CHAPTER 4

PREVENTIVE AND CURATIVE EFFECT OF WOODFORDIA FRUTICOSA KURZ FLOWERS ON THIOACETAMIDE INDUCED OXIDATIVE STRESS IN RATS
4.1. INTRODUCTION

Oxidative stress plays an important role in many diseases including those of the liver and it can be controlled by the antioxidant systems in living organisms (Ravikumar and Gnanadesigan, 2011). Human systems possess enzymatic and non-enzymatic antioxidative mechanisms which minimizes the generation of reactive oxygen species (Sinha et al., 2010). When the generation of the active oxygen-free radical exceeds the scavenging ability many degenerative diseases such as brain dysfunction, cancer, heart diseases, age-related degenerative conditions, declination of the immune system, gastric ulcer and DNA damage will arise. Natural antioxidants present in fruits, vegetables, cereals and medicinal plants act as effective free radical scavengers, by donating hydrogen to highly reactive radicals. Studies reveal that increased consumption of fruits rich in antioxidant polyphenols lower the risk of degenerative diseases (Patel et al., 2011). Recently, interest in finding naturally occurring antioxidants has increased considerably to replace synthetic antioxidants such as butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) which are now used in drug composition. In food industry, the attention of manufactures has been shifted from synthetic to natural antioxidants due to their side effects.

Herbal drugs are playing an important role in health care programmes worldwide, mainly due to the general belief that they are without any side effects besides being cheap and locally available. Lately there is resurgence of interest in herbal medicine for treatment of various ailments including liver disorders. In India, about 40 polyherbal commercial formulations with hepatoprotective action are being used. Hepatoprotective herbal drugs contain a variety of chemical constituents like...
phenols, coumarins, lignins, essential oils, monoterpenes, carotenoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes (Eesha et al., 2011). Our previous study revealed the in vitro antioxidant property exhibited by the methanolic extract of *Woodfordia fruticosa*. Phytochemical profiling (LC-MS) of MEWF identified the presence of compounds with antioxidant and hepatoprotective activity such as ellagic acid, quercetin methyl ether, stigmasterol etc. In view of this the present study was undertaken to evaluate the preventive and curative effect of MEWF against thioacetamide induced oxidative stress in experimental animals.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Thioacetamide (TAA) was purchased from Loba Cheme, Mumbai, India. Assay kits for serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase were purchased from Agappe Diagnostic, India. All other chemicals were of analytical grade.

4.2.2. Animals and diets

Male Wistar rats weighing 150-160 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.
4.2.3. Preparation of plant extract

A 50 g of dried powder was soxhlet extracted with 400 ml of methanol for 48 h. 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 in vivo studies and stored at -20°C.

4.2.4. Preparation of doses and treatments

Thioacetamide suspended in normal saline was administered (100 mg/kg body weight) subcutaneously to induce the oxidative stress in rats (Ahmad et al., 1999). 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. It is reported that the extracts of *W. fruticosa* flowers are safe with LD$_{50}$ more than 2000mg/kg, p.o (Chandan et al., 2008).

4.2.5. Experimental design of pre-treatment evaluation (Roy et al., 2006)

Male Wistar rats weighing 155 ± 5.2 gm (Mean ± S.D, n = 30) were used in this study. Rats were divided into five groups with six rats in each group and that were treated as follows:

- **Group I**: Control rats (vehicle only)
- **Group II**: Thioacetamide control (100 mg/kg, s.c.)
- **Group III**: Thioacetamide (as in group II) + Silymarin (100 mg/kg, p.o.)
- **Group IV**: Thioacetamide (as in group II) + MEWF (100 mg/kg, p.o.)
- **Group V**: Thioacetamide (as in group II) + MEWF (200 mg/kg, p.o.)
All the groups except group I received a single dose of thioacetamide (100 mg/kg; s.c) suspended in normal saline on 9th day of the experiment. Nine days before the thioacetamide challenge, group III, IV and V rats received 100 mg/kg silymarin, 100 mg/kg MEWF and 200 mg/kg MEWF respectively. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of drug and thioacetamide respectively. All the animals were sacrificed 24 h after thioacetamide administration.

4.2.6. **Experimental design of post-treatment evaluation** (Ahmad et al., 2002)

Male Wistar rats weighing 156 ± 4.2 gm (Mean ± S.D, n = 30) were used in this study. Rats were divided into five groups with six rats in each group and that were treated as follows:

- **Group I**: Control rats (vehicle only)
- **Group II**: Thioacetamide control (100 mg/kg, s.c.)
- **Group III**: Thioacetamide (as in group II) + Silymarin (100 mg/kg, p.o.)
- **Group IV**: Thioacetamide (as in group II) + MEWF (100 mg/kg, p.o.)
- **Group V**: Thioacetamide (as in group II) + MEWF (200 mg/kg, p.o.)

All the groups except group I received a single dose of thioacetamide (100 mg/kg; s.c) on 1st day of the experiment. Groups III–V received silymarin and MEWF 2, 24 and 48 h after thioacetamide challenge. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of drug and thioacetamide respectively. All the animals were sacrificed 72 h after thioacetamide administration.
4.2.7. Serum enzyme analysis

Hepatotoxicity was assessed by quantifying the serum levels 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 auto analyzer (RMS, India).

4.2.8. Tissue analysis

Liver and kidney were excised, washed thoroughly in ice-cold saline to remove the blood. Ten percent of homogenate was prepared in 0.1M Tris HCl buffer (pH – 7.4) (Ilavarasan et al., 2003). 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), catalase (CAT), 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 decrease in GSH content after incubating the sample in the presence of H2O2 and NaN3 (Rotruck et al., 1973). CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H2O2 (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)

Percent protection was calculated using the formula,

\[
\text{Percentage protection} = \frac{\text{Toxic control} - \text{Extract treated}}{\text{Toxic control} - \text{Normal control}} \times 100
\]

4.2.9. Histopathological studies

Small pieces of liver tissues fixed in 10% neutral buffered formalin were processed for embedding in paraffin. Sections of 5–6 \(\mu\)m were taken and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam 1000 camera at original magnification of 100\(\times\).

Liver sections were graded numerically to assess the degree of histological features. Acute hepatic injury is indicated by centrilobular necrosis, bridging hepatic necrosis and lymphocyte infiltration. Centrilobular necrosis is the necrosis around the central vein characterized by prominent ballooning and swollen granular cytoplasm with fading nuclei. Bridging hepatic necrosis is a form of confluent necrosis of liver cells linking central veins to portal tracts or portal tracts to one another (Wills and Asha, 2006a). A combined score of centrilobular necrosis, bridging hepatic necrosis and lymphocyte infiltration was given a maximum value of 6 and descriptive modifiers such as mild, moderate, and severe was applied to activity and staging. The
parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1 to 2 indicating mild injury, 3 to 4 indicating moderate injury and 5 to 6 representing severe liver injury.

4.2.10. 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.

4.3. RESULTS

4.3.1. Effect of TAA and MEWF on serum marker enzymes

4.3.1.1. 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 a single dose of TAA, when compared to normal control. The treatment with MEWF at a dose of 100 and 200 mg/kg showed a significant decrease \( (p \leq 0.05) \) in AST, ALT, ALP and LDH levels. Standard control drug, silymarin at a dose of 100 mg/kg also prevented the elevation of serum enzymes. Treatment with 200 mg/kg methanolic extract and silymarin exhibited a protection of 96.5% and 72.3% in AST levels, 97.5% and 67.8% in ALT levels, 98.2% and 62.2% in ALP levels and 90.5% and 68.4% in LDH levels respectively. The preventive effect of the extract in decreasing the elevated levels of serum enzymes was in a dose dependent manner. The results are graphically depicted in Figure 4.1 (A, B, C and D)
4.3.1.2. Post-treatment evaluation

There was a significant \((p \leq 0.05)\) rise in the serum levels of AST, ALT, ALP and LDH after TAA administration in post-treated animals. In contrast, treatment with MEWF (100mg/kg and 200 mg/kg) and silymarin (100mg/kg) exhibited an ability to counteract the TAA induced hepatotoxicity by decreasing the serum enzymes levels \((p \leq 0.05)\) compared to toxic control. 200 mg/kg of methanolic extract showed a protection of 96.8%, 98.0%, 98.6% and 91.5% for AST, ALT, ALP and LDH respectively. Silymarin also showed a remarkable protection of 81.9%, 80.4%, 70.2% and 79.4% for AST, ALT, ALP and LDH respectively towards TAA intoxication \((p \leq 0.05)\). Graphical representations of the results are shown in Figure 4.1 (A, B, C and D).

![Graph showing the effect of MEWF and silymarin on changes in serum enzyme levels of rats pre and post-treated with TAA.](image)

**Fig. 4.1. Effect of MEWF and silymarin on changes in serum enzyme levels of rats pre and post-treated with TAA.**

**A** (A) Aspartate aminotransferase

(I) Normal control, (II) Thioacetamide control, (III) Silymarin, (IV) MEWF – 100 mg/kg, (V) MEWF – 200 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, \(n = 6\) animals. † \(p \leq 0.05\) vs. normal control. *\(p \leq 0.05\) vs. thioacetamide control.
Fig. 4.1. (Cont.) Effect of MEWF and silymarin on changes in serum enzyme levels of rats pre and post-treated with TAA.

(B) Alanine aminotransferase (C) Alkaline phosphatase

(I) Normal control, (II) Thioacetamide control, (III) Silymarin, (IV) MEWF – 100 mg/kg, (V) MEWF – 200 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † \( p \leq 0.05 \) vs. normal control. *\( p \leq 0.05 \) vs. thioacetamide control.
Fig. 4.1. (Cont.) Effect of MEWF and silymarin on changes in serum enzyme levels of rats pre and post-treated with TAA.

(D) Lactate dehydrogenase.

(I) Normal control, (II) Thioacetamide control, (III) Silymarin, (IV) MEWF – 100 mg/kg, (V) MEWF – 200 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 vs. normal control. *p ≤ 0.05 vs. thioacetamide control.

4.3.2. Pre-treatment effect of MEWF on TAA induced changes in the antioxidant status of hepatic and renal tissues

The protective effects of MEWF against TAA induced changes in the liver and kidney antioxidant status are shown in Table 4.1 and 4.2 respectively

4.3.2.1. Reduced glutathione (GSH) level

In the pre-treatment groups, rats administered with TAA alone were found significantly (p ≤ 0.05) lowered level of reduced glutathione (GSH). Treatment with MEWF exhibited significant increase (p ≤ 0.05) in both hepatic and renal glutathione levels. In liver and kidney, 200 mg/kg of MEWF showed a protection of
87.5% and 92.3% respectively. Silymarin-treated rats also showed considerable prevention of GSH and the percentage of protection was 65.6 and 74.7 respectively in liver and kidney.

**4.3.2.2. Glutathione - S - transferase (GST) activity**

When compared to normal control the GST activity of liver and kidney tissues were significantly ($p \leq 0.05$) reduced in TAA intoxicated rats. The MEWF dose dependently increased ($p \leq 0.05$) the activity of GST in both the hepatic and renal tissues. Treatment with 200 mg/kg methanolic extract exhibited a significant increase i.e., 92.7 and 97%, respectively in hepatic and renal tissues. In addition, silymarin treated rats also prevented the TAA induced decrease in GST activity by 69.5 and 76.4% in hepatic and renal tissues respectively.

**4.3.2.3. Glutathione reductase (GR) activity**

GR activity was significantly decreased ($p \leq 0.05$) in TAA treated animals when compared to control in the pre-treatment groups. A significant increase ($p \leq 0.05$) in the level of GR was observed in MEWF (100 and 200 mg/kg) and silymarin (100 mg/kg) treated rats intoxicated with TAA. Both hepatic and renal tissues showed the same pattern of GR activity in all groups treated with MEWF and silymarin. The percentage of protection in liver and kidney were 86.9 and 94.8 respectively for 200 mg/kg of methanolic extract. Silymarin restored the GR activity upto 66.6% in liver and 69.3% in kidney.

**4.3.2.4. Glutathione Peroxidase (GPx) activity**

Activities of hepatic and renal GPx in pre-treatment groups were significantly ($p \leq 0.05$) lowered in TAA treated rats. MEWF dose dependently
prevented the lowering of GPx in both the organs compared to TAA alone treated groups. In liver and kidney, 200 mg/kg of methanolic extract showed a protection of 89.6% and 91.1% respectively. Silymarin-treated rats also prevented the lowering of GPx by 76.8% in hepatic and 87.4% in renal tissues.

4.3.2.5. Catalase (CAT) activity

The CAT activity in liver and kidney showed a significant \( (p \leq 0.05) \) reduction in TAA intoxicated rats. MEWF dose dependently increased the activity of CAT in both hepatic and renal tissues. Treatment with 200 mg/kg methanolic extract exhibited significant increase i.e., 90.5% and 83.8%, respectively in liver and kidney. In addition, silymarin treated rats also prevented \( (p \leq 0.05) \) the TAA induced decrease in CAT activity by 70.3 and 72.5% in hepatic and renal tissues respectively.

4.3.2.6. Lipid peroxidation (MDA) level

A significant increase \( (p \leq 0.05) \) in tissue MDA level was observed in TAA alone treated rats. However, TAA induced elevation of MDA concentration was lowered \( (p \leq 0.05) \) by 92.3% in hepatic and 88.4% in renal tissues of rats treated with MEWF at a dose of 200 mg/kg. Silymarin also showed a protection \( (p \leq 0.05) \) of 72.3% in liver and 67.6% in kidney.
Table 4.1. Pre-treatment (Protective) effects of MEWF against TAA induced changes in the liver antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) + TAA</th>
<th>MEWF (100mg/kg) + TAA</th>
<th>MEWF (200mg/kg) + TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>23.5 ± 0.6</td>
<td>13.9 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>20.2 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>17.2 ± 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>22.3 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST&lt;sup&gt;2&lt;/sup&gt;</td>
<td>68.5 ± 0.7</td>
<td>32.4 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>57.5 ± 0.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>43.5 ± 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>65.9 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>17.3 ± 0.5</td>
<td>6.2 ± 0.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>13.6 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11.6 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.2 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx&lt;sup&gt;4&lt;/sup&gt;</td>
<td>291.4 ± 6.2</td>
<td>167.2 ± 5.9&lt;sup&gt;†&lt;/sup&gt;</td>
<td>262.6 ± 7.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>210.5 ± 6.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>278.5 ± 5.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT&lt;sup&gt;5&lt;/sup&gt;</td>
<td>49.8 ± 2.3</td>
<td>31.9 ± 1.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>44.5 ± 1.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>38.7 ± 1.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>48.1 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>46.2 ± 0.7</td>
<td>78.7 ± 1.1&lt;sup&gt;†&lt;/sup&gt;</td>
<td>55.2 ± 0.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>64.8 ± 1.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>48.7 ± 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
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Table 4.2. Pre-treatment (Protective) effects of MEWF against TAA induced changes in the kidney antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) + TAA</th>
<th>MEWF (100mg/kg) + TAA</th>
<th>MEWF (200mg/kg) + TAA</th>
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<tr>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>17.5 ± 0.6</td>
<td>8.4 ± 0.3&lt;sup&gt;†&lt;/sup&gt;</td>
<td>15.2 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12.7 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.8 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST&lt;sup&gt;2&lt;/sup&gt;</td>
<td>48.6 ± 0.7</td>
<td>25.2 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>43.1 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>38.6 ± 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47.9 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>15.4 ± 0.3</td>
<td>5.6 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>12.4 ± 0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.2 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>14.9 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx&lt;sup&gt;4&lt;/sup&gt;</td>
<td>278.4 ± 7.1</td>
<td>152.3 ± 6.3&lt;sup&gt;†&lt;/sup&gt;</td>
<td>243.5 ± 4.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>189.5 ± 6.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>267.3 ± 4.8&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT&lt;sup&gt;5&lt;/sup&gt;</td>
<td>55.7 ± 0.9</td>
<td>41.5 ± 1.2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>51.8 ± 1.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47.2 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>53.4 ± 0.7&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>41.8 ± 0.3</td>
<td>74.6 ± 0.8&lt;sup&gt;†&lt;/sup&gt;</td>
<td>52.4 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>61.1 ± 0.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>45.6 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
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<sup>1</sup>(nmol/mg protein); <sup>2</sup>(µmol CDNB-GSH conjugate formed/min/mg protein); <sup>3</sup>(nmol of GSSG utilized/min/mg protein); <sup>4</sup>(nmol of GSH oxidized/min/mg protein); <sup>5</sup>(U/mg protein); <sup>6</sup>(nmol/g tissue).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: <i>p</i> ≤ 0.05. <sup>†</sup> TAA group differs significantly from normal control group. <sup>*</sup> Silymarin (100mg/kg) + TAA, MEWF–100 mg/kg + TAA and MEWF–200 mg/kg + TAA groups differ significantly from TAA alone treated group.
4.3.3. Histopathological analysis of pre-treated groups

Normal architecture of liver (Fig. 4.2 A) was completely lost in rats treated with TAA (Fig. 4.2 B) with the appearance of centrilocular necrosis, bridging hepatic necrosis and lymphocyte infiltration with a score of 5.4 ± 0.4 (mean ± S.D.; \( n=3 \)). The animals administered with silymarin and MEWF at 100 and 200 mg/kg showed a significant (\( p \leq 0.05 \)) protection from TAA induced liver damage as evident from hepatic architectural pattern with mild to moderate hepatitis with scores 2.4 ± 0.6; 2.8 ± 1.0; and 2.0 ± 0.5 (mean ± S.D.; \( n=3 \); \( p \leq 0.05 \)), respectively (Fig. 4.2.C–E).

4.3.4. Post-treatment effects of MEWF on TAA induced changes in the antioxidant status of hepatic and renal tissues

The curative effects of MEWF against TAA induced changes in the liver and kidney antioxidant status are shown in Table 4.3 and 4.4 respectively.

4.3.4.1. Reduced glutathione (GSH) level

When compared to TAA alone treated rats, post-treatment with MEWF (100 and 200 mg/kg) and silymarin significantly (\( p \leq 0.05 \)) restored the decreased glutathione levels in liver and kidney. In hepatic tissue, 82.3% reversal in GSH level shown by 200 mg/kg of methanolic extract was comparable with 46% exhibited by 100 mg/kg of silymarin. In renal tissue, 200 mg/kg of methanolic extract and 100 mg/kg of silymarin restored the GSH level by 85.2 and 57.8% respectively.

4.3.4.2. Glutathione - S- transferase (GST) activity

Rats administered with TAA alone showed significant (\( p \leq 0.05 \)) reduction in hepatic and renal GST level. Treatment with MEWF (100 and 200 mg/kg) showed
significant reversal ($p \leq 0.05$) of TAA induced toxicity. Silymarin (100 mg/kg) also markedly ($p \leq 0.05$) inhibited the TAA induced decrease in GST activity. Rats treated with 200 mg/kg methanolic extract and 100 mg/kg silymarin restored the decrease of GST levels by 87.8 and 63.4% in the liver and 89.6 and 62.6% in the kidney respectively (Table 4.3 and 4.4).

Fig. 4.2. Histopathological changes occurred in rat liver due to pre-treatment with MEWF (hematoxylin and eosin, 100×).

(A) Normal control; (B) TAA control, (100 mg/kg s.c.); (C) Silymarin (100 mg/kg) + TAA; (D) MEWF (100 mg/kg) + TAA; (E) MEWF (200 mg/kg) + TAA.
4.3.4.3. Glutathione reductase (GR) activity

Glutathione reductase activity was significantly ($p \leq 0.05$) reduced in TAA control group. Treatment with MEWF exhibited significant increase ($p \leq 0.05$) in both hepatic and renal GR activity. In liver and kidney, 200 mg/kg of MEWF restored the activity of GR by 91.8% and 93.8% respectively. Silymarin-treated rats also restored GR activity by 60.9% in liver and 69.3% in kidney.

4.3.4.4. Glutathione peroxidase (GPx) activity

Compared to TAA alone treated animals, rats treated with MEWF significantly ($p \leq 0.05$) restored the decreased GPx activity in liver and kidney. In hepatic tissue, 94.7% reversal in GPx activity shown by 200 mg/kg of MEWF was comparable with 73.1% exhibited by 100 mg/kg of silymarin. In renal tissue, 200 mg/kg of MEWF and 100 mg/kg of silymarin reinstated the GPx activity by 92.7% and 74.6% respectively.

4.3.4.5. Catalase (CAT) activity

Animals injected with TAA alone showed significant ($p \leq 0.05$) reduction in hepatic and renal CAT activity in post-treatment groups. Treatment with MEWF (100 mg/kg and 200 mg/kg) showed significant reversal ($p \leq 0.05$) of TAA induced hepatotoxicity. Silymarin (100 mg/kg) also markedly ($p \leq 0.05$) restored the TAA induced decrease in CAT activity. Rats treated with 200 mg/kg methanolic extract and 100 mg/kg silymarin restored the decrease of CAT levels by 88.5 and 54.1% in the liver and 88.7 and 56.8% in the kidney respectively.
4.3.4.6. Lipid peroxidation (MDA) level

A significant increase ($p \leq 0.05$) in tissue MDA level was shown in TAA alone treated animals when compared to normal control. MEWF and silymarin significantly ($p \leq 0.05$) reversed the elevation of hepatic and renal MDA formation. MEWF at 200 mg/kg reinstated the MDA formation by 90.8% in hepatic tissue and 89.1% in renal tissue. Silymarin exhibited 67.7 and 71.7% inhibition in MDA formation in liver and kidney respectively.

4.3.5. Histopathological analysis of post-treated groups

In rats treated with TAA, the normal architecture of liver was completely lost with the appearance of centrilobular necrosis, bridging necrosis and lymphocyte infiltration scoring $5.2 \pm 0.8$ (mean $\pm$ S.D.; $n=3$) (Figure 4.3 A and B). Rats treated with silymarin and MEWF (100 and 200 mg/kg) after the establishment of toxic injury showed recovery from centrilobular necrosis, bridging necrosis and lymphocyte infiltration with scores $1.8 \pm 0.6$; $2.2 \pm 0.4$ and $1.1 \pm 0.5$ (Mean $\pm$ S.D.; $n=3$; $p \leq 0.05$), respectively (Fig. 4.3 C–E).
Table 4.3. Post-treatment (Curative) effects of MEWF against TAA induced changes in the liver antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>TAA + Silymarin (100mg/kg)</th>
<th>TAA + MEWF (100mg/kg)</th>
<th>TAA + MEWF (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>24.7 ± 0.6</td>
<td>14.5 ± 0.4(\dagger)</td>
<td>19.2 ± 0.7(*)</td>
<td>17.3 ± 0.5(*)</td>
<td>22.9 ± 0.4(*)</td>
</tr>
<tr>
<td>GST(^2)</td>
<td>73.7 ± 0.3</td>
<td>36.8 ± 0.6(\dagger)</td>
<td>60.5 ± 0.5(*)</td>
<td>57.6 ± 0.8(*)</td>
<td>69.2 ± 0.4(*)</td>
</tr>
<tr>
<td>GR(^3)</td>
<td>20.6 ± 0.5</td>
<td>8.3 ± 0.4(\dagger)</td>
<td>15.8 ± 0.8(*)</td>
<td>12.9 ± 0.6(*)</td>
<td>19.6 ± 0.5(*)</td>
</tr>
<tr>
<td>GPx(^4)</td>
<td>284.7 ± 6.2</td>
<td>169.6 ± 7.5(\dagger)</td>
<td>253.8 ± 5.7(*)</td>
<td>214.1 ± 5.9(*)</td>
<td>278.6 ± 4.8(*)</td>
</tr>
<tr>
<td>CAT(^5)</td>
<td>48.9 ± 1.8</td>
<td>35.8 ± 1.2(\dagger)</td>
<td>42.9 ± 1.5(*)</td>
<td>38.6 ± 1.6(*)</td>
<td>47.4 ± 1.3(*)</td>
</tr>
<tr>
<td>MDA(^6)</td>
<td>47.5 ± 1.2</td>
<td>72.6 ± 0.8(\dagger)</td>
<td>55.6 ± 0.8(*)</td>
<td>61.3 ± 1.4(*)</td>
<td>49.8 ± 0.6(*)</td>
</tr>
</tbody>
</table>

Table 4.4. Post-treatment (Curative) effects of MEWF against TAA induced changes in the kidney antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>TAA + Silymarin (100mg/kg)</th>
<th>TAA + MEWF (100mg/kg)</th>
<th>TAA + MEWF (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>18.6 ± 0.5</td>
<td>9.1 ± 0.4(\dagger)</td>
<td>14.6 ± 0.7(*)</td>
<td>12.8 ± 0.8(*)</td>
<td>17.2 ± 0.6(*)</td>
</tr>
<tr>
<td>GST(^2)</td>
<td>47.8 ± 0.8</td>
<td>28.5 ± 0.6(\dagger)</td>
<td>40.6 ± 1.1(*)</td>
<td>36.4 ± 0.9(*)</td>
<td>45.8 ± 0.5(*)</td>
</tr>
<tr>
<td>GR(^3)</td>
<td>17.1 ± 0.4</td>
<td>7.3 ± 0.6(\dagger)</td>
<td>14.1 ± 0.5(*)</td>
<td>11.6 ± 0.4(*)</td>
<td>16.5 ± 0.3(*)</td>
</tr>
<tr>
<td>GPx(^4)</td>
<td>274.7 ± 6.0</td>
<td>169.6 ± 4.8(\dagger)</td>
<td>248.1 ± 5.4(*)</td>
<td>211.4 ± 6.2(*)</td>
<td>267.5 ± 4.5(*)</td>
</tr>
<tr>
<td>CAT(^5)</td>
<td>56.4 ± 1.3</td>
<td>44.8 ± 1.5(\dagger)</td>
<td>51.4 ± 0.9(*)</td>
<td>48.2 ± 1.4(*)</td>
<td>55.1 ± 1.1(*)</td>
</tr>
<tr>
<td>MDA(^6)</td>
<td>42.3 ± 0.7</td>
<td>70.8 ± 0.8(\dagger)</td>
<td>52.5 ± 0.5(*)</td>
<td>59.7 ± 0.8(*)</td>
<td>45.4 ± 0.6(*)</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); \(^5\) (U/mg protein); \(^6\) (nmol/g tissue).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: \(p \leq 0.05\). \(\dagger\) TAA group differs significantly from normal control group. \(*) TAA + Silymarin (100mg/kg), TAA + MEWF (100 mg/kg) and TAA + MEWF (200 mg/kg) groups differ significantly from TAA alone treated group.
Fig. 4.3. Histopathological changes occurred in rat liver due to post-treatment with MEWF (hematoxylin and eosin, 100×).

(A) Normal control; (B) TAA control, (100 mg/kg s.c.); (C) TAA + Silymarin (100 mg/kg); (D) TAA + MEWF (100 mg/kg); (E) TAA + MEWF (200 mg/kg).
4.4. DISCUSSION

The present study demonstrated that the methanolic extract of _Woodfordia fruticosa_ flower exhibited therapeutic effects on oxidative stress and liver damage induced by TAA exposure in rats. Thioacetamide (TAA) is a compound endowed with liver damaging and carcinogenic activity. It has been used as a model to induce acute liver injury in rats (Gupta and Dixit, 2009). TAA undergoes a two step bioactivation mediated by microsomal cytochrome P450 2E1 (CYP2E1) to thioacetamide sulfoxide (TASO) and further to thioacetamide S,S-dioxide (TASO$_2$). TASO$_2$, an unstable reactive metabolite, covalently binds to liver macromolecules is responsible for the changes of hepatocytes such as an increase in nuclear volume and enlargement of nucleoli, cell permeability changes, rise in intracellular concentration of Ca++, and effects on mitochondrial activity, which leads to cell death (Bautista et al., 2010).

In this study, administration of a single dose of TAA (100 mg/kg body weight) led to hepatic and renal damage, which has been proven by the significant difference in biochemical markers between the TAA control and normal control groups. The increase in the activities of AST, ALT, ALP and LDH in serum level of rats treated with TAA might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Atif M. Al-Attar, 2011) and this showed the stress condition of the TAA treated animals. In preventive and curative models a marked reduction of serum AST, ALT, ALP and LDH levels were observed in rats treated with MEWF. The extract at 200mg/kg produced better results than 100mg/kg, shows the dose response action of
the extract. These results demonstrate the preventive and curative effect of MEWF against TAA intoxication.

Generation of a large amount of ROS due to TAA 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. The intracellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants like GSH. CAT, GST, GPx, and GR constitute the first line of cellular antioxidant defense enzymes. When excess free radicals are produced, the equilibrium is lost and consequently oxidative insult is established (Manna et al., 2007).

Glutathione detoxifies toxic metabolites of drugs, regulates gene expression, apoptosis and transmembrane transport of organic solutes and it is essential to maintain the reduced status of the cell/tissue. Hepatic and renal damage induced by TAA administration is associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH, GST, GR, GPx and CAT activities. In the present study, treatment with MEWF and silymarin significantly \((p \leq 0.05)\) enhanced the hepatic and renal GSH, GST, GR, GPx and CAT level compared to the TAA alone treated animals. This could explain the dose dependent (200 and 100mg/kg) preventive and curative action of the extract. Pre and post-treatment with MEWF significantly \((p \leq 0.05)\) enhanced the GST activity, may be due to the decreased bioactivation of TAA. GST offers protection against lipid peroxidation by promoting the conjugation of toxic electrophiles with GSH (Jakoby, 1988). GR is also essential for the maintenance of GSH levels \textit{in vivo}. The significant \((p \leq 0.05)\)
restoration of GPx activity in MEWF and silymarin in pre and post-treated rats might be due to the antioxidant activity by detoxifying the endogenous metabolic peroxides generated after TAA injury in hepatic and renal tissues.

Catalase is responsible for the breakdown of $\text{H}_2\text{O}_2$, an important ROS, formed during the reaction catalyzed by SOD (Ramanathan et al., 2002). Reduced activity of CAT after exposure to TAA in the present finding could be correlated to increased generation of $\text{H}_2\text{O}_2$. The pre and post-treatment of MEWF significantly ($p \leq 0.05$) aided to maintain the CAT activity near to normal level in both hepatic and renal tissues. This evidently shows the antioxidant property of the extract against oxygen free radicals.

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Celik et al., 2009). The concentration of MDA in tissues of TAA alone exposed group was significantly ($p \leq 0.05$) differed from that of normal control. Pre and post-treatment of rats with MEWF protected the liver and kidney from increased MDA formation. This demonstrates the antilipid peroxidative effect of the extract. Histopathological evaluation showed negligible damage to a few hepatocytes present in the close vicinity of central vein in MEWF treated rats and the improvement of histological scores proved the efficacy of the extract as an antihepatotoxic agent.

LC-MS analysis of MEWF already revealed the presence of phytochemicals with potent antioxidant activities. The reported antioxidant/hepaoprotective activities of these phytochemicals were established by various in vitro and/or in vivo studies. It includes ellagic acid (Das et al., 2007), stigmasterol (Al-Qarawi et al., 2004),
Preventive and curative effect of *Woodfordia fruticosa* Kurz flowers on thioacetamide induced oxidative stress in rats.

quercetin methyl ether (Wei et al., 2001) and Octacosanol (Oliveira et al., 2012).

The identified class of components in single or in combination with other components present in the extract might be responsible for the antihepatotoxic activity in both the treatment groups. The possible mechanism behind the antioxidant and hepatoprotective property of MEWF may be associated with stimulation of antioxidant defense system against the free radicals generated by TAA or by the inhibition of cytochrome-P450 enzyme system responsible for the generation of the toxic free radicals from these chemicals. In conclusion, the result of serum biochemical parameters, level of hepatic and renal lipid peroxides, glutathione antioxidant systems, CAT and histopathological studies support the dose dependent hepatoprotective and antioxidant activity of MEWF. The present study also supports the traditional use of *Woodfordia fruticosa* in derangement of liver. So this can be employed as a main ingredient in medicine for disorders due to oxidative stress.