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Basic Science

Prophylactic and Curative Assessment of Essentiale Forte® On Carbon Tetrachloride-Induced Liver Damage in Wistar Rats

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Abstract

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Key words: Carbon tetrachloride; Curative; Essentiale forte; Prophylactic; Rat.

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AIM: This study was to assess the prophylactic and curative effects of Essentiale forte (ESF) on carbon tetrachloride (CCl₄) induced liver damage in Wistar rats.

MATERIALS AND METHODS: Twenty-four adult Wistar rats were randomly divided into four groups of six rats each. Group I (control group) received 0.3 ml/kg/day of propylene glycol for one month; group II (toxic control) was given 0.7 ml/kg/day of CCl₄ dissolved in olive oil (1:1,v/v) orally for 7 days; group III (prophylactic group) received 4.3 mg/kg/day of ESF for one month followed by CCl₄ for one week; group IV (curative group) was treated with CCl₄ for one week and subsequently received ESF (4.3 mg/kg/day) for one month. Half of the rats were sacrificed at active period, the other half after a 2-week recovery period.

RESULTS: The activities of serum AST, ALT, ALP, total bilirubin level were significantly higher, total protein and GSH levels were significantly reduced in the toxic control group compared to the control group. Group III had significantly higher AST and ALT activities compared to the control rats at active period, whereas after the recovery period no significant differences were observed in almost all the parameters. Moreover, no significant differences in the parameters mentioned above were observed in group IV compared to the control rats at active and recovery period.

CONCLUSION: The results of this study showed that Essential forte was better as a curative agent rather than a prophylactic agent in rats.

Introduction

Hepatotoxicity is defined as an injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent [1].

Hepatotoxic agents like chemicals, drugs, and pesticides are usually encountered by man due to job demands or habits. The incident of liver diseases is on the increase worldwide. The consequences of the toxicity inflicted on the liver by hepatotoxic substances are very grave and constituting a sort of concern for scientist globally [2]. Liver diseases and even their

management methods represent a major global health problem [3]. Toxic chemicals, xenobiotics, alcohol, malnutrition [4], viral and microbial infections are harmful to the liver cells.

Essentiale forte (ESF) is a hepatoprotective drug that has been reported to protect and improve liver function in diabetic subjects with nonalcoholic fatty liver [5] and chronic infections. It basically contains phospholipids from soya-beans, soya-bean oil, hydrogenated castor oil, ethanol, ethyl vanillin, 4-methoxyacetophenone, gelatin colouring agents E171, E172, DL- α -tocopherol. It is also used to treat hepatic cirrhosis, necrosis of the liver cells, liver

failure, liver coma, diseases of the cardiovascular system, hyperlipoproteinemia, hypercholesterolemia, atopic dermatitis, eczema, pyelonephritis [6]. Most of the reported studies on ESF were on human subjects. Studies on animal models of liver damage is also worth embarking upon in view of the fact that animals also suffer from liver ailments just like man and also that information derived from animals studies using the drug could add to the body of the existing knowledge on the use of the drug in the treatment liver diseases. We therefore decided to assess the curative and prophylactic effects of ESF on carbon tetrachloride-induced liver damage in Wistar rats, with a view to providing additional scientific information on the suitability of the drug in the treatment of liver diseases.

Materials and Methods

Animal care and management

Twenty- four adult Wistar rats weighing 150 g - 200 g that were used in this study were obtained from the Animal House of the College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. The rats were kept under normal environmental conditions with a 12/12 h light/dark cycle and had free access to standard rodent pellet diet (Caps Feed PLC, Osogbo, Nigeria) and water *ad libitum*. They were allowed to acclimatize in the laboratory for 2 weeks before the commencement of the study. The experimental procedures adopted in this study were in strict compliance with the guidelines on Experimental Animal Care and Use of Laboratory Animals in Biomedical Research, College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria.

Drugs and chemicals

Essentiale forte[®] was from (A. Natterman and Cie GmbH (Germany). Ltd; batch number 10091); carbon tetrachloride from Hopkins and Williams (Birmingham, England, UK), and propylene glycol from (Biovision, Milpitas, CA, USA).

Drug preparation

Each capsule contains 300 mg of ESF. One capsule was dissolved in 20 ml of propylene glycol. Therefore, 0.04 ml of the solution equivalent to 0.64 mg of ESF was administered to a rat 150 g orally (*p.o.*); this is equivalent to 4.3 mg/kg, which is the therapeutic dose of the drug in humans.

Experimental design

The rats were randomly divided into four groups of six rats each. The rats were treated as

follows: group I (control group received 0.3 ml/kg/day of propylene glycol, the vehicle in which ESF was dissolved, orally for one month); group II (toxic control) was given 0.7 ml/kg/day of carbon-tetrachloride (CCl₄) dissolved in olive oil (1:1,v/v) orally for 7 days so as to induce liver damage [7]; group III (prophylactic group) received 4.3 mg/kg/day of ESF orally for one month and subsequently CCl₄ (0.7 ml/kg/day) was administered for one week; group IV (curative group) was given CCl₄ (0.7 ml/kg/day) for one week and subsequently received 4.3 mg/kg/day of ESF orally for one month.

A day after the last dose of ESF, CCl₄ and propylene glycol as the case may be for each group, three of the rats in each group were sacrificed under chloroform anaesthesia and their blood was obtained by cardiac puncture and drawn into separate plain bottles. The blood was centrifuged for 15 mins at 4000 rpm using a bench centrifuge. The serum was separated and analyzed for the liver enzymes and other organic constituents that are routinely used in the assessment of liver function. Thereafter, the liver of each rat was carefully excised for weight determination. Portion of the liver was homogenized and centrifuged at 4000 rpm for 30 mins to yield a clear supernatant fraction that was used for reduced glutathione (GSH) analysis, while the remaining part was fixed in 10% formo-saline for histopathological studies. The remaining three rats in each group were left for a two week recovery period before they were sacrificed using the same procedure and the same parameters were measured. The entire treatment procedure was summarized in Table 1.

Table 1: Treatment procedure is as shown below:

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Group 1	PG	PG	PG	PG	RP	RP	
Group 2	CCl ₄	RP	RP				
Group 3	ESF	ESF	ESF	ESF	CCl ₄	RP	RP
Group 4	CCl ₄	ESF	ESF	ESF	ESF	RP	RP

PG = Propylene glycol (0.3 ml/kg/day); CCl₄ = Carbon tetrachloride (0.7 ml/kg/day); ESF = Essentiale forte (4.3 mg/kg/day); RP = Recovery period; * = Point of sacrifice of the first half of the group; ** = Point of sacrifice of the second half of the group.

Measurement of body weight

The body weight of the animals were measured once in a week using a (Camry; Zhongshan Guangdong, China) during the experiment to access the weight gain or loss in each group.

Measurement of food consumption and water intake

The food consumption and water intake of the animals were determined daily from the onset of the experiment. The volume of water and weight of food given to each group of rats were measured with a measuring cylinder and a weighing balance respectively. The difference between the previous day volume of water and weight of food and the left-over was taken as the daily food consumption and water intake of the group.

Biochemical analysis

Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, total cholesterol and serum glucose were assayed by the use of appropriate biochemical kits purchased from Randox Laboratories (Crumlin, Co. Antrim UK)

Total protein (TP) was assayed in the serum according to the method of Bradford [8].

Reduced glutathione (GSH) assay was carried out in liver tissue homogenate according to the method of Tietze [9].

Histopathological evaluation

The fixed liver samples were dehydrated in graded alcohol and embedded in paraffin wax. They were then cut into 4-5 μm thick sections and stained with haematoxylin-eosin for photomicroscopic assessment using a Leica DM 750 Camera Microscope at x400 magnification.

Statistical analysis

The results obtained were expressed as mean \pm SEM. The data were analyzed using One-way ANOVA followed by Tukey's multiple comparison tests using GraphPad 5.03 (GraphPad Software Inc., CA, USA). The results were considered significant when $p < 0.05$.

Results

Food and water consumption

The toxic control showed a significant decrease in water intake compared to the control ($p < 0.05$) at active and recovery period (Table 2).

Also, there was a significant decrease in food and water consumption in the prophylactic group compared to the control ($p < 0.05$) at recovery period. A significant decrease in water intake was observed in the curative group compared to the control and prophylactic group at active period, but no significant difference from the control at recovery period.

Table 2: Food consumption, water intake, liver weight and reduced glutathione at active and recovery period.

	Food Consumption (g)		Water Intake (ml)		Liver weight (g)		Reduced glutathione (U/ml)	
	Active	Recovery period	Active	Recovery period	Active	Recovery period	Active	Recovery period
Control (n=3,3)	15.08 \pm 2.43	17.86 \pm 5.16	24.53 \pm 2.81	31.43 \pm 4.30	3.53 \pm 0.08	3.42 \pm 0.03	2.43 \pm 0.56	2.46 \pm 0.58
Toxic control (n=3,3)	14.05 \pm 5.08	16.07 \pm 2.89	18.14 \pm 5.38 ^a	23.93 \pm 2.50 ^a	4.53 \pm 0.33 ^a	4.21 \pm 0.43 ^a	0.61 \pm 0.27 ^a	1.64 \pm 0.25 ^a
Prophylactic (n=3,3)	14.74 \pm 2.15	13.33 \pm 3.98 ^{ab}	24.46 \pm 4.27 ^{ab}	25.36 \pm 5.32	3.94 \pm 0.28	3.33 \pm 0.10 ^a	1.72 \pm 0.24 ^a	2.74 \pm 0.46 ^a
Curative (n=3,3)	16.14 \pm 3.08	17.98 \pm 6.20	21.03 \pm 3.49 ^a	30.21 \pm 5.38 ^a	3.90 \pm 0.16	3.50 \pm 0.33 ^a	2.25 \pm 0.08 ^a	2.17 \pm 0.70 ^a

Significantly different from ^acontrol, ^btoxic control, and from ^ccurative group. Values are mean \pm SEM.

Liver weight and reduced glutathione

There was a significant increase in the relative liver weight of the toxic control compared to the control ($p < 0.05$) at active and recovery period (Table 2). The GSH level in the treated groups was significantly higher than in the toxic control with values being comparable and not significantly different from the control ($p > 0.05$) at active and recovery period (Table 2).

Serum biochemical parameters

The activities of serum AST, ALT, ALP and total bilirubin level were significantly elevated in the toxic control compared to the control at active period (Table 3). An increase in total cholesterol level was also seen in the toxic control group but not significantly different from the control (Table 3). Also, there were significant reductions in total protein, serum glucose (Table 3) and glutathione activity (Table 2) in the toxic control compared to the control ($p < 0.05$) at active period.

The activity of AST, ALT and ALP activities were also found to be significantly increased in the prophylactic group when compared to the control ($p >$

0.05) at active period (Table 3). On the other hand, there were no significant differences in ALT and AST activities of this group compared to the negative control ($p > 0.05$) at recovery period (Table 4). The curative group showed significant reductions in AST, ALT and ALP activities ($p < 0.05$) when compared to the toxic control at active period (Table 3). However, activities of AST and ALP in the curative group were significantly higher than the control, lower but not significantly different from that of the toxic control at recovery period (Table 4).

Liver weight and reduced glutathione activity

The prophylactic group showed significant decrease in glucose level compared to the control and curative group ($p < 0.05$) at active period. However, there was a significant increase in the serum glucose level of curative group compared to the toxic control ($p > 0.05$) with value being comparable and not significantly different from the control at active period (Table 4).

As shown in Table 4, there were no significant

Table 3: Serum biochemical parameters at active period.

	ALT (U/L)	AST (U/L)	ALP (U/L)	TB (μmol/l)	TP (mg/ml)	SG (mg/dl)	TC (mmol/l)
Control	23.50 ± 6.36	25.00 ± 8.46	47.50 ± 3.54	3.50 ± 2.12	1.45 ± 0.02	119.0 ± 5.66	1.17 ± 0.27
Toxic control	106.33 ± 29.94	96.00 ± 11.27 [*]	657.2 ± 42.03 [*]	20.00 ± 5.00 [*]	1.27 ± 0.02 [*]	73.80 ± 21.40 [*]	1.50 ± 0.32
Prophylactic	84.50 ± 7.76 [#]	77.50 ± 19.09 [#]	575.2 ± 105.70 [#]	5.00 ± 0.14 ^a	1.48 ± 0.01 ^a	63.20 ± 1.41 [#]	1.24 ± 0.08
Curative	22.00 ± 1.41 ^a	33.50 ± 3.54 ^a	82.88 ± 23.31 ^a	5.65 ± 0.92 ^a	1.45 ± 0.03 ^a	136.60 ± 31.16 ^a	1.08 ± 0.19 ^a

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin; TP, total protein; SG, serum glucose; TC, total cholesterol. Significantly different from ^{*}control, ^atoxic control, and from [#]curative group. Values are mean ± SEM. (n = 3).

differences in total bilirubin, total cholesterol, serum glucose and total protein levels of the treated groups and toxic control compared to the control (p > 0.05) at recovery period.

Body weight

A significant decrease in body weight was observed in the prophylactic group when compared to the control (p<0.05) at recovery period, as shown in Figure 1.

Table 4: Serum biochemical parameters at recovery period

	ALT (U/L)	AST (U/L)	ALP (U/L)	TB (μmol/l)	TP (mg/ml)	SG (mg/dl)	TC (mmol/l)
Control	26.00 ± 4.24	28.00 ± 12.73	99.85 ± 1.73	4.35 ± 1.20	1.45 ± 0.03	118.6 ± 13.63	1.18 ± 0.15
Toxic control	32.00 ± 26.66	60.00 ± 8.54 [*]	715.6 ± 140.0 [*]	5.67 ± 1.53	1.48 ± 0.01	120.51 ± 10.74	1.50 ± 1.60
Prophylactic	22.00 ± 1.41	21.50 ± 2.12 [#]	185.0 ± 14.14 ^a	5.80 ± 0.85	1.38 ± 0.07	88.00 ± 18.38	1.32 ± 0.02
Curative	22.50 ± 4.24	54.00 ± 18.38 [#]	482.0 ± 164.0 ^a	4.30 ± 1.84	1.43 ± 0.01	93.92 ± 22.18	1.15 ± 0.06

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin; TP, total protein; SG, serum glucose; TC, total cholesterol. Significantly different from ^{*}control, ^atoxic control, and from [#]curative group. Values are mean ± SEM. (n = 3).

Histomorphological observations

Liver

The liver section of toxic control rats shows distortion of the normal hepatic cells arrangement with few perivascular vacuole (Figure 3) compared to the control which shows normal hepatic cells with well-preserved cytoplasm and prominent nucleus (Figure 2). The curative group shows well-preserved hepatocytes which are arranged normally (Figure 5) compared to the toxic control, while prophylactic group appears necrotic with distortion of hepatic cells (Figure 4) compared to the control and curative group at active period.

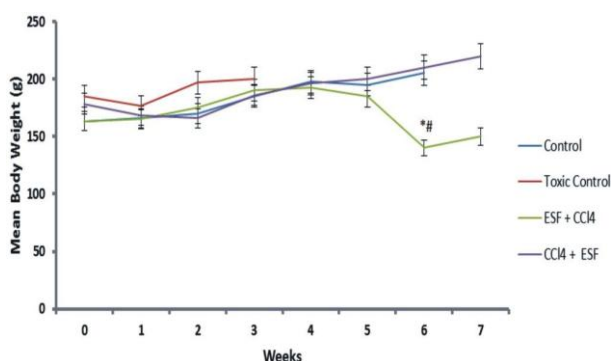


Figure 1: Mean body weights of rats. ^{*} significantly different from control at p < 0.05. [#] significantly different from curative group at p < 0.05.

The liver section of toxic control shows distortion of hepatic cells with slightly ballooned sinusoid (Figure 7) compared to the control which shows normal hepatic cells with distinct and intact central vein (Figure 6). The prophylactic group shows restoration of the normal architecture of the liver as a result of tissue regeneration (Figure 8) compared to the toxic control. Similarly, curative group shows

appreciable tissue regeneration of the hepatic cells (Figure 9) when compared to the toxic control at recovery period.

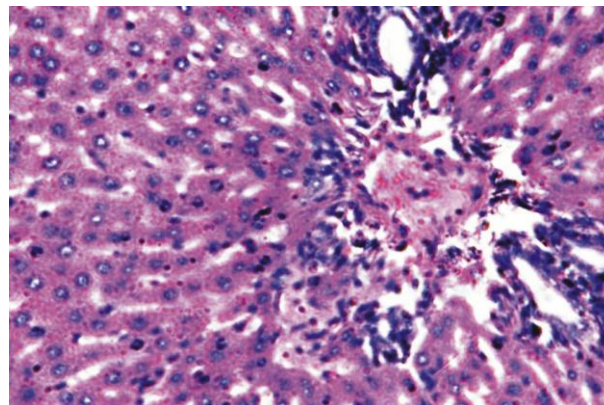


Figure 2: Liver section of control rats sacrificed at active period showing normal hepatocytes with well-preserved cytoplasm and prominent nucleus. The portal vein, hepatic artery, interlobular bile duct are well-defined.

Discussion

Carbon-tetrachloride has been reported to have deleterious effects on the brain of rats [10]. Thus, the significant decrease in water intake observed in the toxic control group and group prophylactically treated with the drug maybe as a result of depression of thirst center located in the lateral hypothalamus [11] by CCl₄. However, this needs further verification.

The significant decrease in food consumption in the prophylactic group compared to the control at recovery period maybe as a result of stupor observed in this group during the period of CCl₄ administration. Similarly, the significant decrease in body weight seen

in the prophylactic group when compared to the control group could be ascribed to the decrease in food consumption observed in this group.

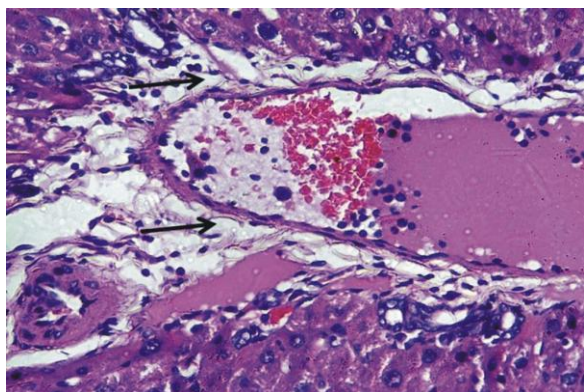


Figure 3: Liver section of toxic control rats sacrificed at active period showing distortion in the arrangement of the hepatic cells with few perivascular vacuole (arrow).

There was a significant increase in enzyme activities of the liver transaminases in the toxic control group, relative to the control group. This may be an indication of cellular leakages and loss of the functional integrity of the cell membranes in the liver which is always associated with hepatonecrosis [12, 13]. The results of the present study indicated that the liver cells of the toxic control group had been damaged by CCl_4 as evidenced by the photomicrograph in Figure. 3. The curative group showed a significant reduction in the activities of the transaminases compared to the toxic control group, while the prophylactic group had high activities of the enzymes which were not significantly different from the toxic control group in rats that were sacrificed a day after the last dose of CCl_4 . The significant decrease in enzyme activities of the liver transaminases in the curative group is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4 . This is supported by the view that serum activities of the transaminases return to normal with the healing of hepatic parenchymal and regeneration of hepatocytes [14].

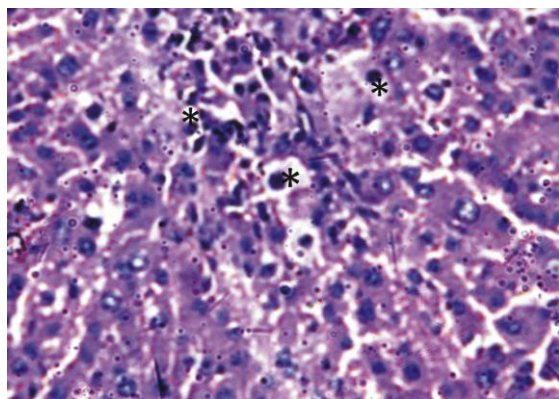


Figure 4: Liver section of prophylactic group sacrificed at active period showing distortion of cellular arrangement, cellular boundaries are obliterated. Some hepatic cells appear degenerating (asterisk).

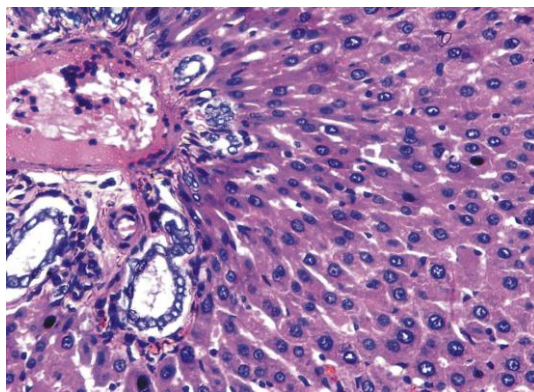


Figure 5: Liver section of curative group sacrificed at active period showing normal hepatic cells and sinusoid (S).

Polyenylphosphatidylcholine (PPC) is a class of phospholipids that is known to protect the liver through the regeneration of liver cells [15]. Therefore, the decrease in enzyme activities of the transaminases in the curative group could be attributed to PPC present in ESF. This assertion is also corroborated by the representative liver photomicrograph of rats in this group (Figure 5). The prophylactic group showed a decrease in the activities of the enzymes that were not significantly different from that of the toxic control group. It might be that PPC contained in ESF does not make the hepatocytes resilient to toxic effect of CCl_4 as much as it facilitates regeneration resulting from hepatotoxin. The photomicrograph of this group is a proof to this affirmation as it appeared more necrotic than that of the curative group (Figure 4).

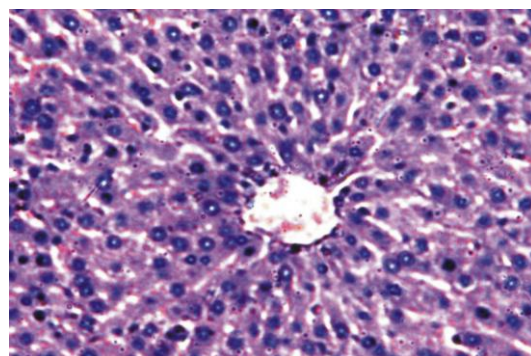


Figure 6: Liver section of control rats sacrificed after the recovery period showing the central vein which is distinct and intact. Hepatocytes appear normal with intact cytoplasm. The sinusoids are normal in size, shape and number and well demarcated.

The activity of AST in group IV was significantly higher than the control group, lower but not significantly different from that of the toxic control at recovery period. The photomicrograph of the liver of this group showed appreciable tissue regeneration. The later upsurge in the activity of AST in this group may be extrahepatic in origin perhaps as a result of leakages from damaged or necrotic tissues of the heart, skeletal muscles and kidneys [16, 17]. There was no significant difference in the activity of ALT in the treated groups compared to the control group at recovery period, due to healing of hepatic parenchymal and regeneration of hepatocytes.

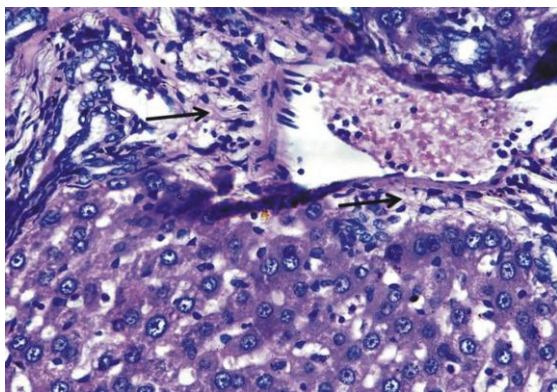


Figure 7: Liver section of toxic control rats sacrificed after the recovery period showing slightly ballooned sinusoids with distortion of hepatic cells. Perivascular vacuole (arrow) are present but not prominent.

The activity of alkaline phosphatase (ALP) was significantly higher in the toxic control group than in the control rats as a result of CCl_4 induced liver damage. Alkaline phosphatase is excreted normally via bile by the liver. In liver injury due to hepatotoxic substances, there is a defective excretion of the bile by the liver, causing increased ALP activity in the serum [18]. The activity of ALP was significantly lower in the curative group than in the toxic control group. Treatment with ESF suppressed the increased activity of ALP in the serum. In the prophylactic group, the activity of this enzyme was not significantly different from the toxic control, indicating that ESF was not able to prevent the cells of the liver from being damaged by CCl_4 .

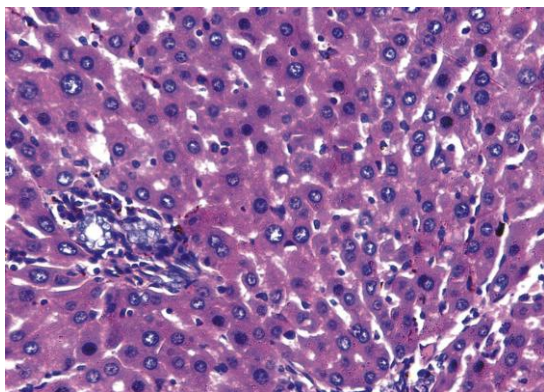


Figure 8: Liver section of prophylactic group sacrificed after the recovery period showing normal hepatic cells arranged in plate. Hepatic vein appears normal.

ALP activity in the treated groups was significantly higher than the control group but significantly lower than the toxic control at recovery period. This suggests that CCl_4 may be inducing the synthesis of ALP in other organs but possibly at a slow rate.

Similarly, total bilirubin level was significantly increased in the toxic control group compared to the control group at active period may have resulted from the inability of the liver to metabolized and then excrete bilirubin through the bile ducts and gallbladder into the intestine indicating a possible hepatic injury.

The bilirubin level in both the curative and prophylactic groups were not significantly different from the control group in rats that were sacrificed after the last dose of ESF and CCl_4 as the case may be for each group. The reduction in bilirubin level in the treated groups in rats that were sacrificed after the recovery period is a strong indication of restoring normal liver function.

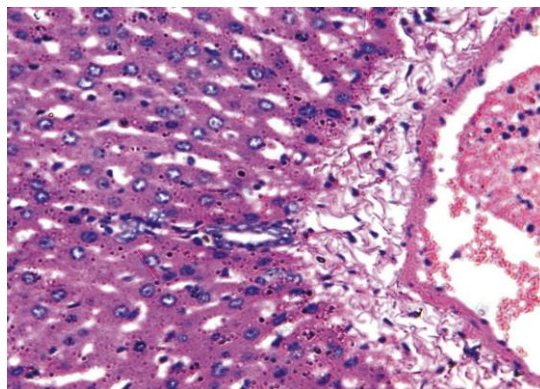


Figure 9: Liver section of curative group sacrificed after the recovery period showing normal hepatocytes. Sinusoids (S) appear slightly dilated.

There was a significant decrease in total protein in the toxic control group compared to the control group and treated groups at active period. This may be due to defects in protein biosynthesis as well as disruption and dissociation of polyribosomes from endoplasmic reticulum following administration of CCl_4 [19]. The increase in total protein level in the treated groups relative to the toxic control group could have been due to the promotion of ribosome assembly on endoplasmic reticulum which facilitates uninterrupted protein biosynthesis [13].

The observed increase in cholesterol level may have resulted from damage to hepatic parenchymal cells that lead to disruption of lipid metabolism in the liver [20]. There was a significant decrease in total cholesterol level of the treated groups compared to the toxic control group, because ESF has been reportedly used in the treatment of hypercholesterolemia [6].

The serum glucose level also dropped significantly in the toxic control group compared to the control group at active period. This could be due to decreased hepatic glycogen content after treatment with CCl_4 , reflecting decreased gluconeogenesis by the liver [21]. The significant decrease in the serum glucose level observed in the prophylactic group may have resulted from decreased food consumption observed in this group, more so as the photomicrograph of the liver of this group showed appreciable tissue regeneration of the hepatocytes (Figure 8).

The toxic control group showed a significant increase in the relative liver weight compared to the control group. This may have resulted from inflammation in response to injury caused by CCl_4 or reduction in protein causing rapid mitosis of the

hepatic cells and growth of the liver to a larger size [11].

The glutathione (GSH) level in the toxic control group was significantly lower than in the control and treated groups. Administration of CCl₄ to rats in order to induce liver damage resulted in decreased level of glutathione (GSH) [22]. The observed decrease in GSH activity in the toxic control group could be attributed to increased use of GSH in preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides [23]. The significant increase in GSH level, both in the curative and prophylactic groups could be ascribed to the presence of vitamin E in ESF. Vitamin E has been reported to protect the polyunsaturated fatty acids of cellular membranes and low-density lipoproteins (LDL) from oxidative damage caused by free radicals. In this manner, vitamin E serves to protect and stabilize the cellular membrane through its role as a powerful antioxidant [24].

In conclusion, the results of the study showed that ESF do not have much membrane stabilizing properties as they facilitate regeneration of the hepatocytes when used as remedies against hepatotoxic substances.

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