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Hepatoprotective Effects of Carissa spinarum Extract on Carbon Tetra Chloride Induced Liver Damage in Zebrafish (Danio rerio)

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Abstract

Background: *Carissa spinarum* L. (Apocynaceae) is a thorny bush found all over India, especially in forests and sub-Himalayan tracts up to 4,000 feet. In the traditional Indian and Chinese medicine system, all parts of the Plant (roots, stems, leaves, and fruit) are used to treat acute and chronic diseases

Objective: A study was performed to check if the Plant *Carissa spinarum* leaf extract exhibits a hepatoprotective effect. As there is an increase in demand for hepatoprotective drugs, this study was an attempt to introduce an alternative to hepatoprotective drugs

Methods: An acute toxicity study was performed on adult wild strain Zebrafish (Danio rerio), and carbon tetra chloride (5 ppm) (parts per million) was utilized to induce hepatic damage. A preliminary study was also performed for the standardization of doses. Animals were divided into seven groups, consisting of 10 each, and treated with an aqua-alcoholic extract prepared from the shadow-dried leaves of the Plant *Carissa spinarum* at doses of 0.5 ppm, 1 ppm, and 2 ppm. Silymarin (50 ppm) was used as a standard hepatoprotective drug.

Results: Groups received plant extract at doses of 0.5 ppm, 1 ppm, and 2 ppm; a hepatic-protective effect was seen in all these groups. The most hepatic-protective effect was seen at the highest dose (2 ppm). Phytochemical studies also verified the presence of various plant secondary metabolites.

Conclusion: The whole experiment was aimed to determine the hepatoprotective effect of an extract prepared from the leaves of the Plant *Carissa spinarum*. After the administration of the plant extract, it helped the liver and other organs heal faster, which proves it also exhibits regenerative properties.

Keywords: Hepatoprotective, Zebrafish, Danio rerio, *Carissa spinarum*, Acute toxicity studies

INTRODUCTION

Carissa spinarum L. (Apocynaceae) is a thorny bush found all over India, especially in forests and sub-Himalayan tracts up to 4,000 feet. In the traditional Indian and Chinese medicine system, all parts of the Plant (roots, stems, leaves, and fruit) are used to treat acute and chronic diseases [1, 2]. A number of studies have already been performed on the Plant *Carissa spinarum* and related species to evaluate and investigate different pharmacological activities possessed by the Plant. Studies have already proven the Plant's anti-diabetic, diuretic, antioxidant, and anticonvulsant properties [3-6]. Studies also reported extract prepared from the roots of *Carissa spinarum exerts* antimicrobial and cytotoxic activities [7, 8]. It was proven that the stem of this Plant possesses anti-cancer and anti-herpetic potential because of inhibition in cell proliferation of various human cancer cell lines and moderate reduction in herpes simplex virus (I and II) was reported in these studies [9, 10]. Leaves of Plants were also investigated, and results revealed they exert anti-inflammatory activity. This might be due to the fact that this Plant must contain a number of phytochemicals. Phytochemical identification tests were performed on leave extract to confirm the presence of various secondary plant metabolites such as alkaloids, tannins, glycosides, flavonoids etc [11]. Roots of Plant show significant hepatoprotective activity against carbon tetra chloride (CCl_4) and Paracetamol-induced liver injury in Albino and Wister rats when compared with standard drug Silymarin [12].

The liver is a vital organ in our body that performs the metabolism of various drugs and toxins. If the liver fails to metabolize the toxins present in the systemic circulation, it may lead to hepatic damage [13]. Hepatoprotective agents are a class of chemical moieties that protect the liver from the damage caused by toxic agents. Many synthetic and herbal drugs available in the market possess hepatoprotective activity. Studies have revealed that a great number of traditional herbal plants and their formulations have been claimed to have hepatoprotective activity [14]. There are different causes of hepatic toxicity, including toxic chemicals, excessive consumption of alcohol, infections, and auto-immune disorders. NSAIDs such as acetaminophen convert into ROS (reactive oxidative species), causing hepatic toxicity, and chemicals like carbon tetra chloride do the same [15]. Our study aimed to find out the hepatoprotective potential exerted by leaves of Plant *Carissa spinarum.* This study utilized Adult Zebrafish (Danio rerio) as an animal model. Zebrafish are vertebrate species, and when it comes to their molecular mechanism and cellular physiology, they exhibit a high degree of similarity with mammals. That's why

Zebrafish nowadays is an emerging model system for human diseases and drug discovery [16]. Liver toxicity was induced in animals with the help of the commonly used hepatotoxic agent Carbon tetra chloride (CCl_4). It metabolizes in the liver to highly reactive trichloromethyl radical, causing lipid peroxidation of the cytoplasmic membrane's phospholipids, leading to liver injury [17].

MATERIAL AND METHODS:

Plant collection: Leaves of Plants were collected in February 2021 from the small town Sainthal, District Mandi, Himachal Pradesh, India. The plant specimen deposited was identified as (Garna) *Carissa spinarum* L. belonging to the family Apocynaceae by Mr. Om Parkash, Senior Technical Officer, High Altitude Biology Division, CSIR-IHBT, Palampur, HP. The specimen was kept at the herbarium of CSIR-IHBT under voucher number (PLP-16486).

Extract preparation: Collected leaves were cleaned under running water, air-dried, and then shadow-dried for about two weeks. Dried leaves were ground in a mechanical grinder to form a powder and sifted with a sieve with mesh size #60. Dried powder (200g) was soaked in 1600 ml of aqua alcoholic solvent Ethanol: Water (50:50) [18] for 48 hours and then filtrated with Whatman filter. Filtrate was heated at 40ºC temperature at 135 MM of mercury of pressure in the Rotary evaporator. The semi-solid extract was found at the end of evaporation. Viscous semi-solid extract was lyophilized to remove the traces of ethanol. The yield of lyophilized extract was found to be 18% w/w.

Phytochemical Screening: The plant extract was screened for the presence of various secondary plant metabolites such as alkaloids, Saponins, terpenoids, anthraquinones, glycosides, coumarins, phlobatannins, flavonoids, and tannins [19-21].

In vivo Studies :

Preliminary study

This study was performed for standardization of Carbon tetra chloride dose to induce toxicity in Zebrafish (*Danio rerio)*. Three graded doses of Carbon tetra chloride at the dose at levels of 10, 20, and 40 ppm (parts per million) were administered to three groups comprising ten (10) Zebrafish (*Danio rerio*) in each group for 96 hours of exposure. The mortality rate among the groups was calculated, and LD_{50} (median lethal dose) level was established accordingly.

Acute toxicity study

Acute toxicity was performed for 96 hours as per OECD (Organization for Economic Co-operation and Development) test guideline (203) [22], and for this, animals were divided into seven groups; each group consisted of 10 Zebrafish (Danio rerio). Hepatotoxicity was induced using

carbon tetra chloride. Then, the effect of the leaf extract prepared from C. spinarum on the hepatic damage produced by carbon tetra chloride was compared against the standard hepatoprotective drug Silymarin. Treatment doses were calculated corresponding to their weights and then dissolved in the water tank, converting the dose into ppm, Table 1. Carbon tetra chloride was dispersed in the water tank to induce hepatic toxicity in fish. As we already know, Carbon tetra chloride is not soluble in water; a substance evaluation report 2019 for Carbon tetra chloride by the European Chemical Agency (ECHA) [23] suggested that data has been generated according to OECD guideline 105 [24]

Table 1 Group plan for carbon tetra chloride Induced Hepatotoxicity in Zebrafish (Danio rerio)
(Note: ppm= parts per million, CCl_4= carbon tetra chloride)

Group		Treatment	Animal count	Dose (ppm)
I	**Negative control (Healthy control)**	-	10	-
II	**Positive control (Disease control)**	Carbon Tetra Chloride (CCl_4) Only	10	5 ppm
III	**Standard control**	CCl_4 +Silymarin (Standard drug)	10	5 + 50 ppm
IV	**Test I**	CCl_4+Extract *(C. spinarum)*	10	5 + 0.5 ppm
V	**Test II**	CCl_4+Extract *(C. spinarum)*	10	5 +1 ppm
VI	**Test III**	CCl_4+Extract *(C. spinarum)*	10	5 +2 ppm
VII	**Test IV**	Extract *(C. spinarum)* only	10	2 ppm

\ and GLP requirements which give Carbon tetra chloride water solubility value of 846.1 mg/L at 20ºC. Another supportive data reported in the CRC Handbook provides a Carbon with tetra chloride water solubility value of 0.65 g/L at 25ºC. As we already know, Silymarin is a flavolignan and it is insoluble in water. So it was first triturated with fish's feed in pestle & mortar and then after proper mixing fed to the fish 3 times a day. Fish were fed in a control manner to maintain the Silymarin dose of 50 ppm per day [25].

Behavioral evaluation

Clinical signs were observed in fish during the experiment. A behavior study was performed during this experiment, and all the parameters were analyzed as per OECD test guidelines (203). Key parameters observed during the study were loss of equilibrium, abnormal swimming behavior, abnormal ventilatory (respiratory) function, abnormal skin pigmentation, and other visible (appearance and behavior) abnormalities . All these parameters were observed from the first day to the last day of the experiment.

Tissue sample collection

Fish was first anesthetized using the rapid chilling method. The container was filled with ice cubes and water at a ratio of 1:6,

and then the fish was inserted in a container for at least 2-6 seconds [26]. Then fish was placed on a Petridis, and a vertical cut was made between the anal fin and caudal fin, and then the caudal fin was amputated, as shown in Figure 1.

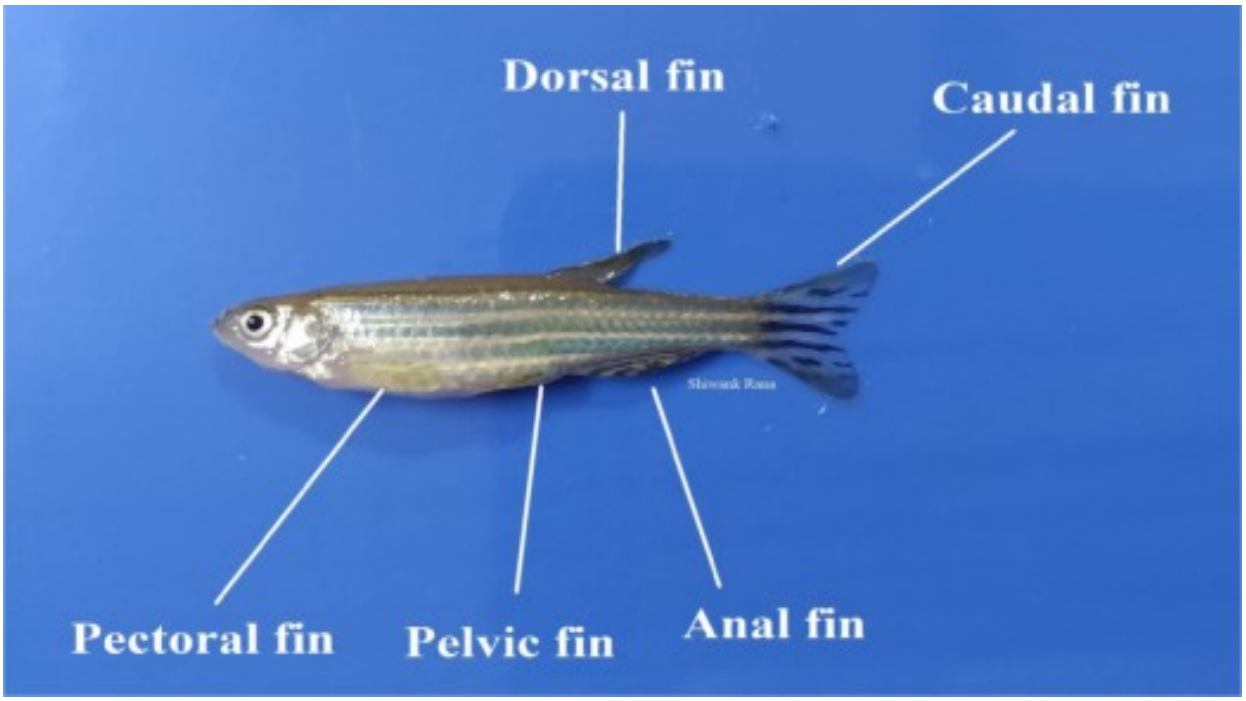


Figure 1: Adult Zebra fish with fins labeled

After making a fine cut on the skin and underlying muscles along the belly from the anal fin operculum and the skin and muscle were removed. All the organs were visible, and liver tissue was isolated and then placed in a formalin solution (10% v/v).

Histological evaluation and examination

At the end of the experimentation, all Zebrafish (Danio rerio) fish from each group were sacrificed, and a systematic necropsy examination was carried out. A representative tissue sample of the internal organ liver was collected and fixed in a 10% neutral buffered formalin solution for at least 48 hours. For histopathological processing, the tissue samples were given overnight washing under tap water and dehydrated in increasing grades of ethyl alcohol, cleared in xylene, and embedded in paraffin. From paraffin-embedded tissue blocks, 3-4 µ thick tissue sections were cut on glass slides, and hematoxylin and eosin (HE) staining was performed. Then, H & E stained sections were examined under a light microscope for pathological alterations, if any. The microphotography was performed using Olympus Microscope, Japan (BX-40).

RESULTS AND DISCUSSION

Phytochemical qualitative screening of leaves extract

Phytochemical quantitative evaluation results of extract prepared from the leaves of Plant Carrissa spinarum confirmed the presence of alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, phlobatannins, terpenoids, saponins, and tannins as in Table 2.

Table 2 Phytochemical analysis of aqua-alcoholic leaves extract of Plant *Carrissa spinarum* (Note: (+) sign represents confirmed presence of component)

Identification test	Aqua-alcoholic extract (Ethanol + Water)
Alkaloids	+
Saponins	+
Coumarins	+
Flavonoids	+
Phlobatannins	+
Tannins	+
Anthraquinones	+
Glycosides	+
Terpenoids	+

Another phytochemical screening study was also performed on various leaf extracts of Plant Carissa spinarum prepared from different solvents. In this study, ethanolic and aqueous extract confirmed the presence of steroids, carbohydrates, glycosides, flavonoids, tannins, and alkaloids. These phyto-constituents exert antioxidant activities by scavenging free radicals that cause lipid peroxidation. Hence, the protective effects of Carissa spinarum leaf extract observed in this study must be due to these secondary plant metabolites [27].

Preliminary study for standardization of the CCl_4 dose

Three graded doses of CCl_4 at the dose levels of 10, 20, and 40 ppm were administered to three groups comprising ten (10) Zebrafish (Danio rerio) in each group for 96 hours of exposure. The highest dose (CCl_4 at 40ppm) revealed 5 (50%) mortality among the group. However, the lower doses (CCl_4 at 20 & 10ppm) revealed 3 (30.0%) and 2 (20%) mortality among the groups, respectively. Therefore, the present dose of 5ppm was selected as 1/8th of LD_{50} dose level for the present study. The pilot study was performed to find out LD_{50} dose level for present studies. No data is available for the use of CCl_4 as a hepatotoxic agent in adult Zebrafish as an animal model. The only data available regarding this was the use of CCl_4 as a liver fibrotic agent in Zebrafish embryos [28]. However, it is a popular hepatotoxic agent in other animal models.

Behavioral studies

The mortality rate in group I (GI) (control) was found to be 0 (0%). As expected in this group, not a single abnormal behavior was observed during the study, the total mean abnormality

score was found to be 0. The mortality rate in group-II (GII) was found to be 2 (20%). The total mean abnormality score in this group was found to be 0.78. Group-III (GIII), the total mean abnormality score in this group was found to be 0.66, and the mortality rate was 0 (0%). Group-IV (GIV), the Mortality rate in this group was found to be 0 (0%), and the total mean abnormality score in this group was found to be 0.36. Group-V (GV), the mortality rate in this group was found to be 0 (0%) and the total mean abnormality score in this group was found to be 0.26. In group-VI (GVI), the mortality rate was found to be 0 (0%), and the mean abnormality score was 0.23. Group-VII (GVII), no abnormality in behavior was observed in this group throughout the study, proving that plant extract has no role in producing abnormal behavior in experimental animals. The mortality rate in this group was found to be 0 (0%). The total mean abnormality score in this group was found to be 0 as shown in Table 3.

The behavioral study was performed as per OECD guidelines (203). As we discussed the intensity of abnormal behavior observed in each group, a number of fishes showing any abnormality in behavior was noted down for every interval of observation during 96 hours of acute toxicity study. The mean abnormal behavior score was calculated for each group. Abnormal Score was found to be 0 in the case of Group-I (GI). Abnormal behavior in all these groups might be induced by drugs administered, as described in a study [29]. The abnormality score was highest in Group-II (GII) (0.78), as we compare it with other groups. The reason for the highest abnormality score in this group might be the toxicities induced by the CCl_4. Mortality was only observed in this group, 2 (20%) fishes died during the experiment as shown in Table 3. The mortality rate of Group-II (GII) was similar to the group of Zebrafish in pilot study received CCl_4 at the dose of 10ppm. The abnormal behavior score of Group-III (GIII) was less (0.66) as compared to the Group-II (GII). This suggests that the protective effect possessed by the Silymarin might be the cause of the reduction in abnormal behavior. A study has already reported that the use of liver protective drug (N-acetylcysteine) causes a reduction in the degree of abnormal behaviors in Zebrafish [30]. Also, no mortality was seen in this group, which hints towards the protective action of Silymarin. Abnormality scores in Group-IV (GIV) (0.36), Group-V (GV) (0.26), and Group-VI (GVI) (0.23) were significantly less as compared to Group-II (GII) and Group-III (GIII), Table 3. The score calculated as following

$$\textbf{Abnormality Score Mean} = \frac{\sum x_i}{\text{ni } (\textit{Total number of alive fishes in each set})}$$

The results of this study suggested that dose-dependent reduction in abnormal behavior scores in test animal groups might be due to the protective action of

Table 3 Behavioral changes observed in all groups of experimental Zebrafish (*Danio rerio*)

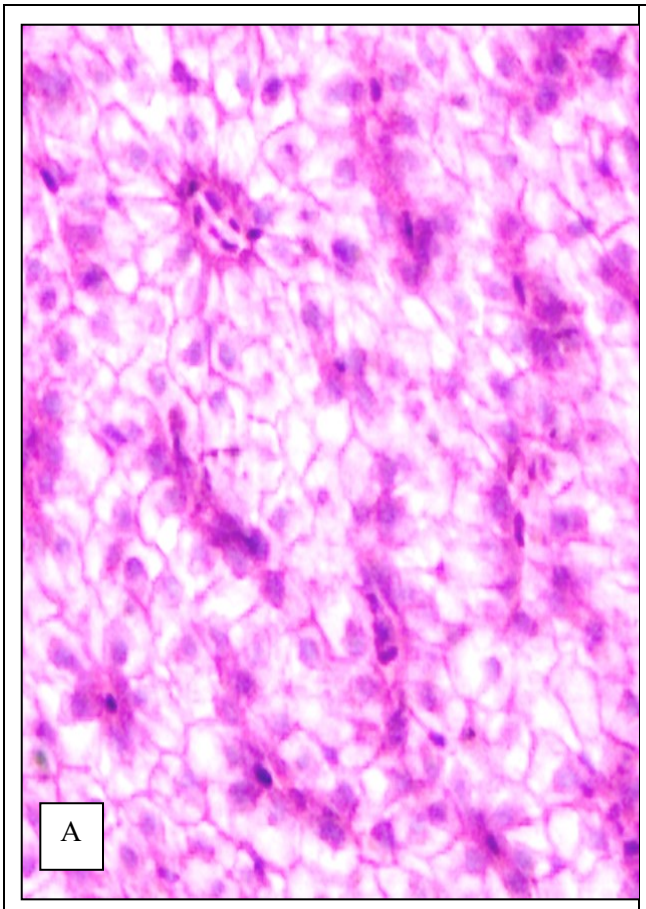
Observations	GI	GII	GIII	GIV	GV	GVI	GVII
Loss of equilibrium	0	0.36	0.18	0.13	0.07	0.04	0
Abnormal swimming behavior	0	0.2	0.22	0.16	0.13	0.12	0
Abnormal respiratory function	0	0.22	0.26	0.07	0.06	0.07	0
Abnormal skin pigmentation	0	0	0	0	0	0	0
Other	0	0	0	0	0	0	0
Total	**0**	**0.78**	**0.66**	**0.36**	**0.26**	**0.23**	**0**
Mortality (n_i=10)	**0**	**2**	**0**	**0**	**0**	**0**	**0**

(Note: Each column represents one set of observations of different abnormal behavior. (Here 0= No abnormalities observed and n_i= total number of fish in a group, x_i = number of fishes showing abnormal behavior in each set of observations; G = group (GI group I)

plant extract. The least abnormal behavior was seen in the group receiving the highest dose of plant extract (2 ppm). Hence, it proves that the abnormal behavior seen in all these groups was dose-dependent. Results also reveal that the abnormality score in the group receiving the lowest dose (0.5ppm) of plant extract was less as we compare it with the group receiving the standard drug Silymarin (50ppm), Table 3. No abnormality was observed in Group-VII (GVII), proving that plant extract itself does not affect the normal physical activity of animals as some drugs might induce abnormal behavior in Zebrafish described in a study [31].

Microscopic studies

The microscopic findings in the liver section of all groups reveal the possible pharmacological effect of plant extract prepared from the leaves of *Carissa spinarum*. Microscopy of the liver tissue section of the control group showed no abnormality, and the morphology of hepatocytes appears normal,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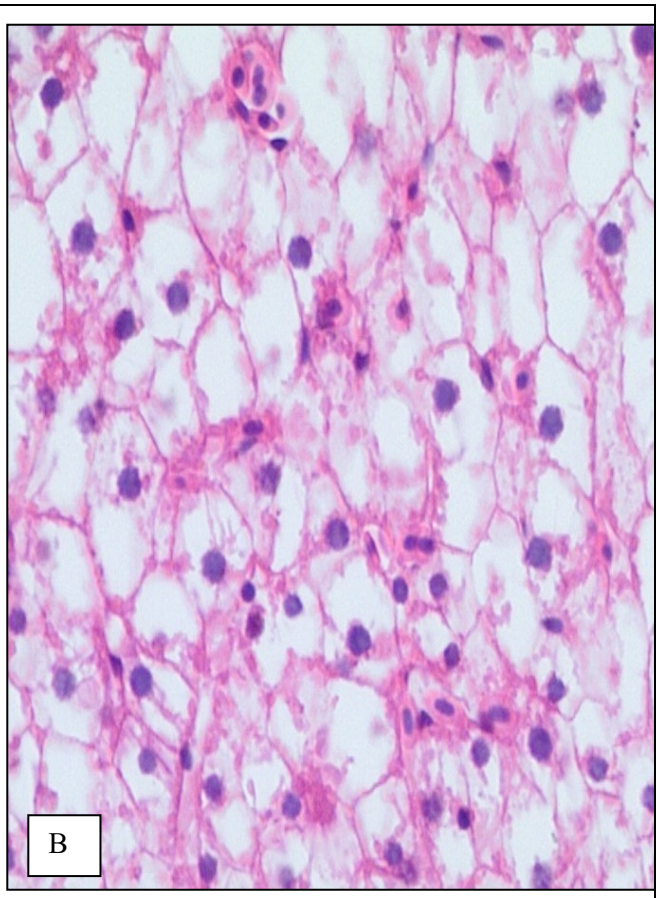
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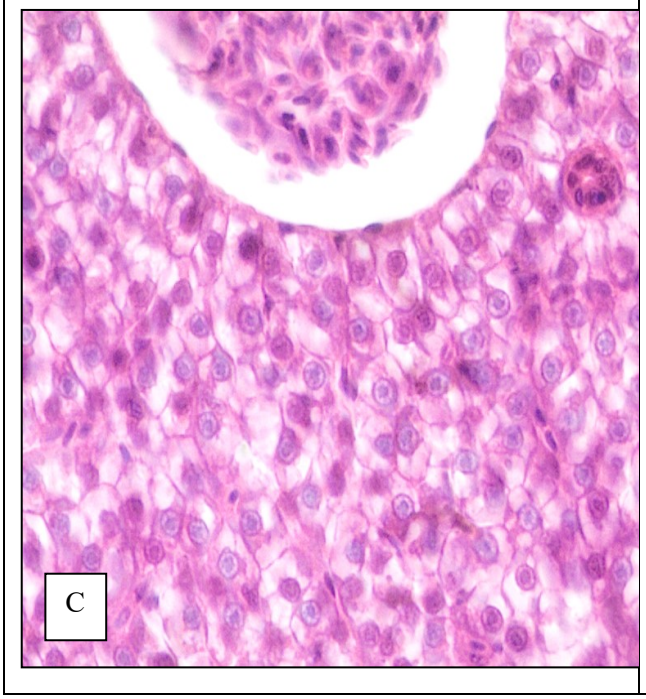
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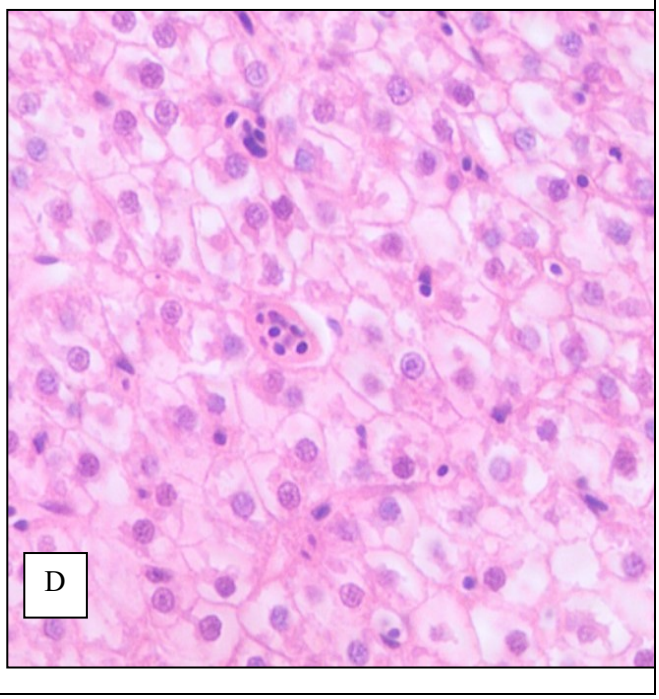
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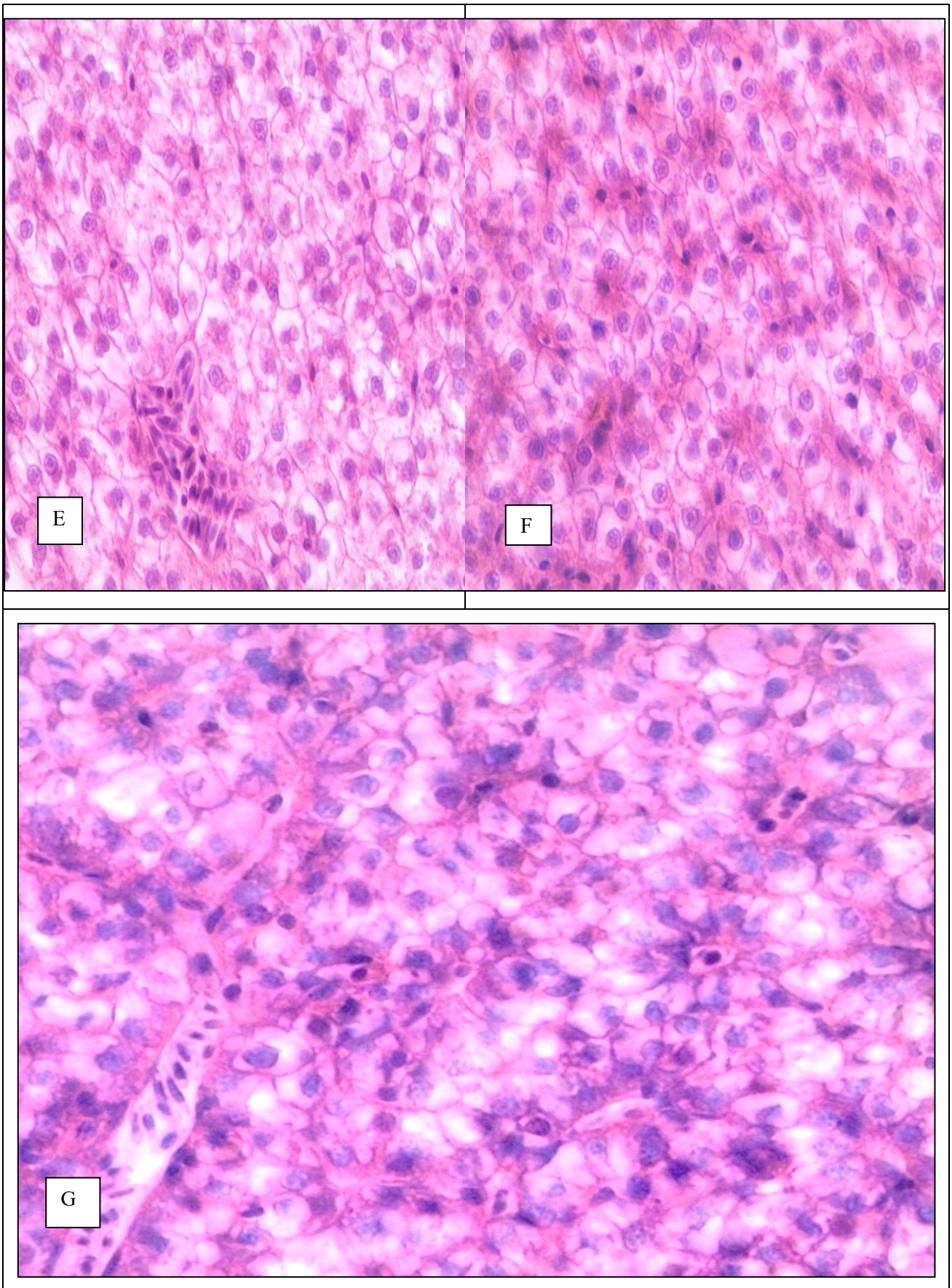

Figure 2 Microscopic changes in the liver ; Figure 2A: Microscopic changes in the liver of Group-I (Control) The liver architecture was normal in all the fish samples of group-I. B; **Figure 2B** (Group II) there is Increased cytoplasmic

granularity, vacuolar degeneration including fatty vacuoles, increased Kupffer's cell activity, and nuclear changes such as pyknosis, karyorrhexis, or karyolysis; **Figure 2C:** Microscopic changes in the liver of Group-III (Carbon tetra chloride at 5ppm + Silymarin at 50ppm), in figure 2c mild histopathological changes as compared to the Group-II fishes, which include dilated and engorged blood vasculature, mildly swollen and degeneration of hepatocytes, with or without increased Kupffer's cell activity in few fishes. **Figure 2D:** Microscopic changes in the liver of Group-IV (Carbon tetra chloride at 5ppm + Plant extract at 0.5ppm) less severe as compared to the Group II fishes, which include variably dilated and engorged blood vasculature, acute cellular swelling of the hepatocytes, increased cytoplasmic granularity, hepatocytic degeneration with or without vacuolations **Figure 2E:** Microscopic changes in the liver of Group-V (Carbon tetra chloride at 5ppm + Plant Extract at 1ppm) Increased Kupffer's cell activity and nuclear changes such as pyknosis, karyorrhexis or karyolysis were also observed. . The histopathological changes of the liver in group-V were mild to moderate in nature as compared to the Group-II fishes, which include mildly dilated and engorged blood vasculature, mild swelling of the hepatocytes and mild hepatocytic degeneration with or without increased Kupffer's cell activity **Figure 2F**: Microscopic changes in the liver of Group-VI (Carbon tetra chloride at 5ppm + Plant Extract at 2ppm) includes mildly dilated and engorged blood vasculature, mild swelling of the hepatocytes, and mild hepatocytic degeneration with or without increased Kupffer's cell activity, Figure 2F. The liver architecture was as good as normal in all the fish samples of group-VII, **Figure 2G**: Microscopic changes in the liver of Group-VII (Plant Extract at 2ppm only)

Figure 2A**.** On the other hand, when we compare the microscopic findings of Group-I (control) with Group-II, we come to know that severe histopathological abnormalities were induced due to hepatic damage caused by CCl_4. Dilated and engorged blood vasculature, hemorrhage, acute cellular swelling of the hepatocytes, increased cytoplasmic granularity, vacuolar degeneration, including fatty vacuoles, were seen in Figure 2B. A similar study was performed to check the hepatoprotective effect of *Salvia plebeian* on adult Zebrafish and its larvae [32]. In a similar study, thioacetamide, which produces liver damage, was used as a hepatotoxic agent. As we compare this experiment with the present study, the liver damage induced by the CCl_4 is identical to the liver damage induced by thioacetamide. Microscopy findings or the current study also showed increased Kupffer's cell activity, which is a marker of hepatic inflammation due to antigens. Condensation of chromatin in the nucleus was also observed, which suggests hepatic cells are undergoing necrosis or apoptosis. However, similar studies were performed to check the hepatoprotective effect of ethanolic extract prepared from the roots of *Carissa spinarum* and methanolic extract prepared from the leaves of a similar species *Carissa opaca,* in a different animal model (Albino rat). If we compare these two studies with our present experiment, we come to know that CCl_4 induced identical hepatic damage in rat liver, as it produces in Zebrafish. All these findings prove that CCl_4 successfully induce hepatic damage in Zebrafish, and it can be utilized as a hepatotoxic agent in acute toxicity studies of Zebrafish as an animal model.

Microscopic findings of Group-III show fewer histopathological abnormalities as compared to the diseased group. In this group, the animals were first treated with CCl_4 to induce hepatic damage and then

treated with standard hepatoprotective drug Silymarin. It is a well-known hepatoprotective agent being used in various hepatotoxicity studies as a standard drug. Another study described the hepatoprotective effect of Silymarin with various combinations of drugs. The microscopic findings in this study reveal that the morphology of the hepatic cell appears normal as compared to the diseased control group [33]. In another study, rats were pretreated with Silymarin at the dose of 25mg/kg of body weight as a standard hepatoprotective agent, which showed normal architecture of hepatic cells and less fatty changes. Similar results were also observed in the present study. There was less dilated blood vasculature, and lower Kupffer's cell activity was observed, which suggests the hepatoprotective effect of Silymarin, Figure 2C. Histopathological changes in Group-IV (were less severe as compared to Group-II, but when compared with Group-III, hepatic cell degeneration and increased Kupffer's cell activity were seen, which hints apoptosis or necrosis. These findings conclude that plant extract at a lower dose (0.5ppm) has a heaptoprotective effect but less as compared to the standard drug Silymarin, Figure 2D. When we compare these results with another similar study, the plant extract prepared from *Salvia plebeian* was able to inhibit apoptosis, while thioacetamide significantly increased apoptosis levels in the liver. Microscopic findings of Group-V show mild to moderate histopathological changes as shown in Figure 2E, while Group-VI shows mild hepatocyte swelling and degeneration with or without increased Kupffer's cell activity, Figure 2F. These results suggest that extract prepared from the leaves of the Plant *Carissa spinarum* possesses hepatoprotective activity. The most hepatoprotective effect was observed in Group-VI at the highest dose (2ppm). A dose-dependent hepatoprotective effect was observed when graded doses of plant extract were administered. A similar dose-dependent hepatoprotective effect was seen in a study [12] in which plant extract was given in graded doses (100, 200, and 400 mg/kg BW), producing a dose-dependent hepatoprotective effect. Microscopic findings of Group-VII show no histopathological abnormalities or changes, which proves that plant extract does not possess any hepatotoxic activity itself, Figure 2G.

CONCLUSION

The whole experiment was aimed to find out the hepatoprotective effect of extract prepared from the leaves of the Plant *Carissa spinarum.* After the administration of the plant extract, it helped the liver and other organs to heal faster, which proves it also exhibits regenerative properties. Phytochemical screening of leave extract also verified the presence of various Plant secondary metabolites that might have prevented hepatocytes from oxidative damage.

Figure 1 demonstrates the drug utilization by the university students. Out of 254 students a total of 238 (93.70%) students took self-medication, while the remaining 16 (6.30%) students have never taken self-medication.

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Ethical Statement: This experiment was performed as per the rules and guidelines of the Institutional Animal Ethical Committee (IAEC), SPES, BUEST, Baddi, Solan, Himachal Pradesh-173205, and Registered Number: 1421/PO/Re/S/11/CPCSEA; Dated: 28th March, 2018. IAEC approval certificate (Approval Number: BUEST/SPES/IAEC/2021/001) was issued for this experimentation.

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

high percentage of pharmacy students aware to read the package inserts and labels and followed the instructions written (83.61 %). Approximately 82.35% of participants were aware of the significance of medicine expiry dates (Table 6).

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