Original Article

Antiproliferative effect of *Woodfordia fruticosa* Kurz flowers on experimentally induced hepatocellular carcinoma in rats and in human hepatoma cell line

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A B S T R A C T

Hepatocellular carcinoma is the fifth most common cancer worldwide and one of the most lethal. Chemoprevention involving the use of synthetic or natural products to inhibit or reverse the carcinogenic process is an effective approach to control cancer. *Woodfordia fruticosa* is an important medicinal plant and its flowers have been reported to possess hepatoprotective and antitumor properties.

Objective: To study the effect of methanolic extract of *W. fruticosa* (MEWF) flowers on hepatocellular carcinoma.

Methods: In this study the effect of MEWF was tested by following the serum parameters like AFP, ALP, LDH and Bilirubin; tissue level of GSH, CAT and MDA; histopathology of liver and immunohistochemical analysis of vascular endothelial growth factor (VEGF). Antiproliferative effect of MEWF was studied in human hepatoma PLC/PRF/5 cells by MTT assay. The chemotherapeutic drug, 5-flurouracil (5-FU) was used as positive control.

Results: This study validates the potential chemopreventive efficacy of MEWF.

Conclusion: The potential antioxidant and chemopreventive activities of MEWF may be due to its free radical scavenging and antiproliferative effect. Therefore, *W. fruticosa* may serve as a novel therapeutic agent for the treatment of radical mediated liver diseases particularly hepatocellular carcinoma.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common pathology worldwide and the most common type of liver cancer. 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. Intake of acetaminophen
like drugs and certain chemicals may also lead to hepatocellular carcinoma.

N-nitrosodiethylamine (NDEA) is a potent carcinogenic dialky nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals and in a number of alcoholic beverages. It is a hepatocarcinogen producing reproducible HCC after repeated administration. The formation of reactive oxygen species (ROS) during the metabolism of NDEA may be one of the key factors in the etiology of cancer. HCC is associated with over expression of vascular endothelial growth factor (VEGF) which are produced by hepatocytes in the portal area of liver tissue. In addition to the animal experimental models of cancer, human cancer cell lines have been widely used to study the antiproliferative effect.

Numerous components of plants, collectively termed “phytochemicals” have been reported to possess substantial chemopreventive properties. Development of nontoxic and biologically safe anticarcinogenic agent has been highlighted as a promising way to treat carcinogenesis. 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.

Woodfordia fruticosa (Lythraceae) is a traditional medicinal plant and its dried flowers are used as tonic in disorders of mucous membrane, hemorrhoids and in derangement of liver. Phenolics, particularly hydroxylizable tannins and flavonoids were identified as major components of W. fruticosa flowers. In view of these the present work was undertaken to evaluate the protective effect of W. fruticosa against NDEA induced hepatocellular carcinoma in experimental rats and in human hepatoma PLC/PRF/5 cell lines.

2. Materials and methods

2.1. Chemicals

NDEA, Silymarin, anti-mouse IgG horseradish peroxidase, streptavidin horseradish peroxidase conjugate, diaminobenzidine, Fetal bovine serum (FBS) and N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid (HEPES) were purchased from Sigma Chemical Co., St. Louis, MO, USA. VEGF antibody from Santa Cruz Biotecnology, Santa Cruz, CA, USA. Alpha fetoprotein, mouse monoclonal antibody against human alpha fetoprotein (AFP) assay kit was purchased from Creative diagnostics, USA. Assay kits for serum alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and bilirubin were purchased from Agappe Diagnostics, India. 5-flourouracil (5-FU) was purchased from Biochem Pharmaceutical Industries, Mumbai, India. RPMI Medium and antibiotic-antimycotic were purchased from Gibco, Grand Island, N.Y, USA. Cell Proliferation Assay kit [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)] was purchased from HiMedia, India. Dimethyl sulfoxide (DMSO) was obtained from Merck, Mumbai, India. All other chemicals were of analytical grade.

2.2. Cell lines

PLC/PRF/5 cell line was purchased from National Centre for Cell Science (NCCS), Pune, India and grown as a monolayer in DMEM medium containing HEPES and sodium bicarbonate supplemented with 10% FBS and 1X antibiotic-antimycotics. Cells were maintained in a tissue culture flask and kept in a humidified incubator (5% CO2 in air at 37°C) with a medium change every 2–3 days. When the cells reached 70–80% confluence, they were harvested with trypsin—EDTA (ethylene diamine tetra acetate) and seeded into a new tissue culture flask.

2.3. Collection of plant material and preparation of plant extracts

W. fruticosa flowers were collected from natural habitat during November–January. Plant material was identified by Dr. V.T Antony and a voucher specimen (Acc. No. 7566) was deposited at the herbarium of the Department of Botany, S.B College, Changanassery, Kottayam, Kerala. Flowers were shade-dried, powdered and 50 g of dried powder was soxhlet extracted with 400 mL of methanol for 48 h. The extract was concentrated under reduced pressure using a rotary evaporator and was kept under refrigeration. The yield of methanolic extract of Woodfordia fruticosa (MEWF) was 12.5% (w/w). The concentrate was suspended in 5% Tween 80 for in vitro study and in DMSO for in vitro antiproliferative study.

For in vitro antiproliferative study, MEWF was dissolved in DMSO at a concentration of 25 mg/ml. The test solution was prepared freshly on the day of use, diluted to two different concentrations of MEWF (100 μg/ml, 50 μg/ml) and 5-flourouracil, the standard control (50 μg/ml) with DMEM medium containing 10% (v/v) FBS and 1x antibiotic-antimycotics.

2.4. Animals and diets

Male Wistar rats weighing 160–180 g were used for this study. The animals were housed in polypropylene cages and had free access to standard pellet diet (Sai Durga Feeds, Bangalore, India) and drinking water. 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 Institute Animal Ethics Committee 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.

2.5. Induction of hepatocellular carcinoma

HCC was induced by oral administration of 0.02% NDEA (2 ml, 5 days/week for 20 weeks). Silymarin at an oral dose of 100 mg/kg body weight was used as standard control. Two different doses of MEWF (100 mg/kg and 200 mg/kg) were also prepared for oral administration to the animals. The lethal dose of W. fruticosa was found to be more than 2000 mg/kg p.o.

2.6. Experimental design

Thirty six rats were divided into six groups,

Group I – Normal control
Group II – NDEA control (0.02% NDEA, 2 ml, 5 days/week, p.o)
Group III – Silymarin (100 mg/kg b.w) + NDEA
Group IV – MEWF (100 mg/kg, b.w) + NDEA
Group V – MEWF (200 mg/kg, b.w) + NDEA
Group VI – MEWF (200 mg/kg) alone

Daily doses of Silymarin and MEWF treatments were started in group III–V animals 1 week before the onset of NDEA administration and continued up to 20 weeks. Group VI served as drug control received MEWF alone for the entire period. The rats were sacrificed 48 h after the last dose of NDEA administration.

2.7. Morphometry evaluation

Rat livers were blotted dry and examined on the surface for visible macroscopic liver lesions (neoplastic nodules). The grayish white lesions 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.

2.8. Biochemical analysis

Serum was separated from collected blood using centrifuge at 3000 g for 15 min and used for estimation of AFP, ALP and LDH. The excised liver was then weighed and homogenized in chilled Tris buffer (0.1 M, pH 7.4) at a concentration of 10% w/v. The homogenates were centrifuged at 10,000 g for 20 min. The clear supernatants were used for the assays of reduced glutathione (GSH), Catalase (CAT), MDA and total protein.

2.9. Histopathological and immunohistochemical studies

Small pieces of liver fixed in 10% buffered formalin and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were then embedded in paraffin blocks and cut into 5 μm thick sections. Sections were stained with hematoxylin and eosin.

For Immunohistochemistry VEGF monoclonal antibody was used and was done by the method of Wills et al with some modifications. Here after deparaffinization the slides were placed in citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven for antigen retrieval. Images were taken at original magnification of 100× (Motic AE 21, Germany and Moticam 1000 camera).

2.10. Cytotoxicity study by MTT assay

The cell viability was assessed by MTT assay, which determines the metabolically active mitochondria of cells. PLC/PRF/5 cells were seeded in 96-well plates (Greiner, Frickenhausen, Germany) with 5 × 10^3 cells/100 μL and incubated for 24 h at 37 °C. The cells were then treated with MEWF (100 μg/mL and 50 μg/mL), 5-FU (50 μg/mL) and DMSO (0.1% v/v) and incubated for different time intervals (12 h, 24 h, 48 h and 72 h) at 37 °C in a 5% CO₂ atmosphere. The assay was performed by the addition of premixed MTT reagent, to a final concentration of 10% of total volume, to culture wells containing various concentrations of the test substance and incubated for further 4 h. During 4 h incubation, living cells converted the tetrazolium component of the dye solution into a formazan product. The solubilization/stop solution was then added to the culture wells to solubilize the formazan product and the absorbance at 570 nm was recorded using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate. Percentage inhibition was calculated using the formula,

\[
\text{Percentage growth inhibition} = \left( \frac{\text{Mean absorbance of the control cells} - \text{Mean absorbance of treated cells}}{\text{Mean absorbance of control cells}} \right) \times 100
\]

2.11. Statistical analysis

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

3. Results

The changes in body weights of rats among the experimental group 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. Pretreatment with Silymarin and MEWF (100 mg/kg, 200 mg/kg) prevented the decline in animal body weight due to NDEA treatment. Pretreatment with Silymarin and MEWF exhibited significant ($p \leq 0.05$) elevation in the body weight compared to NDEA treated group (Fig. 1).

Liver weight of NDEA alone treated rats increased significantly ($p \leq 0.05$) at the end of the 20th week of exposure when compared with normal rats. But treatment with MEWF prevented the increase in liver weight in rats exposed to NDEA. MEWF alone treated rats did not show any significant changes when compared to normal control (Table 1).

NDEA treated rats showed significantly ($p \leq 0.05$) elevated serum levels of AFP, ALP, LDH and bilirubin when compared to normal control. A significant ($p \leq 0.05$) reduction was observed in serum markers in the animals treated with Silymarin (100 mg/kg), MEWF (100 mg/kg and 200 mg/kg) compared to NDEA treated group (Fig. 2).

In morphology and morphometry evaluation, NDEA treated rat liver become very large in size and a large number of

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Liver weight</th>
</tr>
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<tbody>
<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 (100 mg/kg, b.w)</td>
<td>6.8 ± 1.2**</td>
</tr>
<tr>
<td>NDEA + MEWF (100 mg/kg, b.w)</td>
<td>8.3 ± 0.8**</td>
</tr>
<tr>
<td>NDEA + MEWF (200 mg/kg, b.w)</td>
<td>7.2 ± 1.0**</td>
</tr>
<tr>
<td>MEWF alone (200 mg/kg, b.w)</td>
<td>7.8 ± 0.8**</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., $n = 6$.

* $p \leq 0.05$ versus normal control.

** $p \leq 0.05$ versus NDEA control.

![Fig. 2 – Effects of MEWF on changes in serum enzyme levels of rats treated with NDEA. (A) AFP (B) ALP (C) LDH (D) 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, * $p \leq 0.05$ versus normal control, ** $p \leq 0.05$ versus NDEA control.](image-url)
hepatic nodules were observed (Fig. 3). 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 2).

Tissue biochemical analysis showed a significant \((p < 0.05)\) reduction in GSH, CAT and increased levels of MDA in NDEA treated group compared to normal control. A significant \((p < 0.05)\) elevation in GSH, CAT and MDA were observed in animals treated with Silymarin (100 mg/kg), MEWF (100 mg/kg and 200 mg/kg) compared to NDEA treated group (Table 3).

In NDEA intoxicated rat tissue enlarged nuclei, hyperchromatism, scattered masses of necrotic tissues, proliferating hepatocytes and mild congestion of sinusoids with central vein dilation were detected in histopathological studies. However, treatment with MEWF at a dose of 200 mg/kg
showed almost normal architecture with normal hepatocytes and uniform sinusoids (Fig. 4).

In immunohistochemical analysis NDEA intoxicated rat tissue showed localization of VEGF around periportal area (arrow heads). A significant down regulation of VEGF was spotted in MEWF at a dose of 200 mg/kg treated group (Fig. 5).

The dose-dependent cytotoxic effect of MEWF on PLC/PRF/5 cells was evaluated by MTT assay. The cells were treated with 50 and 100 μg/ml of MEWF and the inhibition of cell proliferation was assessed after 12 h, 24 h, 48 h and 72 h. MEWF exerted cytotoxic effect on PLC/PRF/5 cells in a dose-dependent manner with percentage of cell inhibition values 12.4 ± 0.8, 23.1 ± 0.9, 44.4 ± 1.7 and 55.8 ± 2.2 for 50 μg/ml and 24.2 ± 1.3, 33.8 ± 1.2, 56.8 ± 2.0 and 65.3 ± 2.5 for 100 μg/ml after 12 h, 24 h, 48 h and 72 h respectively. 5-flourouracil, used as positive control, showed an inhibition of 26.8 ± 1.0, 36.2 ± 1.5, 59.2 ± 2.3 and 70.2 ± 2.8 for 50 μg/ml and 14.7 ± 1.1, 25.2 ± 0.8, 47.9 ± 1.8 and 59.1 ± 2.3 for 25 μg/ml after 12 h, 24 h, 48 h and 72 h respectively. Treatment with MEWF exhibited significant cytotoxic effect on PLC/PRF/5 cells (p < 0.05) when compared to the cells treated alone with DMSO. The results were graphically expressed in Fig. 6.

4. Discussion

The results of the present study showed that the MEWF had marked effect in hepatocellular carcinoma in in vitro and in vivo models. Generation of large amount of ROS is apparent during the metabolic biotransformation of NDEA resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression. A significant elevation in liver marker enzymes is an indication of abnormal functioning of liver. The enzymes are cytoplasmic in nature; upon liver injury these enzymes enter into the circulatory system due to altered permeability of the membrane. Administration of NDEA to rats significantly increased serum AFP, ALP, LDH and bilirubin levels. Treatment with MEWF at a dose of 200 mg/kg normalized the altered serum parameters.

In our study a significant decrease in the concentration of GSH and CAT and an increase in the levels of MDA in NDEA treated group was observed. Catalase is responsible for the breakdown of H₂O₂, an important ROS. Increased MDA content is an important indicator of lipid peroxidation. MEWF significantly and dose-dependently reversed the changes in antioxidant levels. It has already been reported the liver protective efficacy of Woodfordia fruticosa in experimental animals.[7,17,18]

Histopathological data indicates that NDEA treated rat liver showed enlarged nuclei and necrotic tissues which are the characteristic features of HCC. Treatment with MEWF dose-dependently prevented the toxic effects of NDEA on hepatic tissues.

Vascular endothelial growth factor overexpresses in HCC tissues relative to noncancerous liver tissues. It is secreted by hepatoma cells and hepatic stellate cells, which is up regulated during tumor dedifferentiation and vascular development of HCC. In the present study, immunohistochemical analysis showed the localization of overexpressed VEGF around the periportal area in NDEA intoxicated rats.

<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>
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<tbody>
<tr>
<td>Normal control</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</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 (100 mg/kg) + NDEA</td>
<td>1/6</td>
<td>16</td>
<td>8 ± 0</td>
<td>8</td>
</tr>
<tr>
<td>MEWF (100 mg/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 (200 mg/kg b.w) + NDEA</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>MEWF alone (200 mg/kg, b.w)</td>
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<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (nmol/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>MDA (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>22.1 ± 0.5</td>
<td>47 ± 1.2</td>
<td>43.8 ± 1.2</td>
</tr>
<tr>
<td>NDEA control (0.02%)</td>
<td>12.1 ± 0.4*</td>
<td>32.1 ± 1.7*</td>
<td>74.5 ± 1.5*</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg) + NDEA</td>
<td>18.2 ± 0.4**</td>
<td>41.2 ± 1.7**</td>
<td>56.5 ± 1.7**</td>
</tr>
<tr>
<td>MEWF (100 mg/kg b.w) + NDEA</td>
<td>15.8 ± 0.5**</td>
<td>38.1 ± 1.4**</td>
<td>64.3 ± 1.1**</td>
</tr>
<tr>
<td>MEWF (200 mg/kg b.w) + NDEA</td>
<td>21 ± 0.4**</td>
<td>43.5 ± 1.4**</td>
<td>46.6 ± 1.7**</td>
</tr>
<tr>
<td>MEWF alone (200 mg/kg, b.w)</td>
<td>22.6 ± 0.6**</td>
<td>46.2 ± 1.5**</td>
<td>44.9 ± 1.6**</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6.
*p ≤ 0.05 versus normal control.
*p ≤ 0.05 versus NDEA control.
Treatment with MEWF significantly and dose-dependently inhibited the over expression of VEGF indicating the inhibitory role of MEWF in neo-vasculature formation.

MTT assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. In the present study, cytotoxic effect of the MEWF on PLC/PRF/5 cell was determined based on reduction of the yellow colored water soluble tetrazolium dye 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) to formazan crystals. Mitochondrial dehydrogenase produced by live cells reduces MTT to blue formazan product, which reflects the normal function of mitochondria and cell viability. A dose-dependent reduction of MTT (or color change from yellow to purple) observed in 5-FU and extracts treated cells indicate their cytotoxic potential against human hepatoma PLC/PRF/5 cells.

Fig. 4 – Histopathological changes occurred in rat liver due to pretreatment 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 (200 mg/kg) alone.
Phytochemical analysis of MEWF showed positive test for saponins (steroids and terpenes), phenolics, alkaloids, flavonoids, tannins etc. The compounds reported from W. fruticosa flowers were β-sitosterol, kaempferol, ellagic acid, octacosanol, meso-inositol, quercetin, woodfordin A, B, C, D and eonothein A and B. Ellagic acid is an anticarcinogenic agent, it inhibits DNA topoisomerase. Quercetin is an antioxidant possesses antiinflammatory and anticarcinogenic properties. Woodfordin C and eonothein B, a class of macrocyclic hydrolysable tannins exhibited potent host-mediated antitumor activity against sarcoma 180 in mice. Woodfordin C showed remarkable inhibition of DNA topoisomerase II. Woodfordin D and eonothein A, trimeric hydrolysable tannins also have antitumor activity. The identified class of components in single or in combination with other components present in the extract

Fig. 5 – Immunohistochemical localizations of VEGF in control and treated. Liver tissue was immunostained for VEGF (arrow heads) followed by staining with hematoxylin (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 (200 mg/kg) alone.
might be responsible for the prevention of hepatocellular carcinoma.

5. Conclusion

The results in the present study validate the potential anticancer activity of MEWF. HCC induced by NDEA was effectively inhibited by the treatment with MEWF at a dose of 200 mg/kg, b.w. The potential antiproliferative effect of MEWF was also evidenced by human hepatoma PLC/PRF/5 cell line. The potential chemoprevention observed in this study might be due to synergistic effect of the phytomolecules present in the extract. This finding suggested a possible basis for the potential use of the flowers of *W. fruticosa* in the inhibition of hepatic cancer. These findings might also provide a pharmacological background on the traditional use of the plant for the treatment of liver diseases. However further work is required for the fractionation of MEWF and identification of the active compound which is underway.

Conflicts of interest

All authors have none to declare.

Acknowledgments

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