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Histological Investigation to Verify the Protective Advantages of Carissa Spinarum Leaves Extract in Zebrafish (Danio rerio)

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Abstract

Background: *Carissa spinarum*, a traditional medicinal plant, is known for its therapeutic properties, including hepatoprotective and antioxidant effects.

Objective: A study was conducted to determine whether the herb *Carissa spinarum* leaf extract has a protective impact on certain organs.

Methods: Adult wild strain zebrafish (*Danio rerio*) were used in an acute toxicity study (96 hours), and a dose of 5 ppm (parts per million) of carbon tetra chloride was used to cause multiple organ damage. The healthy control group was fed only while the diseased control group was treated with carbon tetra chloride at the dose of 5 ppm. A hydro-alcoholic extract made from the shadow-dried leaves of the plant *Carissa spinarum* was administered to the animals by dissolving it in fish cabinet in seven groups of ten each at concentrations of 0.5 ppm, 1 ppm, and 2 ppm. Standard protective medication utilized was silymarin at a dose of 50 ppm mixed with food.

Results: Plant extract was administered to the groups at levels of 0.5 ppm, 1 ppm, and 2 ppm; in all of these groups, the multiple organ protective effect was seen. At the highest dose of 2 ppm, the greatest protective effect (68-71%) was observed. Microscopic analysis revealed that the extract had no protective effects on the skeletal muscle tissue.

Conclusion: : The goal of the entire experiment was to determine Protective effects of the extract made from *Carissa spinarum* plant leaves had any protective effects on organs. The kidney, gut, and gills of zebrafish recovered more quickly after the plant extract was administered, demonstrating that it also had regenerative qualities in addition to hepatoprotective effects.

Keywords: Multiple organ protective effect, Zebrafish, *Danio rerio*, *Carissa spinarum* extract, Acute toxicity studies

INTRODUCTION

India's forests and sub-Himalayan regions are home to the prickly Carissa spinarum, a member of the Apocynaceae familyIt has long been used as a treatment for a number of illnesses, and more recent research has demonstrated its anticonvulsant, wound-healing, antimicrobial, diuretic, hepatoprotective, and anti-diabetic properties [1–5]. Numerous phytochemicals, including alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, phlobatannins, terpenoids, saponins, and tannins, have already been shown to be present in leaf extract through studies. The same study also demonstrated the hepatoprotective potential of leaf extract made from the Carissa spinarum plant, using a wild strain of zebrafish (Danio rerio) as the animal model [6,7]. The purpose of this study is to investigate the preventive effect of Carissa spinarum hydro-alcoholic leaf extract against various organ damage in zebrafish (Danio rerio) produced by carbon tetra chloride (CCl4). Adult zebrafish were used as the animal model in this investigation. Zebrafish are vertebrate animals that share many characteristics with mammals in terms of their cellular physiology and molecular mechanisms. For this reason, zebrafish are an emerging model system for human diseases and medication discovery [8–11].

MATERIALS AND METHODS

2.1 Plant Identification

The High Altitude Biology Division, CSIR-IHBT, Palampur, HP, recognized leaves of plants taken from Himachal Pradesh, India, as (Garna) *Carissa spinarum* L., is belonging to the Apocynaceae family, under voucher number (PLP-16486).

2.2 Extract preparation

The collected leaves were cleaned under running water, let to dry in shadow, crushed into a powder using a mechanical grinder, and then sieved using a 60 mesh sized screen. Hydro-alcoholic solvent (ethanol:water, 50:50) was used for extraction, and any residual ethanol was subsequently lyophilized out of the extract.

2.3 *in vivo* Studies

2.3.1 Acute Toxicity Study

According to OECD (Organisation for Economic Co-operation and Development) 203 test guideline, acute toxicity was assessed for 96 hours. To do this, animals were split into seven groups, each of which had ten zebrafish (*Danio rerio*). Carbon tetra chloride was utilized to produce toxicity. Subsequently, the impact of the *C. spinarum* leaf extract on the organ damage caused by carbon tetra chloride was evaluated in comparison to the conventional protective medication silymarin. Table 1 shows how treatment doses were determined based on weights and then dissolved in the water tank to convert the dose to parts per million. As per the guidelines provided by the European Chemical Agency (ECHA) and

the OECD guideline 105, carbon tetra chloride was utilised as a toxicant and distributed throughout the water tank. One flavolignan that is not soluble in water is silymarin. Thus, it was fed to the fish three times a day after being properly mixed and first triturated with fish feed in a pestle and mortar. In order to maintain the 50 ppm (parts per million) daily silymarin dose, as was done in a prior study, fish were fed in a controlled way [12–14].

Table 1 Animal grouping plan for carbon tetra chloride induced multi organ toxicity in zebrafish (*Danio rerio)*

Animal group		Treated with	Animal count	Dose (ppm)
A	**Healthy control**	-	10	-
B	**Disease control**	Carbon Tetra Chloride (CCl_4) Only	10	5 ppm
C	**Standard control**	CCl_4 & Silymarin (Standard drug)	10	5 & 50 ppm
D	**Sample 1**	CCl_4 & Extract *(C. spinarum)*	10	5 & 0.5 ppm
E	**Sample 2**	CCl_4 & Extract *(C. spinarum)*	10	5 & 1 ppm
F	**Sample 3**	CCl_4 & Extract *(C. spinarum)*	10	5 & 2 ppm
G	**Sample 4**	Only Extract *(C. spinarum)*	10	2 ppm

2.3.2 Tissue sample collection

First, the fish was rapidly chilled to induce anaesthesia. After adding ice cubes and water to the container in a 1:6 ratio, the fish was placed inside for a minimum of two to six seconds [11]. The fish was then placed on a Petridis, with a vertical cut made between its anal and caudal fins, and its caudal fin was sliced off. After the anal fin operculum was expertly cut, the skin and muscles beneath it were removed from the abdomen. The tissue from the kidney, colon, gills, and muscles was isolated and kept in a 10% v/v formalin solution.

2.3.3 Histological evaluation and examination

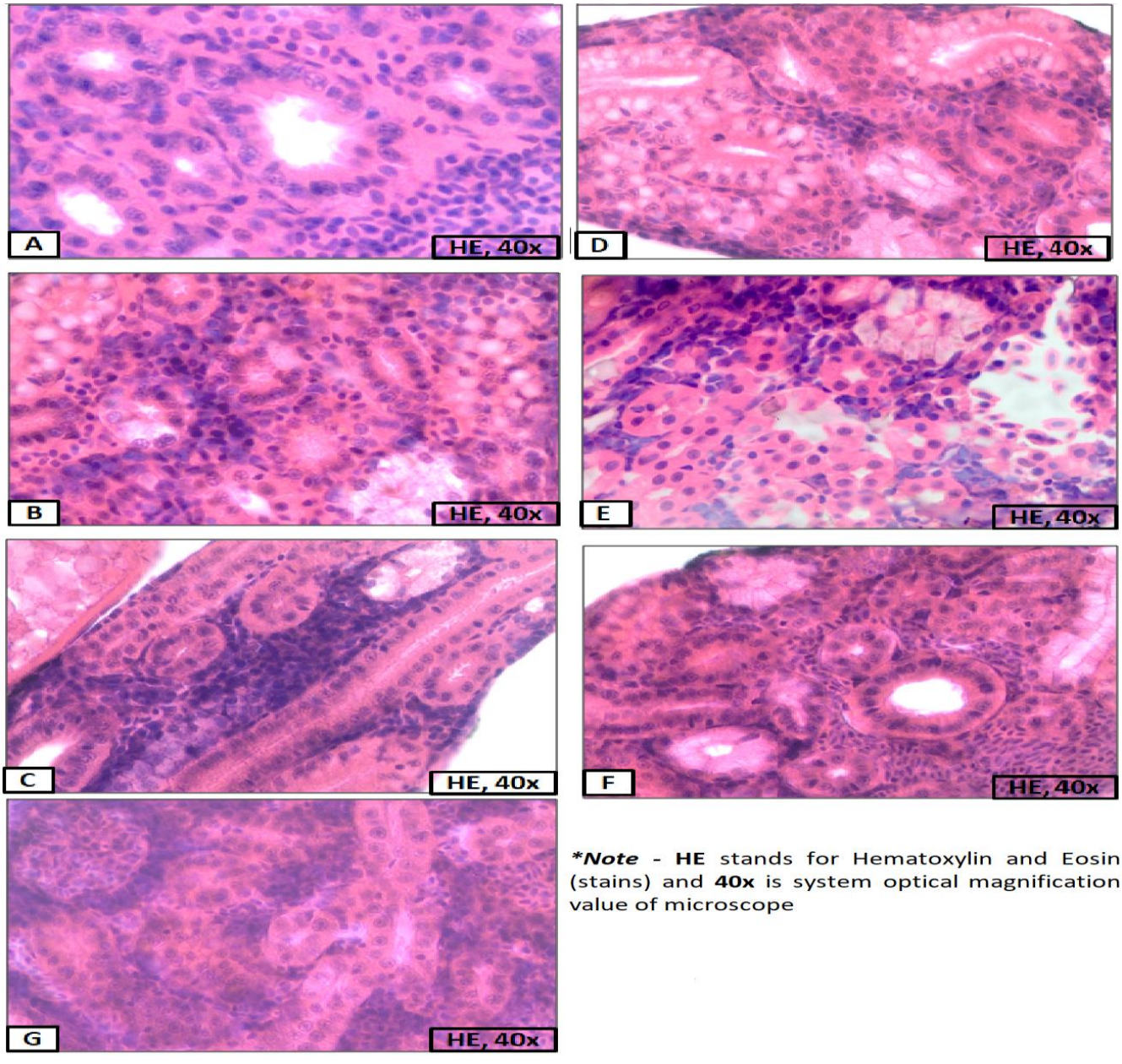
All of the fish in each group were sacrificed at the completion of the experiment, and a thorough necropsy study was performed. For at least 48 hours, a representative tissue sample of the internal organs such as; kidney, colon, gills, and muscles was obtained and preserved in a 10% neutral buffered formalin solution. The tissue samples were cleaned under running water for the entire night before being dehydrated in progressively stronger ethyl alcohol, clarified in xylene, and embedded in

paraffin for histological processing. Haematoxylin and eosin (HE) staining was carried out on 3–4 µ thick tissue sections that were cut from paraffin-embedded tissue blocks and placed on glass slides. Subsequently, H&E-stained slices were inspected for any pathological changes using a light microscope. The Olympus Microscope, Japan (BX-40) was used for the microphotography. To assess the extent of damage induced by the toxicant carbon tetra chloride, tissues were examined. Standard protective medication silymarin (50 ppm) and plant leaf extract at different dosages (0.5 ppm, 1 ppm, and 2 ppm) were compared for reduction in tissue damage.

RESULTS

3.1 Histological evaluation of kidney tissue

All of the fish samples from group A had normal anterior and posterior kidney architecture and normal haemopoietic activity, as shown in Figure 1.



***Note** - **HE** stands for Hematoxylin and Eosin (stains) and **40x** is system optical magnification value of microscope*

Figure 1 Microscopic changes in the kidney of animal group A (healthy control), **1(b)** Microscopic changes in the kidney of animal group B (CCl_4 at 5ppm only), **1(c)** Microscopic changes in the kidney of animal group C (CCl_4 and silymarin at 5ppm and 50ppm), **1(d)** Microscopic changes in the kidney of animal group D (CCl_4 and plant extract at 5ppm and 0.5ppm), **1(e)** Microscopic changes in the kidney of animal group E (CCl_4 and plant extract at 5ppm and 1ppm), **1(f)** Microscopic changes in the kidney of animal group F (CCl_4 and plant extract at 5ppm and 2ppm), and **1(g)** Microscopic changes in the kidney of animal group G (Plant extract at 2ppm only)

According to group B's histological analysis, the group had consistent severe renal damage, as shown by Figure 1(b), which shows areas of haemorrhage, swollen and vacuolar degeneration of the renal epithelium, nuclear pyknosis, karyorrhexis or karyolysis, and depletion of hemopoetic activity in the anterior portion of the kidney. Compared to group B fish, the kidney sections from group C showed milder histological alterations, such as slightly enlarged and dilated vasculature and enlarged or deteriorated epithelium in a few tubules, Figure 1(c). The reduction in damage was found to be 72% in group C as shown in Figure 5. When compared to the group B fish, the histological alterations in the kidney were less severe in the group D fish. The reduction in damage was found to be 36% in group D as shown in Figure 5. These changes included nuclear pyknosis, karyorrhexis or karyolysis in the renal tubules, as well as variably dilated and engorged vasculature, swelling and vacuolar degeneration of epithelium, Figure 1(d). When compared to the group B fish, the histological alterations in the kidneys of group-E were minor to moderate.The reduction in damage was found to be 51% in group E as shown in Figure 5. These changes included swelling and vacuolar degeneration of the renal tubule epithelium, as well as mild to moderately dilated and engorged vasculature, Figure 1(e). The histological alterations observed in the kidneys of group F fishes were found to be less severe than those of group B fishes. The reduction in damage was found to be 68% in group F as shown in Figure 5. These changes included mildly dilated and engorged vasculature, as well as swelling and vacuolar degeneration of the epithelium of a few renal tubules, Figure 1(f). Each fish sample in group G had kidney architecture that was as good as normal, as seen in Figure 1(g).

3.2 Histological evaluation of intestinal tissue

All of the fish samples in group A had normal intestinal architecture, as seen in Figure 2. The intestinal microstructure of group B exhibited severe and consistent alterations, as illustrated in Figure 2(b), including shorter villi and increased activity of goblet cells at the mucosal lining of the intestine, as well as engorged and dilated blood vessels of the mucosa or sub mucosaIt was also possible to see the necrotic and deteriorated epithelium inside the lumen, together with desquamated cellular debris. When compared to the group B fish, the microscopic alterations in the group C fish's intestine showed mild histopathological changes, such as slightly dilated and engorged blood vessels in the mucosa or submucosa and

slightly elevated goblet cell activity at the mucosal lining of the intestine, as seen in Figure 2(c).

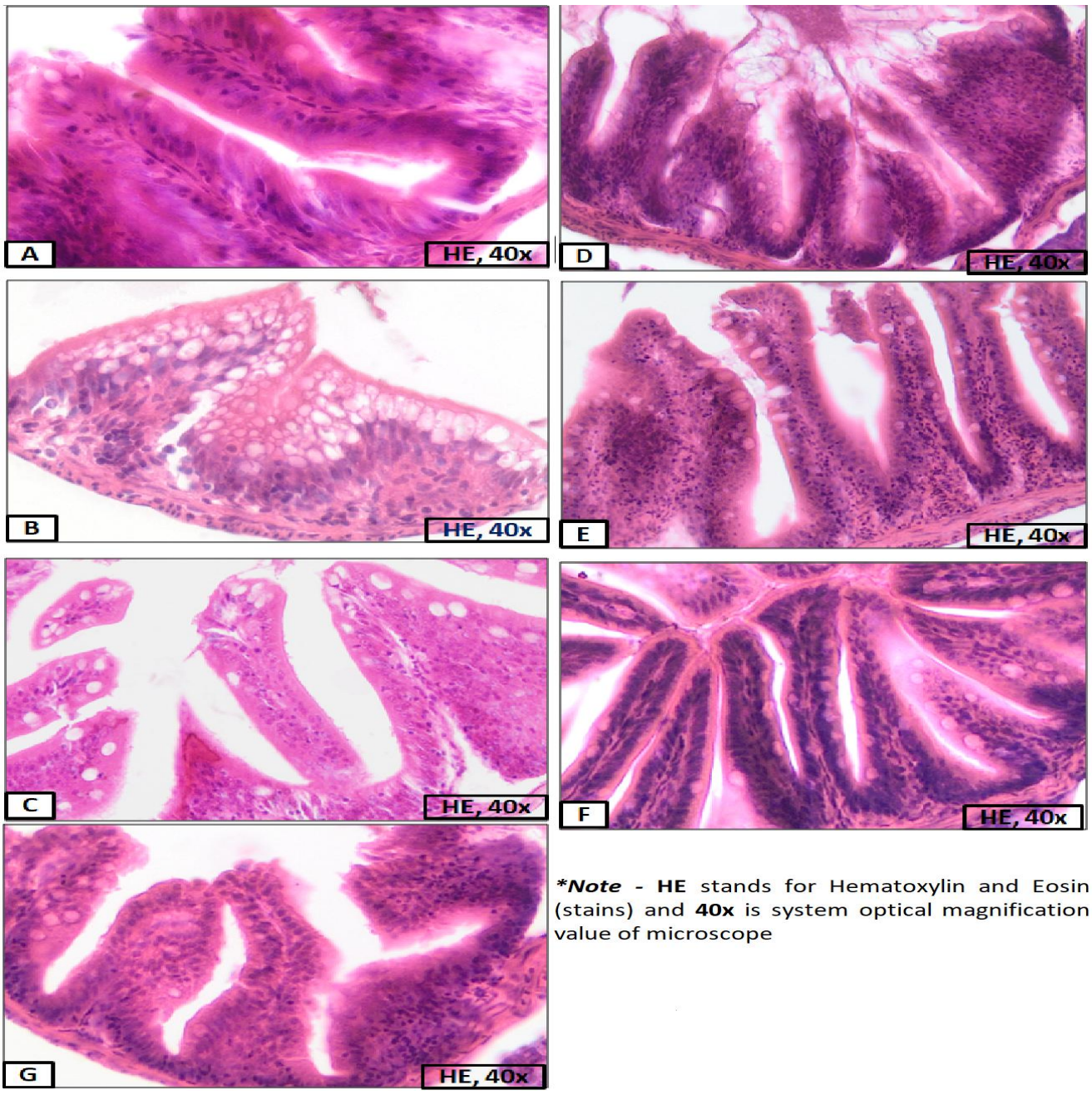


Figure 2 Microscopic changes in the intestine of animal group A (healthy control), **2(b)** Microscopic changes in the intestine of animal group B (CCl_4 at 5ppm only), **2(c)** Microscopic changes in the intestine of animal group C (CCl_4 and silymarin at 5ppm and 50ppm), **2(d)** Microscopic changes in the intestine of animal group D (CCl_4 and plant extract at 5ppm and 0.5ppm), **2(e)** Microscopic changes in the intestine of animal group E (CCl_4 and plant extract at 5ppm and 1ppm), **2(f)** Microscopic changes in the intestine of animal group F (CCl_4 and plant extract at 5ppm and 2ppm), and **2(g)** Microscopic changes in the intestine of animal group G (Plant extract at 2ppm only)

The reduction in damage was found to be 69% in group C as shown in Figure 5 When compared to the group B fishes, the group D fishes' intestine displayed less severe microscopic alterations. These included variable engorgement and dilation of the mucosa or submucosa blood vessels, increased goblet cell activity at the mucosal lining of the intestine, and desquamated cellular debris inside the lumen, as seen in Figure 2(d). When compared to the group B fish, the microscopic alterations in the group E's intestine were mild to moderate in nature. These included slightly increased activity of goblet cells at the mucosal lining of the intestine and mildly engorged and dilated blood vessels of the mucosa or submucosa, as illustrated in Figure 2(e).The reduction in damage was found to be 49% in group E as shown in Figure 5. When compared to the group B fish, the microscopic alterations in the group F fish's intestine were uneven and mild. These included slightly dilated and engorged blood vessels in the mucosa or submucosa as well as slightly elevated goblet cell activity at the mucosal lining of the intestine, as seen in Figure 2(f). The reduction in damage was found to be 71% in group F as shown in Figure 5. As seen in Figure 2(g), the intestinal architecture was as good as usual in every fish sample from this group G.

3.3 Histological evaluation of gills tissue

As shown in Figure 3(a), the architecture of the gills, which display main and secondary lamellae, was normal in all fish samples from group A. Group B exhibited severe and consistent microscopic gill alterations, including dilated and enlarged blood vessels or capillaries in the primary and secondary lamellae, swelling, and degenerated gill epithelium. Additionally, the secondary lamellar structures were distorted, causing them to curl and even fuse together, as illustrated in Figure 3(b). In a small number of fish, there were also visible signs of gill filament and secondary lamellae degeneration and necrosis, as well as pillar cell pyknotic nuclei. When comparing the group C fishes' gills to those of the group B fish, the microscopic changes showed milder histopathological changes, such as swelling and degenerated gill epithelium, mildly dilated and engorged capillaries or blood vasculature in the primary and secondary lamellae, and mildly distorted secondary lamellar structures that at times resulted in curling and even fusion of them, as shown in Figure 3(c).The reduction in damage was found to be 71% in group C as shown in Figure 5. Group D fishes had less severe microscopic gill alterations than group B fishes. These alterations included swelling and degeneration of the gill epithelium, dilated and variably engorged capillaries or blood vasculature in the primary and secondary lamellae, and distorted secondary lamellar structures that at times resulted in curling and even fusion of them, as shown in Figure 3(d). The reduction in damage was found to be 41% in group D as shown in Figure 5. Compared to group B fish, the microscopic gill alterations in group E were mild to moderate in character. These alterations included swelling and degeneration of the gill epithelium as depicted in Figure 3(e) as well as moderately engorged and dilated capillaries or blood vasculature in the

primary and secondary lamellae. The reduction in damage was found to be 47% in group E as shown in Figure 5. When compared to the group B fish, the histopathological alterations in the kidney of group F were less severe and more uneven. These changes included engorged and dilated capillaries or blood vasculature in the primary and secondary lamellae, as well as swelling and degeneration of the gill epithelium, as seen in Figure 3(f). The reduction in damage was found to be 69% in group F as shown in Figure 5. As seen in Figure 3(g), the usual architecture exhibiting the primary and secondary gill lamellae was noted in all fish samples belonging to group G.

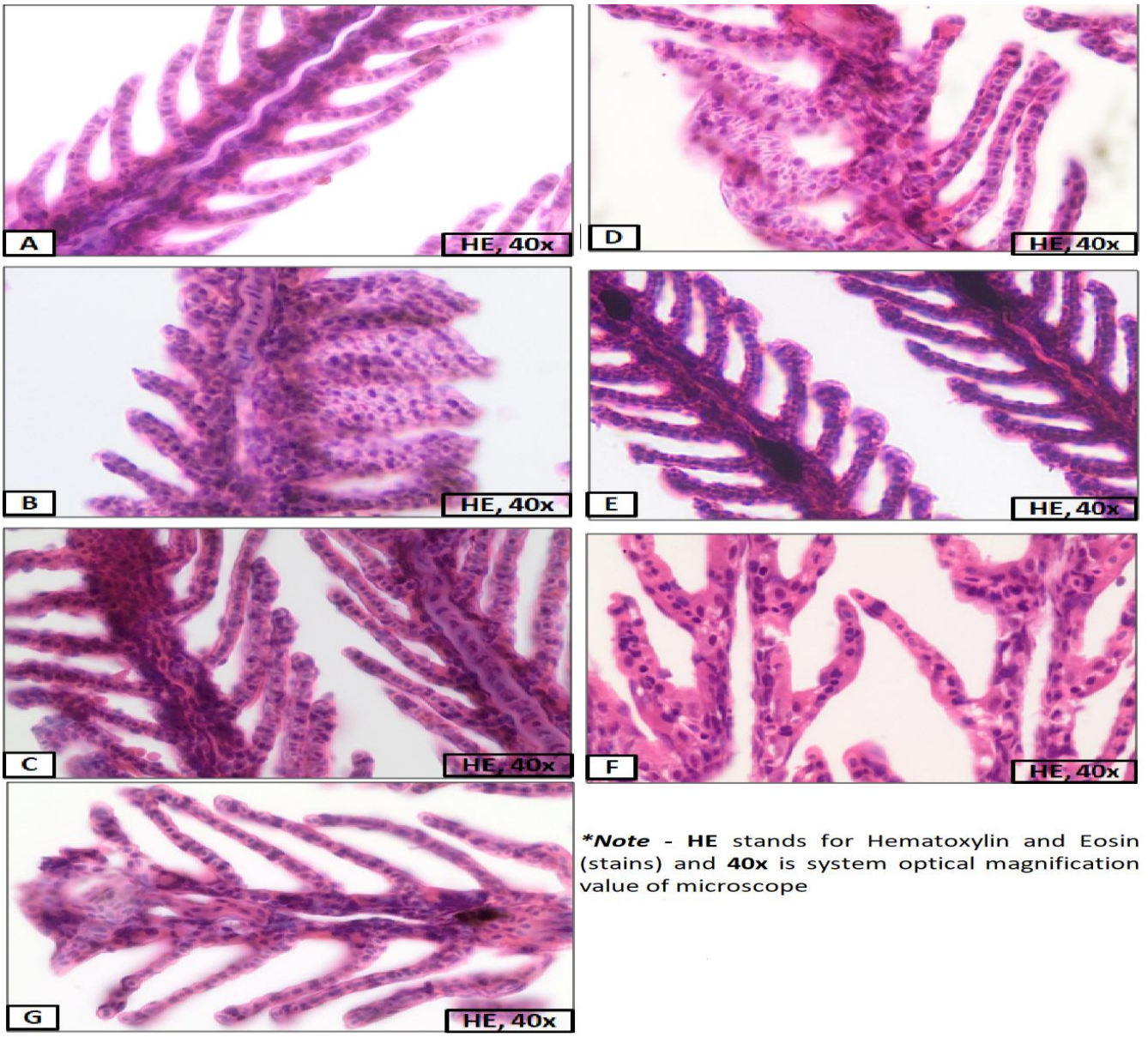


Figure 3(a) Microscopic changes in the gills of animal group A (healthy control), **3(b)** Microscopic changes in the gills of animal group B (CCl_4 at 5ppm only), **3(c)** Microscopic changes in the gills of animal group C (CCl_4 and silymarin at 5ppm and 50ppm), **3(d)** Microscopic changes in the gills of animal group D (CCl_4 and plant extract at 5ppm and 0.5ppm), **3(e)** Microscopic changes in the gills of animal group E (CCl_4 and plant extract at 5ppm and 1ppm), **3(f)** Microscopic changes in the gills of animal group F (CCl_4 and plant extract at 5ppm and 2ppm), and **3(g)** Microscopic changes in the gills of animal group G (Plant extract at 2ppm only)

3.4 Histological evaluation of skeletal muscle tissue

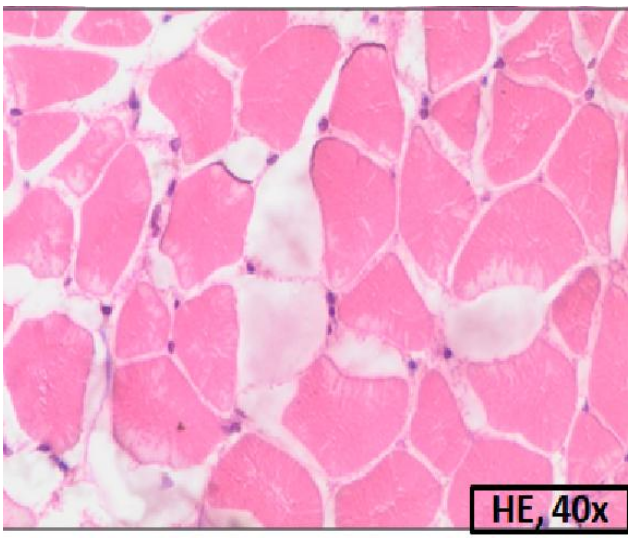


Figure 4: Microscopic changes in the skeletal muscle of animal group B (CCl_4 at 5ppm only) *Note HE stands for Hematoxylin and Eosin (stains) and 40x is system optical magnification value of microscope

The fish belonging to all the groups (A, C, D, E, F, and G) had normal skeletal muscle architecture. A small number of fish in group B did, however, exhibit microscopic skeletal muscle abnormalities, such as dilated and enlarged blood vessels, enlarged and degraded muscle fibers, and the presence of edema inside or between muscle bundles, as depicted in Figure 4.

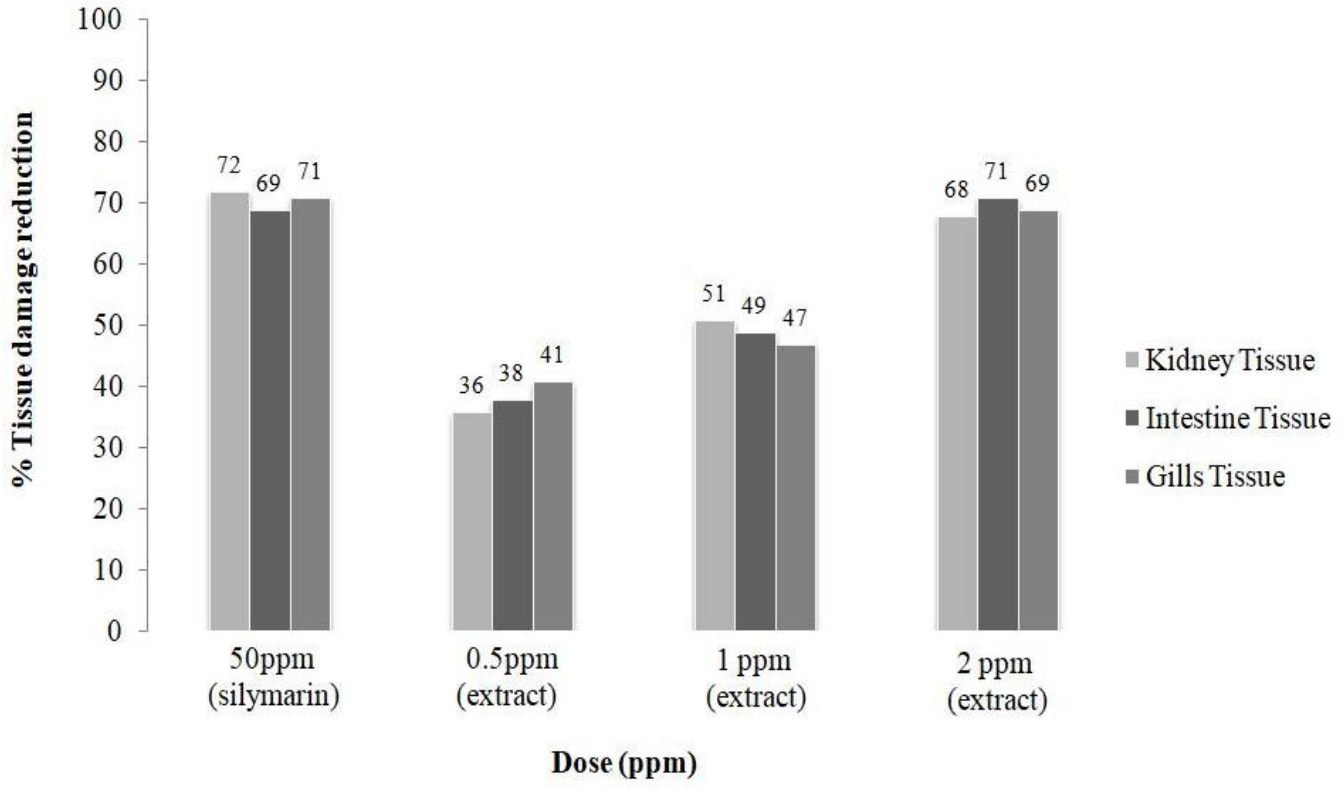


Figure 5: Protective effect comparison between silymarin and plant extract at various doses

DISCUSSION

The purpose of this study is to determine the numerous organ protection effects of an extract made from *Carissa spinarum* leaves. Previous investigations have demonstrated the hepatoprotective effect. A plant's phytochemical composition necessitates a range of pharmacological effects [16–18]. The findings of the study indicated that an extract made from *Carissa spinarum* leaves must have some pharmacological properties. Following the delivery of plant extract, microscopy of several organ tissues, including the kidney, colon, and gills, demonstrated a variety of structural and functional alterations. All of group A's tissues have normal microscopy results. All of group B's tissues underwent microscopy, which showed tissue damage and an excess of inflammatory mediators and immune cells, including goblet cells in the colon. Microscopy of group C, where animals were given the conventional protective medication silymarin, revealed slight histological alterations, demonstrating the protective action of silymarin [19–23]. Group D microscopy revealed a modest protective effect of the plant extract in the zebrafish kidney, gut, and gill tissue. The kidney, gut, and gill tissue of zebrafish exhibited a significant protective effect of the plant extract, as demonstrated by microscopy in groups E and F. Plant extract does not have a harmful impact of its own, as demonstrated by the microscopy of group E, which did not change at all after the plant extract was administered at the greatest dose. As the dose of extract was raised, the protective effect increased. The highest level of organ protection was seen at 2ppm of dosage as shown in figure 5. Muscle tissue examined under a microscope showed that plant extract had neither harmful nor protective effects on it. Numerous comparable investigations have already demonstrated the preventive benefits of extracts made from other plant sections, including the roots, leaves, and branches [24,25].

CONCLUSION

The goal of the entire experiment was to determine whether an extract made from *Carissa spinarum* plant leaves had any protective effects on organs. The kidney, gut, and gills of zebrafish recovered more quickly after the plant extract was administered, demonstrating that it also had regenerative qualities. Lastly, the results showed that the extract made from the leaves of the *Carissa spinarum* plant had several organ protective effects in addition to hepatoprotective effects.

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Ethical Statement: The Institutional Animal Ethical Committee (IAEC), SPES, BUEST, Baddi, Solan, Himachal Pradesh-173205, and its registered number, 1421/PO/Re/S/11/CPCSEA, were followed in conducting this experiment. The date of the approval was March 28, 2018. For this experiment, an IAEC approval certificate (Approval Number: BUEST/SPES/IAEC/2021/001) was given.

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Disclosure of interest: Authors declare that they have no competing interest.

Author contribution

All Authors contributed equally in this research

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