CHAPTER 5

METHANOLIC EXTRACT OF WOODFORDIA FRUTICOSA KURZ FLOWERS AMELIORATES CARBON TETRACHLORIDE INDUCED CHRONIC HEPATIC FIBROSIS IN RATS
5.1. INTRODUCTION

Reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis (Geesin et al., 1990). Hepatic fibrosis, a precursor of cirrhosis, developing in response to chronic hepatocellular injury and show the general features of wound repair process. It is characterized by the excessive deposition of extracellular matrix (ECM) proteins including collagen, fibronectin, laminin and proteoglycans (Wills and Asha, 2006b). In the injured liver these ECM components are produced in hepatic stellate cells (HSCs) or lipocytes and transform it into myofibroblasts (Wang et al., 2012). Excess depositions of ECM proteins disrupt the normal functioning of the liver, ultimately leading to patho-physiological damage to the organ, which has high mortality rate (Wills and Asha, 2007). Wide ranges of pathogenic factors, such as hepatitis B virus (HBV), hepatitis C virus (HCV), hepatotoxins, metabolic disorders, alcoholisms and schistosomiasis are closely related with hepatic fibrogenesis or progression of hepatic cirrhosis. Emerging antifibrotic therapies are aimed at inhibiting the accumulation of fibrogenic cells and/or preventing the deposition of extracellular matrix proteins in the liver (Bataller and Brenner, 2005). Traditional plant drugs have been found to be effective in preventing fibrogenesis and other chronic liver injury which develops a more hopeful future in controlling liver fibrosis, cirrhosis and hepatocarcinogenesis (Inao et al., 2004; Lee et al., 2003; Shahjahan et al., 2005; Yao et al., 2005). Several herbal drugs have been investigated for their antifibrotic effects on carbon tetrachloride induced hepatic fibrosis in rats (Wills and Asha, 2006b; Wang et al., 2012).
This chapter deals with the antifibrotic activity of methanolic extract of *Woodfordia fruticosa* in preventive and curative models. As the methanolic extract of Woodfordia exhibited a promising antioxidant and hepatoprotective activity against thioacetamide induced oxidative stress, it was further confirmed for its antifibrotic activity against CCl₄ induced hepatic fibrosis.

**5.2. MATERIALS AND METHODS**

**5.2.1. Chemicals**

Carbon tetrachloride was purchased from Merck, Mumbai. Liquid paraffin, chloramine-T, bovine serum albumin, monoclonal anti-collagen type III (mouse IgG1 isotype), antimouse IgG peroxidase conjugate (whole molecule), streptavidin–horseradish peroxidase conjugate, antimouse IgG (whole molecule) and 3, 3’-Diaminobenzidine tetrahydrochloride (DAB) were procured from Sigma Chemical Co., MO, USA. Assay kits for serum AST, ALT, ALP and LDH were purchased from Agappe Diagnostics, India. All other chemicals were of analytical grade.

**5.2.2. Animals and diets**

Male Wistar rats weighing 140-150 gm were used for this study. The animals were housed in polypropylene cages and given standard rat chow (Sai Feeds, Bangalore, India) and drinking water *ad libitum*. The animals were maintained at a controlled condition of temperature of 26–28°C with a 12 h light: 12 h dark cycle. Animal studies were followed according to Institutional Animal Ethics Committee (IAEC) regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No. B 2442009/4) and conducted humanely.
5.2.3. Preparation of plant extract

A 50 g of dried powder was soxhlet extracted with 400 ml of methanol for 48h. The extracts were concentrated under reduced pressure using a rotary evaporator and were kept under refrigeration. The yield of methanolic extract was 12.5 % (w/w). The concentrate was suspended in 5% Tween 80 to respective dosages for this study and stored at -20°C.

5.2.4. Preparation of doses

Silymarin at an oral dose of 100 mg/kg body weight was used as standard control in the experiment (Shyamal et al., 2010). Two different doses (100 and 200 mg/kg) of MEWF suspended in 5% Tween 80 were also prepared for oral administration to the animals.

5.2.5. Induction of hepatic fibrosis

Hepatic fibrosis was induced by oral administration of CCl$_4$ at a dose of 150µl/100g rat weight mixed with liquid paraffin at a ratio of 1:1 twice a week for 10 weeks (Wasser et al., 1998).

5.2.6. Experimental design

5.2.6.1. Pre-treatment evaluation

Male Wistar rats weighing 144 ± 6.4 gm (Mean ± S.D, n = 36) were used in this study. Rats were divided into six groups with six rats in each group and that were treated as follows

- Group I - Normal control (vehicle only)
- Group II - CCl$_4$ control (150µl/100g b.w twice a week, p.o)
- Group III - CCl$_4$ (as in group II) + silymarin (100mg/kg b.w, p.o)
• Group IV  - CCl$_4$ (as in group II) + MEWF (100mg/kg, b.w, p.o)
• Group V  - CCl$_4$ (as in group II) + MEWF (200mg/kg, b.w, p.o)
• Group VI  - MEWF (200 mg/kg) alone.

Rats of Groups II, III, IV and V were given CCl$_4$ at a dose of 150µl/100 g mixed with liquid paraffin at a ratio of 1:1 twice a week for 10 weeks orally. In Group III, IV and V rats, daily doses of silymarin and MEWF (100 and 200mg/kg) treatments were started respectively 1 week before the onset of CCl$_4$ administration and continued for 10 weeks. Group VI animals served as drug control received MEWF at 200mg/kg for the entire period. Group I animals treated as vehicle control received 5% Tween 80 and liquid paraffin instead of drug and CCl$_4$ respectively. The rats were sacrificed 48 h after the last dose of CCl$_4$ administration.

5.2.6.2. Post-treatment evaluation

Male Wistar rats weighing 146 ± 3.5 gm (Mean ± S.D, n = 36) were used in this study. Rats were divided into six groups with six rats in each group and that were treated as follows:

• Group I  - Normal control (vehicle only)
• Group II  - CCl$_4$ control (150µl/100g b.w twice a week, p.o)
• Group III  - CCl$_4$ (as in group II) + silymarin (100mg/kg b.w, p.o)
• Group IV  - CCl$_4$ (as in group II) + MEWF (100mg/kg, b.w, p.o)
• Group V  - CCl$_4$ (as in group II) + MEWF (200mg/kg, b.w, p.o)
• Group VI  - MEWF (200 mg/kg) alone.

After the intoxication with CCl$_4$ for 10 weeks, group III, IV and V were treated with daily doses of silymarin and MEWF (100 and 200mg/kg) respectively for 2 weeks. Group II received 1ml of 5% tween 80 daily for 2 weeks. Group VI
animals received MEWF at 200mg/kg, only for the last two weeks. Group I animals treated as vehicle control received 5% Tween 80 and liquid paraffin instead of drug and CCl₄ respectively. Animals were sacrificed on the 15th day after the last dose of CCl₄ administration.

5.2.7. Serum enzyme analysis

Serum was separated from collected blood by centrifugation at 2500 rpm at 4°C for 15 min. and used for the estimation of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), ALP (EC 3.1.3.1) and LDH (EC 1.1.1.27) by kinetic method using a standard diagnostic kit (Agappe Diagnostic Ltd., India). Activities of these serum enzymes were measured by using semi autoanalyzer (RMS, India).

5.2.8. Tissue analysis

The excised liver was washed, weighed and homogenized in chilled Tris buffer (0.1M, pH 7.4) at a concentration of 10% w/v. The homogenate was centrifuged at 3000 rpm for 20 min at 4°C and the supernatant was used for the estimation of reduced glutathione (GSH), glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and concentration of MDA (Thiobarbituric Acid Reactive Substances – TBARS) and total protein.

GSH levels in tissues were determined based on the formation of a yellow colored complex with DTNB (Ellman, 1959). GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB (Habig et al., 1974). GR (EC 1.6.4.2) activity was assayed at 37°C and 340 nm by following the oxidation of NADPH by GSSG (Carlberg and Mannervik, 1985). GPx (EC 1.11.1.9) activity was determined by measuring the
increase in GSH content after incubating the sample in the presence of H\textsubscript{2}O\textsubscript{2} and NaN\textsubscript{3} (Rotruck et al., 1973). CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H\textsubscript{2}O\textsubscript{2} (Beers and Sizer, 1952). The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1’1’3’3’ tetramethoxypropane as standard (Niehuis and Samuelsson, 1968). Protein contents of the tissues were determined using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

(Detailed procedures are explained under chapter 2, section 2.2.14. Procedures for \textit{in vivo} antioxidant assays)

5.2.8.1. \textit{Hydroxyproline content in the liver}

The hydroxyproline content in the liver tissue was measured by the method of Jamall et al., (1981).

(Detailed procedure is described in the chapter 2, section 2.2.8.)

5.2.9. \textit{Histopathological studies}

Small pieces of liver tissues fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 5–6 \( \mu \)m were cut and stained with hematoxylin and eosin and examined for histopathological changes.

Liver sections were graded numerically based on Knodell’s histological activity index to assess the degree of histologic injury in hepatic fibrosis (Knodell, 1981). 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.
5.2.10. Immunohistochemical analysis of collagen III

For immunohistochemical analysis of collagen III the primary antibody used was anticollagen type III antibody diluted at a concentration of 1:200 with 1% BSA. After incubation with primary antibody, washed the sections with PBS and incubated with secondary antibody - anti-mouse IgG (whole molecule) peroxidase at a dilution 1:200.

(Detailed procedure is explained under chapter 2, section 2.2.10. Immunohistochemical analysis)

5.2.11. Statistical analysis

Results are expressed as mean ± standard deviation (SD). 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.

5.3. RESULTS

5.3.1. Body weight and liver weight

A sharp decline in body weight and liver weight were recorded after 10 weeks in $\text{CCl}_4$ alone treated animals. MEWF treatment prevented the weight loss of body and liver due to $\text{CCl}_4$ intoxication in preventive treatment groups. In curative treatment groups, MEWF administration for last two weeks after the establishment of hepatic fibrosis enhanced the body weight and liver weight (Table 5.1).
Table 5.1. Body weight and liver weight pattern of different group of rats pre and post- treated with MEWF

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Liver weight (g)</td>
</tr>
<tr>
<td>Normal control</td>
<td>279.1±3.4</td>
<td>6.9±0.18</td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>161.6±2.9</td>
<td>4±0.17</td>
</tr>
<tr>
<td>CCl₄ + Silymarin 100mg/kg</td>
<td>253.2±3.6</td>
<td>6.01±0.18</td>
</tr>
<tr>
<td>CCl₄ + MEWF 100mg/kg</td>
<td>210±3.4</td>
<td>5.6±0.16</td>
</tr>
<tr>
<td>CCl₄ + MEWF 200mg/kg</td>
<td>276.1±3.7</td>
<td>6.8±0.14</td>
</tr>
<tr>
<td>MEWF 200mg/kg alone</td>
<td>283±2.6</td>
<td>7.1±0.16</td>
</tr>
</tbody>
</table>

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. "CCl₄ control differs significantly from normal control. "Silymarin 100mg/kg + CCl₄, MEWF 100mg/kg + CCl₄, MEWF 200mg/kg + CCl₄ and MEWF 200mg/kg alone were significantly different CCl₄ control. "MEWF 200mg/kg + CCl₄ treated group differs significantly from MEWF 100mg/kg + CCl₄ treated group. "MEWF 200mg/kg alone treated group non significantly different from normal control.

5.3.2. Serum enzymes of pre and post -treatment groups

5.3.2.1. Pre- treatment evaluation

The serum levels of AST, ALT, ALP and LDH in group II were significantly $(p \leq 0.05)$ elevated by the administration of CCl₄ when compared to normal control. The pre-treatment with MEWF at a dose of 100mg/kg and 200 mg/kg, b.w showed a significant decrease $(p \leq 0.05)$ in AST, ALT, ALP and LDH levels (Fig.5.1). Standard control drug, silymarin at a dose of 100 mg/kg also showed a concomitant result. Treatment with MEWF at a dose of 200 mg/kg, b.w and silymarin exhibited a protection of 94% and 76% in AST levels, 95% and 83.5% in ALT levels, 94.4% and 85.1% in ALP levels and 97.3% and 71.4% in LDH levels, respectively.
5.3.2.2. Post-treatment evaluation

CCL₄ treated rats showed significantly \( (p \leq 0.05) \) elevated serum levels of AST, ALT, ALP and LDH when compared to normal control. In contrast, post-treatment with MEWF (100 and 200 mg/kg) exhibited an ability to counteract the CCL₄ induced hepatic fibrosis by decreasing the serum enzyme levels \( (p \leq 0.05) \) compared to CCL₄ control. MEWF at a dose of 200 mg/kg, b.w showed a protection of 96%, 97.3%, 98.1% and 97.9% for AST, ALT, ALP and LDH respectively. Silymarin also showed a remarkable protection of 76%, 83.9%, 89.1% and 70.8% for AST, ALT, ALP and LDH respectively towards CCL₄ induced fibrosis \( (p \leq 0.05) \) (Fig.5.1).

5.3.3. Tissue antioxidants and other biochemical constituents of pre-treated groups

5.3.3.1. Reduced glutathione (GSH) level

Rats administered with CCL₄ alone were found significantly \( (p \leq 0.05) \) lowered level of reduced glutathione (GSH). Pre-treatment with MEWF exhibited significant increase \( (p \leq 0.05) \) in hepatic glutathione levels. MEWF at a dose of 200 mg/kg showed a protection of 85%. Silymarin treated rats also showed considerable prevention of GSH and the percentage of protection was 69% (Table 5.2).

5.3.3.2. Glutathione - S - transferase (GST) activity

GST activity in liver was significantly \( (p \leq 0.05) \) reduced in CCL₄ intoxicated rats compared to normal control. Treatment with MEWF dose dependently increased \( (p \leq 0.05) \) the GST activity, MEWF at a dose of 200 mg/kg exhibited prominently increased activity i.e., 85.3%. In addition, silymarin treated rats also prevented the CCL₄ induced decrease in GST activity by 62.2% (Table 5.2).
Fig. 5.1. Changes in serum enzyme levels of rats pre and post-treated with MEWF

(A) Aspartate aminotransferase (B) Alanine aminotransferase

(I) Normal control, (II) CCl₄ control, (III) Silymarin (100 mg/kg) + CCl₄; (IV) MEWF (100 mg/kg) + CCl₄; (V) MEWF (200 mg/kg) + CCl₄; (V) MEWF (200 mg/kg) alone.

Values are mean ± S.D from 6 rats in each group. Statistical significance: \( p \leq 0.05 \). ¹ CCl₄ control differs significantly from normal control. ² Silymarin 100mg/kg + CCl₄, MEWF 100mg/kg + CCl₄, MEWF 200mg/kg + CCl₄ and MEWF 200mg/kg alone were significantly different CCl₄ control. ³ MEWF 200mg/kg + CCl₄ treated group differs significantly from MEWF 100mg/kg + CCl₄ treated group. ⁴ MEWF 200mg/kg alone treated group non significantly different from normal control.
Methanolic extract of *Woodfordia fruticosa* Kurz flowers ameliorates carbon tetrachloride induced chronic hepatic fibrosis in rats

![Graph C: ALKALINE PHOSPHATASE](image)

![Graph D: LACTATE DEHYDROGENASE](image)

Fig. 5.1. (Cont.) Changes in serum enzyme levels of rats pre and post-treated with MEWF

(C) Alkaline phosphatase  (D) Lactate dehydrogenase.

(I) Normal control, (II) CCl₄ control, (III) Silymarin (100 mg/kg) + CCl₄; (IV) MEWF (100 mg/kg) + CCl₄; (V) MEWF (200 mg/kg) + CCl₄; (V) MEWF (200mg/kg) alone.

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. a CCl₄ control differs significantly from normal control. b Silymarin 100mg/kg + CCl₄, MEWF 100mg/kg + CCl₄, MEWF 200mg/kg + CCl₄ and MEWF 200mg/kg alone were significantly different CCl₄ control. c MEWF 200mg/kg + CCl₄ treated group differs significantly from MEWF 100mg/kg + CCl₄ treated group. d MEWF 200mg/kg alone treated group non significantly different from normal control.
5.3.3.3. Glutathione reductase (GR) activity

GR activity was significantly decreased ($p \leq 0.05$) in CCl$_4$ treated animals when compared to control. Pre-treated with MEWF (100 mg/kg, 200 mg/kg) and silymarin (100 mg/kg) showed significant increase ($p \leq 0.05$) in the level of GR. The percentage of protection in liver was 90.8 for 200 mg/kg of methanolic extract. Silymarin restored the GR activity up to 59.1% in rat liver (Table 5.2).

5.3.3.4. Glutathione peroxidase (GPx) activity

In pre-treatment groups, GPx activity was significantly ($p \leq 0.05$) lowered in CCl$_4$ treated rats. MEWF treatment dose dependently prevented the lowering of GPx, MEWF at a dose of 200 mg/kg showed a protection of 89% and silymarin-treated rats prevented the lowering of GPx by 74.5% (Table 5.2).

5.3.3.5. Lipid peroxidation (MDA) level

A significant increase ($p \leq 0.05$) in tissue MDA level was observed in CCl$_4$ alone treated rats. The elevated levels of MDA concentration was lowered by 83% on treatment with MEWF at a dose of 200 mg/kg and silymarin showed a protection ($p \leq 0.05$) of 61.7% (Table 5.2).

5.3.3.6. Hydroxyproline content of liver tissue

Collagen content of liver tissue was estimated in terms of hydroxyproline. CCl$_4$ treatment dramatically increased the concentration of hydroxyproline as compared with normal control ($p \leq 0.05$). MEWF treatment (100 and 200 mg/kg) significantly reduced the elevation of hydroxyproline concentration in a dose dependent manner as compared with CCl$_4$ control group. In pretreatment groups the
Methanolic extract of *Woodfordia fruticosa* Kurz flowers ameliorates carbon tetrachloride induced chronic hepatic fibrosis in rats

decrease in hydroxyproline content induced by MEWF 200mg/kg was 97.4% and in silymarin it was 88.8% (Table 5.2).

### 5.3.4. Tissue antioxidants and other biochemical constituents of post-treated groups

#### 5.3.4.1. Reduced glutathione (GSH) level

In the post-treatment groups, rats treated with MEWF 100mg/kg and 200 mg/kg significantly ($p \leq 0.05$) restored the decreased glutathione levels (Table 5.3). In hepatic tissue, 80.8% reversal in GSH level shown by 200 mg/kg of MEWF was comparable with 64.3% exhibited by 100 mg/kg of silymarin.

### Table 5.2. Pre-treatment effect of MEWF on antioxidant status and hydroxyproline content of liver

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>CCl₄ control</th>
<th>Silymarin (100mg/kg) + CCl₄</th>
<th>MEWF (100mg/kg) + CCl₄</th>
<th>MEWF (200mg/kg) + CCl₄</th>
<th>MEWF 200mg/kg</th>
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</thead>
<tbody>
<tr>
<td>GSH¹</td>
<td>22.3±0.3</td>
<td>12.3±0.4a</td>
<td>19.25±0.47b</td>
<td>17.15±0.30b</td>
<td>20.8±0.36bc</td>
<td>22.8±0.53bd</td>
</tr>
<tr>
<td>GST²</td>
<td>70.16±1.0</td>
<td>30.46±0.5a</td>
<td>55.18±0.87b</td>
<td>46.23±1.13b</td>
<td>64.3±0.89bc</td>
<td>68.9±0.78bd</td>
</tr>
<tr>
<td>GR³</td>
<td>18.1±0.70</td>
<td>6.1±0.6a</td>
<td>13.2±0.60b</td>
<td>10.78±0.62b</td>
<td>17.05±0.6bc</td>
<td>17.4±0.54bd</td>
</tr>
<tr>
<td>GPx⁴</td>
<td>296.3±4.7</td>
<td>149.9±4.5a</td>
<td>259.1±3.4b</td>
<td>202.6±6.6b</td>
<td>280.3±4.9bc</td>
<td>293.1±3.9bd</td>
</tr>
<tr>
<td>MDA⁵</td>
<td>45.3±2.08</td>
<td>77.2±2.76a</td>
<td>57.5±2.58b</td>
<td>66.1±2.73b</td>
<td>50.7±4.2bc</td>
<td>47.9±2.7bd</td>
</tr>
<tr>
<td>Hydroxy Proline⁶</td>
<td>0.35±0.05</td>
<td>1.52±0.14a</td>
<td>0.48±0.05b</td>
<td>0.60±0.10b</td>
<td>0.38±0.06bc</td>
<td>0.34±0.08bd</td>
</tr>
</tbody>
</table>

¹(nmol/mg protein); ²(µmol CDNB-GSH conjugate formed/min/mg protein); ³(nmol of GSSG utilized/min/mg protein); ⁴(nmol of GSH oxidized/min/mg protein); ⁵(nmol/g tissue); ⁶(nmol/mg tissue).

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. a CCl₄ control differs significantly from normal control. b Silymarin 100mg/kg + CCl₄, MEWF 100mg/kg + CCl₄, MEWF 200mg/kg + CCl₄ and MEWF 200mg/kg alone were significantly different from CCl₄ control. c MEWF 200mg/kg + CCl₄ treated group differs significantly from MEWF 100mg/kg + CCl₄ treated group. d MEWF 200mg/kg alone treated group non significantly different from normal control.
5.3.4.2. Glutathione - S - transferase (GST) activity

Rats administered with CCl₄ alone showed significant ($p \leq 0.05$) reduction in hepatic GST level. Treatment with MEWF at a dose of 200 mg/kg showed significant reversal ($p \leq 0.05$) of GST level by 89%. Silymarin (100 mg/kg) also markedly ($p \leq 0.05$) inhibited the CCl₄ induced decrease in GST activity by 69.7%. (Table 5.3).

5.3.4.3. Glutathione reductase (GR) activity

In rats treated with CCl₄ alone, a significantly ($p \leq 0.05$) reduced GR activity was observed. In liver, 200 mg/kg of methanolic extract restored the GR activity by 93.6% and silymarin by 60% (Table 5.3).

5.3.4.4. Glutathione peroxidase (GPx) activity

In the post-treatment groups, rats treated with MEWF at a dose of 100 and 200 mg/kg, significantly ($p \leq 0.05$) restored the decreased GPx activity in liver (Table 5.3). Treatment with MEWF at a dose of 200mg/kg showed 93% reversal in GPx activity which was comparable with 75% exhibited by silymarin at a dose of 100 mg/kg.

5.3.4.5. Lipid peroxidation (MDA) level

In Table 5.3 a significant increase ($p \leq 0.05$) in tissue MDA level was shown in CCl₄ alone treated animals when compared to normal control. MEWF and silymarin significantly ($p \leq 0.05$) reversed the elevation of hepatic MDA formation. Treatment with MEWF at a dose of 200 mg/kg reinstated the MDA formation by 87.8% and silymarin exhibited 64.9% inhibition.
5.3.4.6. Hydroxyproline content

In post treatment, hydroxyproline content of liver tissue was significantly reduced in CCl₄ alone treated rats. The percentage protection induced by the treatment with MEWF at a dose of 200mg/kg was 98.1% and 91.8% in silymarin treated groups (Table 5.3). Hydroxyproline levels of MEWF alone treated group were not significantly different from normal control.

Table 5.3. Post-treatment effect of MEWF on antioxidant status and hydroxyproline content of liver

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>CCl₄ control</th>
<th>CCl₄+Silymarin (100mg/kg)</th>
<th>CCl₄+MEWF (100mg/kg)</th>
<th>CCl₄+MEWF (200mg/kg)</th>
<th>MEWF 200mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>20.8±1.1</td>
<td>13.5±0.82ᵃ</td>
<td>18.2±1.0ᵇ</td>
<td>16.7±0.9ᵇ</td>
<td>19.4±0.86ᵇᶜ</td>
<td>21.5±1.3ᵇᵈ</td>
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<tr>
<td>GST</td>
<td>72.06±2.2</td>
<td>33.7±0.95ᵃ</td>
<td>60.4±1.42ᵇ</td>
<td>49.5±1.13ᵇ</td>
<td>67.8±1.41ᵇᶜ</td>
<td>70.8±1.70ᵇᵈ</td>
</tr>
<tr>
<td>GR</td>
<td>18.5±0.8</td>
<td>7.5±0.79ᵃ</td>
<td>14.1±0.66ᵇ</td>
<td>12.0 ±0.58ᵇ</td>
<td>17.8±1.03ᵇᶜ</td>
<td>18.2±0.76ᵇᵈ</td>
</tr>
<tr>
<td>GPx</td>
<td>295.6±5.4</td>
<td>154.7±3.2ᵃ</td>
<td>260.5±3.8ᵇ</td>
<td>210.6±4.2ᵇ</td>
<td>285.8±4.9ᵇᶜ</td>
<td>292.8±5.0ᵇᵈ</td>
</tr>
<tr>
<td>MDA</td>
<td>43.5±1.92</td>
<td>75.7±4.1ᵃ</td>
<td>54.8±3.2ᵇ</td>
<td>63.5±2.9ᵇ</td>
<td>47.2±3.8ᵇᶜ</td>
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<tr>
<td>Hydroxyproline</td>
<td>0.37±0.08</td>
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<td>0.58±0.10ᵇ</td>
<td>0.39±0.08ᵇᶜ</td>
<td>0.36±0.05ᵇᵈ</td>
</tr>
</tbody>
</table>

¹(nmol/mg protein); ²(µmol CDNB-GSH conjugate formed/min/mg protein); ³(nmol of GSSG utilized/min/mg protein); ⁴(nmol of GSH oxidized/min/mg protein); ⁵(nmol/g tissue); ⁶(nmol/mg tissue).

Values are mean ± S.D from 6 rats in each group. Statistical significance: p ≤ 0.05. ᵃ CCl₄ control differs significantly from normal control. ᵇ CCl₄+Silymarin 100mg/kg, CCl₄+MEWF 100mg/kg, CCl₄+MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from CCl₄ control. ᶜ CCl₄+MEWF 200mg/kg treated group differs significantly from CCl₄+MEWF 100mg/kg treated group. ᵈ MEWF 200mg/kg alone treated group non significantly different from normal control.
5.3.5. Histopathology

CCl₄ control group had a high degree of fibrosis. The two treatment groups, both preventive and curative had significantly \( p \leq 0.05 \) improved histological scores (Fig. 5.2) compared to toxic control.

Fig. 5.2. Histological scores of CCl₄ treated rat livers and their improvement upon treatment with MEWF.

(I) Normal control; (II) CCl₄ control; (III) CCl₄ + silymarin (100mg/kg); (IV) CCl₄ + MEWF (100mg/kg); (V) CCl₄ + MEWF (200mg/kg); (VI) MEWF alone.

Values are mean ± S.D from 6 rats in each group. Statistical significance: \( p \leq 0.05 \).

- a CCl₄ control differs significantly from normal control.
- b Silymarin 100mg/kg + CCl₄, MEWF 100mg/kg + CCl₄, MEWF 200mg/kg + CCl₄ and MEWF 200mg/kg alone were significantly different from CCl₄ control.
- c MEWF 200mg/kg + CCl₄ treated group differs significantly from MEWF 100mg/kg + CCl₄ treated group.
- d MEWF 200mg/kg alone treated group non significantly different from normal control.
Comparing the effectiveness of MEWF in preventive (Fig. 5.3) and curative group (Fig. 5.4), the latter had more marked reduction in histological grading. Rats treated with MEWF alone were comparable with untreated normal control which had no adverse effect in liver tissue and in histological scores. These two groups were not significantly ($p \leq 0.05$) different from each other.

Fig. 5.3. Histopathological features of liver in pre-treated groups. Liver tissue was stained with H&E (100×).

(A) Normal rat liver (B) CCl$_4$ control (C) Silymarin (100 mg/kg) + CCl$_4$ (D) MEWF (100 mg/kg) + CCl$_4$ (E) MEWF (200 mg/kg) + CCl$_4$ (F) MEWF (200 mg/kg) alone.
Fig. 5.4. Histopathological features of liver in post- treatment groups. Liver tissue was stained with H&E (100×).

(A) Normal rat liver; (B) CCl₄ control; (C) CCl₄ + Silymarin (100 mg/kg); (D) CCl₄ + MEWF (100 mg/kg); (E) CCl₄ + MEWF (200 mg/kg); (F) MEWF (200 mg/kg) alone.
5.3.6. Immunohistochemical localization of Collagen-III

Immunohistochemical analysis showed that Collagen-III was predominantly expressed along fibrous septa in CCl₄ treated rats. Reduced level of Collagen-III in MEWF treated rats co-exposed with CCl₄ was detected in preventive (Fig. 5.5) and curative groups (Fig. 5.6).

Fig. 5.5. Immunohistochemical localization of Collagen-III in the preventive group. Liver tissue was immunostained for Collagen-III followed by staining with hematoxylin (100×).

(A) Normal rat liver; (B) CCl₄ control; (C) Silymarin (100 mg/kg) + CCl₄; (D) MEWF (100 mg/kg) + CCl₄; (E) MEWF (200 mg/kg) + CCl₄; (F) MEWF (200 mg/kg) alone.
Fig. 5.6. Immunohistochemical localization of Collagen-III in the reversal group. Liver tissue was immunostained for Collagen-III followed by staining with hematoxylin (100×).

(A) Normal rat liver; (B) CCl₄ control; (C) CCl₄ + Silymarin (100 mg/kg); (D) CCl₄ +MEWF (100 mg/kg); (E) CCl₄ +MEWF (200 mg/kg); (F) MEWF (200mg/kg) alone.
5.4. DISCUSSION

The CCl\textsubscript{4} treated rats is frequently used as an experimental model to study hepatic fibrosis (Inao et al., 2004; Wang et al 2012; Wills and Asha 2006b) CCl\textsubscript{4} intoxication generates free radicals that trigger a cascade of events resulting in hepatic fibrosis (Bissel and Maher, 1996). CCl\textsubscript{4} being metabolized by cytochrome P450, leading to the production of trichloromethyl radicals (CCl\textsubscript{3}’) and reactive oxygen species (ROS). (Recknagel et al., 1989). Generally, administration of CCl\textsubscript{4} over a 4 week period induces early fibrosis, over an 8 week period causes cirrhosis, and over a 12-week period leads to micro-nodular cirrhosis (Natarajan et al., 2006). The 10-week CCl\textsubscript{4} treatment induced classical advanced fibrosis or early cirrhosis characteristics such as shrunken liver with nodular surface.

In the present study, treatment with CCl\textsubscript{4} for 10 weeks results in a significant decrease ($p \leq 0.05$) in body weight and liver weight and small fibrotic nodules were observed in morphological analysis of liver. The evidence of liver dysfunction was reflected by elevated levels of serum AST, ALT, ALP, LDH and tissue GSH, GST, GR, GPx and MDA. Pre and post-treatment with MEWF effectively and dose dependently prevented or reversed the weight loss of body and liver, lowered the elevated levels of serum AST, ALT, ALP, LDH and tissue GSH, GST, GR, GPx and MDA. Generation of a large amount of ROS due to CCl\textsubscript{4} intoxication can overwhelm the antioxidant defense mechanism and damage cellular ingredients such as lipids, proteins and DNA; this in turn can impair cellular structure and function.

Continuous accumulation of extracellular matrix (ECM) proteins results in hepatic fibrosis; collagen is the main component of the ECM in fibrotic tissue
(Friedman, 2008). Hydroxyproline, which is a major component of collagen, was used as an indicator for evaluating the extent of liver fibrosis (Toyoki et al., 1998). In the present study, CCl$_4$ induced fibrotic rat liver exhibited a marked increase in ECM content as evidenced by hydroxyproline levels, histologically and immunohistochemically displayed accumulation of collagen surrounding the central vein with larger fibrous septa. Treatment with MEWF at 200mg/kg remarkably prevented or reversed the elevated hydroxyproline content. High degree of fibrosis evidenced by histopathological analysis and over expression of collagen III. MEWF treatment decreased the severe hepatocyte necrosis, lymphocyte infiltration, formation of portal to portal septa, and fatty infiltration.

The hepatic stellate cells (HSC) or lipocytes lying beneath the endothelial layer were usually quiescent with a low proliferation rate. During liver injury, these lipocytes gets activated and transform into myofibroblasts cells with high proliferative capacity. HSC activation plays a critical role in the process of hepatic fibrogenesis (Kisseleva and Brenner, 2008). Most of the antifibrotic herbal drugs reverses fibrosis by increased removal of deposited collagen, enhanced collagenolytic activity and enhanced apoptosis of hepatic stellate cells (Luo et al., 2004).

Preliminary phytochemical analysis of MEWF revealed the presence of saponins (steroids and terpenes), phenolics, alkaloids, flavanoids, tannins etc. The phytochemical profiling of MEWF by LC-MS analysis also already revealed the presence of phytochemicals with potent antioxidant/hepatoprotective/chemopreventive properties. The reported antioxidant/hepaoprotective activities of these phytochemicals were established by various *in vitro* and/or *in vivo* studies. It
Methanolic extract of *Woodfordia fruticosa* Kurz flowers ameliorates carbon tetrachloride induced chronic hepatic fibrosis in rats

includes ellagic acid (Das et al., 2007), stigmasterol (Al-Qarawi et al., 2004), quercetin methyl ether (Wei et al., 2001) and Octacosanol (Oliveira et al., 2012). The identified class of components with liver protective efficacy might also be responsible for the antifibrotic activity.

These results indicated that treatment with MEWF reduces the effects of chronic liver fibrosis by promoting extracellular matrix degradation and effectively reverses hepatic fibrosis after the establishment in rats through suppression of HSC activation and increased removal of collagen by enhanced collagenolytic activity. The hepatic fibrosis or cirrhosis may sometimes leads to hepatocellular carcinoma. Many of the compounds identified in MEWF have potent antioxidant, chemopreventive and anticancer properties. So the components in single or in combination with other components present in the extract might also be responsible for the antifibrotic activity. This finding might provide a pharmacological background on the traditional use of the plant for the treatment of liver diseases.