

# Antifibrotic activity of *Phyllanthus maderaspatensis* L. in Wistar rats

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## Abstract

This study evaluates the effectiveness of *Phyllanthus maderaspatensis* L. against liver fibrosis in Wistar rats. Hepatic fibrosis was experimentally induced in male rats with CCl<sub>4</sub> administration. Animals were then post treated with 200 mg/kg dose of *P. maderaspatensis* hexane extract (PmHE). Fibrosis was studied using biochemical assays and histopathological techniques. The molecular mechanism of reversion of hepatic fibrosis by PmHE was studied, using semiquantitative RT-PCR, western blotting and gelatine zymography. PmHE was analyzed using high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC). PmHE was subjected to toxicity evaluation in rats. Post treatment with PmHE almost normalized CCl<sub>4</sub> - induced fibrotic changes. PmHE treatment normalized serum parameters, reduced lipid peroxidation and maintained the normal level of reduced glutathione pool. The increased levels of alpha-smooth muscle actin ( $\alpha$ SMA), collagen I and collagen III and mRNA transcripts were effectively reversed with PmHE post treatment. Histopathology of the liver confirmed its recovery from hepatic fibrosis. Moreover, the post treatment with PmHE, showed a significant reduction in the hydroxyproline level in the liver. Further, matrix metalloproteinase (MMP) activity of MMP-9 and MMP-2 was also reduced. HPTLC and HPLC analysis showed the presence of phyllanthin and quercetin in the extract. The mechanism of action of PmHE against hepatic fibrosis could be at least partly, due to the inhibition of oxidative stress. PmHE was found to be safe, based on the toxicity evaluation. Therefore, PmHE is a very promising antifibrotic agent.

**Key words:** Hepatic fibrosis, lipid peroxidation, hydroxyl proline, alpha-smooth muscle actin, oxidative stress

## 1. Introduction

Hepatic fibrosis or 'scarring' is a wound healing response of the liver, against a variety of insults, regardless of their mechanism of action. Hepatic fibrosis develops due to chronic hepatocellular injury, induced by various agents, such as viral infections, autoimmune reactions, toxicity especially due to alcohol and drugs, metabolic disorders and non-alcoholic fatty liver diseases *etc.* (Friedman, 2003; Iredale, 2008). Liver cirrhosis, the end stage of fibrosis, is a major health problem (Henderson and Forbes, 2008) and one of the leading causes of death worldwide (Gluud, 2005). Cirrhosis is characterized by an increased accumulation of extra cellular matrix (ECM), proteins (fibrogenesis) and a decreased rate of their degradation (fibrolysis), resulting in fibrotic septation, nodules formation and resistance to intrahepatic blood flow, leading to portal hypertension and risk of liver failure (Cho *et al.*, 2009). The mechanism of fibrogenesis is almost common irrespective of the underlying etiology. The most important event in fibrosis is the activation of hepatic stellate cells (HSC), the principal cell type responsible for accumulation of ECM in the liver (Iredale, 2007).

Experimentally induced hepatic fibrosis, using carbon tetrachloride (CCl<sub>4</sub>) in rodents, is a frequently used model for the screening of antifibrotic agents (Weber *et al.*, 2003). The mechanism by which CCl<sub>4</sub> imparts its hepatotoxicity, involves oxidative stress initiated by CCl<sub>4</sub>-derived reactive metabolite trichloromethyl free radical, CCl<sub>3</sub><sup>\*</sup> (Stoyanovsky and Cederbaum, 1999). CCl<sub>3</sub><sup>\*</sup> is biotransformed to peroxynitrite, a strong oxidant able to disrupt cellular structure and function through peroxidation of lipids, proteins and DNA (Recknagel and Ghoshal, 1966). Lipid peroxidation of polyunsaturated fatty acids by peroxynitrite, generates thiobarbituric acid reactive substances (TBARS) in the tissue (Zhou *et al.*, 2002). TBARS is an indicator of oxidative stress induced tissue injury (Wang *et al.*, 2007). The exposure of lipid peroxidation products of damaged hepatocytes results in the activation of HSC, causing excessive accumulation of ECM (Novo *et al.*, 2006).

There are over 100 million people with hepatic fibrosis in the world (Hayashi *et al.*, 2008). The experimental evidence suggests that fibrosis and cirrhosis are reversible with effective antifibrotic therapy. Liver transplantation is the only currently available treatment for advanced fibrosis and cirrhosis (Iredale, 2008). However, shortage of healthy donor, concurrent occurrence of disease, affecting other tissues in the recipient, and recurrence of the original disease in transplant recipients limit, the impact of this treatment (Henderson and Forbes, 2008; Iredale, 2007). Prevention, the most effective approach towards cirrhosis often fails; therefore, antifibrotic treatment that halts the progression of cirrhosis is important.

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*P. maderaspatensis* L. (Euphorbiaceae) is a widely distributed medicinal plant with a well flourished traditional history in the treatment of liver diseases (Ali *et al.*, 2008; Schmelzer and Gurib-Fakim, 2008). Previous experiments from our laboratory have shown that 200 mg/kg dose of PmHE is a good hepatoprotective agent against acetaminophen, CCl<sub>4</sub> and thioacetamide induced acute hepatocellular damage in rats (Asha *et al.*, 2004; Asha *et al.*, 2007). Moreover, the plant has got remarkable antioxidant property (Bommu *et al.*, 2008; Kumaran and Joel Karunakaran, 2007). In the present study, we investigated the potential of (PmHE) in alleviating CCl<sub>4</sub> induced hepatic fibrosis in wistar rats.

## 2. Materials and Methods

### 2.1 Materials

#### 2.1.1 Reagents

Carbon tetrachloride, haematoxylin, eosin and n-hexane were obtained from Merck, Mumbai, India. Corn oil, silymarin, 2-thiobarbituric acid, 5,5'-dithiobis nitrobenzoic acid, chloramine-T, dimethylbenzaldehyde, sodium dodecyl sulphate, sodium deoxycholate, PMSF, proteinase inhibitor cocktail, Masson's Trichome stain, monoclonal anti  $\beta$ -actin (mouse IgG isotype), monoclonal anti-collagen type-III (mouse IgG isotype) and BCIP/NBT were purchased from Sigma Chemicals Co, St. Louis, MO, USA. Trizol reagent was from Invitrogen, Carlsbad, CA, USA. AMV reverse transcriptase, oligo dT primer, RNasin ribonuclease inhibitor, RNase free DNase free dNTPs, PCR master mix, pGME<sup>®</sup>-T vector and T4 DNA ligase were obtained from Promega, Madison, WI, USA. 100 base pair DNA ladder was obtained from New England Biolabs, Beverly, MA, USA. Kaleidoscope prestained protein standard was purchased from BioRad laboratories, Hercules, CA, USA. Mouse monoclonal  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) was obtained from Abcam, Cambridge, UK. Alkaline phosphatase conjugated donkey antimouse IgG was obtained from Jackson ImmunoResearch, West Baltimore, PA. All other chemicals used were of high purity grade.

#### 2.1.2 Plant material and preparation of extract

Fresh whole *P. maderaspatensis* L. collected from Chennai, India, during the month of March, was authenticated by Mrs. Padmaja, Taxonomist, Regional Research Institute, Thiruvananthapuram. A voucher specimen (Ethno-51) was deposited in our institute herbarium. Plants were cleaned with fresh water, shade dried at room temperature and powdered. The powder was soxhlet extracted with n-hexane (10 g/400 ml). The extraction was continued until a drop of solvent from the soxhlet siphon tube did not leave any residue when evaporated on a clean glass plate. Solvent was removed from the extract under reduced pressure, using a rotary evaporator (VV2000, Heidolph, and Schwabach, Germany). The average yield of n-hexane extract was 5.1% (w/w) of powder. Extract was stored in -20°C freezer until use.

#### 2.1.3 Animals and experimental liver fibrosis

Male wistar rats weighing 200-220 g were housed in conventional cages under 25  $\pm$  2 °C, 12 h light/dark cycles and fed on commercial rat pellet (Saidurga Feeds and Foods, Bangalore, India) and water *ad libitum*. All procedures of animal handling were conducted according to the Institute Animal Ethics Committee (IAEC) rules which are approved by the committee for the purpose of control and supervision of experiments on animals (CPCSEA). Thirty male

rats were divided into five groups of six animals in each group. Hepatic fibrosis was induced in rats as described previously (Iredale, 2008) with some modifications. Briefly, rats (groups II, III and V) were treated with CCl<sub>4</sub> at a dose of 150  $\mu$ l/100 g mixed with corn oil in 1:1 ratio, twice a week for 5 weeks. During this period, rats of group I (normal control) and IV (PmHE control) were given 150  $\mu$ l/100 g saline. After 5 weeks of toxication with CCl<sub>4</sub>, rats in group III were treated with PmHE (200 mg/kg suspended in 5% Tween-80) for two weeks daily. In our previous experiments, we established that 200 mg/kg of PmHE was the best therapeutic dose (Asha *et al.*, 2004; Asha *et al.*, 2007). Rats in group V were treated with 50 mg/kg Silymarin, dissolved in distilled water for two weeks daily. During the extract treatment period, rats in group II were given 1 ml of 5% Tween-80 for two weeks. In the mean time, rats in group IV were treated with PmHE (200 mg/kg in 5% tween 80). Animals were sacrificed under ketamine (60 mg/kg), xylazine (7 mg/kg) anesthesia after 15 days from the last dose of CCl<sub>4</sub> administration. Blood was collected from the neck vein for biological assays. Livers were removed immediately after exsanguinations. A small portion of the liver was stored at -80°C for determinations of malondialdehyde (MDA), reduced glutathione (GSH) and hydroxyproline. While another portion was fixed in 10% buffered formalin for histological studies and the residual portion was snap frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation.

### 2.2 Methods

#### 2.2.1 Biological assays

Serum was isolated from the blood sample according to usual clinical procedure as reported elsewhere (Asha *et al.*, 2004). Serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and  $\gamma$ -glutamyl transferase ( $\gamma$ -GT) were estimated spectrophotometrically, using commercially available kits, according to manufacturer's instructions.

#### 2.2.2 Estimation of malondialdehyde (MDA)

Liver tissue was homogenized in 30 mM KCl and the homogenate was reacted with 2-thiobarbituric acid, resulting in the formation of a red fluorescent derivative. The intensity of the red derivative was estimated spectrophotometrically at 532 nm. The levels of MDA in the liver were expressed as nano-moles/mg of tissue (Okhawa *et al.*, 1979).

#### 2.2.3 Estimation of reduced glutathione (GSH)

Level of GSH in the liver was estimated as described elsewhere (Moron *et al.*, 1979). Briefly, liver tissue was homogenized in phosphate buffered saline (pH 7.4) and the protein in the homogenate was precipitated out with trichloro acetic acid. The residual portion was then reacted with Ellman's reagent (5, 5'-Dithiobis-(2-nitrobenzoic acid)) at an alkaline pH that gave a yellow colored complex with absorption maxima at 412 nm. The amount of GSH was expressed as nano-moles/mg of wet tissue.

#### 2.2.4 Estimation of hydroxyproline

Hydroxyproline in the liver sample was estimated according to the procedure of (Jamall *et al.*, 1981). Liver tissue was homogenized in 6N HCl by heating at 110°C for 16 h. The homogenate was then reacted with chloroamine-T. Hydroxyproline present in the homogenate oxidises chloramine-T to release a pink coloured derivative. Excess chloramine-T in the reaction was neutralized

with 2, 4- $\alpha$  dimethylbenzaldehyde. The intensity of the pink colour formed was estimated spectrophotometrically at 560 nm and expressed as nano-moles/mg of wet tissue.

### 2.2.5 Histopathology

Histological studies of the liver specimens were carried out, using differential staining techniques. Liver tissue (n=6, from each group) fixed in 10% buffered formaldehyde, was dehydrated through alcohol series, cleared in xylem and embedded in paraffin blocks. Sections of 4  $\mu$ m thickness were cut with a rotary microtome and the sections were stained with Mayer's haematoxylin and eosin according to standard protocols (Hung *et al.*, 2005). Masson's trichome staining of liver sections was also done. Fibrosis scoring was done according to The French Metavir scoring system (The French METAVIR cooperative study group, 1994).

### 2.2.6 Total RNA isolation, synthesis of cDNA and semi-quantitative reverse transcription PCR analysis

Total RNA was isolated from snap frozen liver tissue, using TRIzol® reagent, according to the protocol (Sreejith *et al.*, 2012). 3  $\mu$ g of total RNA was reverse transcribed to cDNA, using Avian Myeloblastosis Virus Reverse transcriptase (AMV-RT) with oligo dT 15-mer primer in a reaction mix containing 100 units MMV-RT, 100 units RNasin RNase inhibitor and ready to use dNTPs. The following genes were used in subsequent PCR:  $\alpha$ SMA, Pro-collagen-I, Pro-collagen-III.  $\beta$ -actin was used as the internal control for normalizing the PCR products in semiquantitative analysis. Primers of the specific genes (Table 1) were synthesized from Sigma Genosys, Bangalore, India according to published references (Wills and Asha, 2007). PCR amplification was done in a 25  $\mu$ l reaction, consisting of 2  $\times$  PCR, ready to use Master Mix, containing 50 units/ml of Taq DNA polymerase, 400  $\mu$ M dNTPs and 3 mM MgCl<sub>2</sub>, 10  $\mu$ M each primers and 4  $\mu$ l cDNA. PCR reaction was carried out on a programmable iCycler PCR machine (BioRad, Hercules, USA) with following conditions: denaturation at 94°C for 30 seconds, primer annealing for 1 min and extension for 1 min at 72°C. An initial denaturation at 94°C for 4 min and a final extension for 5 min at 72°C were also performed. Annealing temperature, number of PCR cycle and size of amplicon are given in Table 1. PCR products along with 100 base pair standard were run on 1.7% agarose gel and visualized by ethidium bromide staining. Gel images were captured by Gel Doc XR (BioRad, Hercules, USA) and quantified by Quantity One Gel Doc XR software (BioRad, Hercules, USA). The identities of PCR products were further confirmed by cloning to pGEM®-T vector and automated DNA sequencing of the inserts with T7 primer (ABI-310 Prism, Perkin Elmer, Foster City, CA, USA).

### 2.2.7 Western blotting

Total protein was extracted from snap frozen liver tissue, using Radio Immuno Precipitation Assay (RIPA) buffer, containing 50 mM Tris HCl (pH 7.8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 1 mM sodium orthovanadate and 15  $\mu$ l/ml of proteinase inhibitor cocktail. Quantification of the protein was done, using Bradford protein assay reagent. 30  $\mu$ g total protein was resolved on 10% SDS-PAGE, using Mini-PROTEAN® 3Cell (BioRad, Hercules, USA) and electroblotted to PVDF membrane (Immunobilon-P, Millipore, Bedford, USA), using Trans-Blot® SD-SemiDry transfer apparatus

(BioRad, Hercules, USA). Transblotted membrane was then blocked with 5% skimmed milk protein for 1 h at room temperature. After brief washing with Tris Buffered Saline Tween-20 (pH 7.4), the membrane was incubated with primary antibody at 4°C, overnight. Monoclonal anti  $\beta$ -actin in 1:2000 dilutions, monoclonal anti Collagen type III in 1:1000 dilution and monoclonal anti  $\alpha$ -Smooth muscle actin in 1:1500 dilutions were used as the primary antibodies. After washing, the membrane was incubated with anti-mouse IgG alkaline phosphatase conjugate and the bands were visualized by incubation with BCIP/NBT ready to use substrate solution. The intensity of the bands was scanned using BioRad density One Gel Doc XR software.  $\beta$ -actin was used as the internal control.

### 2.2.8 Gelatine zymography

Levels of MMP-2 and MMP-9 in the liver tissue were analyzed by gelatine zymography assay (Koyama *et al.*, 2000). Briefly, 100 mg snap frozen liver sample was homogenized in RIPA as given under Western blotting. 20  $\mu$ g of total protein was subjected to Poly-Acrylamide Gel Electrophoretic (PAGE) separation on 10% gel containing 0.1% gelatine (Porcine Type-A) in denaturing (SDS) but nonreducing condition at 165 V. Following electrophoresis, the PAGE was washed in freshly prepared 2.5% Triton-x-100 solution for 30 min to remove the SDS. Then, the gel was incubated in reaction buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM CaCl<sub>2</sub> and 0.02% sodium azide for 20-24 h at 37°C with gentle agitation. Gels were stained with 0.1% coomassie brilliant blue G-250 and destained with methanol-acetic acid-water (4.5:1:4.5 v: v). Pre-stained molecular weight ladders were also run along with the protein sample to locate the respective pro-MMPs and active MMPs in the gel. The densities of the bands were quantified, using Density One Gel Doc XR software (BioRad, Hercules, USA). The relative activity of MMP-9 and MMP-2 was calculated as the sum of the densities of proenzyme and the active enzyme and expressed as percentage of control.

### 2.2.9 Acute and subacute toxicity study

Acute and subacute toxicity evaluation of PmHE was done in Wistar rats as described (Witthawaskul *et al.*, 2003) with some modifications. In acute toxicity study, 12 rats of either sex (180-200 g) were divided into two groups of six each. Group I (control) rats received 5% Tween-80 and group II rats received a single dose (2 g/kg) of PmHE (ten times of therapeutic dose) in 5% Tween-80 and were kept under observation for 14 days. General behavior, initial and final body weights, intake of water and food, state of faecal matter and body temperature were monitored. On the 15<sup>th</sup> day, animals were sacrificed under ketamine (60 mg/kg) xylazine (7 mg/kg) anesthesia. Plasma AST, ALT, LDH, glucose, urea, cholesterol and creatine were estimated. In subacute toxicity study, rats (n=6) were fed with a daily dose of 500 mg/kg PmHE for 14 days and kept for another 14 days for observation. Behavioral and other parameters were determined as in the case of acute toxicity evaluation (Witthawaskul *et al.*, 2003).

### 2.2.10 HPTLC and HPLC analysis of PmHE

High performance thin layer chromatography (HPTLC) analysis was done according to the standard protocol described elsewhere (Beena and Radhakrishnan, 2012; Rajeshwary *et al.*, 2013). For HPTLC (CAMAG, Switzerland) analysis, fully automated Linomat V sample applicator was used for applying PmHE to precoated

silica gel 60 F<sub>254</sub> plates under a constant flow of nitrogen. HPTLC profile of PmHE was developed in hexane: ethyl acetate (7:3 v/v) mobile phase. The chromatograms were scanned at 366 nm, using CAMAG-twin-through plate development chamber with CAMAG TLC scanner 3 and WinCATS software 4.03. For HPLC analysis of PmHE, 5 µl of 5 mg/ml of each sample was analyzed, using Gilson analytical HPLC with 321 Binary gradient pump and 156 UV-Vis detectors (Gilson, U.S.A). Fractions were analyzed using a kromasil 100 C18 column (250 × 4.6 nm) with particle size of 5 µm. Acetonitrile: methanol gradient were used as the mobile phase with a flow rate of 1 ml/minute. Peaks were detected at 254 nm and 266 nm. Phyllanthin, hypophyllanthin and quercetin was used as standard (LGC Promochem).

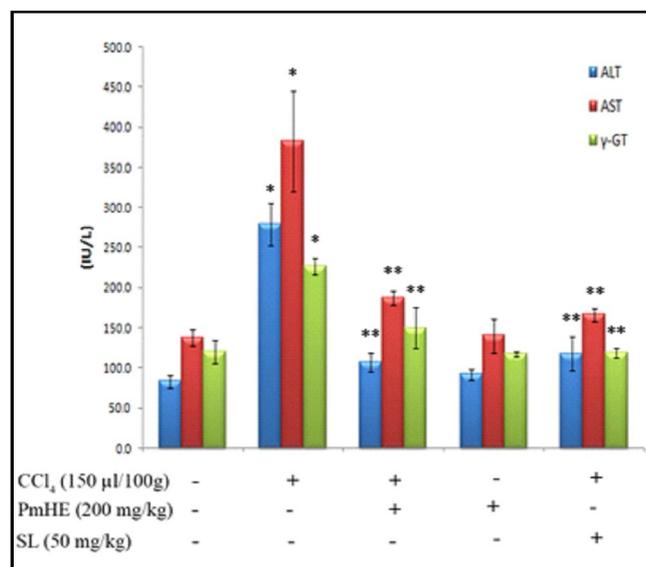
### 2.2.11 Statistical analysis

Results are expressed as Mean ± Standard Deviation and all statistical comparisons were made by one-way-analysis of variance (ANOVA) test, followed by *Tukey post-hoc* analysis and p-values less than or equal to 0.05 were considered significant.

## 3. Results

### 3.1 Effect of PmHE on the plasma levels of liver enzymes

Analysis of blood plasma of CCl<sub>4</sub> control rats showed higher levels of ALT, AST and γ-GT when compared to normal control rats, suggesting hepatic injury and inflammation due to CCl<sub>4</sub> toxication. Post treatment with PmHE (200 mg/kg) has decreased the levels of plasma ALT, AST and γ-GT to near normal levels (Figure 1). Similarly, post treatment with silymarin (50 mg/kg) also reduced the hepatic damage, caused by CCl<sub>4</sub> toxication as judged from the reduced levels of plasma ALT, AST and γ-GT when compared to CCl<sub>4</sub> control group. There were no significant changes in the levels of these parameters in normal control group and PmHE alone treated groups.

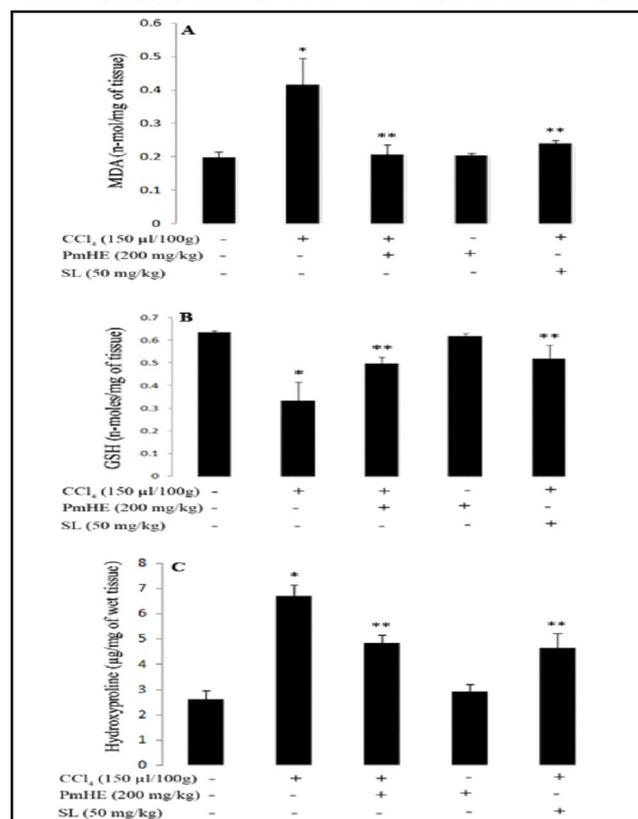


**Figure 1 :** Effect of PmHE on CCl<sub>4</sub> induced hepatic fibrosis and on the plasma levels of liver enzymes

**Legend :** CCl<sub>4</sub>, carbon tetrachloride; PmHE, *Phyllanthus maderaspatensis* hexane extract; SL, silymarin (positive control); Results are expressed as the Mean ± SD, n= 6 in each group; \**p* ≤ 0.05 are significant when compare to normal.

### 3.2 Effect PmHE on MDA, GSH and hydroxyproline

Increased levels of MDA in CCl<sub>4</sub> control group when compared to normal control group, suggest severe oxidative stress in this group (Figure 2A). Treatment with PmHE or silymarin for two weeks significantly reduced the oxidative stress in the rats as evidenced by decreased levels of MDA. There were no significant changes in the level of MDA between normal control rats and PmHE alone treated rats. Five week toxication of rats with CCl<sub>4</sub>, reduced the level of GSH when compared to normal control group (Figure 2B). Post treatment with PmHE for two weeks, significantly increased the level of GSH in the liver. This effect was comparable to that of silymarin, a standard hepatoprotective drug (Figure 2B). There were no significant differences in GSH levels between normal control group and PmHE alone treated animals. Toxication with CCl<sub>4</sub>, resulted in about three fold increase of total hydroxyproline when compared to untreated normal control rats (Figure 2C). This increase in hydroxyproline content in the liver was partly restored to normal level with PmHE post treatment. Similarly, post treatment with silymarin also showed a decrease in the total hydroxyproline content (Figure 2C). There were no noticeable changes in the hydroxyproline content of PmHE alone treated rats and untreated normal control.



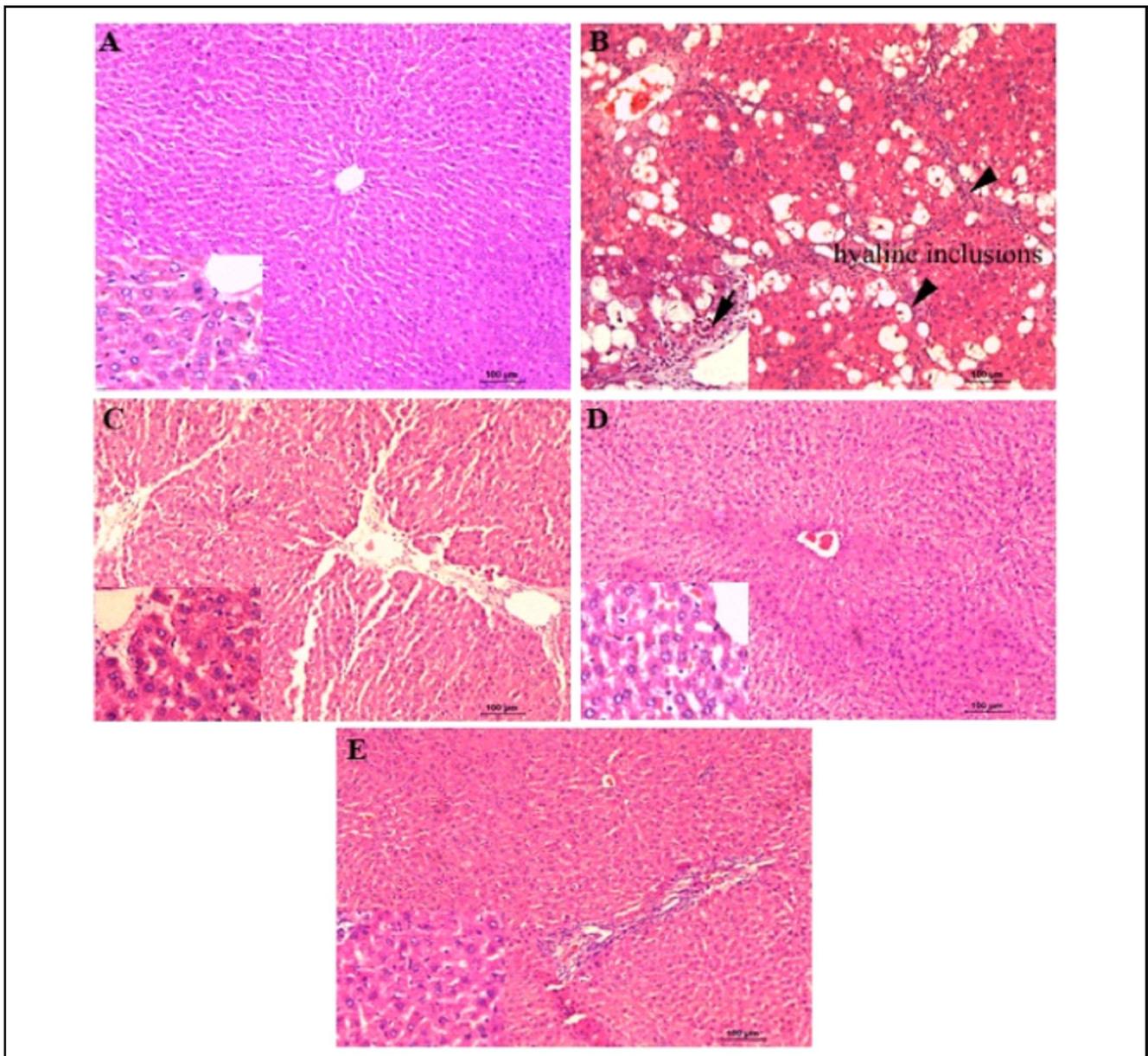
**Figure 2 :** (A), Effect of PmHE on the plasma levels of malondialdehyde (MDA). (B), Effect of PmHE on the plasma levels of reduced glutathione (GSH). (C), Effect of PmHE on the plasma levels of hydroxyproline

**Legend :** CCl<sub>4</sub>, carbon tetrachloride; PmHE, *Phyllanthus maderaspatensis* hexane extract; SL, silymarin (positive control); Values are Mean ± SD, n= 6 (six animals were used in each group); \**p* ≤ 0.05 are significant when compare to normal.

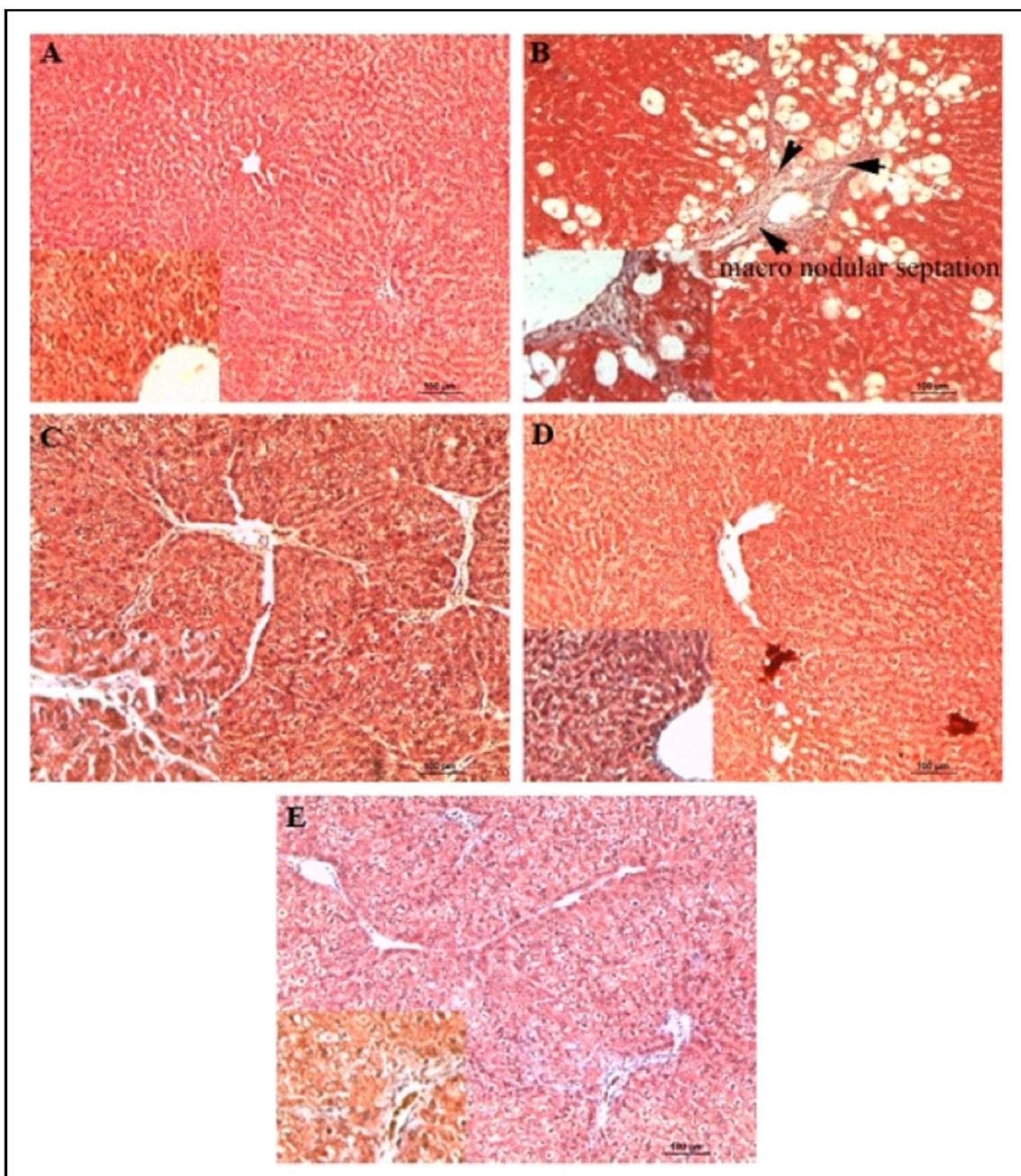
### 3.3 Histoapthological analysis of the liver

Liver sections of normal control rats and PmHE alone treated rats showed normal histological architecture, having intact central vein and radially arranged hepatocytes around the central vein (Figures 3A and 3D). Liver sections in rats treated with  $\text{CCl}_4$  ( $\text{CCl}_4$  control) showed severe neutrophil infiltration and centrilobular necrosis around the central vein and portal tract. Hepatocytes with severe fatty changes and ballooning degeneration were abundantly distributed. Moreover, hyaline inclusions, pyknotic hepatic cells, degeneration of nucleoli and multinuclear condition were also observed in  $\text{CCl}_4$  group (Figure 3B). Post treatment with PmHE showed a remarkable decrease in the hepatocellular damage, induced

by  $\text{CCl}_4$  toxication (Figure 3C). The normal histological architecture of the liver was recovered with complete reversal of the fatty changes. Similarly, post treatment with silymarin (50 mg/kg) reversed the hepatic fibrosis (Figure 3E). To analyse the intensity of collagen deposition, liver sections were stained with Masson's Trichome stain. There was very little/or no deposition of collagen in the normal control group and PmHE alone treated group (Figures 4A and 4D). Intensity of collagen deposition in  $\text{CCl}_4$  control rats was very severe (Figure 4B, at arrow). Moreover, macronodular septation of the liver was also observed. Treatment with PmHE or silymarin remarkably reduced the collagen deposition and nodulation of the liver (Figures 4C and 4E).



**Figure 3:** Effect of PmHE on the histological architecture of the liver (haematoxylin and eosin staining). (A) Liver sections of normal control rats and PmHE alone treated rats. (B) Liver sections in rats treated with  $\text{CCl}_4$  (C) Post treatment with PmHE (200 mg/kg). (D) Post treatment with silymarin. (E) The normal histological architecture of the liver



**Figure 4:** Effect of PmHE on the histological architecture of the liver, trichrome staining. (A) Normal control group and PmHE alone treated group. (B) Liver section in rats treated with  $\text{CCl}_4$ . (C) Post treatment with PmHE. (D) Post treatment with silymarin. (E) The normal histological architecture of the liver

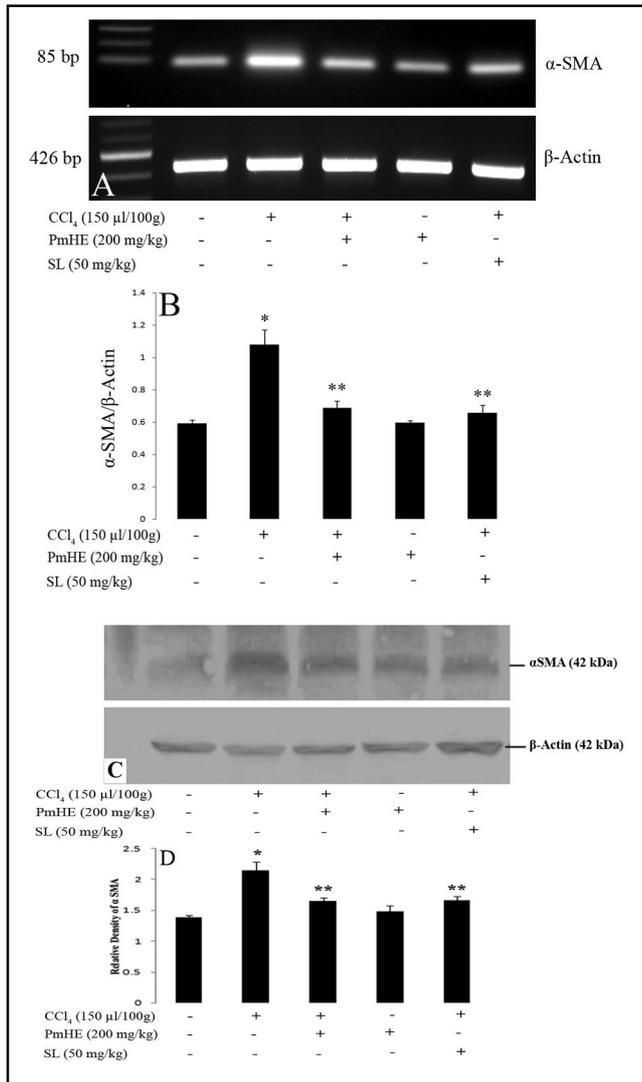
### 3.4 Effect of PmHE on alpha SMA and fibrillar collagens

$\text{CCl}_4$  toxication (group II rats) resulted in a remarkable increase in mRNA transcripts of  $\alpha\text{SMA}$ , the marker protein of activated HSC in the liver; the increase was  $83.3 \pm 4.1\%$  more when compared to control rats (Figures 5A and 5B). Post treatment with PmHE significantly reduced the level of  $\alpha\text{SMA}$  transcripts when compared to  $\text{CCl}_4$  control rats. Similarly, treatment with silymarin also reduced the levels of mRNA transcripts of  $\alpha\text{SMA}$  (Figure 5).

$\text{CCl}_4$  toxicity caused a sharp hike in the level of mRNA transcripts for fibrillar collagen type I (Figure 6A1 and 6A2) and type III (Figure 6B1 and 6B2); the increase in the mRNA transcripts of type I and type III collagen was about  $102.5 \pm 4.2\%$  and  $52.4 \pm 3.2\%$  respectively, when compared to untreated  $\text{CCl}_4$ -control rats (Figures 6 A1 and A2; Fig. 6 B1 and B2). Post treatment with PmHE significantly reduced this increase in fibrillar collagen. Semi-quantitative analysis of the transcripts showed  $29.3 \pm 6.3\%$  and  $27.5 \pm 2.0\%$  reduction in transcription of collagen-I and collagen-III,

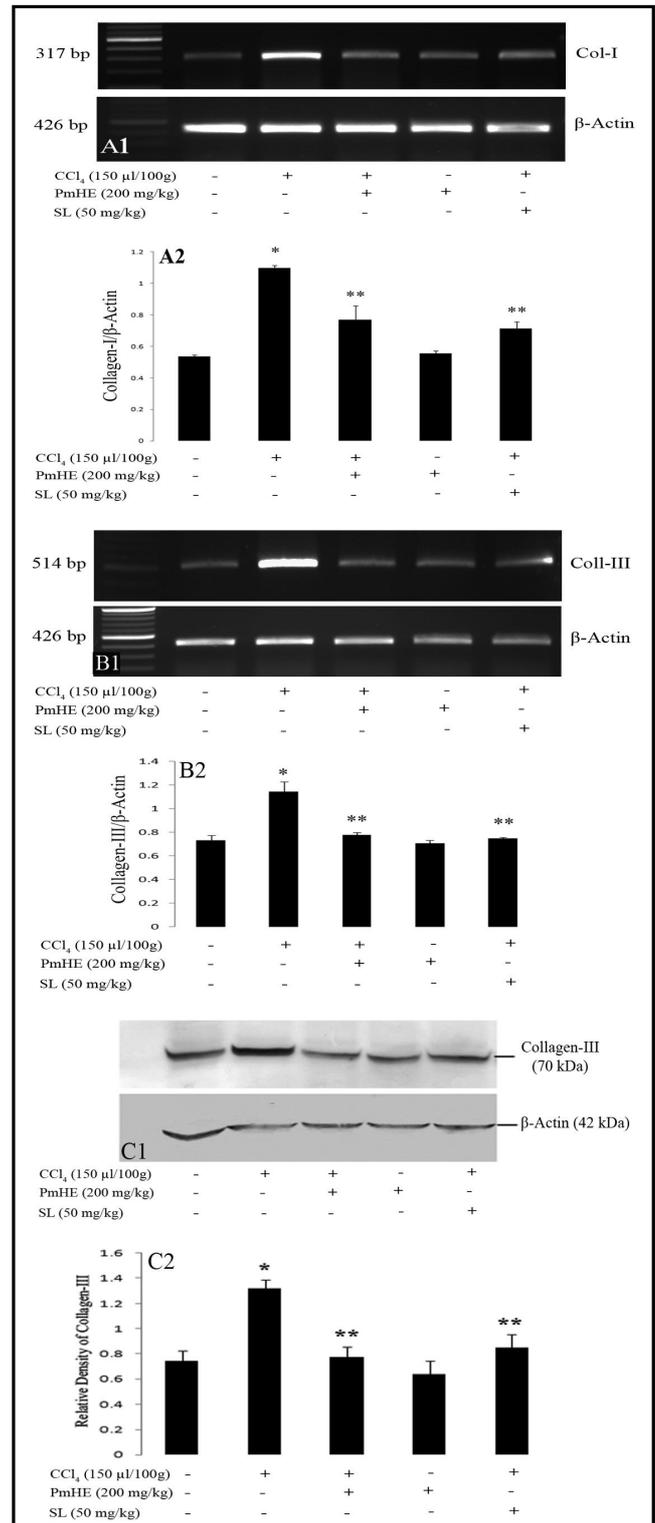
respectively in PmHE treated rats when compared to CCl<sub>4</sub> control group. Post treatment with silymarin also reduced the expression level of collagen-I and collagen-III transcripts almost to the same level (Figures 6. A1 and A2; B1 and B2).

Western blot analysis of αSMA (Figure 5C and 5D) and collagen-III (Figures 6C1 and C2), further confirmed the ability of PmHE to reduce the elevated levels of these proteins, observed in CCl<sub>4</sub> intoxicated rats. There was very little or no expression of αSMA in normal control rats and PmHE alone treated rats (Figures 5C and 5D). Treatment with PmHE reduced the elevated level of αSMA in the liver of CCl<sub>4</sub> challenged rats. Similarly, treatment with silymarin also reduced the elevated αSMA (Figures 5 C and D). Similarly the treatment attenuated CCl<sub>4</sub>-induced increase in the levels of collagen



**Figure 5 :** Effect of PmHE on the mRNA transcript and protein expression of alpha-SMA during liver fibrosis

**Legend :** CCl<sub>4</sub>, carbon tetrachloride; PmHE, *Phyllanthus maderaspatensis* hexane extract; SL, silymarin (positive control); Values are Mean ± SD, n= 6 in each group; \*p ≤ 0.05 are significant when compare to normal



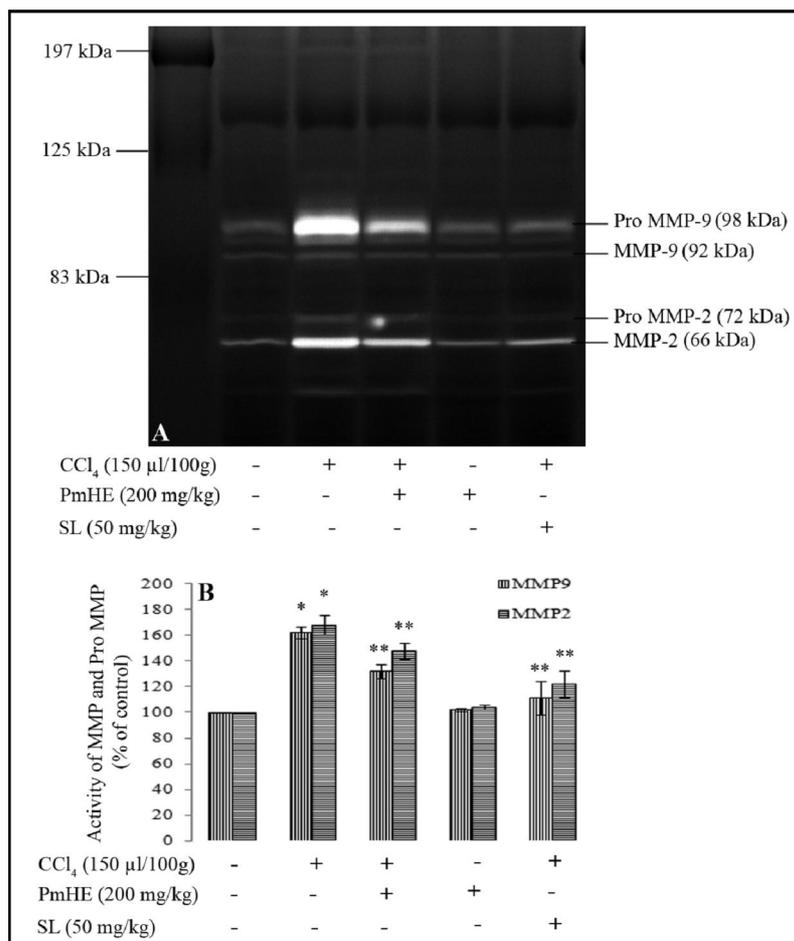
**Figure 6 :** Effect of PmHE on mRNA transcript and protein expression of collagen during liver fibrosis

**Legend :** CCl<sub>4</sub>, carbon tetrachloride; PmHE, *Phyllanthus maderaspatensis* hexane extract; SL, silymarin (positive control); Values are Mean ± SD, n= 6 in each group; \*p ≤ 0.05 are significant when compare to normal

### 3.5 Zymographic analysis of the PmHE

To assess the effect of PmHE post treatment on CCl<sub>4</sub> induced hepatic fibrogenesis, we tested the activities of MMP-2 and MMP-9 in different treatment groups, using gelatine zymography. As shown in Figure 7A, four prominent bands were obtained in gelatine zymography which corresponds to pro-MMP-9 (98 kDa), active MMP-9 (92 kDa), pro-MMP-2 (72 kDa) and active MMP-2 (66 kDa). The intensity of both pro-MMPs and active MMPs was found to be more in CCl<sub>4</sub> control group when compared to

untreated normal control. Post treatment with PmHE (200 mg/kg) or silymarin (50 mg/kg) significantly reduced activity of both MMP-9 and MMP-2. There were no changes in basal MMPs level in PmHE alone treated rats. Quantitative densitometric analysis of the sum of pro and active forms of MMPs showed that CCl<sub>4</sub> treatment increased the activity of MMP-9 and MMP-2 (162 ± 5% and 168 ± 8%, respectively of normal controls). Treatment with PmHE significantly reduced the activity of MMP-9 and MMP-2 compared to CCl<sub>4</sub>-control rats (Figure 7B).



**Figure 7:** Zymographic analysis of the effect of PmHE on MMPs in CCl<sub>4</sub>-treated rats. (A) Zymogram showing the effect of PmHE on MMP-9 and MMP-2 expressions. (B) Showing the activity of MMP and pro MMP. Values are Mean ±SD, n = 6 in each group, \**p* ≤ 0.05 are significant when compare to normal

Sl.No	Gene	Primer sequence 5'-3'	Tm (°C)	PCR cycles	Product size(bp)
1	αSMA	5' CGAGAGGACGTTGTTAGCATAGAG 3' 5' GGGCATCCACGAAACCA 3'	64	36	85
2	Col-I	5' TACTACCGGGCCGATGATGC 3' 5' TCCTTGGGGTTCGGGCTGATGTA 3'	68	31	317
3	Col-III	5' CCCCTGGTCCCTGCTGTGG 3' 5' GAGGCCCGGCTGGAAAGAA 3'	69	32	514
4	β-Actin	5' CGATATGGAGAAGATTTGGCAC 3' 5' TCTAGGGCAACATAGCACAG 3'	59	30	426

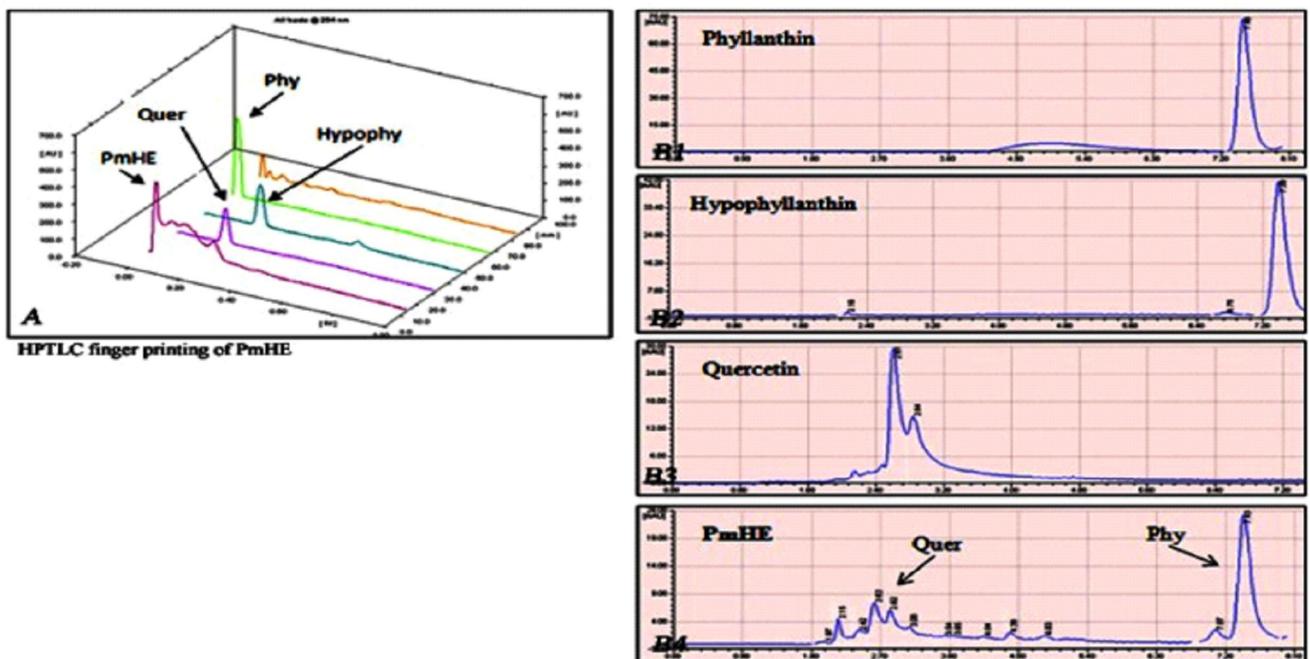
**Table 1:** Sequence of oligo nucleotide primers, annealing temperature (Tm), number of PCR cycles and size of the amplified PCR products of different transcripts analyzed by SQ-RT-PCR

### 3.6 *In vivo* toxicity studies of PmHE

In acute and subacute toxicity studies, oral administration of PmHE did not cause any change in physiological and behavioral parameters studied. Analysis of serum parameters did not show any change in their normal level when compared to untreated normal control rats (Table 2). There was no mortality of animals.

	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Glucose (mg/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	Creatine (mg/dl)
Control	56.57 ± 8.10	142.65 ± 22.24	45.46 ± 23.47	107.92 ± 16.51	22.56 ± 9.92	92.30 ± 5.31	2.40 ± 0.14
Acute Toxicity Study (PmHE, 2 g/kg)	56.88 ± 3.20	128.05 ± 14.84	57.48 ± 4.48	103.15 ± 3.04	31.95 ± 1.10	90.19 ± 3.89	2.50 ± 0.23
Subacute Toxicity Study (PmHE 200 mg/kg)	50.83 ± 8.11	132.50 ± 24.74	41.25 ± 10.33	127.30 ± 0.84	14.29 ± 0.34	79.96 ± 8.21	2.39 ± 0.46

**Table 2:** Effect of acute and subacute treatment of rats with PmHE on serum biochemical parameters



**Figure 8:** HPTLC and HPLC analysis of PmHE. (A), HPTLC finger printing analysis showing the presence of phyllanthin and quercetin in the PmHE extract. (B1) HPLC analysis showing the peak for Phyllanthin. (B2) HPLC analysis showing the peak for Hypophyllanthin. (B3) Showing the peak for Quercetin. (B4) HPLC analysis showing the presence of quercetin and phyllanthin in the extract

### 3.7 HPTLC and HPLC analysis of PmHE

HPLC analysis of PmHE confirmed the presence of phyllanthin and quercetin in the extract. We were not able to detect any trace of hypophyllanthin (Figures 8 A and B)

## 4. Discussion

Carbon tetrachloride ( $CCl_4$ ), one of the most extensively used chemicals to experimentally induce hepatic fibrosis, exerts its toxic effects on liver by oxidative stress (Weber *et al.*, 2003). The free radicals generated after the metabolism of  $CCl_4$  mainly in smooth

endoplasmic reticulum (Recknagel *et al.*, 1989) and mitochondria (Boll *et al.*, 2001), impart the toxic properties of  $CCl_4$ . Our studies showed that  $CCl_4$  toxication induced chronic hepatic damage leading to fibrosis as evidenced by the high level of plasma transaminases. The increase in the level of plasma AST, ALT and  $\gamma$ -GT due to  $CCl_4$  toxication, were successfully reduced to normal level after post treatment with PmHE (200 mg/kg) for two weeks. As we reported earlier, the present study shows the hepatoprotective activity of PmHE against  $CCl_4$  induced hepatic damage (Asha *et al.*, 2004; Asha *et al.*, 2007). Since biochemical parameters always do not reflect the degree of tissue damage (Murayama *et al.*, 2007), we

evaluated the extent of hepatic fibrosis in different treatment groups by histological staining of the liver sections. Histological evaluation of the liver specimens showed the formation of typical fibrotic scarring of the liver by  $\text{CCl}_4$  administration. Post treatment with PmHE significantly reversed the fibrotic condition of the liver.

There are many lines of experimental evidence to support the fact that oxidative stress plays an important role in the development of fibrosis in many organs including liver (Liu and Gaston, 2010). The reactive species generated by the metabolism of  $\text{CCl}_4$  in the hepatocytes or Kupffer cells can directly activate hepatic stellate cells (HSC) (Svegliati *et al.*, 1998). Activation of HSC is associated with oxidative stress and may be prevented by treatment with antioxidants (Lotersztajn *et al.*, 2005). Our results showed that  $\text{CCl}_4$  induced elevated levels of MDA due to reactive free radicals, was significantly reduced by post treatment with PmHE. The antioxidant and free radical scavenging potential of *P. maderaspatensis* was previously reported from our laboratory and elsewhere (Asha *et al.*, 2004; Asha *et al.*, 2007; Bommu *et al.*, 2008; Kumaran and Karunakaran, 2007). The antifibrotic potential of antioxidants like silymarin is further confirmed by our study (Boigk *et al.*, 1997). Moreover, increase in oxidative stress is associated with a decrease in the level of reduced glutathione (GSH) in the liver. GSH, a tripeptide, is the most abundant intracellular free thiol and one of the important resident antioxidants (Liu and Gaston, 2010). The level of GSH in the liver decreases in the experimental fibrosis models (Liu and Gaston, 2010). The most important and well known function of GSH is inhibition of lipid peroxidation and reduction of oxidative stress (Biteau *et al.*, 2003). The maintenance of intracellular homeostasis of GSH is vital for normal cell functions (Biteau *et al.*, 2003).  $\text{CCl}_4$  toxication results in the rapid decrease of GSH in the liver (Cabre *et al.*, 2000) which will result in exponential production of Reactive Oxygen Species, lipid peroxidation and finally fibrotic progression (Vendemiale *et al.*, 2001). Analysis of the total glutathione pool in different treatment groups, have shown that  $\text{CCl}_4$  administration caused a rapid decline in GSH in the liver which was significantly reversed by the treatment with PmHE (200 mg/kg) or silymarin (50 mg/kg). This showed the antioxidant and free radical scavenging efficiency of PmHE. The efficiency of PmHE to reduce the lipid peroxidation may be partly due to the increased GSH level associated with PmHE treatment. PmHE can also directly scavenge reactive free radicals, and thereby, it can reduce the oxidative stress (Asha *et al.*, 2004).

HSCs are the quiescent vitamin A storing cells in the normal liver. In response to fibrogenic stimuli, such as  $\text{CCl}_4$  toxicity, the quiescent HSCs are transformed to a rapidly proliferating myofibroblast like cells which lose its vitamin A storing capacity and show increased expression of  $\alpha$  smooth muscle actin ( $\alpha$ SMA). Since  $\alpha$ SMA is considered as a marker of activated HSC, the expression level variation of this cytoskeletal protein can be used to evaluate the efficiency of antifibrotic agents screening (Carpino *et al.*, 2005). Our results from SQ-RT-PCR and Western blot analysis of  $\alpha$ SMA, showed that PmHE considerably reduced the amount of  $\alpha$ SMA in the liver of  $\text{CCl}_4$  treated rats. This suggests the gradual reversal of  $\text{CCl}_4$  induced hepatic fibrosis by post treatment with PmHE. Furthermore, the expression of the fibrillar collagen-I and collagen-III and intensity of collagen deposition as evidenced by staining of the liver sections and level of total hydroxyproline in the liver support, the therapeutic potential of PmHE and silymarin in  $\text{CCl}_4$  induced

hepatic fibrosis. Proteolytic degradation of ECM is an important physiological process that controls the cellular behavior and survival (Werb, 1997). Matrix metalloproteinases (MMPs) promote the degradation of ECM and the activities of MMPs are negatively regulated by Tissue Inhibitor of Metalloproteinase (TIMPs) (Murphy and Docherty, 1992). The regulation of MMPs and TIMPs is essential for the normal ECM homeostasis. Among the members of metalloproteinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase-B) play a major role in many physiological and pathological processes including liver fibrosis (Lewi *et al.*, 1996). HSCs are the major source of MMP-2 and these can also express MMP-9 (Arthur, 2000; Han *et al.*, 2007). Increased expression of MMP-2 is characteristic to hepatic fibrosis progression (Benyon *et al.*, 1996). The principal cellular source of MMP-9 is Kupffer cells (Winwood *et al.*, 1995). In the present study, we revealed that the levels of MMP-2 and MMP-9 activity were significantly reduced, when compared to  $\text{CCl}_4$  control, by the post treatment with PmHE or silymarin. Previous reports regarding the MMP activity in transformed fibroblast cells have shown that GSH can inhibit MMP-2 activity (Tyagi *et al.*, 1996). Moreover GSH appears to be an effective inhibitor of MMP in cold preserved liver allografts (Upadhy and Strasberg, 2000). Thus, the reduction of MMP activity after PmHE treatment of  $\text{CCl}_4$  toxication may be due to the attenuation of oxidative stress and increased the level of GSH in the liver.

Preliminary phytochemical analysis of PmHE indicated the presence of resins, phenolic compounds, tannins and saponins in different solvent fractions. The plant is also reported to contain low concentrations of the lignansphyllanthin and hypophyllanthin which are responsible for hepatoprotective activity (Schmelzer and Gurib-Fakim, 2008). In our study, the HPTLC and HPLC analysis was able to detect the presence of phyllanthin and quercetin in the hexane extract. We were not able to detect the presence of hypophyllanthin in the hexane extract. Polyphenolic compounds having significant biological properties such as free radical scavenging activity, inhibition of lipid peroxidation and anti-inflammatory activity were also reported to be present in *P. maderaspatensis* (Bagul *et al.*, 2005). The seeds of the plant contain a deep yellow oil rich in myristic, palmitic, stearic, oleic and linolenic acids (Schmelzer and Gurib-Fakim, 2008). The presence of these saturated and unsaturated fatty acids in the esterified PmHE was also confirmed by gas chromatographic study from our laboratory. The fatty acids are also reported to have antioxidant properties (Henry *et al.*, 2002) and, thus, the presence of these compounds in PmHE may impart the antifibrotic properties of PmHE. Further, studies are in progress in our laboratory to elucidate the details of the molecular mechanism of action and isolate the active component(s) from PmHE.

## 5. Conclusion

As a conclusion, the findings of the study suggest the antifibrotic activity of PmHE in Wistar rats. We studied both preventive and curative treatment strategies. Post treatment with PmHE (200 mg/kg) reversed  $\text{CCl}_4$  induced hepatic fibrosis in rats. The HPLC and HPTLC fingerprint profiles of PmHE was analyzed and confirmed the presence of phyllanthin and quercetin. The present study also grants scientific evidence to validate the traditional use *P. maderaspatensis* in the treatment of liver problems. The effect may exert *via* scavenging the reactive oxygen species, produced

from inflammatory cells and liver cells, reducing collagen deposition and down regulating the over expression of  $\alpha$ SMA and collagen III at proteins and mRNA levels. Post treatment provides an effective curative strategy against hepatic fibrosis possibly by inhibit oxidative stress.

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### Conflict of interest

We declare that we have no conflict of interest.

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