CHAPTER 6

PREVENTIVE AND CURATIVE EFFECT OF METHANOLIC EXTRACT OF WOODFORDIA FRUTICOSA KURZ FLOWERS ON N-NITROSODIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOMA IN RATS
6.1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with poor diagnosis and the third most common cause of mortality with a continuously increasing incidence annually (Parkin et al., 2005). Liver is an organ of paramount importance and it plays an essential role in drug and xenobiotic metabolism. Chronic infection of hepatitis B and/ C, toxic industrial chemicals, aflatoxin exposure in diets, cigarette smoking, alcohol consumption, air and water pollutants etc are the major risk factors of liver diseases. Moreover, due to the high tolerance of liver, HCC is seldom detected at the early stage and once detected treatment faces a poor prognosis in most cases (Singh et al., 2009).

N-nitrosodiethylamine (NDEA) is a potent carcinogenic dialkynitrosoamine present in tobacco smoke, cheddar cheese, cured and fried meals and in a number of alcoholic beverages. It is a hepatocarcinogen producing reproducible HCC after repeated administration and is the most important environmental carcinogen among N-nitroso compounds (Singh et al., 2009). Administration of NDEA to animals causes cancer in liver and at low incidence in other organs also. The formation of reactive oxygen species (ROS) during the metabolism of NDEA may be one of the key factors in the etiology of cancer (Bansal et al., 2005).

The mechanism of action is due to metabolism of NDEA to alkylating agents and reactive oxygen species and further interaction with DNA molecule, forming various DNA adducts that can lead to mutations (Jeena et al.,1999). The $O_4$ – ethyl deoxythymidine adduct ($O_4$- Etdt) accumulates in hepatocyte DNA following NDEA administration which is thought to be important in tumor initiation (Sivalokanathan et al., 2006). Metabolism of certain therapeutic drugs is also reported to produce
N-nitrosodiethylamine (Akintonwa, 1985). NDEA became metabolically active by the action of cytochrome P450 enzymes to produce reactive electrophiles, which increase oxidative stress level leading to cytotoxicity, mutagenecity and carcinogenicity (Archer, 1989).

Chemoprevention, which is referred to as the use of nontoxic natural or synthetic chemicals to intervene in multistage carcinogenesis, has emerged as a promising and pragmatic medical approach to reduce the risk of cancer. Numerous components of plants, collectively termed “phytochemicals” have been reported to possess substantial chemopreventive properties. For many years cancer chemotherapy has been dominated by potent drugs that either interrupt the synthesis of DNA or destroy its structure once it has formed. Unfortunately, their toxicity is not limited to cancer cells and normal cells are also harmed (Wills et al., 2006). Development of nontoxic and biologically safe anticarcinogenic agent has been highlighted as a promising way to treat carcinogenesis (Jeon et al., 2005). Several herbal drugs like, *Acacia nilotica, Achyranthes aspera, Scutia myrtina* etc have been evaluated for its potential as liver protectant against NDEA induced hepatotoxicity in rats (Singh et al., 2009; Kartik et al., 2010b; Ramanathan et al., 2011).

*Woodfordia fruticosa* is an important medicinal plant traditionally used in India for the treatment of various liver diseases and the flowers have been reported to possess antitumour activity. Our previous study revealed the antioxidant and hepatoprotective efficacy of methanolic extract of *Woodfordia fruticosa*. More than that the phytochemical constituents identified by LC-MS analysis such as confertin, ellagic acid and quercetin methyl ether are well known for its chemopreventive/anticancer properties. In view of these the present study was undertaken to evaluate the preventive
Preventive and curative effect of methanolic extract of *Woodfordia fruticosa* Kurz flowers on *N*-nitrosodiethylamine induced hepatocellular carcinoma in rats.

6.2. MATERIALS AND METHODS

6.2.1. Chemicals

*N*-nitrosodiethyamine (NDEA), silymarin, proliferating cell nuclear antigen (PCNA), cyclin D1, anti-mouse IgG (whole molecule) peroxidase, streptavidin horse radish peroxidase conjugate and diaminobenzidine were purchased from Sigma Chemical Co., USA. Monoclonal anti-vascular endothelial growth factor (VEGF) antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. All other chemicals were of analytical grade.

6.2.2. 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.

6.2.3. 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 this study and stored at -20°C.

6.2.4. 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.

6.2.5. Induction of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) was induced by oral administration of 0.02% NDEA (2ml, 5 days/week) for 20 weeks (Wills et al., 2006).

6.2.6. Experimental design

6.2.6.1. Preventive effect of the extract

Male Wistar rats weighing 155 ± 5.4 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 control)
- Group II - NDEA control (0.02% NDEA, 2 ml, 5days/week, p.o)
- Group III - NDEA (as in group II) + Silymarin (100mg/kg b.w)
- Group IV - NDEA (as in group II) + MEWF (100mg/kg, b.w )
- Group V - NDEA (as in group II) + MEWF (200mg/kg, b.w)
- Group VI - MEWF (200 mg/kg) alone

Group II-V animals received 0.02% NDEA, 2 ml, 5days/week, p.o for 20 weeks. Daily doses of silymarin and MEWF treatments were started in group III to V
animals 1 week before the onset of NDEA administration and continued up to 20 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 instead of drug. The rats were sacrificed 48 h after the last dose of NDEA administration. Duration of this entire study was 20 weeks.

6.2.6.2. Curative effect of the extract

Male Wistar rats weighing 156 ± 3.8 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 control)
- **Group II** - NDEA control (0.02% NDEA, 2 ml, 5days/week, p.o)
- **Group III** - NDEA (as in group II) + Silymarin (100mg/kg b.w)
- **Group IV** - NDEA (as in group II) + MEWF (100mg/kg, b.w )
- **Group V** - NDEA (as in group II) + MEWF (200mg/kg, b.w)
- **Group VI** - MEWF (200 mg/kg) alone

After the intoxication with NDEA for 20 weeks, group III, IV and V were treated with daily doses of silymarin and MEWF 100mg/kg, b.w and 200mg/kg, b.w respectively for 28 days. Group VI served as drug control received MEWF (200mg/kg) only for last 28 days of the experiment. Group II received normal diet and 1ml of 5% tween 80 daily for last 28 days. Group I animals treated as vehicle control received 5% Tween 80 instead of drug. Animals were sacrificed 48h after the last dose of MEWF administration. So duration of the entire study was 24 weeks.
6.2.7. Serum enzyme analysis

In both preventive and curative treatment groups blood was collected from neck blood vessels and kept for 30 min at 4°C. Serum was separated by centrifugation at 2500rpm at 4°C for 15 min. The extent of liver damage was analysed by quantifying the serum levels of AST, ALT, ALP, LDH, AFP, GGT and bilirubin by kinetic method using a standard diagnostic kit. Alpha feto-protein (AFP) assay kit was purchased from Yuvraj biobiz, Chennai, India.

6.2.8. Morphometry evaluation

Liver tissue was excised, washed thoroughly in ice-cold saline to remove the blood then blotted dry and examined on the surface for visible macroscopic liver lesions (neoplastic nodules). Nodules were easily recognized and distinguished from the surrounding non-nodular reddish brown liver parenchyma. The nodules were spherical in shape. The percentage of nodule incidence and the total number of nodules were calculated in both preventive and curative groups (Ramanathan et al., 2011)

6.2.9. Tissue biochemical analysis

Ten percent of liver tissue homogenate was prepared in 0.1M Tris HCl buffer (pH – 7.4). 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), malondialdehyde (MDA) and total protein.

(Detailed procedures are explained under chapter 2, section 2.2.14. Procedures for *in vivo* antioxidant assays)
6.2.10. Histopathological analysis

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

6.2.11. Immunohistochemical analysis

Immunohistochemical analysis for three cancer markers like vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA) and Cyclin D1 were done for preventive and curative treatment groups (Detailed procedure is explained under chapter 2, section 2.2.10. Immunohistochemical analysis)

6.2.11.1. Immunohistochemical analysis of VEGF

For immunohistochemical analysis of VEGF the primary antibody used was vascular endothelial growth factor diluted at a concentration of 1:80 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 with 1% BSA.

6.2.11.2. Immunohistochemical analysis of PCNA

For immunohistochemical analysis of PCNA the primary antibody used was monoclonal antiproliferating cell nuclear antigen diluted at a concentration of 1:500 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 with 1% BSA.
6.2.11.3. Immunohistochemical analysis of Cyclin D1

For immunohistochemical analysis of Cyclin D1 the primary antibody used was monoclonal anti-proliferating cell nuclear antigen diluted at a concentration of 1:80 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 with 1% BSA.

6.2.12. 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.

6.3. RESULTS

6.3.1. Body weight

6.3.1.1. Body weight of pre-treatment groups

The changes in body weights of rats among the experimental groups after 20 weeks were found to be significant. Significant reduction ($p \leq 0.05$) was observed in the body weight of NDEA treated group compared to normal control group. Pre-treatment with silymarin and MEWF (100 mg/kg, 200mg/kg) prevented the decline in body weight of animals due to NDEA treatment. Pre-treatment with silymarin and MEWF exhibited significant ($p \leq 0.05$) elevation in the body weight compared to NDEA treated group (Fig.6.1).
Fig. 6.1. Graph showing the body weight pattern of rats administered with NDEA and pre-treatment with MEWF. The mean of each of the groups represented, with error bar indicating the standard deviation.

6.3.1.2. Body weight of post-treatment groups

Body weight of NDEA treated animals declined significantly ($p \leq 0.05$) at the end of the 20th week of exposure when compared with the normal rats. After 20 weeks, treatment with MEWF for 28 days dose dependently reversed the body weight. Silymarin also showed good results. MEWF alone treated rats did not show any significant change in body weight when compared to normal control (Fig. 6.2).
Fig. 6.2. Graph showing the body weight pattern of rats administered with NDEA and post-treatment with MEWF. The mean of each of the groups represented, with error bar indicating the standard deviation.

6.3.2. Liver weight

In pre-treatment groups liver weight of NDEA alone treated rats increased significantly \( (p \leq 0.05) \) at the end of the 20\(^{th}\) week of exposure when compared with normal rats. But treatment with MEWF prevented the increase in liver weight of rats exposed to NDEA. MEWF alone treated rats did not show any significant change when compared to normal control (Table 6.1).

In post treatment groups also liver weight of NDEA alone treated animals declined significantly \( (p \leq 0.05) \) at the end of the 20\(^{th}\) week of exposure. After intoxication with NDEA for 20 weeks, treatment with MEWF for four weeks dose dependently reversed the liver weight. Silymarin also showed good results. MEWF alone
treated rats did not show any significant changes in liver weight compared to normal control (Table 6.1).

Table 6.1. Final liver weight pattern of different group of rats pre and post-treated with MEWF

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Final liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>Normal control</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>NDEA control</td>
<td>19.4±2.1</td>
</tr>
<tr>
<td>NDEA + Silymarin (100mg/kg)</td>
<td>6.8±1.2 b</td>
</tr>
<tr>
<td>NDEA + MEWF (100mg/kg)</td>
<td>8.3±0.8 b</td>
</tr>
<tr>
<td>NDEA + MEWF (200mg/kg)</td>
<td>7.2±1.0 b,c</td>
</tr>
<tr>
<td>MEWF alone</td>
<td>7.8±0.8 b,d</td>
</tr>
</tbody>
</table>

Values are mean ± S.D from 6 rats in each group. Statistical significance: \( p \leq 0.05 \). \(^a\) NDEA control differs significantly from normal control. \(^b\) NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from \( \text{CCl}_4 \) control. \(^c\) NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. \(^d\) MEWF 200mg/kg alone treated group non significantly different from normal control.

6.3.3. Serum enzymes

6.3.3.1. Pre – treatment evaluation

NDEA treated rats showed significantly \( (p \leq 0.05) \) elevated levels of serum AST, ALT, ALP, LDH, AFP, GGT and bilirubin when compared to normal control. A significant \( (p \leq 0.05) \) reduction was observed in serum markers in the animals pre-treated with silymarin (100mg/kg) and MEWF (100mg/kg and 200mg/kg) when compared to NDEA treated group (Fig. 6.3)
Fig. 6.3. Changes in serum enzyme levels of rats pre-treated with MEWF.

(A) Aspartate aminotransferase (B) Alanine aminotransferase

(I) Normal control, (II) NDEA control (III) Silymarin + NDEA, (IV) MEWF (100 mg/kg) + NDEA (V) MEWF (200 mg/kg) + NDEA (VI) MEWF alone. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. * p ≤ 0.05 vs. normal control. ** p ≤ 0.05 vs. NDEA control.
Preventive and curative effect of methanolic extract of *Woodfordia fruticosa* Kurz flowers on *N*-nitrosodiethylamine induced hepato-cellular carcinoma in rats

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**Fig. 6.3. (Cont.) Changes in serum enzyme levels of rats pre-treated with MEWF.**

**(C) Alkalinephosphatase**

**(D) Lactate dehydrogenase**

(I) Normal control, (II) NDEA control (III) Silymarin + NDEA, (IV) MEWF (100 mg/kg) + NDEA (V) MEWF (200 mg/kg) + NDEA (VI) MEWF alone. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. * p ≤ 0.05 vs. normal control. ** p ≤ 0.05 vs. NDEA control.
Fig. 6.3. (Cont.) Changes in serum enzyme levels of rats pre-treated with MEWF.

(E) Alpha feto-protein, (F) Gama glutamyl transferase

(I) Normal control, (II) NDEA control (III) Silymarin + NDEA, (IV) MEWF (100 mg/kg) + NDEA (V) MEWF (200 mg/kg) + NDEA (VI) MEWF alone. 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. NDEA control.
Preventive and curative effect of methanolic extract of Wosforia fruticosa Kurz flowers on N-nitrosodiethylamine induced hepato-cellular carcinoma in rats

Fig. 6.3. (Cont.) Changes in serum enzyme levels of rats pre-treated with MEWF.

(G) Bilirubin.

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

6.3.3.2. Post-treatment evaluation

The serum levels of AST, ALT, ALP, LDH, AFP, GGT and bilirubin in group II were significantly ($p \leq 0.05$) elevated by the administration of NDEA, when compared to normal control. The treatment with MEWF at a dose of 100mg/kg and 200 mg/kg showed a dose dependently significant decrease ($p \leq 0.05$) in AST, ALT, ALP, LDH, AFP, GGT and bilirubin levels (Fig.6.4). MEWF at 200 mg/kg, b.w showed better results than the standard drug, silymarin treated groups.
Fig. 6.4. Changes in serum enzyme levels of rats post-treated with MEWF.

(A) Aspartate aminotransferase, (B) Alanine aminotransferase

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

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. $^a$ NDEA control differs significantly from normal control. $^b$ NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from CCl$_4$ control. $^c$ NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. $^d$ MEWF 200mg/kg alone treated group non significantly different from normal control.
Fig. 6.4. (Cont.) Changes in serum enzyme levels of rats post-treated with MEWF.
(C) Alkaline phosphatase (D) Lactate dehydrogenase
(I) Normal control, (II) NDEA control (III) NDEA + Silymarin, (IV) NDEA + MEWF (100 mg/kg) (V) NDEA + MEWF (200 mg/kg) (VI) MEWF alone.

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. a NDEA control differs significantly from normal control. b NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg , NDEA + MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from CCl$_4$ control. c NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. d MEWF 200mg/kg alone treated group non significantly different from normal control.
Fig. 6.4. (Cont.) Changes in serum enzyme levels of rats post-treated with MEWF.

(E) Alpha feto-protein, (F) Gama glutamyl transferase

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

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. $^a$ NDEA control differs significantly from normal control. $^b$ NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from CCl$_4$ control. $^c$ NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. $^d$ MEWF 200mg/kg alone treated group non significantly different from normal control.
Preventive and curative effect of methanolic extract of *Woolffaria fruticosa* Kurz flowers on *N*-nitrosodiethylamine induced hepato-cellular carcinoma in rats

6.4. Changes in serum enzyme levels of rats post-treated with MEWF.

(G) Bilirubin.

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

Values are mean ± S.D from 6 rats in each group. Statistical significance: $p \leq 0.05$. $^a$ NDEA control differs significantly from normal control. $^b$ NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg and MEWF 200mg/kg alone were significantly different from CCl$_4$ control. $^c$ NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. $^d$ MEWF 200mg/kg alone treated group non significantly different from normal control.

6.3.4. Liver morphology

Fig. 6.5 shows the morphological variations of rat livers in pre-treatment groups. NDEA treated rat liver becomes very large in size and a large number of hepatic nodules were observed. In MEWF 200mg/kg treated group and in MEWF alone treated groups liver morphology is very similar to normal rats.

In post-treatment groups also NDEA treated rat liver becomes morphologically different from normal rat liver. In NDEA alone treated group rat liver become very large in size and a large number of hepatic nodules were observed. Treatment with MEWF at a dose of 200mg/kg and reinstated the liver morphology as that of normal rat liver (Fig. 6.6)
Chapter 6

6.3.5. Morphometry

6.3.5.1. Pre-treatment evaluation

In pre-treatment groups a large number of hepatic nodules were observed in NDEA alone treated rats. Administration of silymarin and MEWF (100 mg/kg b.w, 200 mg/kg) showed significant reduction in the nodule incidence in NDEA induced hepatocarcinogenesis (Table 6.2).

Fig. 6.5. Morphological variations of liver in control and pre-treated groups.

(A) Normal control; (B) NDEA control; (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA (F) MEWF (200 mg/kg) alone.
Fig. 6.6. Morphological variations of liver in control and post-treated groups.

(A) Normal control; (B) NDEA control (0.02%), (C) NDEA + Silymarin (100 mg/kg), (D) NDEA + MEWF (100 mg/kg) (E) NDEA + MEWF (200 mg/kg) (F) MEWF (200 mg/kg) alone.
Table 6.2. Pre-treatment effect of MEWF on the development of NDEA induced hepatic nodules in rat liver

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of rats with nodule/ Total no. of rats</th>
<th>Nodule incidence</th>
<th>Total no. of nodules</th>
<th>Average no. of nodules/ Nodule bearing liver (Nodule multiplicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDEA control (0.02%)</td>
<td>6/6</td>
<td>100</td>
<td>139.5±20.2</td>
<td>23.25</td>
</tr>
<tr>
<td>Silymarin(100mg/kg) + NDEA</td>
<td>1/6</td>
<td>16</td>
<td>8±0</td>
<td>8</td>
</tr>
<tr>
<td>MEWF (100mg/kg b.w) + NDEA</td>
<td>3/6</td>
<td>50</td>
<td>28±4.5</td>
<td>9.3</td>
</tr>
<tr>
<td>MEWF (200mg/kg b.w) + NDEA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MEWF alone (200mg/kg, b.w)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

6.3.5.2. Post-treatment evaluation

In post-treatment groups administration of MEWF at a dose of 100 mg/kg b.w and 200 mg/kg b.w for last 28 days showed a significant reduction in the nodule incidence in NDEA induced hepatocarcinogenesis in rats (Table 6.3).

6.3.6. Tissue antioxidants and other biochemical constituents

6.3.6.1. Reduced glutathione (GSH) level

(i) Pre-treatment evaluation

Reduced glutathione (GSH) levels were lowered significantly ($p \leq 0.05$) in NDEA intoxicated rats. Treatment with MEWF at a dose of 200mg/kg increased the
levels of GSH by 90.3% when compared to NDEA control. In silymarin treated groups it was 60.5% (Table 6.4).

(ii) Post-treatment evaluation

Reduced glutathione (GSH) levels were lowered significantly \((p \leq 0.05)\) in NDEA intoxicated rats. Treatment with MEWF at a dose of 200mg/kg increased the levels of GSH by 86.1% when compared to NDEA control. In silymarin treated groups it was 57.4% (Table 6.5).

Table 6.3. Post-treatment effect of MEWF on the development of NDEA induced hepatic nodules in rat liver

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of rats with nodule/ Total no. of rats</th>
<th>Nodule incidence</th>
<th>Total no. of nodules</th>
<th>Average no. of nodules/ Nodule bearing liver (Nodule multiplicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NDEA control (0.02%)</td>
<td>6/6</td>
<td>100</td>
<td>124±27</td>
<td>20.7</td>
</tr>
<tr>
<td>Silymarin(100mg/kg) + NDEA</td>
<td>2/6</td>
<td>33</td>
<td>11±4</td>
<td>5.5</td>
</tr>
<tr>
<td>MEWF (100mg/kg b.w) + NDEA</td>
<td>4/6</td>
<td>50</td>
<td>16±5</td>
<td>4</td>
</tr>
<tr>
<td>MEWF(200mg/kg b.w)+ NDEA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MEWF alone (200mg/kg, b.w)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
6.3.6.2. Activity of glutathione - S - transferase (GST)

(i) Pre-treatment evaluation

The GST activity of liver tissues were significantly \((p \leq 0.05)\) reduced in NDEA intoxicated rats of pre-treatment groups compared to normal control. The MEWF dose dependently increased \((p \leq 0.05)\) the activity of GST in hepatic tissues (Table 6.4). Treatment with 200 mg/kg methanolic extract exhibited prominently increased i.e., 89.9% GST levels. In addition, silymarin treated rats also prevented the NDEA induced decrease in GST activity by 69.9% in hepatic tissue.

(ii) Post-treatment evaluation

In post-treatment GST activity of liver tissues were significantly \((p \leq 0.05)\) reduced in NDEA intoxicated rats compared to normal control. The MEWF dose dependently increased \((p \leq 0.05)\) the activity of GST in hepatic tissues (Table 6.5). Treatment with MEWF 200 mg/kg exhibited prominently increased the GST levels (95%). In addition, in silymarin treated rats the GST activity was 67%.

6.3.6.3. Activity of glutathione reductase (GR)

(i) Pre-treatment evaluation

GR activity was significantly decreased \((p \leq 0.05)\) in NDEA treated animals when compared to control. 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 NDEA (Table 6.4). The percentage of protection in liver tissue was 83.8% for 200 mg/kg of MEWF. Silymarin restored the GR activity up to 62.6% in rat liver.

(ii) Post-treatment evaluation

In post-treatment also GR activity was significantly decreased \((p \leq 0.05)\) in NDEA treated animals. When NDEA intoxicated rats were treated with MEWF (100
Preventive and curative effect of methanolic extract of Woodfordia fruticosa Kurz flowers on N-nitrosodiethylamine induced hepatocellular carcinoma in rats

and 200 mg/kg) and silymarin (100 mg/kg) significant increase \((p \leq 0.05)\) in the GR level was observed (Table 6.5). The percentage of protection in liver tissue was 89% for 200 mg/kg of MEWF and 67% for silymarin.

6.3.6.4. Activity of glutathione peroxidase (GPx)

(i) Pre-treatment evaluation

Activities of hepatic GPx was significantly \((p \leq 0.05)\) lowered in NDEA treated rats (Table 6.4). MEWF dose dependently prevented the lowering of GPx compared to NDEA alone treated groups. In liver, 200 mg/kg of methanolic extract showed a protection of 94.5%. Silymarin-treated rats also prevented the lowering of GPx by 73.3% in hepatic tissues.

(ii) Post-treatment evaluation

In NDEA alone treated rats the activities of GPx was significantly \((p \leq 0.05)\) lowered. MEWF treatment dose dependently restored the decreased GPx activity compared to NDEA alone treated group. Treatment with MEWF at a dose of 200 mg/kg showed a prominently increased GPx level (97%) and in silymarin treated rats the glutathione peroxidase was 76.3% (Table 6.5)

6.3.6.5. Activity of catalase (CAT)

(i) Pre-treatment evaluation

The CAT activity in liver showed a significant \((p \leq 0.05)\) reduction in NDEA intoxicated rats compared to normal control. MEWF treatment increased the activity of CAT in hepatic tissue (Table 6.4). Treatment with 200 mg/kg methanolic extract exhibited significant increase i.e., 75.5%. In addition, silymarin treated rats also prevented \((p \leq 0.05)\) the NDEA induced decrease in CAT activity by 60% (Table 6.4).
(ii) Post-treatment evaluation

In post-treatment also NDEA intoxicated rat liver showed a significant ($p \leq 0.05$) reduction in CAT activity compared to normal control. Treatment with MEWF increased the activity of CAT (Table 6.5). Treatment with MEWF at a dose of 200 mg/kg exhibited significant increase i.e., 85.7% and in silymarin treatment restored the CAT activity by 69.9%.

6.3.6.6. Lipid peroxidation (MDA) level

(i) Pre-treatment evaluation

A significant ($p \leq 0.05$) elevation of malondialdehyde levels was observed in NDEA intoxicated rats. In silymarin treated group the percentage of protection was 58.6%. MEWF treatment exerted its protection by 33.2% and 90.8% in 100mg/kg and 200mg/kg treated groups respectively compared to NDEA control (Table 6.4).

(ii) Post-treatment evaluation

In post-treatment groups a significant ($p \leq 0.05$) elevation of malondialdehyde levels was observed in NDEA intoxicated rats. In silymarin treated group the percentage of protection was 60.2%. MEWF treatment exerted its protection by 95% in 200mg/kg treated group compared to NDEA control (Table 6.5).
### Table 6.4. Pre-treatment effects of MEWF against NDEA induced changes in the liver antioxidant status.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>NDEA control</th>
<th>Silymarin (100mg/kg) + NDEA</th>
<th>MEWF (100mg/kg) + NDEA</th>
<th>MEWF (200mg/kg) + NDEA</th>
<th>MEWF (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH¹</td>
<td>22.1±0.5</td>
<td>12.1±0.4</td>
<td>18.2±0.4</td>
<td>15.8±0.5</td>
<td>21±0.4</td>
<td>22.6±0.6</td>
</tr>
<tr>
<td>GST²</td>
<td>72.5±0.70</td>
<td>33.1±0.43</td>
<td>58.3±0.52</td>
<td>53.9±0.78</td>
<td>68.5±0.51</td>
<td>70.2±0.5</td>
</tr>
<tr>
<td>GR³</td>
<td>21.4±0.77</td>
<td>7.2±0.38</td>
<td>16.16±0.4</td>
<td>12.3±0.45</td>
<td>19.1±0.8</td>
<td>21.7±0.6</td>
</tr>
<tr>
<td>GPx⁴</td>
<td>283.1±2.8</td>
<td>162.4±3.0</td>
<td>250.8±3.0</td>
<td>217.5±2.5</td>
<td>276.4±3.8</td>
<td>281.8±3.0</td>
</tr>
<tr>
<td>CAT⁵</td>
<td>47±1.2</td>
<td>32.1±1.7</td>
<td>41.2±1.7</td>
<td>38.1±1.4</td>
<td>43.5±1.4</td>
<td>46.2±1.5</td>
</tr>
<tr>
<td>MDA⁶</td>
<td>43.8±1.2</td>
<td>74.5±1.5</td>
<td>56.5±1.7</td>
<td>64.3±1.1</td>
<td>46.6±1.7</td>
<td>44.9±1.6</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 mean ± S.D from 6 rats in each group. Statistical significance: p ≤ 0.05. ¹ NDEA control differs significantly from normal control. ² NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg, and MEWF 200mg/kg alone were significantly different from CCl₄ control. ³ NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. ⁴ MEWF 200mg/kg alone treated group non significantly different from normal control.

### Table 6.5. Post-treatment effects of MEWF against NDEA induced changes in the liver antioxidant status.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>NDEA control</th>
<th>NDEA + Silymarin (100mg/kg)</th>
<th>NDEA + MEWF (100mg/kg)</th>
<th>NDEA + MEWF (200mg/kg)</th>
<th>MEWF (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH¹</td>
<td>23.5±0.9</td>
<td>13.4±1.5</td>
<td>19.2±1.8</td>
<td>16.9±1.0</td>
<td>22.1±0.8</td>
<td>23.2±1.4</td>
</tr>
<tr>
<td>GST²</td>
<td>70.4±1.6</td>
<td>35.7±0.9</td>
<td>59.1±1.2</td>
<td>52.8±1.9</td>
<td>68.7±2.1</td>
<td>71.2±1.5</td>
</tr>
<tr>
<td>GR³</td>
<td>21.8±1.1</td>
<td>8.8±0.9</td>
<td>17.6±1.4</td>
<td>13.9±0.8</td>
<td>20.4±1.6</td>
<td>22.2±0.8</td>
</tr>
<tr>
<td>GPx⁴</td>
<td>281.2±5.8</td>
<td>165.1±4.9</td>
<td>253.8±6.1</td>
<td>220.2±3.8</td>
<td>278.5±5.2</td>
<td>280.6±4.2</td>
</tr>
<tr>
<td>CAT⁵</td>
<td>47.1±1.5</td>
<td>33.8±1.8</td>
<td>43.1±1.4</td>
<td>38.1±1.6</td>
<td>45.2±0.9</td>
<td>47.8±1.2</td>
</tr>
<tr>
<td>MDA⁶</td>
<td>42.3±1.3</td>
<td>72.5±2.3</td>
<td>54.3±1.9</td>
<td>61.5±1.5</td>
<td>43.8±0.8</td>
<td>44.1±1.2</td>
</tr>
</tbody>
</table>

Values are mean ± S.D from 6 rats in each group. Statistical significance: p ≤ 0.05. ¹ NDEA control differs significantly from normal control. ² NDEA + Silymarin 100mg/kg, NDEA + MEWF 100mg/kg, NDEA + MEWF 200mg/kg, and MEWF 200mg/kg alone were significantly different from CCl₄ control. ³ NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100mg/kg treated group. ⁴ MEWF 200mg/kg alone treated group non significantly different from normal control.
6.3.7. Histopathology

Fig. 6.7 and Fig. 6.8 depict the histological analysis of pre and post-treatment groups. The normal rat liver showed normal architecture. Here the cells are uniformly arranged with granulated cytoplasm, oval hepatocytes and small uniform nuclei. In NDEA intoxicated rats enlarged nuclei, hyperchromatism, scattered masses of necrotic tissue, proliferating hepatocytes and mild congestion of sinusoids with central vein dilation were detected in most areas. Microscopic examination also revealed the trabecular structure of liver tissue, which is a typical feature of hepatocellular carcinoma. In pre and post-treatment evaluation, silymarin at a dose of 100mg/kg b.w treated groups showed necrotic tissues in certain areas. However, in MEWF at a dose of 100mg/kg treated groups some degenerating hepatic cells were detected. In MEWF at a dose of 200mg/kg b.w treated group the liver tissue showed almost normal architecture - normal hepatocytes with uniform sinusoids. The liver of MEWF alone treated group showed no appreciable changes or histological abnormalities.

6.3.8. Immunohistochemical localization of VEGF

Immunohistochemical analysis of VEGF demonstrated that hepatocytes located in the periportal areas were the main source of VEGF production.

In pre and post-treatment evaluation immunohistochemical analysis of the normal rat tissue showed regularly stained nucleus. In NDEA intoxicated rat liver tissue, localization of VEGF around periportal area (arrow heads) was prominent. A significant down regulation of VEGF was spotted in MEWF treated groups. MEWF at a dose of 200mg/kg dose dependently down regulates VEGF expression. Silymarin treated group also showed concomitant result. Fig. 6.9 and Fig 6.10 showed the pre and post-treatment effects of MEWF in immunohistochemical analysis of VEGF.
Preventive and curative effect of methanolic extract of *Woodfordia fruticosa* Kurz flowers on *N*-nitrosodiethylamine induced hepato-cellular carcinoma in rats

Fig. 6.7. Histopathological changes occurred in rat liver due to pre-treatment with MEWF (hematoxylin and eosin, 100×).

(A) Normal control; (B) NDEA control; (C) Silymarin (100 mg/kg) + NDEA; D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA (F) MEWF (200mg/kg) alone.
Fig. 6.8. Histopathological changes occurred in rat liver due to post-treatment with MEWF (hematoxylin and eosin, 100×).

(A) Normal control; (B) NDEA control, (0.02%); (C) NDEA + Silymarin (100 mg/kg); (D) NDEA + MEWF (100 mg/kg); (E) NDEA + MEWF (200 mg/kg) and (F) MEWF (200 mg/kg) alone.
6.3.9. Immunohistochemical localization of PCNA

In pre and post-treatment evaluation normal rat tissue showed regularly stained nucleus. NDEA administered rat liver tissue showed over expression of proliferating cell nuclear antigen (PCNA). Administration of MEWF showed a reduction in the expression of PCNA. PCNA is a common index for proliferation of hepatocytes at late G1 stage and early S stage. HCC bearing group stained positively for PCNA indicating active cell proliferation when compared with normal control. The MEWF given at a concentration of 200mg/kg showed a remarkable lowering of the expression of PCNA and was comparable to the effect rendered by silymarin at a concentration of 100mg/kg. Fig. 6.11 and Fig. 6.12 showed the pre and post-treatment effects of MEWF in immunohistochemical analysis of PCNA.

6.3.10. Immunohistochemical localization of Cyclin D1

Fig. 6.13 and Fig. 6.14 show the immunohistochemical analysis of Cyclin D1 in pre and post-treatment groups respectively. Here in both the experimental groups normal rat tissue showed regularly stained nucleus. NDEA administered rat liver tissue showed over expression of Cyclin D1. Administration of MEWF showed a reduction in the expression Cyclin D1 in a dose-dependent manner. MEWF 200mg/kg treated group showed good results than MEWF 100mg/kg treated group and silymarin treated groups.
Fig. 6.9. Immunohistochemical localizations of VEGF in control and pre-treated groups. Liver tissue was immunostained for VEGF (arrow heads) followed by staining with hematoxylin (100x).

(A) Normal control; (B) NDEA control; (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA; (F) MEWF (200mg/kg) alone.
Fig. 6.10. Immunohistochemical localizations of VEGF in post-treated groups. Liver tissue was immunostained for VEGF followed by staining with hematoxylin (100x).

(A) Normal control; (B) NDEA control (0.02%); (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA and (F) MEWF (200mg/kg) alone.
Fig. 6.11. Immunohistochemical localizations of PCNA in control and pre-treated groups. Liver tissue was immunostained for PCNA followed by staining with hematoxylin (100x).

(A) Normal control; (B) NDEA control (0.02%); (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA and (F) MEWF (200mg/kg) alone.
Preventive and curative effect of methanolic extract of Woodfordia fruticosa Kurz flowers on N-nitrosodiethylamine induced hepato-cellular carcinoma in rats

Fig. 6.12. Immunohistochemical localizations of PCNA in control and post-treated groups. Liver tissue was immunostained for PCNA followed by staining with hematoxylin (100 x).

(A) Normal control; (B) NDEA control (0.02%); (C) NDEA + Silymarin (100 mg/kg); (D) NDEA + MEWF (100 mg/kg); (E) NDEA + MEWF (200 mg/kg) and (F) MEWF (200mg/kg) alone.
Fig. 6.13. Immunohistochemical localizations of Cyclin D1 in control and pre-treated groups. Liver tissue was immunostained for Cyclin D1 (indicated by brown stained nuclei) followed by staining with hematoxilin (100x).

(A) Normal control; (B) NDEA control (0.02%); (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA and (F) MEWF (200mg/kg) alone.
Preventive and curative effect of methanolic extract of *Woodsorla fruticosa* Kurz flowers on *N*-nitrosodiethylamine induced hepato-cellular carcinoma in rats

Fig. 6.14. Immunohistochemical localizations of Cyclin D1 in control and post-treated groups. Liver tissue was immunostained for Cyclin D1 (indicated by brown stained nuclei) followed by staining with hematoxylin (100x).

(A); Normal control; (B) NDEA control (0.02%); (C) NDEA + Silymarin (100 mg/kg); (D) NDEA + MEWF (100 mg/kg); (E) NDEA + MEWF (200 mg/kg) and (F) MEWF (200mg/kg) alone.
6.4. DISCUSSION

N-nitrosodiethylamine (NDEA) is a major environmental carcinogen suggested to increase the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Shaarawy et al., 2009). Since liver is the main site of NDEA metabolism, the production of ROS in the liver may be the mechanism responsible for its carcinogenic effects (Bansal et al., 2005). NDEA is mainly metabolized in the liver by the action of cytochrome P450 enzyme and the reactive metabolites are primarily responsible for its hepatotoxic effects (Verna et al., 1996). ROS are continuously generated as a result of NDEA administration causing oxidative stress that wreak havoc in biological system by damaging tissues, altering biochemical compounds causing chromosomal instability, corroding cell membranes and mutation which play an important role in development of cancer (Harman, 1980)

This study demonstrated that NDEA intoxication to rats for a period of 20 weeks leads to a drastic loss in body weight of animals, increase in liver weight, formation of a large number of hepatic nodules and marked elevation in the levels of serum AST, ALT, ALP, LDH, AFP, GGT and Bilirubin. Serum transaminases, ALP and GGT are representative of liver function and their increased levels are indicators of liver damage. The increase in the activities of these serum enzymes of rats might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Atef M. Al-Attar, 2011) Pre and post-treatment with MEWF prevented the decline in body weight and increase in liver weight. There was a remarkable suppression in nodule development in MEWF treated groups, nodule development was practically nil or if nodules developed they were very much reduced in size. In preventive and curative models a marked reduction of serum
AST, ALT, ALP and LDH levels were observed in rats treated with MEWF. Elevation of serum level of α-feto protein (AFP) has been reported in several diseases, however it is the most widely used tumor marker for diagnosis of HCC (Banker, 2003). AFP is a unique immunomodulatory glycoprotein (65 kDa) normally made by the immature liver cells in the fetus (Sell and Becker, 1978). It has also been recognized that exposure of rats to certain carcinogens like NDEA increases the circulating AFP level. In this study also there was an increased level of AFP in the carcinogen administered animals confirming the presence of HCC, where as treatment with NDEA significantly reduced its elevation. The increased serum GGT activity is also considered to be one of the best indicators of liver damage. It is an enzyme embedded in the hepatocyte plasma membrane mainly in the canalicular domain and its liberation into serum indicates damage of cells and thus injury to liver (Sivaramakrishnan et al., 2008). MEWF at a dose of 200mg/kg produced better results than 100mg/kg, showed the dose response action of the extract.

NDEA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis (Gey, 1993). Generation of a large amount of ROS due to NDEA 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. The intra cellular antioxidant system comprises of different free radical scavenging antioxidant enzymes GST, GR, GPx and CAT along with some non-enzyme antioxidants like GSH, constitute the first line of cellular antioxidant defense system. When excess free radicals are produced, the equilibrium is lost and consequently oxidative insult is established (Manna et al., 2007).
Pre and post-treatment with MEWF increased the hepatic GSH level significantly. 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. GST offers protection against lipid peroxidation by promoting the conjugation of toxic electrophiles with GSH (Jackoby 1988). GR is also essential for the maintenance of GSH levels \textit{in vivo}. The significant \((p \leq 0.05)\) restoration of GPx activity due to pre and post-treatment with MEWF and silymarin treatment might be due to the antioxidant activity by detoxifying the endogenous metabolic peroxides generated after NDEA intoxication in hepatic 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 NDEA intoxication 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 hepatic tissues. This evidently showed the antioxidant property of the extract against oxygen free radicals. Hepatic damage induced by NDEA administration is associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH, GST, GR, GPx and CAT activities.

Lipid peroxidation plays an important role in carcinogenesis (Banakar et al., 2004) and may lead to the formation of several toxic products such as MDA. Hepatic MDA levels in NDEA intoxicated rats were elevated. Increased MDA content is an important indicator of lipid peroxidation (Celik et al, 2009). In this study the increased MDA level can be prevented/cured by the treatment with MEWF, also revealed the antioxidant potential of the extract. So these results indicated that administration of
NDEA leads to the induction of HCC and augmentation of oxidative stress. This in turn alleviated by the treatment with methanolic extract of *Woodfordia fruticosa*.

Histopathological analysis of NDEA administered rat liver tissue showed variations in cell architecture such as cytomegaly, vacuolated hepatocytes, irregular blabbing of nuclei towards the central vein region. These symptoms were reduced in MEWF treated groups both in preventive and curative models, showed negligible damage to a few hepatocytes present in the close vicinity of central vein proved the efficacy of the extract. The recovery towards normalization of histological architecture by pre and post-treatment with MEWF were almost similar to that of normal control. Indeed there was remarkable reduction in the characteristic features of HCC such as formation of necrotic tissues, enlarged nuclei and lymphocyte infiltration.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that induces and promotes angiogenesis and endothelial cell proliferation, which plays an important role in regulating angiogenesis. It is secreted by hepatoma cells and hepatic stellate cells, which is up regulated during tumor dedifferentiation and vascular development of HCC (Mitsuhashi et al., 2003). In the present study, immunohistochemical analysis showed the localization of over expressed VEGF around the periportal area in NDEA intoxicated rats. Pre and post-treatment with MEWF showed significant downregulation of VEGF indicating its inhibitory role of neo-vasculature formation in rats during NDEA administration.

Abnormal proliferation of cells is the main feature of carcinogenesis, and there for exploration of drugs that can affect malignant proliferation of liver cells is of primary importance in chemical prevention of liver cancer. Proliferating cell nuclear
antigen (PCNA) present in cell nucleus is directly involved in DNA replication. It has been found that the positive expression of PCNA were a common index for proliferation of hepatocytes at late G1 stage and early S stage. The positive expression of proteins was mainly found in the precancerous proliferation focus and cancerous liver tissue during NDEA induced hepatocarcinogenesis. The high expression of PCNA in the present study suggested that the ability of cell proliferation become stronger, and this was closely related to malignant cell proliferation and carcinogenesis (Chodon et al., 2007). PCNA immunostaining conducted in the present study clearly depicted an upregulation of the protein which appeared as brown stained nuclei in the sections of NDEA treated rat livers while administration of MEWF in preventive and curative models could remarkably decrease the PCNA in HCC bearing tissue, suggesting the efficacy in suppressing the malignant proliferation of hepatocytes.

The cyclin families of proteins are highly conserved nuclear cell cycle regulatory proteins of which the cyclin D family (Cyclin D1, D2 and D3) play pivotal roles in progression through the G1 phase of the cell cycle. Over expression of Cyclin D1 has been associated with a variety of cancers including HCC. Amplification or over expression of cyclin D1 plays an important role in the development of a subset of human tumorigenesis and cellular metastasis (Fu et al., 2004). In the present study pre and post-treatment with MEWF dose dependently inhibit the expression of cyclin D1 in NDEA induced HCC tissue.

LCMS analysis of MEWF revealed the presence of octacosanol, malonic acid, oxaloacetic acid, octanoic acid, isocaryophyllene, confertin, Quercetin methyl ether, ellagic acid, ursolic acid, stigmasterol, hydroxy methyl flavan etc. Confertin showed antiproliferative effect on DLA cell lines (Thara and Zuhara, 2012). Quercetin methyl
ether suppresses proliferation of mouse epidermal JB6P+ cells by targeting ERKs (Li et al., 2012). It showed hepatoprotective effect on copper induced oxidative damage in hepatocytes and it also have in vitro anti inflammatory effect (Wei et al., 2001). Ellagic acid is a polyphenol antioxidant and a chemopreventive agent. It has antiproliferative activity, it slows the growth of some tumors caused by certain carcinogens and it inhibits two topoisomerases (Constantinou et al., 1995). Stigmasterol (β sitosterol) is a component reported as hepatoprotective agent (Al-Qurawi et al., 2004).

In conclusion the results presented in this study indicated the anticancer efficacy of MEWF. HCC induced by N-nitrosodiethylamine was effectively inhibited by the treatment with MEWF at a dose of 200 mg/kg, b.w. MEWF showed better results than the silymarin treated group. Many of the compounds reported from the dried flowers of Woodfordia fruticosa were present in MEWF which might be responsible for the anticancer activity. This finding suggested a possible basis for the potential use of the flowers of Woodfordia fruticosa for the treatment of hepatocellular carcinoma. This finding might also provide a pharmacological background on the traditional use of the plant in the treatment of liver cancer.