide (300 mg/kg+0.71 ml) has no alterations. The results revealed no blood vessels congestion and necrosis. The excessive hemorrhage, hypertrophy of pillar cells and excessive hyperplasia of epithelial cells in branchial secondary lamellae were actively recovered and appear same as in control group fish gills. No mucous accumulation was observed at the ends of branchial secondary lamellae. Atrophy in epithelial cells and pillar cells of branchial secondary lamellae and shrinkage of branchial blood vessels and intensive lamellar disorganization cause by glyphosate-based herbicide were ameliorated (Fig. 2.Q-S).



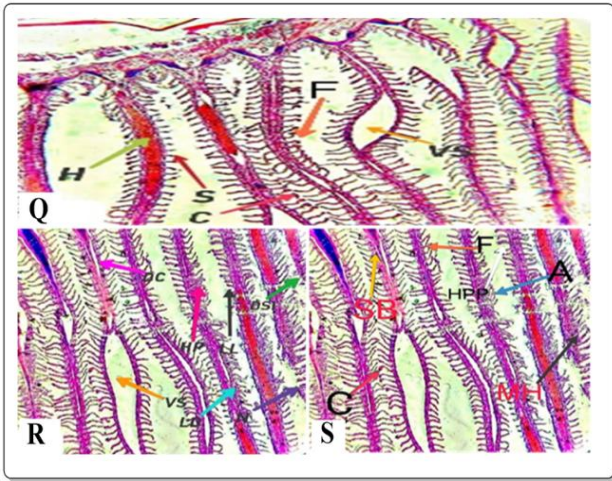


Fig. 2: Photomicrographs of the gills of control group (Group-I), GBH-0.71 (Group-II) and EGBH-300 mg/kg (Group=III). (**K & L**) gills histology of the control group (Group-I); primary gill lamellae are indicated by red arrows; they are long and slender in appearance and resemble thin plates. Secondary gill lamellae are indicated by yellow arrows, they have finger like or filamentous appearance and has cartilaginous core indicated by pink arrows. The flattened or elongated epithelial cells that resemble a network of interconnected cartilaginous branches and are indicated by orange arrows. The gill filament that is elongated and slender is indicated by sky blue arrow. Mucous cells are indicated by purple arrow and are spherical or oval in shape. The central venous sinus is indicated by black arrow, it is large blood vessel located in the center of the gill filament & water channel. (**M-P**) gills histo-physiology of GBH-0.71 (Group-II); the blue arrow indicates epithelial lifting; hyperplasia in epithelium is indicated by pink arrow. Dilation of the central venous with blood congestion is indicated by green arrow. Fusion of the secondary lamellae is indicated by orange arrow. Necrosis is indicated by purple arrow. Hemorrhage,

detachment of gill filament, cellular atrophy and fusion at the terminal portion of secondary lamellae are indicated green, sky blue, red and black arrows respectively. Proliferation of the gill epithelium is indicated by yellow arrow, disruption of cartilaginous core and infiltration of cells & fusion at the base of secondary lamellae are indicated by light pink and white arrow. Lamellar curling is indicated by light red color. (**Q-S**) gills sections of EGBH-300 mg/kg (Group-III); recovery of severe hypertrophy of the pillar cells and necrosis indicated by white and purple arrows. The black arrow indicates normal mucous membrane; light red arrow indicates uncurling at the end of branchial secondary lamellae. Orange arrow indicates recovery of the fused branchial secondary lamellae at the ends. Amelioration of the cellular atrophy and lamellar lifting is indicated by blue and black arrows, and vasodilation of blood vessels is indicated by light yellow arrow. Red arrow indicates the regenerated secondary lamellae. Recovery of hyperplasia of epithelial cell and damaged secondary lamellae by pink and green arrows.

4.0 DISCUSSION

Glyphosate-based herbicide is accumulated in water bodies because it is washed off from fields by water. Temperature changes have also effects on aquatic organisms. Therefore, in the current study histopathological effects of glyphosate-based herbicide and its amelioration by using medicinal plant were investigated on the kidney and gills of common carp. This study founds potential toxic effects of GBH on the kidney and gills. Nephron is the structural and functional unit of kidneys which was observed in microscopic examination of the normal fish or control group. It consists of the renal

corpuse and the renal tubules which have a single layer of epithelial cells. The renal corpuscles are surrounded by proximal convoluted tubules. The lumen of the distal tubule is more rounded and the apical surface of the cells is sharper. The cells forming the collecting tubules are cuboidal and smaller than those of the proximal tubule. The spaces of the tubules are enriched with hematopoietic tissues. The current findings of the histopathology of the normal control group were in accordance to previously reported studies [25]. Cloudy swelling and coagulative necrosis in renal tubules along with interstitial hemorrhages has been reported when common carp fish was exposed to GBH [26]. In our findings the group exposed to GBH only (Group-II), the microscopic examination revealed glomerular disintegration (G) and necrosis. Infiltration of mononuclear inflammatory cells in the hematopoietic tissues (HT). Swelling of renal tubules, necrosis and interstitial hemorrhages were observed as shown in Fig. 2. The above mentioned effects were also noted in a previously reported study that validates the current findings [27-30]. The findings of this study when fish were treated with extract and GBH (EGBH-300 mg/kg) recovered the degenerated glomerulus along with hemorrhage. Swelling and expansion of Bowman's space and coagulative necrosis in the epithelial cells lining of renal tubules, hypertrophy, infiltration of mononuclear inflammatory cells in the hematopoietic tissues were ameliorated indicating the protective potential of the methanolic extract of *Celtis occidentalis* as shown in Fig. 3. (K & L) [23]. The results were in accordance to previously reported studies. Studies have revealed that the gills of the normal group were found with no changes in histopathological architecture of gills [31-34] and validated the findings of our study shown in Fig.4, regarding the control group.

Lethal and sub-lethal exposure to GBH caused histopathological fluctuations in gills such as hypertrophy, hyperplasia, secondary lamellar adhesion, swollen primary gill, necrosis, and hemorrhage after exposure to lethal and sub-lethal concentrations of GBH (50,100 and 150 mL/L) for 4 days [35]. In our study, same histopathological variations were seen in Group-II (GBH-0.71 mL) exposed to GBH such as hyperplasia, necrosis, hemorrhage hypertrophy, secondary lamellar adhesion, and fusion of gill lamellae as shown in (Fig. 2.M-P). In this work, the microscopic examination of gills of the Group-III (EGBH-300 mg/kg) revealed recovery of the cellular atrophy, excessive lamellar lifting along with greater shrinkage of blood vessels, severe hypertrophy of the pillar cells, and dense abundance of mucous substance with hemorrhage. Mostly curling and fusion at the ends of branchial secondary lamellae, lamellar disorganization, and disruption of the cartilaginous core of the gills of fish, clear vasodilation of the central venous sinus, and damage and hyperplasia of epithelial pillar cells of branchial secondary lamellae were actively attenuated by extract treatment as shown in (Fig. 2.Q-S).

CONCLUSION

The present study were aimed to assess the toxic effect of glyphosate-based herbicide and high temperature on kidney and gills of common carp fish, as GBH is the most commonly used herbicide and it is washed from fields by water and then accumulates in aquatic organisms. The results of this study confirmed that exposure to glyphosate causes toxicity in kidney and gills. The *Celtis occidentalis* has strong histo-protective role in attenuating GBH toxicity. Further experimental work and clinical trials should be recommended to isolate the phytochemicals and to investigate as antioxidant.

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