IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 1 Ver. I (Jan. 2014), PP 71-76
www.iosrjournals.org

Curative efficacy of methanolic extract of Monochoria vaginalis against Carbontetra chloride (CCl_4) induced liver fibrosis in male wistar rats

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Abstract: *The traditional medicine involves the use of different plant extracts or the bioactive constituents and provide cure to many ailments of mankind. Carbontetra chloride (CCl_4) induced hepatic fibrosis is a well-established animal model to study the pathogenesis and therapy of chronic liver injury.* ***Objectives****: This study was aimed to evaluate antifibrotic efficacy of methanolic extract of Monochoria vaginalis (MEMV) on CCl_4 induced liver injury in rats.* ***Methods:*** *Hepatic fibrosis was induced in male wistar rats by oral administration of CCl_4 (150µl/100g rat weight) twice a week for 10 weeks. In curative treatment M.vaginals and Silymarin at doses of 100mg, 200mg and 50mg/kg b.wt respectively were administered orally for 2 weeks after the establishment of fibrosis for 10 weeks.* ***Results****: CCl_4 administration caused a significant decrease in body weight and resulted in significantly increased levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP),thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and hydroxyproline (HP). MEMV at the dose of 200mg/kg b.wt prevented or reversed the decline in body weight and the levels of marker enzymes. The treatment reduced the tissue levels of TBARS, CD & hydroxyproline, and increased the levels of tissue GSH, serum total protein and albumin in chronically treated rats. Histopathological changes of hepatic lesions induced were significantly ($p \leq 0.05$) improved by treatment with M.vaginalis.* ***Conclusions:****.The results indicate the antifibrotic effect of MEMV and thus scientifically validated the use of root part in traditional medicine for hepatic disorders.*

Key words*: Carbontetra choloride, hepatic fibrosis, histopathology, hydroxyproline, Monochoria vaginalis*

I. Introduction

Herbal drugs are being proved as effective as synthetic drugs with lesser side-effects. Monochoria vaginalis (M.vaginalis) is a plant belonging to the pontederiaceae family known to have several medicinal properties[1]. It is a weed found in rice (oryza sativa) fields and is used as a vegetable. The leaf juice of M.vaginalis is used to treat cough and that of roots is used to treat stomach and liver problems, asthma and tooth ache[2].The phytochemical studies reveal the presence of flavanoids (3-0-beta-glucopyranoside). Fraction of n-butanol from M.vaginalis exhibited anti-oxidant activity[3].

M.vaginalis leaf and root extracts were evaluated for their antioxidant, anti-inflammatory and anti-nephrotoxic activities[4-5].Tail immersion and hot plate studies [6] of alcoholic extract of M.vaginalis showed significant analgesic activity of the extract.

Liver fibrosis refers to the accumulation of extracellular matrix (ECM) proteins, which occurs in most types of chronic diseases. Of these proteins, collagen, which is the body's self-repairing protein induced by inflammation, most commonly accumulates.Hepatic stellate cells (HSCs) are the primary ECM producing cell type during hepatic fibrogenesis[7-8].HSC activation, characterized by enhanced cell growth and over production of ECM, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSC and fibrogenic transforming growth factor (TGF)- β, mostly from kupffer cells.

Medicinally plants have made a significant contribution to the treatment of liver fibrosis [9-10].Traditional plant drugs have been found to be effective in preventing fibrogenesis and other chronic liver injury which develops a more hopeful future for controlling liver fibrosis, cirrhosis and hepatocarcinogenesis[11-12].

Hepatic fibrosis caused by CCl_4 has been extensively used in experimental models in rats.In the present study CCl_4 induced hepatic fibrosis was used to elucidate the antifibrotic effect of *M.vaginalis* extracts at two different doses. Histopathological and immunohistochemical changes, enzymatic alterations of serum marker enzymes, hydroxyproline and lipid peroxide levels were examined and compared with Silymarin, a standard hepatoprotective drug.

II. Materials And Methods

11.1.Chemicals

Carbontetra chloride was purchased from Merck, Mumbai. Chloramine-T, P- dimethyl amino benzaldehyde and Silymarin were purchased from sigma chemical Co., St. Louis, MO, USA. AST, ALT, ALP, total protein (TP) and albumin assay kit were purchased from Span diagnostics Ltd., Surat, India. All other chemicals were of analytical grade.

11.2. Animals

Male wistar rats (150-200g) were used for the study. The animals were housed in well ventilated cages and given standard rat chow (Sai Feeds ,Bangalore, India), and water adlibitum.The animals were maintained at a controlled condition of temperature of 26-28^oC with a 12 hr light: 12 hr dark cycle. Animal studies were followed according to Institute Animal Ethics Committee (IAEC) regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No.B 2442009/5) and conducted humanely.

11.3. Plant material and preparation of extracts

The plant Monochoria vaginals was collected from its natural habitat (aquatic) and authenticated. A voucher specimen (SBSBRL-15) is maintained in the institute. The root part of the plant was soxhlet extracted with methanol and the extract was dried and stored.

11.4. CCl_4 induced liver fibrosis

11.4.1. Curative effect of the extract

Thirty rats were divided into five groups. Group I served as normal control, Group II was CCl_4 control, Group III & IV received CCl_4 and methanolic extract of *M.vaginalis (*MEMV*)* at doses of 100mg and 200mg/kg and Group V received CCl_4 and Silymarin at a dose of 50mg/kg. Fibrosis was induced in group (II-V) by oral administration of CCl_4 at a dose of 150µl/100g mixed with ground nut oil at a ratio of 1:1 twice a week for 10 weeks. After 10 weeks of intoxication with CCl_4, rats in group III & IV were given MEMV at doses of 100 and 200mg body weight respectively and Group V was treated with Silymarin at a dose of 50mg/kg orally, for 2 weeks. Group II animals received 1 ml of 5% Tween 80 daily for 2 weeks. Animals were sacrificed on 15th day after the last dose of CCl_4 administration.

11.5. Biochemical Assays

Blood was collected from the neck blood vessels and kept for 20 min at 4^{0}C. Serum was separated by centrifugation at 2500 rpm at 4^{0}C for 10 min. Dissected livers were cut into separate portions for estimations of lipid peroxidation, hydroxyproline (HP) and histopathological examination. Routine biochemical analysis including AST, ALT, ALP, total protein and albumin were estimated by using semi autoanalyzer (RMS, India).

500mg liver tissue was homogenized with 5ml, 0.025M Tris-HCl buffer at pH7. Liver homogenate was centrifuged at 2500rpm for 15 min and the supernatant was used for the assays of thiobarbituricacid reactive substances (TBARS)[13], conjugated dienes(CD)[14], glutathione (GSH)[15] and hydroxyproline (HP)[16]. An extra sample of liver was excised and fixed in 10% formalin solution for histopathological and immunohistochemical analysis[17].

11.5.1. Estimation of hydroxyproline in liver

The hydroxyproline content in the liver was measured [16].Briefly, the liver tissue (200 mg) was homogenized and 2 ml of the homogenate was mixed with 6 N HCl and was then hydrolyzed at 110^oC for 16 hours. After cooling 100 µl of the sample was made up to 2 ml with acetate-citrate buffer. 1 ml of chloramine-T reagent (0.56% buffered) was added and kept for 20 min at room temperature. Following this 1 ml of freshly prepared Ehrlich's reagent was added and the mixture was incubated at 60^oC for 15 min and cooled. Samples were read at 560 nm against a reagent blank which contained the complete system without the added tissue.

11.5.2. Histopathological studies

Liver specimens were preserved in 10% neutral buffered formalin and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were then embedded in paraffin blocks and cut into 5 µm thick sections. Sections were stained with hematoxylin and eosin (H&E).Liver sections were graded numerically based on Knodell's histological activity index[18] to assess the degree of histologic injury in hepatic fibrosis.The parameters were graded from score 0 to 4, with 0 indicating no abnormality, 1 indicating periportal with or without bridging necrosis, 2 indicating interlobular degeneration and focal necrosis and 3 with portal inflammation and score 4 indicating fibrosis.

11.5.3. Immunohistochemical analysis

Liver samples were collected, washed with phosphate-buffered saline (PBS) and fixed overnight in 10% buffered formalin. Serial sections (5 µm) were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin wax.

For immunohistochemistry, mouse anti rat Collagen III monoclonal antibody was used. The sections were immunostained with Collagen III antibody diluted 1:200 with 3% BSA in PBS and incubated overnight at 4^oC. Sections were then washed thrice in PBS and incubated with anti-mouse horseradish peroxidase for 45 minutes. After triplicate washing with PBS, sections were incubated for 30 minutes with streptavidin-HRP complex. Sections were then washed with PBS and incubated for 5 – 10 minutes in a solution of 0.02% diaminobenzidine containing 0.01% H_2O_2. Counterstaining was performed with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The micrographs were taken using Moticam 1000 camera at original magnification of 100X.

11.6. Statistical analysis

Results were expressed as Mean $\pm$ S.D and all statistical comparisons were made by means of one way ANOVA test followed by Tukey's post hoc analysis and p-values less than or equal to 0.05 were considered significant.

III. Results

A sharp decline in body weight was recorded during the 10 week treatment in CCl_4 treated animals (Fig. 1). *M.vaginalis* extract treatment after the establishment of hepatic fibrosis for 10 weeks enhanced the body weight (Fig.2**).**The extent of liver fibrosis was assessed in terms of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) (Table 1). Statistically significant increase in biochemical parameters viz. AST, ALT, ALP, TBARS, CD and decrease in total protein, albumin and GSH were observed after CCl_4 treatment (Table 1).MELA at two doses and Silymarin administration resulted in significant decrease in AST, ALT, ALP, HP, TBARS and (CD) levels and an increase in levels of serum protein, albumin and tissue GSH .

Hydroxyproline content is a good marker of fibrosis. HP level in the liver was remarkably reduced by the plant extract treatment (200mg dose) indicating its efficacy in reversing fibrosis and 96.21% decrease in HP content was observed by the extract treatment. Histopathological studies showed the integrity of the liver cell membrane as was evidenced by reduced degree of fibrosis and normal liver pattern in the extract treated groups (Fig.1). Immunohiistochemical analysis showed that collagen – III was predominantly expressed along fibrous septa in CCl_4 treated rats(Fig.2).

IV. Discussion

Histopathological analysis and hydroxyproline measurements are the traditional methods for assessing hepatic fibrosis. Medicinally plants have made a significant contribution to the treatment of liver fibrosis [9-10]. Traditional plant drugs have been found to be effective in preventing fibrogenesis and other chronic liver injury. The baicalin treatment significantly lowered the levels of serum ALT, AST and liver index, reduced histological changes of liver fibrosis [19].

N-hexane extract of L. flexuosum[20] showed the antifibrotic efficacy against CCl_4 induced hepatic fibrosis in rats. The study also helped the Protective mechanism of *Lygodim flexuosum* extract in treating and preventing CCl_4 induced hepatic fibrosis in rats.

V. Conclusion

Preliminary phytochemical analysis demonstrated the presence of flavonoids and alkaloids. The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggested that the potential use of the methanolic extract of *M. vaginalis* as a novel therapeutically useful antifibrotic agent. Therefore further studies to elucidate their mechanisms of action should be conducted to aid the discovery of new therapeutic agents for the treatment of liver diseases.

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TABLES AND FIGURES

Table 1:Levels of Marker enzymes, TBARS, CD and HP

	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TBARS (mM/100g)	CD (mM/100g)	HP (nM/mg)
Group 1	150.83±8.89	60.58±4.33	157±6.45	1.213±o.05	56.86±3.96	0.26±0.01
Group 2	924.02±.44.02$^{\#}$	342.72±12.7$^{\#}$	429.03±21.23$^{\#}$	22.2±.33$^{\#}$	113.66±7.35$^{\#}$	1.242±0.02$^{\#}$
Group 3	371.25±.13.1*	114.58±.8.5*	276.1±8.3*	12.19±.0.56*	80.23±.3.9*	0.50±0.01*
Group4	183.21±.7.14*	78.21±7.9*	214.12±12.1*	2.76±.0.21*	59.12±1.46*	0.31±0.01*
Group 5	175.73±6.26*	82.82±6.7*	224.45±9.55*	2.3±0.06*	63.87±4.26*	0.286±0.01*

$^{\#}$P≤0.05 versus normal control, *p≤ 0.5 versus CCl_4 control. Values are mean ± S.D, n =6

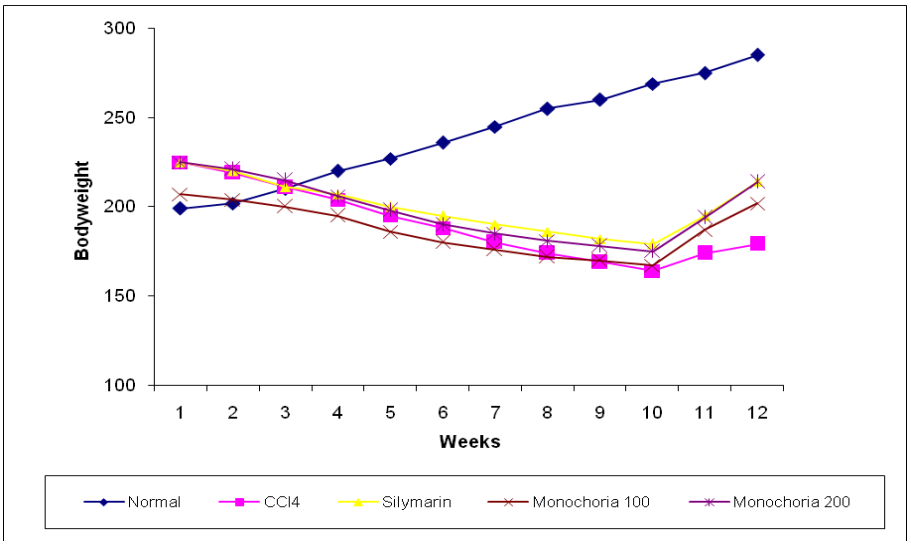

Fig. 1 Body weight pattern of curatively treated rats

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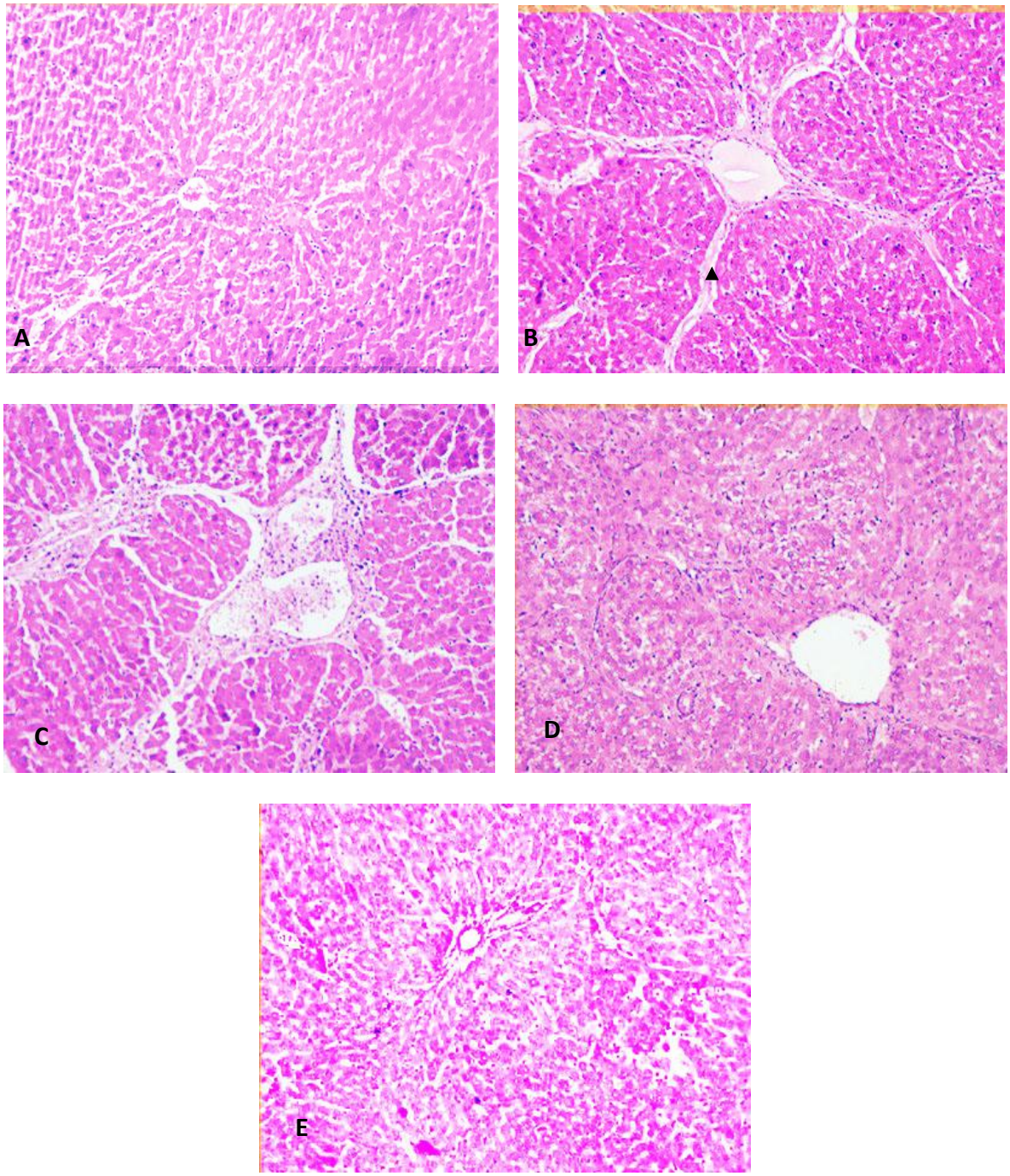

Fig.2: Histopathological features of liver in the curative treatment group. Liver tissue was stained with H & E (X 200). (A) Normal rat liver; (B) CCl_4 control; (C) CCl_4 + *M* 100; (D) CCl_4 + *M.* 200 alone (E) CCl_4 + Silymarin. Arrow head represent fibrous septa

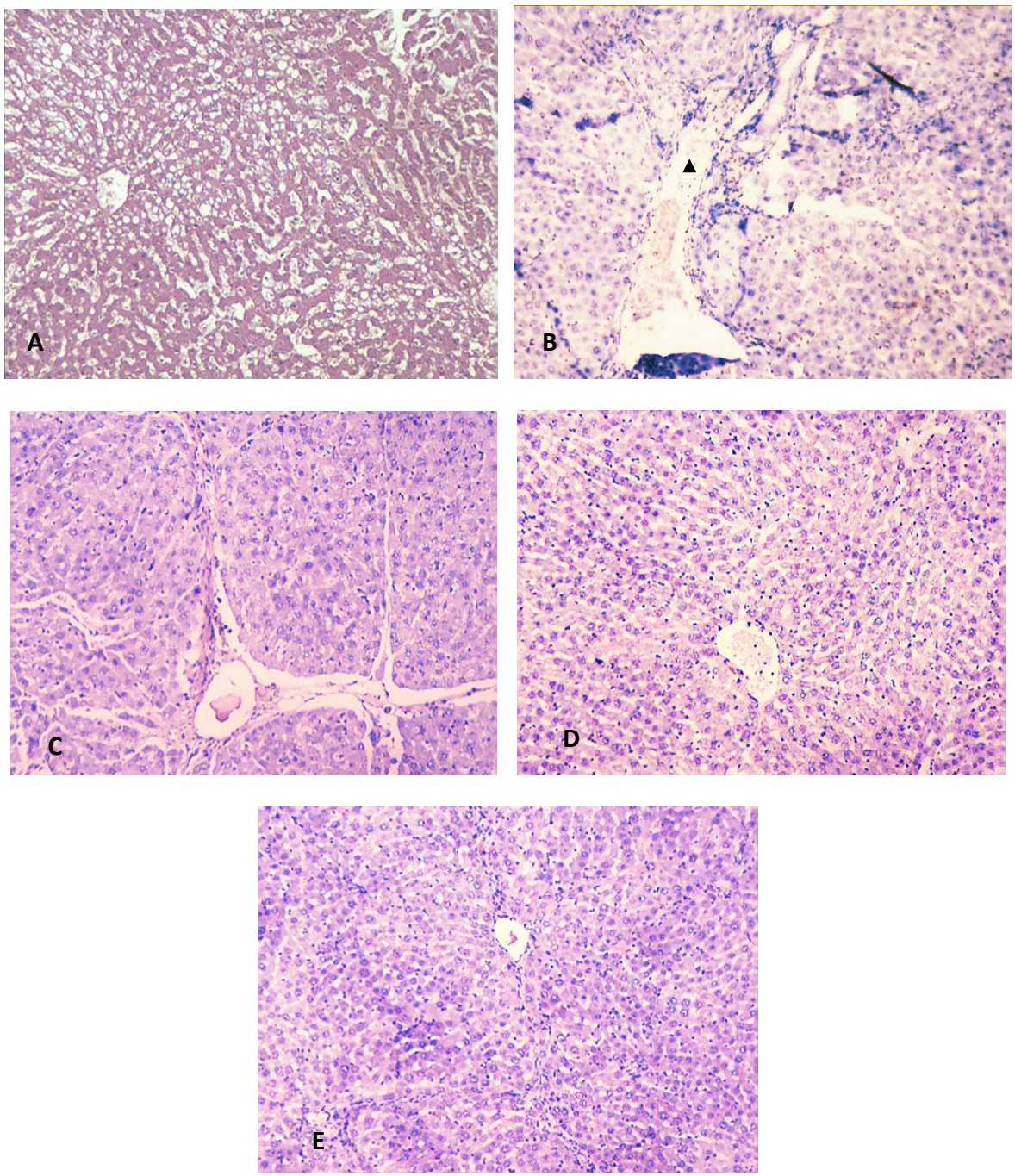

Fig.3: Immunohistochemical localization of Collagen-III in the curative treatment group. Liver tissue was stained with H & E (X 200). (A) Normal rat liver; (B) CCl_4 control; (C) CCl_4 + *L.* 100; (D) CCl_4 + *L.* 200 alone (E) CCl_4 + Silymarin. Arrow head represent fibrous septa