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HEPATOPROTECTIVE ACTIVITY OF METHANOL EXTRACT OF *FENUGREEK SEEDS* ON RATS

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ABSTRACT: The present study was conducted to evaluate the hepato-protective activity of methanol extract of seeds of *Trigonella foenum graecum* (TFG) in CCl_4 induced toxicity in Wistar albino rats. Seeds of TFG were collected, and subjected to continuous cold maceration in a macerator, for 72 h with solvents like petroleum ether (60 – 80) and methanol separately. Hepatic injury in rats was induced separately by administration of equal mixture of CCl_4 and olive oil (50% v/v, 1.25 ml/kg, i.p.). Liver damaged was monitored by raised biochemical marker enzymes (SGOT, SGPT, and alkaline phosphatase). CCl_4 was administered twice a week, on every first and fourth day of all 14 days. The extract at the dose of 250 mg/kg b. wt. was evaluated by inducing hepatotoxicity with CCl_4 and using silymarin (100 mg/kg) as the reference standard. Biochemical parameters like, SGOT, SGPT and serum bilirubin level were analysed. A section of liver was subjected to histopathological studies. Based on the above studies, it is reported that the methanol extract of TFG possess significant hepato-protection against CCl_4 induced hepatotoxicity in albino rats.

INTRODUCTION: Hepatoprotection or anti-hepatotoxicity is the ability to prevent damage to the liver. A number of medicinal plants are used in traditional system of medicine for the management of liver disorders. Fenugreek (*Trigonella foenum* Graecum) [1] is an annual Mediterranean and Asiatic herb with aromatic seeds (**Figure 1**). It grows to two feet in height with brownish seeds contained in sickle shaped pods. It is used worldwide as a culinary spice as well as a medicinal herb to soothe the stomach and help maintain blood sugar levels. The seeds are rich in protein and contain about 50% fiber and 25% soothing mucilage.

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FIGURE 1: FENUGREEK SEEDS

Fenugreek is one of the primary supplements used to support type II diabetics or noninsulin-dependent diabetes mellitus (NIDDM). Most NIDDM patients typically have enough insulin but it is not used effectively. Research as to the cause seems to indicate high levels of body fat, too many calorie consumed from refined foods, lack of polyunsaturated fats and chromium deficiencies. Fenugreek Seed helps by not only reducing blood sugar levels with its high concentrations of phytochemicals, but it has also helped reduce low density cholesterols and triacylglycerol [2]. This plant is used in the treatment of liver disorders in folk medicine also.

This has triggered the authors and the present study was conducted to evaluate the hepato-protective activity of methanol extract of TFG against liver disorders induced by CCl_4 in Wistar albino rats.

Biochemical parameters like, SGOT, SGPT, SALP and serum bilirubin were determined to assess the hepato-protective effect of methanol extract against CCl_4 induced liver disorders. The study revealed that methanol extract significantly reduced SGOT, SGPT, SALP and serum bilirubin levels. The preliminary findings suggest that the seeds TFG possess potential hepato-protective activity.

The present study scientifically validated the traditional use of *Trigonella foenum* Graecum for liver disorders.

MATERIALS AND METHODS:

Plant material: Plants sample were collected from vegetable growing area of West Bengal. Collected plant seeds were washed under running tap water, dried, powdered and stored in polythene bags at 40°C. Water soaked TFG seed were germinated for three days and then powdered for experimental purpose.

Plant extracts preparation: Air-dried seeds (2 Kg) were finely ground and soaked in petroleum ether 60- 80 (1.0lit.) at room temperature (28°C) for 3 days, after which the slurry was filtered. This process was repeated two times. The filtrate evaporated to dryness at 50°C and under reduced pressure, yielding reddish-brown syrup (7.5gm). Next, the residue was soaked in methanol (1.2 lit) at room temperature (28°C) for 3 days. The slurry was filtered. This process was repeated two times. The filtrate evaporated to dryness at 50 °C and under reduced pressure to give yellowish brown syrup (35gm) [3, 4].

Phytochemical study: The crude residues were employed for further investigation. The extracts were subjected to qualitative chemical tests for the detection of various plant constituents like carbohydrates, glycosides, proteins and amino acids, fixed oils and fats, gums and mucilage, alkaloids, phytosterols, flavonoids, tannins and phenolic compounds, saponins, triterpenoids, etc.

Animals: Wistar albino rats (female) were used throughout the experiments (Figure 2). Weight of each animal was between 95-120gm. Before initiation of experiment, the rats were acclimatized for a period of 10days. Standard environmental conditions such as temperature (25 ± 2°C), relative humidity (45-55%) and 12 hours dark/light cycle were maintained in the quarantine.

All the animals were fed with normal diet & water was allowed *ad libitum* under strict hygienic conditions. Ethical experiments on animals were obtained from Institutional Animal Ethics Committee (IAEC). Animal were caged according to their weight in separate cage [7, 8, 9, 10, 11].

FIGURE 2: WISTAR ALBINO RATS

Preparation of Standard dose: Here silymarin was used as standard drug for evaluating the hepato-protective activity. The drug was powdered and weighted as per calculation of 100mg/kg b.w and then made into suspension in 1% gum acacia (suspending agent) [7, 8, 9, 10, 11].

Induction of hepatic injury: Hepatic injury in rats was induced separately by administration of equal mixture of CCl_4 and olive oil (50% v/v, 1.25 ml/kg, i.p.). Liver damage was monitored by raised biochemical marker enzymes (SGOT, SGPT, and alkaline phosphatase) [7, 8, 9, 10, 11].

Experimental Design: The rats were divided into four groups of six animals each [7, 8, 9, 10, 11].

Group 1: Saline (0.9 % isotonic saline solution, ISS), 0.1 mL

Group 2: Hepatic control (only CCl_4) on the 14th day.

Group 3: Silymarin [100mg/kg, b.w, p.o. per day respectively for 14 days] + CCl_4 [1.25ml/kg, i.p. on the 14th day].

Group 4: Test compound suspension [250mg/kg, b.w, p.o. per day respectively for 4 days]+ CCl_4 [1.25ml/kg, i.p. on the 14th day].

Preparation of serum: Twenty-four hours after the animals were administered with a single dose of CCl_4, they were sacrificed by cervical dislocation [7, 8, 9, 10, 11]. Blood samples of each animal were collected by heart puncture and were allowed to clot for 45mins at room temperature.

Serum was separated by centrifugation at 2000Xg for 15mins and analysed for various biochemical parameters (serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), and serum lactate dehydrogenase (LDH). The liver samples were cut into small pieces and stored into 10% formalin for histopathological study.

Statistical analysis: All data were expressed as mean ± standard error of the mean (SEM) or as percentages. Analysis of variance (ANOVA) was used for the statistical analysis of data [7, 8, 9, 10, 11].

Dunnett's test, Tukey's HSD test (Tukey's honestly significant difference test) and LSD test (least significant difference test) were used for determining significance. Results with $p<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION:

Phytochemical screening of two extracts:

Tests	Pet. Ether extract	Methanol extract
Alkaloids		
Dragendroff's	+	++
Wagner's	+	++
Mayer's	+	++
Hager's	+	++
Flavonoids		
$FeCl_3$	+	++
Lead acetate	+	++
NaOH	+	++
Shinoda	+	++
Phenols		
$FeCl_3$	+	++
Lead acetate	+	++
Gelatine	+	++
Magnesium and hydrochloric acid reduction	+	++
Tannins		
Phlobatannins	-	-
Anthraquinones	-	-
Triterpenoids		
Liberman's	-	+
Salkowski	-	+
Glycosides		
Borntrager's	+	+
Legal's test	+	+
STEROIDS	+	+
SAPONINS	++	+++
Carbohydrate		
Molish's	+	+
Fehling's	+	+
Barfoed's	+	+
Benedict's	+	+
Amino Acids		
Millon's	+	+
Biuret test	+	+
Ninhydrin	+	+
Napthoquinone	-	-

Note: + = Less precipitation, ++ = Moderate precipitation, +++ = Higher precipitation, - = Negative test

Estimation of biochemical parameters: Carbon tetrachloride (CCl_4) is a highly toxic chemical agent. The toxic effects of CCl_4 on liver have been known for years and been studied extensively. Furthermore, CCl_4 treatment has been used as a model to induce fatty liver for studying possible interacting effects of a compound or a treatment. The effects of CCl_4 on hepatocytes, depending on dose and exposure time, are manifested histologically as hepatic steatosis (e.g., fatty infiltration), centrilobular necrosis and ultimately cirrhosis.

Hepatic steatosis of the liver is a multi-factorial phenomenon thought to be caused by a blockage of lipoprotein secretion; impaired synthesis or peroxidation of phospholipids. It may also due to the toxic effects of free alkyl radicals on cell membranes and disturbances in methylation reactions. The endoplasmic reticulum and mitochondria have been shown to be involved in cell damage. The metabolic effects of CCl_4 inside mitochondria have been described and it has been found that damage to the calcium pump in mitochondria is dependent upon halo alkylation. However, the profound accumulation of fat following CCl_4 poisoning is considered to be independent of mitochondrial damage. The fatty infiltration of the liver is thought to develop as a result of the action of free alkyl radicals on bio membranes that in turn cause halo alkylation-dependent blocking at the exit of the lipoprotein micelles from the Golgi apparatus.

It is well known that carbon tetrachloride is converted by cytochrome P_{450} mixed function oxygenases in smooth endoplasmic reticulum of liver into toxic metabolite, mainly trichloromethyl radical (CCl_3·). This free radical in the presence of oxygen may cause peroxidation of lipids on target cell resulting in extensive damage to liver. The results of biochemical parameters revealed to the alteration of enzyme levels in CCl_4 treated group indicating that CCl_4 induces damage to the liver. Table 2 shows that CCl_4 causes significant increase in SGOT level from control 35.56 ±0.873 IU/L to 161.52 ± 0.735 IU/L after CCl_4 intoxication. Administration of methanol extract of TFG seeds in CCl_4 intoxicated rats caused reduction in SGOT level to 62.75 ± 1.731 IU/L and 43.10 ± 1.390 IU/L respectively (P<0.001). The extract in control animals does not cause any significant changes in SGOT.

Further, table 2 reveals that CCl_4 causes a significant increase in SGPT level from control 45.74 ± 0.620 IU/L to 169.66 ± 1.025 IU/L after intoxication. However, administration of methanol extract of TFG seeds in CCl_4 intoxicated rats led to reduction of SGPT level to 76.08 ± 1.134 IU/L and 53.18 ± 1.596 IU/L respectively (P<0.001). The extract in control animals showed no such significant alteration in SGPT level.

SALP level in the control group increased from 77.62 ± 1.114 IU/L to 191.28± 2.216 IU/L in CCl_4 intoxicated rat as shown in **Table 2**. Administration of methanol extract of TFG seeds in CCl_4 intoxicated rats led to lowering of the SALP level to 104.97 ± 1.245 IU/L and 86.17 ± 0.931 IU/L respectively (P<0.001). The extract in control animals showed no significant alteration in SALP level.

CCl_4 also caused a significant decrease in total cholesterol, HDL and LDL levels from their control values of 97.40 ± 1.227 mg/dL, 34.62 ± 0.897 mg/dL and 47.67 ± 0.472 mg/dL to 60.75 ± 0.833 mg/dL, 24.50 ± 0.993 mg/dL and 15.25 ±1.172 mg/dL respectively after intoxication as shown in **Table 3**. Administration of methanol extract of TFG seeds in CCl_4 intoxicated rats led to increase of the total cholesterol, HDL and LDL levels to 75.60 ± 1.019 mg/dL, 28.64 ± 0.613 mg/dL and 35.36 ± 0.994 mg/dL respectively.

TABLE 2: BIOCHEMICAL CHANGES IN ALBINO RATS TREATED WITH METHANOL EXTRACT OF TFG SEEDS, AGAINST CCl_4 INDUCED HEPATIC INJURY:

Groups	Treatment and dose (mg/kg)	SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)
Group 1	Normal	35.56 ± 0.873	45.74 ± 0.620	77.62 ± 1.114
Group 2	CCl_4	161.52 ± 0.735^{a3}	169.66 ± 1.025^{a3}	191.28 ± 2.216^{a3}
Group 3	CCl_4 + Silymarin	38.91 ± 0.932^{b3}	49.47 ± 1.172^{b3}	80.91 ± 1.299^{b3}
Group 4	CCl_4 + methanol extract of TFG seeds	62.75 ± 1.003^{b3}	76.08 ± 1.134^{b3}	104.97 ± 1.245^{b3}

The values represent the mean ± S.E.M. (standard error of the mean). Post-hoc LSD (least significant difference) test: a^3: P<0.001 compared with control. b^3: P<0.001, compared with CCl_4 treated group.

TABLE 3: LIPID PROFILE CHANGES IN ALBINO RATS TREATED WITH METHANOL EXTRACT OF TFG SEEDS AGAINST CCl_4 INDUCED HEPATIC INJURY

Groups	Treatment and dose (mg/kg)	Total cholesterol (mg/dL)	HDL (mg/dL)	LDH (IU/L)
Groups 1	Control	97.40 ± 1.227	34.62 ± 0.897	47.67 ± 0.472
Groups 2	CCl_4	60.75 ± 0.833^{a3}	24.50 ± 0.993^{a3}	15.25 ± 1.172^{a3}
Groups 3	CCl_4 + Silymarin	93.96 ± 0.662^{b3}	33.45 ± 0.749^{b3}	44.76 ± 1.491^{b3}
Groups 4	CCl_4 + methanol extract of TFG seeds	75.60 ± 1.019^{b3}	28.64 ± 0.613^{b2}	35.36 ± 0.994^{b3}

Values are expressed as mean ± SEM of 6 animals each. a^3 P<0.001 compared with control, b^2 P<0.01, b^3 P<0.001, compared with CCl_4 treated group.

Histopathology study: Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. Histology is an essential tool of biology and medicine. Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of liver diseases usually requires histopathological examination of samples. Sheets of connective tissue divide the liver into thousands of small units called lobules.

A lobule is roughly hexagonal in shape, with portal triads at the vertices and a central vein in the middle. The lobule is the structural unit of the liver and rather easy to observe. In contrast, the hepatic acinus is more difficult to visualize, but represents a unit that is of more relevance to hepatic function because it is oriented around the afferent vascular system. The parenchyma cells of the liver are hepatocytes. These polygonal cells are joined to one another in anastomosing plates, with borders that face either the sinusoids or adjacent hepatocytes.

The ultra-structure appearance of hepatocytes reflects their function as metabolic superstars, with abundant rough and smooth endoplasmic reticulum, and Golgi membranes. Glycogen granules and vesicles containing very low density lipoproteins are readily observed. Hepatocytes make contact with blood in sinusoids, which are distensible vascularchannels lined with highly fenestrated endothelial cells and populated with phagocytic Kupffer cells.

The space between endothelium and hepatocytes is called the 'Space of Disse' which collects lymph for delivery to lymphatic capillaries. Bile originates as secretions from the basal surface of hepatocytes, which collect in channels called canaliculi. These secretions flow towards the periphery of lobules and into bile ductules and interlobular bile ducts, ultimately collecting in the hepatic duct outside the liver.

The hepatic duct is continuous with the common bile duct, which delivers bile into the duodenum. In most species, bile is diverted through the cystic duct into the gall bladder. The columnar epithelium of the gall bladder is devoted largely to absorption of water and electrolytes.

As the liver is the major site for drug metabolism, it is not surprising that drug toxicity and adverse drug reactions would incite variable functional, histological and ultra-structural hepatic abnormalities. The type of liver cell injury may be intrinsic and dose dependent, whereby the mechanism may relate either to the formation of free radicals or electrophilic intermediates, or to the production of active oxygen species, and destruction of liver cell membranes. On the other hand, liver cell damage may be idiosyncratic and dose independent. A wide variety of hepatic histological changes have been documented as secondary to drugs and toxins; in addition, up to 1000 drugs and toxins have been implicated in causing these histological changes.

This type of liver cell injury is usually related to direct effects of the drug (e.g.carbon tetrachloride, acetaminophen) or toxin (e.g. mushrooms) itself or its metabolites. Unlike drug-induced hypersensitivity reactions, the type of liver cell necrosis can be predicted, and is most often zonal in distribution. Usually, the liver cell injury is coagulative in type, whereby the damaged liver cells become shrunken, with eosinophilic cytoplasm and hyperchromatic nuclei with eventual nuclear pyknosis and karyorrhexis.

Although an inflammatory reaction is not characteristic of this type of liver cell injury, a histological response to the necrotic hepatocytes may secondarily occur, with this type of inflammatory reaction both neutrophilic and histiocytic. A zonal nature is often characteristic of specific drugs. Most frequently, the injury is perivenular, but other zones may be specifically affected. Often the borders of the areas of necrosis are sharply defined and distinct from the adjacent viable hepatocytes. The spared liver cells with time may show a hydropic change not representing liver cell injury but instead representing regenerative activity.

Sometimes fatty change secondary to intrinsic damage may also occur. When there is impediment to bile flow, cholestasis may also be present. The distinction in the vast majority of cases rests upon eliminating other causes of liver disease, as no reliable approach outside of discontinuing the medication and observing improvement of liver tests is feasible.

Usually, the degree of active liver disease manifested by monitoring of hepatic function will resolve within one to two weeks in hepatitic reactions but may take months in cholestatic reactions, although in some instances the abnormal liver tests may persist for considerable time periods with either.

The hepatoprotective effect of methanol extract of TFG seed was confirmed by histological examination. The liver sections of normal control animals showed hepatic cells with well-preserved cytoplasm, prominent nucleus and central vein (Fig. 3). The normal architecture of liver was completely lost in rats treated with CCl_4 (Fig. 4) with the appearance of vacuolated hepatocytes and degenerated nuclei. Vacuolization, fatty metamorphosis in the adjacent hepatocytes, cell infiltration of lymphocytes and Kupffer cells and necrosis of hepatocytes were severe in the centrilobular region and these changes were also observed in areas other than the centrilobular regions.

The livers of rats treated with methanol extract of TFG seed (Fig. 6), or silymarin 100 mg/kg (Fig. 5) showed a significant attenuation from CCl_4-induced liver damage as evident from normal hepatocytes with well-defined nuclei. Vacuolization and fatty degeneration were remarkably prevented by the treatment with extract and silymarin. All the above histopathological findings are well correlating with the biochemical estimations (Tables 2 to 3). These results suggest that the methanol extract of TFG seed has potential clinical applications for treating liver disorders.

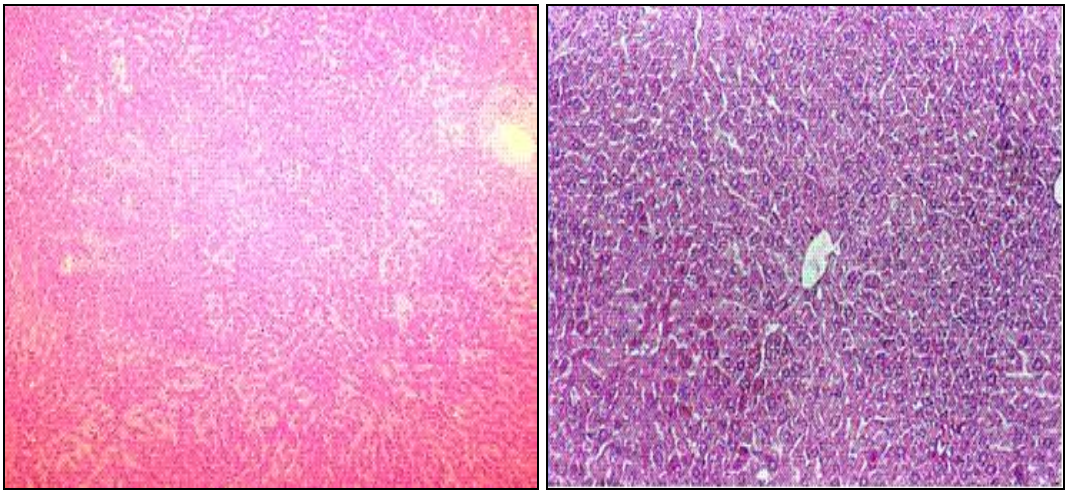

FIG. 3: NORMAL HEPATIC CELL WHERE CENTRAL VAIN IS PRESENT PROPERLY

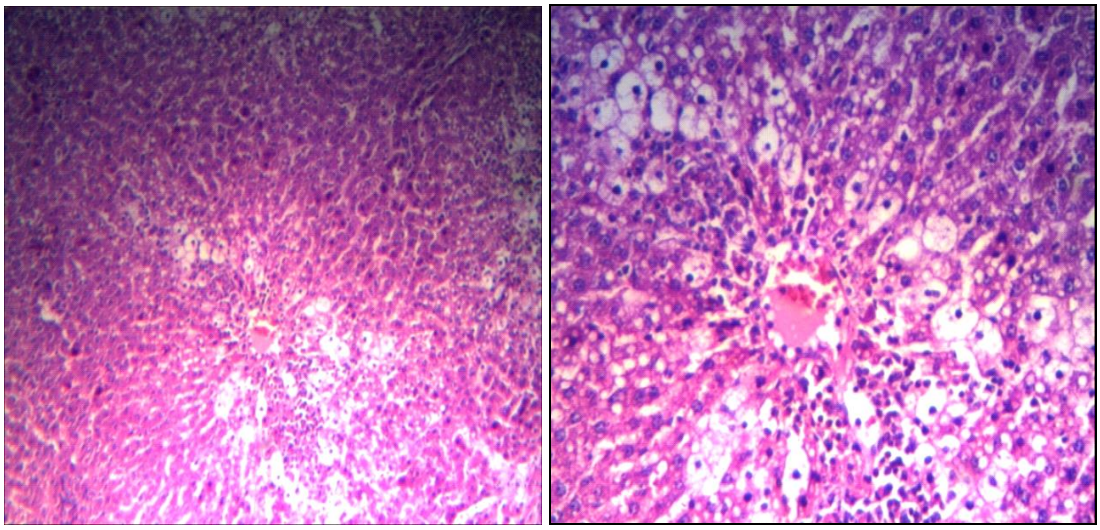

FIG. 4: DAMAGE CELLS AND CENTRAL VAIN IS APPEARED FOR ACTION OF CCl_4

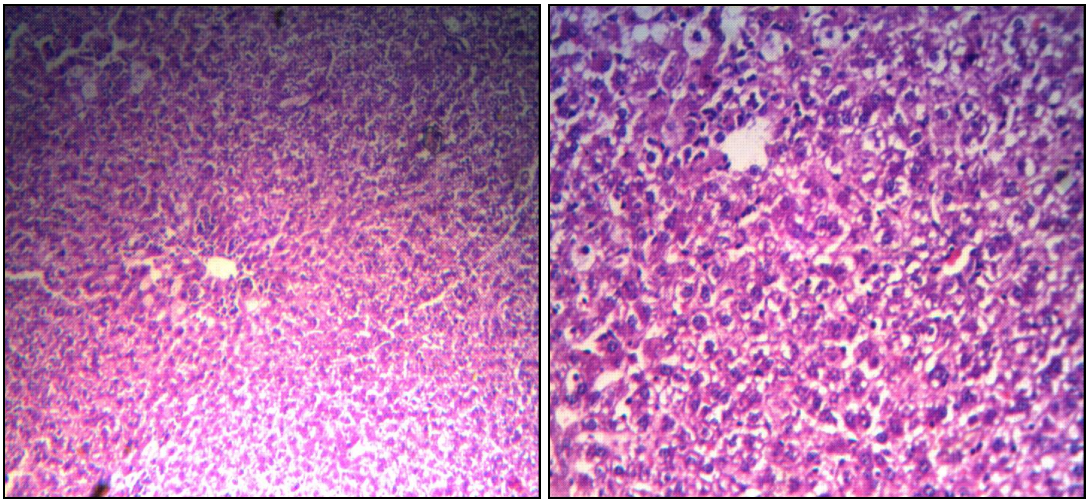

FIG. 5: DAMAGED CELLS AFTER CCL_4 TREATMENT IS RECOVERED BY SILYMARIN TREATMENT

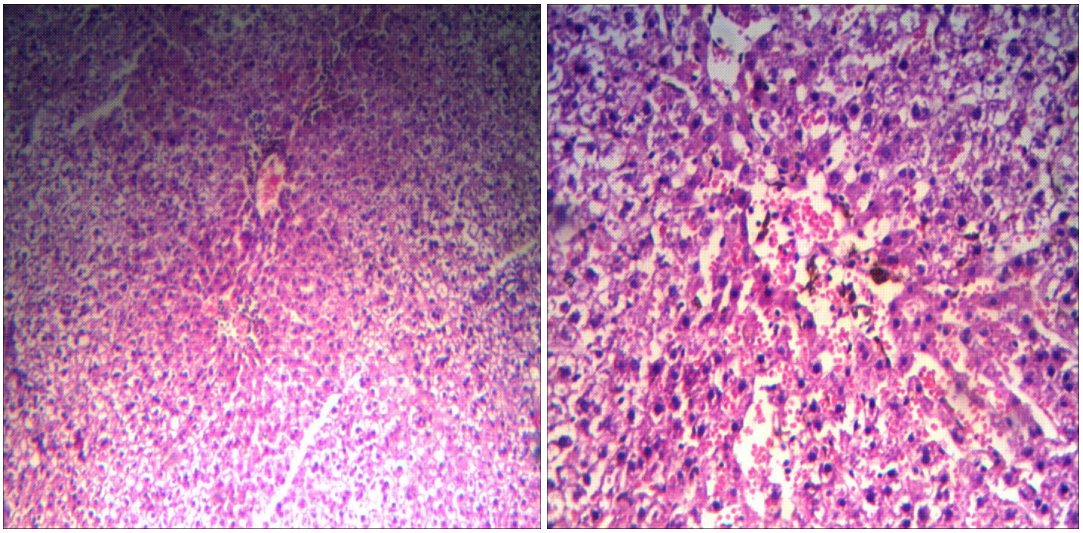

FIG. 6: DAMAGED CELLS AFTER CCl_4 TREATMENT IS RECOVERED BY METHANOL EXTRACT OF TFG SEED TREATMENT

CONCLUSION: Based on the results obtained, it may be concluded that the methanol extract of TFG seed is non-toxic and is safe. As the results indicated, the extract possesses significant hepato-protective activity. A study of effect of extract on immunological parameters, like TNF-alpha, interleukin, etc is required to be conducted. Also, a thorough study of clinical trials is required to be performed. After carrying out these studies, the plant may be considered as a low cost, potent, herbal medicine for liver disorders.

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