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Original Article:

AMELIORATION OF CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN RATS BY STANDARDIZED *FERONIA LIMONIA*. LINN LEAF EXTRACTS

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ABSTRACT

The hepatoprotective potential of standardized *Feronia limonia* (Family, Rutaceae) methanolic extract (FL-7) and chloroform soluble fraction (FL-9) were assessed against carbon tetrachloride (CCl_4) induced oxidative stress and hepatotoxicity in rats. Rats treated with CCl_4 recorded significant elevation in plasma markers of hepatic injury, alteration in hepatic antioxidant status and histopathological damages. However, rats pretreated with FL-7 (200 or 400 mg/kg, *p.o.*) and FL-9 (100 or 200 mg/kg, *p.o.*) for 7 days and later administered CCl_4 (0.5 ml/kg, *i.p.*) recorded lowered indices of the above mentioned parameters and minimal histological damage in a dose dependent manner. These results were comparable to that of CCl_4+silymarin treated rats. The results obtained with FL-7 and FL-9 are attributable to their free radical scavenging potential due to high contents of polyphenols and flavonols recorded herein. Overall, this study establishes the efficacy of FL-7 and FL-9 as hepatoprotective agents against CCl_4 induced hepatotoxicity in rats.

Keywords: carbon tetrachloride, silymarin, *Feronia limonia,* histopathology

Abbreviations: - AST: Aspartate amino transferase, ALT: Alanine amino transferase, CCL_4: Carbon tetrachloride, BW: Bodyweight, w/w: Weight/weight, OECD: Organization for Economic Co-operation and Development, ALP: Alkaline phosphatase, SOD: Superoxide dismutase, CAT: Catalase. GSH: Reduced glutathione, LPO: Lipid peroxidation, MDA: Malonaldehyde, AA: Ascorbic acid

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INTRODUCTION

Liver carries out three major physiological processes viz. production of bile, metabolism of nutrients and elimination of metabolic waste. Additionally, it is involved in detoxification and subsequent elimination of drugs and xenobiotics (Saleem et al., 2010). Incidences of chemical/drug induced hepatotoxicity are on the rise and provoke a need for a safe hepatoprotective agent in lieu of synthetic counterparts. Traditional Indian systems of medicine such as Ayurveda, Siddha and Unani have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness (Kumar, 2012). Several Indian medicinal plants have been listed in the Indian traditional system of medicine with claims of treating disorders associated with liver and also imparting hepatoprotection (Saleem et al., 2010, Kumar et al., 2012).

Standardization of herbal formulations is essential in order to assess their quality, efficacy and possible side effects (Jain et al., 2010). But, use of folklore herbal preparations as an alternative medicine is seriously hampered because they often lack scientific validity, phytochemical characterization and reproducibility of results (Sahoo et al., 2010). Identification and characterization of markers/bioactive compounds present in herbal extract is therefore mandatory as per the regulatory guidelines (Choudhary and Sekhon, 2011). Chemical fingerprints obtained by chromatographic, spectroscopic, thermo-gravimetric analysis, capillary electrophoresis and polarography techniques have become the most potent tools for quality control of traditional herbal medicines (Jain et al., 2011a).

Feronia limonia. L (Family Rutaceae, subfamily Aurantioideae), commonly known as wood-apple, elephant apple, monkey fruit, curd fruit etc. is widely distributed in dry warm regions of India, Bangladesh, Myanmar, Java & Srilanka (Qureshi et al., 2010). Fruit, gum, leaves, bark and pulp of FL have been reported to possess wide array of pharmacological properties (Qureshi et al., 2010). Phytochemical characterization of FL leaves has shown presence of coumarins (luvangetin, xanthotoxin and marmesin), triterpenoids (lupeol and limonin), steroids (sitosterol and sitosterol-O- β-D-glucoside) and essential oil (methyl chavicol, linalool etc.) (Qureshi et al., 2010).

Previous studies have reported hepatoprotective potential of crude FL leaf extract against thioacetamide, allyl alcohol (Manjusha et al., 2004), paracetamol (Ahamed et al., 2010), D-galactosamine and lipopolysaccharide (Ahamed et al., 2011) induced hepatotoxicity in rats. However, these studies lack details pertaining to the characterization and phytochemical constituents of FL extract. Hence, it is not possible to predict the compound/s or their mixtures responsible for imparting hepatoprotection. Recently, we had reported *in vitro* hepatoprotective potential of bioassay guided characterized extract/fractions of FL leaf (Jain et al., 2011b). In continuation of this study, this inventory is an effort to evaluate efficacy of standardized FL leaf extracts (methanolic and chloroform soluble fractions) in ameliorating carbon tetrachloride induced oxidative stress and hepatotoxicity in rats.

MATERIAL AND METHODS

Chemicals

CCl_4 and others solvents, were of analytical grade purchased from Merck India Ltd, Mumbai, India. Silymarin was obtained from Admac Pharma, India. All the diagnostic kits were purchased from Reckon Diagnostics, Baroda, India.

Plant material

Feronia limonia leaves were collected in the months of September–October 2008 from campus of The M. S. University of Baroda, Vadodara, India, authenticated in the Botany Department and a voucher specimen (No. Pharmacy/FL/ 08-09/01/MJ) was

deposited in the Pharmacy Department of the university.

Extraction and fractionation

The leaves were shade dried, powdered (500 g) and extracted three times with petroleum ether (3X1.5 L) in a soxhlet apparatus. The filtrates were then combined and filtered and concentrated to dryness in a rotary evaporator (Buchi-R-215, Germany) to obtain a crude petroleum ether extract [9.5 g (1.90 % w/w)]. The remaining marc was then dried and again exhaustively extracted at temperature (60-80 °C) with methanol (3 × 1.5 L) in a soxhlet apparatus. The pooled extracts obtained were then concentrated under vacuum to give methanolic extract (FL-7) [27 g (5.07 % w/w]. This extract was re-dissolved in water: methanol and partitioned with organic solvents to provide a chloroform fraction (FL-9) [4.6 g (0.92 % w/w)].

Preparation of extract, fraction and standard solutions for HPLC

Standard marmesin (5 mg) was weighed in separate volumetric flasks and dissolved in 5 ml methanol to prepare a stock solution of 1000 µg/ml. The same were serially diluted with methanol to obtain a range of 20–100 µg/ml. Sample extract and fraction (10 mg each) were weighed in separate volumetric flasks and dissolved in 10 ml methanol to obtain a stock concentrations of 1000 µg/ml.

HPLC fingerprinting of extract and fraction

Methanolic extract and chloroform fraction of FL leaves along with marmesin were subjected to HPLC analysis (Spinchrom Chromatographic Station® CFR Version 2.4.0.193, Chennai, India) with a SPD M20A photo-diode array (PDA) detector to obtain a chromatogram. Hypersil C18 column (particle size 5 mm; 250 ´ 4.6 mm id; Thermoquest, Cheshire, UK) proceeded by an ODS (Thermoquest) guard column (10 mm, 10 ´ 5 mm id) at an ambient temperature was used. FL-7 and FL-9 were filtered through a 0.45 µM filter disk. Each sample (20 µl) was injected onto the column and monitored at 280 nm. Peak obtained in the UV spectra thus confirmed purity. The mobile phase consisting of methanol: water (1:1) was run at room temperature (flow rate = 2 Ml/min) for 20 min. The procedure was repeated three times for each sample. Calibration curve for marmesin was prepared using calibration solution (in range of 10-50 µg/ml) (Jain et al., 2011a). Each solution was injected in triplicate and a calibration curve was prepared using mean values. Concentration of marmesin was expressed in terms of % w/w.

In vitro antioxidant activity of FL-7 and FL-9 by DPPH assay

Free radical-scavenging activity of FL-7 and Fl-9 was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assay as described previously by Jadeja et al. (2009). Briefly, the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength (517 nm) in the presence of the FL-7 and FL-9. One ml of DPPH (0.08 mM) was added to 0.3 ml of FL-7, FL-9 or AA (10-100 µg/ml). The reaction mixture was mixed thoroughly and allowed to stand at room temperature for 30 min, and later was read at 517 nm on UV-VIS spectrophotometer against distilled water blank.

Experimental animals

Age matched (8-10 weeks old) male *Wistar* rats weighing 280±20 (obtained from Zydus Research Centre, Ahmedabad, Gujarat, India) were housed and maintained in clean polypropylene cages and fed with laboratory chow (Pranav Agro Ltd., India). Experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the institutional animal ethical committee (IAEC) of the Department of Pharmacy, Faculty of Technology

and Engineering, The M. S. University of Baroda, Vadodara, INDIA.

Acute toxicity studies

Acute oral toxicity study was conducted using the limit test procedure as per the OECD test guidelines on acute oral toxicity test 401 (OECD, 2001). Sixty four Wistar rats of either sex were divided into eight groups (n=8) and were orally administered with a single dose of 1000 mg, 2000 mg, 3000 mg or 5000 mg body weight of FL-7 or FL-9. Animals were observed for possible behavioral changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somato-motor activities and diarrhea.

CCl_4 induced hepatotoxicity in rats

Forty two rats were randomly divided into 7 groups of 6 each. Group – I (CON) served as normal control and was orally given 0.5 % carboxy methyl cellulose (CMC) solution (0.1 ml) once daily for 7 days. Group – II (CCl_4) was given 0.5 % CMC solution (0.1 ml) once daily for 7 days. Group – III and IV (CCl_4+FL-7A and CCl_4+FL-7B) were orally given 200 or 400 mg/kg BW of FL-7 extract once daily for 7 days respectively. Group – V and VI (CCl_4+FL-9A and CCl_4+FL-9B) were orally given 100 or 200 mg/kg BW once daily for 7 days, respectively. Group – VII (CCl_4+SYL) was orally given 25 mg/kg of silymarin (Jadeja et al., 2011) once daily for 7 days. Groups II, III, IV, V, VI and VII were injected with a single dose of CCl_4 (0.5 ml/kg i.p.) on 7^{th} day of the study (Jadeja et al., 2011) whereas, Group I was given equal volume of olive oil (vehicle).

On the 8^{th} day, blood samples were collected from overnight fasted rats via retro-orbital sinus puncture under mild ether anesthesia and the plasma separated for biochemical analysis. Later, animals were sacrificed by cervical dislocation under mild ether anesthesia and liver was excised and stored at -80 °C for further estimations.

Plasma biochemical analysis

Plasma AST, ALT, ALP, total bilirubin and total protein were assayed using commercially available kits (Reckon diagnostics, Baroda, India).

Hepatic antioxidants and lipid peroxidation

Liver of control and treated animal were excised, weighed and homogenized in chilled tris buffer (10 mM, pH 7.4) at a concentration of 10 % (w/v). The homogenates were then centrifuged at 10,000×g at 0 °C for 20 min in high speed cooling centrifuge. Supernatants were used for the assay of SOD (Kakkar et al., 1984), CAT (Sinha, 1972), GSH (Beutler et al., 1963), total protein (Lowry et al., 1951) and LPO (Buege and Aust, 1978) whereas, ascorbic acid content was determined in sediment by Roe and Küether, 1943.

Microscopic evaluation of hepatic tissue

Liver samples were fixed in 4 % buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five µm thick sections were cut and stained with hematoxyline and eosin and examined for gross structural changes. Observations were done under Leica microscope and photographs were taken with Canon power shot S72 digital Camera (200 X).

Statistical analysis

Data was analyzed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, CA, USA.

RESULTS

HPLC fingerprinting of extract and fraction

The methanolic extract (FL-7) showed 11 peaks with varying retention times (0.737, 2.183, 2.443, 2.827, 3.657, 4.117,

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5.193, 7.887, 12.610, 13.710 and 17.643) (Figure 1). The acetone fraction (FL-9) showed 9 peaks at varying retention times, (0.147, 2.143, 2.443, 2.827, 3.657, 5.193, 7.887, 12.610 and 17. 643) (Figure 1). The chromatogram obtained for FL-7 and FL-9 revealed multiple peaks including that of marmesin (Retention time, 7.847).

Quantification of marmesin by HPLC

Developed methods were applied to determine the marmesin in the leaf extract (FL-7) and chloroform fraction (FL-9) of F. limonia. The chromatogram obtained for marmesin (Figure 1A), FL-7 (Figure 1B) and FL-9 (Figure 1C) showed a separate distinct peak for marmesin (Retention time, 7.847 min). The calibration curve was prepared with marmesin and was found to be linear (R2 = 0.988) in the concentration range used (10–50 mg/ml). The presence of marmesin in FL-7 and FL-9 were found to be 4.71 ± 0.02 and 6.82 ± 0.04 mg/g.

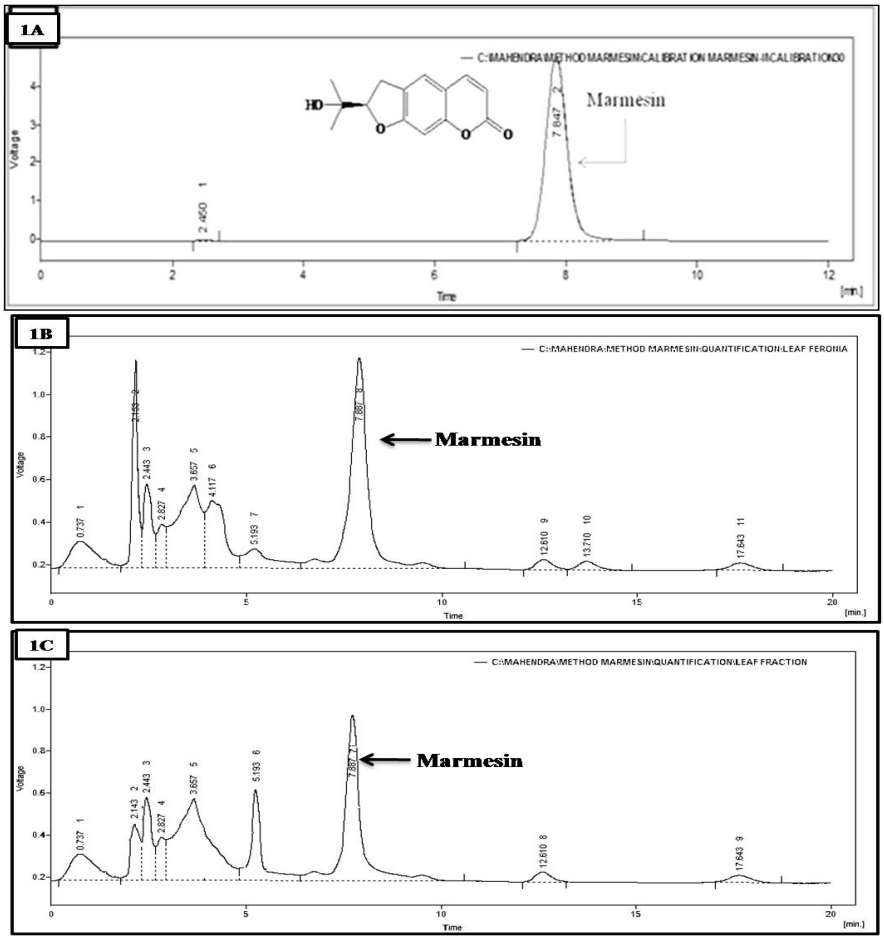

Figure 1: HPLC chromatogram of marmesin (A) *F. limonia* leaf methanolic extract (B), chloroform fraction (C) showing presence of marmesin (Retention time; 7.847 min).

HPLC process parameter of marmesin, FL-7 and FL-9 (mobile phase, Methanol-water; 50: 50, flow rate; 2 ml/min, detection; UV at 280 nm, Retention time 7.847 minutes

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DPPH radical scavenging assay

As shown in Figure 2, FL-7 and FL-9 demonstrated dose dependent DPPH radical scavenging activity with inhibition concentration (IC_{50}) of 60±1.40 and 76±1.52 µg/ml, respectively, whereas IC_{50} of AA was found to be 24.32±0.98 µg/ml (Figure 2).

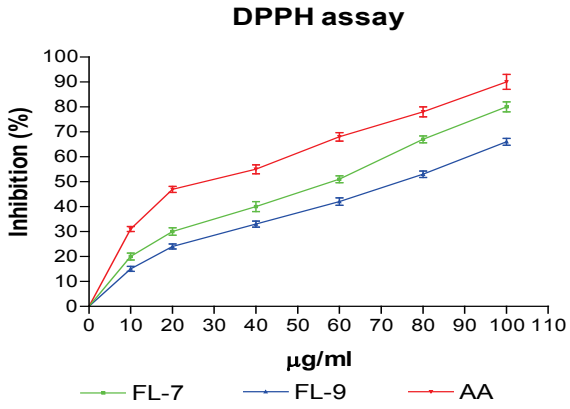

Figure 2: DPPH radical scavenging potential of *F. imonia* leaf methanolic extract (FL-7), chloroform fraction (FL-9).
Results are expressed as mean ± S.D for n=3 and error bar represents standard deviation.

Acute toxicity studies

No mortality was recorded in animals that were orally administered with single dose (1000, 3000 or 5000 mg/kg) of FL-7 or FL-9. There were also no adverse behavioral changes or food aversion was observed. Also, other ailments like excessive salivation or diarrhea were not observed in all the treatment groups.

Hepatic antioxidants and lipid peroxidation

As shown in Table 1, there was significant decrement in activity levels of hepatic SOD (60.26 %) and CAT (52.40 %) and contents of GSH (62.50 %) and AA (52.26 %) in CCl_4 treated rats compared to control. However, CCl_4 treatment to FL7 or FL-9 pretreated rats recorded minimal changes in these parameters comparable to that of control rats. Hepatic LPO levels were significantly high (%) in CCl_4 treated groups compared to control rats. However, LPO levels of rats pretreated with FL-7, FL-9 or SYL was comparable to that of the control (Table 1).

Table 1: Effect of *F. limonia* leaf methanolic extract (FL-7), chloroform fraction (FL-9) and silymarin (SYL) on hepatic antioxidants and lipid peroxidation

Experimental groups	SOD (U/min/mg protein)	CAT (U/min/mg protein)	GSH (mg/g)	AA (mg/g)	LPO (nmol of MDA /mg protein)
Control	65.11 ± 3.13	32.21 ± 1.13	6.00 ± 0.31	4.19 ± 0.07	2.98 ± 0.04
0.5 ml/kg CCl_4	25.87 ± 1.17^{C}	15.33±0.66^{C}	2.25 ± 0.03^{C}	2.00 ± 0.05^{C}	5.42 ± 0.05^{C}
SYL (25 mg/kg) +0.5 ml/kg CCl_4	57.01 ± 2.33^{c}	30.68 ± 1.60^{c}	5.00 ± 0.05^{b}	3.78 ± 0.06^{c}	2.29 ± 0.03^{c}
FL-7 (200mg/kg) +0.5 ml/kg CCl_4	44.33 ± 1.20^{c}	24.00 ± 0.58^{b}	3.91 ± 0.02^{b}	2.96 ± 0.01^{c}	3.15 ± 0.02^{b}
FL-7 (400mg/kg) +0.5 ml/kg CCl_4	48.23 ± 0.88^{c}	26.00 ± 0.31^{c}	4.54 ± 0.03^{c}	2.65 ± 0.28^{c}	2.67 ± 0.03^{c}
FL-9 (100mg/kg) +0.5 ml/kg CCl_4	46.35 ± 0.88^{c}	24.33 ± 0.33^{b}	4.13 ± 0.07^{c}	3.25 ± 0.01^{c}	2.67 ± 0.02^{c}
FL-9 (200mg/kg) +0.5 ml/kg CCl_4	50.10 ± 0.52^{c}	27.00 ± 0.59^{c}	4.85 ± 0.04^{c}	3.72 ± 0.02^{c}	2.37 ± 0.04^{c}

Data expressed as mean ± S.E.M. for n=6, where $^{C}p<0.001$ compared to control and $^{b}p<0.01$ and $^{c}p<0.001$ compared to CCl_4

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Table 2: Effect of *F. imonia* leaf methanolic extract (FL-7), acetone fraction (FL-9) and silymarin (SYL) on hepatic antioxidants and lipid peroxidation

Experimental groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Total protein (g/dl)	Bilirubin (g/dl)
Control	47.52 ± 1.23	83.11 ± 1.65	1.55 ± 0.02	7.62 ± 0.03	1.30 ± 0.01
0.5 ml/kg CCl_4	141.10 ± 2.25^C	321.13 ± 4.99^C	3.34 ± 0.02^C	4.31 ± 0.04^C	4.00 ± 0.04^C
SYL (25 mg/kg) +0.5 ml/kg CCl_4	51.12 ± 1.55^c	90.22 ± 4.67^c	1.51 ± 0.01^b	7.31 ± 0.03^c	1.39 ± 0.02^c
FL-7 (200mg/kg) +0.5 ml/kg CCl_4	67.83 ± 2.26^c	131.33 ± 4.06^b	2.38 ± 0.04^b	6.35 ± 0.03^b	2.22 ± 0.02^a
FL-7 (400mg/kg) +0.5 ml/kg CCl_4	58.5 ± 3.00^c	112.00 ± 2.06^c	2.21 ± 0.02^b	6.86 ± 0.08^c	1.94 ± 0.01^b
FL-9 (100mg/kg) +0.5 ml/kg CCl_4	59.66 ± 4.14^c	111.83 ± 2.56^b	2.10 ± 0.04^b	6.87 ± 0.03^b	1.84 ± 0.03^c
FL-9 (200mg/kg) +0.5 ml/kg CCl_4	52.00 ± 3.81^c	99.16 ± 4.69^c	1.91 ± 0.02^c	7.58 ± 0.04^c	1.48 ± 0.02^c

Data expressed as mean ± S.E.M. for n=6, where ^{C}p<0.001 compared to control and ^{a}p<0.05, ^{b}p<0.01 and ^{c}p<0.001 compared to CCl_4

Plasma markers of hepatic injury

Treatment of CCl_4 to rats recorded significantly elevated level of plasma AST (74.11 %), ALT (66.32 %), ALP (53.59 %) and total bilirubin (67.5 %) whereas, the total protein (43.43 %) content was significantly reduced compared to control rats (Table 2). However, FL7 or FL-9 pretreatment significantly (p<0.05) prevented CCl_4 induced elevation in plasma markers of hepatic injury and decrement in plasma total protein. The results were comparable to that of SYL+CCl_4 rats (Table 2).

Microscopic evaluation of liver

Intra cellular lipid accumulation, appearance of ballooning hepatocytes, heaptocytes necrosis and infiltration of inflammatory cells were the characteristic features of liver section of CCl_4 treated group (Figure 3).

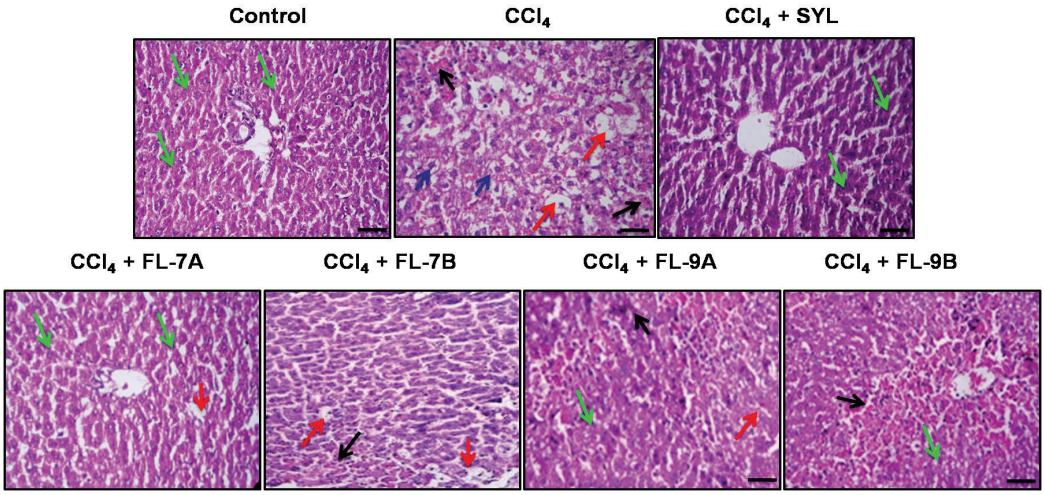

Figure 3: Haematoxyline-eosin stained photomicrographs of rat liver treated with either vehicle (control) or CCl_4 (0.5 ml/kg) alone or *F. limonia* 200 or 400 mg/kg leaf methanolic extract (FL-7A and FL-7B), 100 or 200 mg/kg chloroform fraction (FL-9A and FL-9B) or 50 mg/kg silymarin (SYL) pretreatment followed by CCl_4 (0.5 ml/kg). Where scale bar represents 50 µm, green arrow; healthy population of hepatocytes, black arrow; infiltration of inflammatory cells and red arrow; fatty degeneration of hepatocytes

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These cellular changes were greatly reduced in FL-7 and FL-9 pre-treated rats wherein, few scattered ballooning hepatocytes and mild evidence of hepatocyte lipid accumulation and inflammation was seen. The same was comparable with the liver sections of SYL+CCl_4 rats (Figure 3).

DISCUSSION

A wide variety of phytochemical compounds has been isolated from roots and aerial parts of FL leaves. Recently, studies from our laboratory had reported presence of marmesin (a coumarin) in FL extract that can be used for standardization of extracts prepared from different parts of FL (Jain et al., 2010, 2011a, 2012). Also, *in vitro* screening of bioassay guided fractions of FL had revealed that methanolic extract and its chloroform soluble fractions were biologically potent in imparting hepatoprotection (Jain et al., 2011b). Hence, it was thought pertinent to evaluate *in vivo* hepatoprotective potential of FL leaf methanolic extract and chloroform soluble fraction. Carbon tetrachloride induced hepatotoxicity in rats is a well established experimental model for screening of hepatoprotective herbals and the same was used in our study. The hepatotoxic effect of CCl_4 is reported to be initiated as a result of its reductive dehalogenation by hepatic cytochrome P450 2E1 forming highly reactive trichloromethyl radical ($^{\cdot}CCl_3$). In the presence of oxygen $^{\cdot}CCl_3$ gets converted into a trichloromethyl peroxyl radical ($^{\cdot}OOCCl_3$). These metabolites of CCl_4 in turn results in hepatic oxidative stress, severe damage to mitochondria and nuclei, hepatic fibrosis ultimately culminating in necrotic cell death (Ferreira et al., 2010). Abstraction of hydrogen atoms from poly unsaturated fatty acids of cell membrane by CCl3 and $OOCCl_3$ initiates the process of lipid peroxidation (LPO). Further, $^{\cdot}CCl_3$ is also capable of reacting with the sulfhydryl group of reduced glutathione (GSH) resulting in abnormal function (Recknagel et al., 1992; Halim et al., 1997). This condition of diminished antioxidants and heightened lipid peroxidation results in consumption of enzymatic antioxidants (SOD and CAT) to maintain cellular oxidative stress. Presently recorded significantly lowered levels of hepatic antioxidants coupled with high indices of lipid peroxidation are in agreement with earlier reports (Jadeja et al., 2011; Desai et al., 2012) and justify occurrence of hepatotoxicity in CCl_4 treated rats. However, FL-7 and FL-9 pre-supplementation was capable of imparting significant protection against CCl_4 induced antioxidant imbalance in a dose dependent manner. These results can be attributed to potent free radical scavenging ability of FL-7 and FL-9 reported herein.

CCl_4 causes altered permeability of membrane resulting in leakage of hepatic marker enzymes (AST and ALT) from cells into the circulation. Hence, elevation in levels of these enzymes in plasma acts as a reliable marker for assessing hepatotoxicity (Firdous et al., 2012). Significantly elevated levels of plasma ALT, AST, ALP and bilirubin and decrement in the total protein content were recorded following CCl_4 treatment. Increase in plasma level of ALP in CCl_4 treated rats could be due to its increased synthesis in presence of elevated biliary pressure and subsequent increment in bilirubin. Decrement in total serum protein recorded herein following CCl_4 treatment results due to decreased number of functional hepatocytes (Firdous et al., 2012). However, pretreatment with FL-7 and FL-9 significantly minimized these set of changes indicating at their membrane stabilizing potential. High content of flavonoids, polyphenols and flavonol in the FL extract could be the possible causative agents responsible for the observed effect.

Liver sections of CCl_4 treated rats were characterized by significant intracellular lipid accumulation, formation of ballooning hepatocytes, infiltration of inflammatory cells and hepatocyte necrosis. These histopathological changes are in agreement with previous reports on CCl_4 induced hepatotoxicity (Jadeja et al., 2011; Desai et al.,

2012) and also corroborate the biochemical findings envisaged herein. However, FL-7+CCl_4 and FL-9+CCl_4 groups recorded minimal changes in the structure of hepatocytes and were comparable with SYL+CCl_4 group.

CONCLUSION

It can be concluded from the present study that standardized methanolic extract and chloroform soluble fraction of FL leaves is capable of imparting hepatoprotection against CCl_4 induced hepatotoxicity in rats and can be developed as an herbal hepatoprotective formulation.

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