

CURATIVE EFFECT OF *WOODFORDIA FRUTICOSA* KURZ FLOWERS ON *N*-NITROSODIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOMA IN RATS

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ABSTRACT

Objective: To evaluate the antioxidant and anticancer effect of methanolic extract of *Woodfordia fruticosa* (MEWF) dried flowers on *N*-nitrosodiethylamine induced hepatocellular carcinoma in experimental rats.

Methods: Six groups of six rats in each were selected for the study. Hepatocellular carcinoma was induced by the oral administration of NDEA (0.02%, 2ml, 5days/weeks) for 20 weeks to all groups except group I and VI. Group I animals treated as normal control and group VI served as drug control. After the intoxication with NDEA for 20 weeks, group III, IV and V animals were treated with daily doses of Silymarin and MEWF 100mg/kg, b.w and 200mg/kg, b.w respectively for 28 days. Serum and tissue biochemical analysis and immunohistochemical analysis were done to evaluate the antioxidant and anticancer effect of MEWF.

Results: MEWF significantly ($p \leq 0.05$) prevented the elevation of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) levels and reduced glutathione (GSH), glutathione-S-transferase (GST) and glutathione reductase (GR) levels. Immunohistochemical analysis revealed the overexpression of proliferating cell nuclear antigen (PCNA) and cyclin D1 in the liver tissue of rat intoxicated with NDEA. The over expression was effectively reduced by the treatment with MEWF.

Conclusion: These results suggest the curative effect of MEWF against NDEA induced hepatocellular carcinoma and thus scientifically supports the use of this plant in traditional medicine for the treatment of liver cancer.

Keywords: Antioxidant, Cyclin D1, *N*- nitrosodiethylamine (NDEA), Proliferating cell nuclear antigen (PCNA), Silymarin, *Woodfordia fruticosa*.

INTRODUCTION

Malignant cancers are the second leading cause of death, among them hepatocellular carcinoma (HCC) is the most malignant tumor with very high morbidity and mortality rates, and poor prognosis [1]. 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. *N*-nitrosodiethylamine (NDEA) is a potent carcinogenic dialkyl nitrosoamine used to induce liver cancer in animal models [2]. It is found in a wide variety of foods such as cheese, soybeans, smoked, salted and dried fish, cured meat and alcoholic beverages and producing reproducible hepatocellular carcinoma after repeated administration [3]. NDEA became metabolically active by the action of cytochrome P450 enzymes to produce reactive electrophiles, which increase oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity [4]. Oxidative stress is considered as critical mechanism contributing to NDEA-induced hepatotoxicity [5].

Numerous components of plants, collectively termed "phytochemicals" have been reported to possess chemopreventive properties. *Woodfordia fruticosa* is a traditional medicinal plant belongs to the family Lythraceae is used against a wide variety of diseases. All parts of the plant possess valuable medicinal properties such as anti inflammatory, antitumor, hepatoprotective and free radical scavenging activity but flowers are of maximum demand. Dried flowers are used as tonic in disorders of mucous membrane, hemorrhoids and in derangement of liver [6].

Phenolics, particularly hydrolysable tannins and flavonoids were identified as major components of *Woodfordia fruticosa* flowers. Our preliminary study supported the hepatoprotective and antiproliferative effect of the methanolic extract of *Woodfordia fruticosa* in experimental animals [7, 8]. In view of these, a detailed study was undertaken to evaluate the curative effect of *Woodfordia fruticosa* flowers on NDEA induced hepatocellular carcinoma in experimental rats.

MATERIALS AND METHODS

Chemicals

N-nitrosodiethylamine (NDEA), silymarin, proliferating cell nuclear antigen (PCNA), cyclin D1, anti-mouse IgG horse radish peroxidase, streptavidin horse radish peroxidase conjugate and diaminobenzidine were purchased from Sigma Chemical Co., USA. Assay kits for serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) were purchased from Agappe Diagnostics, India. All other chemicals were of analytical grade.

Collection of plant material and preparation of plant extracts

Woodfordia 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 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 the present study.

Animals and diets

Male Wistar rats weighing 160-180 gm were used in 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 (Reg. No. B 2442009/4) and conducted humanely.

Induction of hepatocellular carcinoma

HCC was induced by oral administration of 0.02% NDEA (2ml, 5 days/week) for 20 weeks [9]. Silymarin at an oral dose of 100 mg/kg body weight was used as standard control in the experiment [10]. Two different doses of MEWF (100mg/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 2000mg/kg p.o [11].

Experimental design

Thirty six rats were divided into six groups

Group I - Normal control

Group II - NDEA control (0.02% NDEA, 2 ml, 5days/week, p.o)

Group III - NDEA + Silymarin

Group IV- NDEA + MEWF (100mg/kg, b.w)

Group V - NDEA + 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 only MEWF (200mg/kg) alone for last 28 days. Group II received normal diet and 1ml of 5% tween 80 daily for 28 weeks. Animals were sacrificed 48 h after the last dose of MEWF administration. So duration of the entire study was 24 weeks.

Serum enzyme analysis

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. Quantifying the serum levels of AST, ALT and GGT by kinetic method using a standard diagnostic kit. Activities of these serum enzymes were measured by using semi autoanalyzer (RMS, India).

Tissue analysis

Liver tissue was excised, washed thoroughly in ice-cold saline to remove the blood. Firstly morphometry evaluation was made and then the dissected livers were cut into separate portions for biochemical assays and immunohistochemical analysis.

Morphometry evaluation

Rat livers were 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.

Biochemical assays

Ten percent of 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) and total protein.

GSH was determined based on the formation of a yellow colored complex with dithio-bis-2 nitrobenzoic acid (DTNB) [11]. GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB [12]. GR (EC 1.6.4.2) activity was assayed at 37 °C and 340 nm by following the oxidation of NADPH by GSSG [13]. Protein content in the tissue was determined using bovine serum albumin (BSA) as the standard [14].

Immunohistochemical analysis

Immunohistochemical analysis for two cancer markers, namely proliferating cell nuclear antigen (PCNA) and Cyclin D1 were conducted. Tissue sections were deparaffinised in three changes of xylene at 60 °C for 10min each and hydrated through a graded series of alcohol. For antigen retrieval evaluation slides were incubated in citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven. The sections were then allowed to cool to room temperature and then rinsed with 1x tris buffered saline (TBS), and treated with 0.3% H₂O₂ in water for 10 min to block endogenous peroxidase activity. Non specific binding was blocked with 3% BSA at room temperature for 1 h and then incubated with PCNA antibody diluted 1:500 / Cyclin D1 antibody diluted 1:80 with 1% BSA in PBS for overnight at 4°C. Sections were then washed thrice in PBS and incubated with anti-mouse horseradish peroxidase for 45 min. After triplicate washing with PBS, sections were incubated for 30 min with streptavidin-HRP complex. Sections were then washed with PBS and incubated for 5-10 min in a solution of diaminobenzidine (6 mg/10mL 50mM Tris-HCl, pH 7.6) containing 0.01% H₂O₂. Counterstaining was performed with hematoxylin. Images were taken at original magnification of 100× (Motic AE 21, Germany and Moticam 1000 camera).

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.

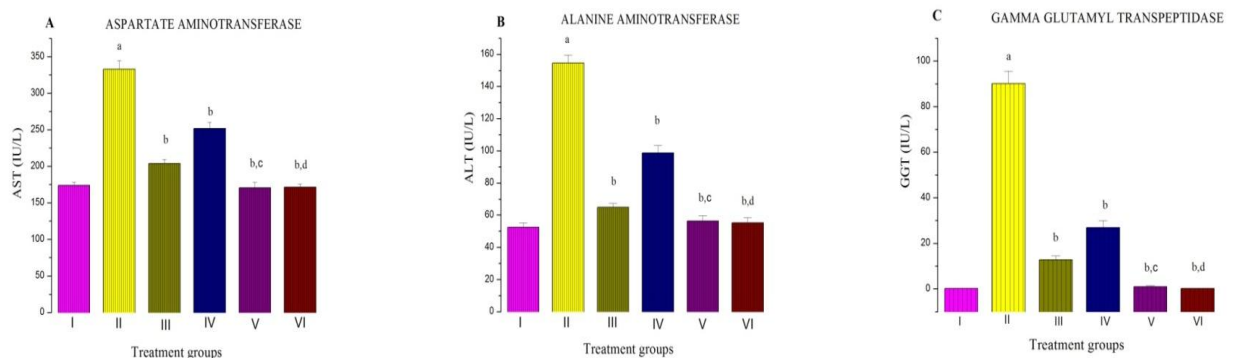


Fig. 1: Effects of MEWF on changes in serum enzyme levels of rats post-treated with MEWF. (A) Aspartate aminotransferase, (B) Alanine aminotransferase (C) Gama glutamyl transferase (I) Normal control, (II) NDEA control (III) NDEA + Silymarin, (IV) NDEA + MEWF (100 mg/kg) (V) NDEA + MEWF (200 mg/kg) (VI) MEWF alone. Values are mean ± S.D from 6 rats in each group. Statistical significance: *p* ≤ 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 NDEA 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.

Table 1: Effect of MEWF on the development of NDEA induced hepatic nodules in rat

Groups	No of rats with nodule/ Nodule incidence	Total no. of nodule/ Nodule/ Total no. of rats	Average no. of	Nodule bearing liver	Nodule multiplicity
Normal control	-	-	-	-	-
NDEA control(0.02%)	6/6	100		124±27	20.7
Silymarin (100mg/kg) + NDEA	2/6	33		11 ±4	5.5
MEWF(100mg/kg) + NDEA	4/6	50		16±5	4
MEWF (200mg/kg) + NDEA	-	-		-	-
MEWF alone (200mg/kg)	-	-		-	-

RESULTS

Serum analysis

The serum levels of AST, ALT and GGT 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 100 and 200 mg/kg showed a dose depended decrease ($p \leq 0.05$) in AST, ALT and GGT levels (Fig.1). MEWF at a dose of 200 mg/kg, b.w showed better results when compared to the standard drug silymarin.

Morphological evaluation

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 (Table1). In NDEA control group the nodule incidence was 100% and it dropped to 50% by the treatment with MEWF at a dose of 100mg/kg, b.w. and to 33% due to the treatment with standard control drug silymarin. Administration of MEWF at a dose of 200mg/kg and in MEWF alone treated group the nodule formation was significantly down regulated.

Tissue biochemical analysis

Estimation of reduced Glutathione (GSH)

Reduced glutathione (GSH) levels were lowered significantly ($p \leq 0.05$) in NDEA intoxicated rats.

Treatment with MEWF at a dose of 200mg/kg, b.w increased the levels of GSH by 86.1% when compared to NDEA control. In silymarin treated groups it was 57.4% (Table 2).

Estimation of Glutathione - S - transferase (GST)

The 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 2). Treatment with MEWF 200 mg/kg exhibited prominently increased i.e., 95% GST levels.

In addition, In silymarin treated rats the GST activity was 67%.

Estimation of Glutathione Reductase (GR)

GR activity was significantly decreased ($p \leq 0.05$) in NDEA treated animals when compared to normal control. When NDEA intoxicated rats were treated with MEWF (100 and 200 mg/kg) and Silymarin (100 mg/kg) significant increase ($p \leq 0.05$) in the GR level was observed (Table 2). The percentage of protection in liver tissue was 89% for 200 mg/kg of MEWF and 67% for silymarin.

Table 2: Effect of MEWF on GSH, GST and GR activities in liver tissue of different experimental groups

Treatment groups	GSH ¹	GST ²	GR ³
Normal control	23.5±0.9	70.4±1.6	21.8±1.1
NDEAControl (0.02%)	13.4±1.5 ^a	35.7±0.9 ^a	8.8±0.9 ^a
NDEA + Silymarin(100mg/kg)	19.2±1.8 ^b	59.1±1.2 ^b	17.6±1.4 ^b
NDEA + MEWF (100mg/kg)	16.9±1.0 ^b	52.8±1.9 ^b	13.9±0.8 ^b
NDEA + MEWF (200mg/kg)	22.1±0.8 ^{b,c}	68.7±2.1 ^{b,c}	20.4±1.6 ^{b,c}
MEWF alone	23.2±1.4 ^{b,d}	71.2±1.5 ^{b,d}	22.2±0.8 ^{b,d}

¹: nmol/mg protein; ²: μmol CDNB-GSH conjugate formed/min/mg protein; ³: nmol of GSSG utilized/min/mg protein

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

Immunohistochemical analysis

Immunohistochemical analysis of the normal rat tissue showed regularly stained nucleus.

In Fig. 2 NDEA administered rat liver tissue showed over expression of Proliferating Cell Nuclear Antigen (PCNA). PCNA is a common index for proliferation of hepatocytes at late G1 stage and early S stage. Administration of MEWF showed a dose dependent reduction in the expression of PCNA in Fig. 2B (HCC bearing) stained positively for PCNA indicating active cell proliferation when compared with normal control.

The plant extract 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. In Fig. 2 no significant difference was noted between normal control and MEWF alone (200mg/kg) treated rat liver.

In Fig. 3 NDEA administered rat liver tissue showed over expression of Cyclin D1. Administration of MEWF showed a dose dependent reduction in the expression of Cyclin D1. Fig. 3B (HCC bearing) stained positively for Cyclin D1 indicating active cell proliferation when compared with normal control. The plant extract given at a concentration of 200mg/kg showed a remarkable lowering of the expression of cyclin D1 and was comparable to the effect rendered by silymarin at a concentration of 100mg/kg. In Fig. 3 no significant difference was noted between normal control and MEWF alone (200mg/kg) treated rat liver.

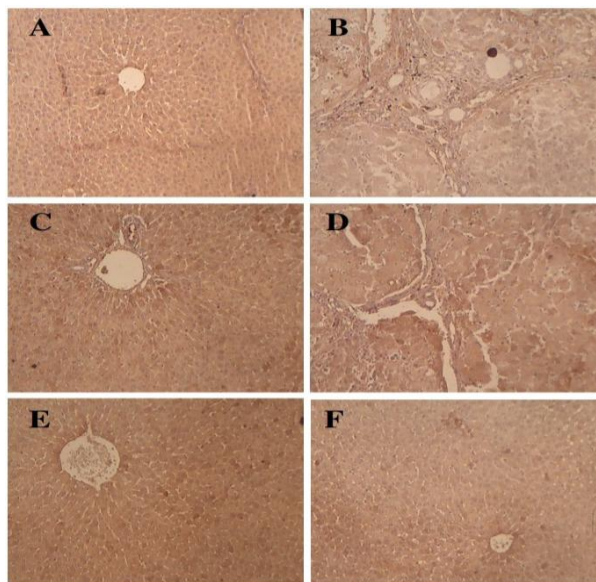


Fig. 2: Immunohistochemical localizations of PCNA in control and treated groups. Liver tissue was immunostained for PCNA followed by staining with hematoxylin (100x). (A) Normal control; (B) NDEA control (0.02%); (C) NDEA + Silymarin (100 mg/kg); (D) NDEA + MEWF (100 mg/kg); (E) NDEA + MEWF (200 mg/kg); (F) MEWF (200mg/kg) alone

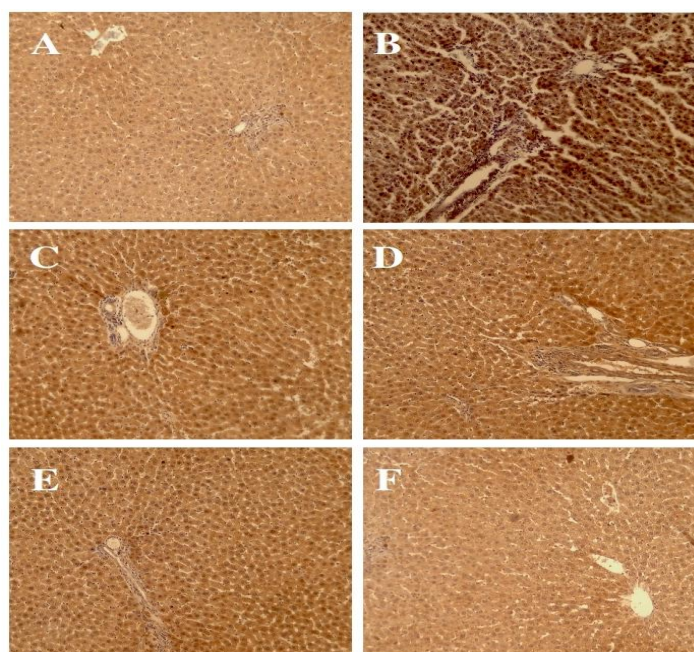


Fig. 3: Immunohistochemical localizations of Cyclin D1 in control and treated groups. Liver tissue was immunostained for Cyclin D1 (indicated by brown stained nuclei) followed by staining with hematoxylin (100x). (A) Normal control; (B) NDEA control (0.02%); (C) Silymarin (100 mg/kg) + NDEA; (D) MEWF (100 mg/kg) + NDEA; (E) MEWF (200 mg/kg) + NDEA and (F) MEWF (200mg/kg) alone.

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 [15]. 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 [16].

In this study the NDEA administration to the rats for a period of 20 weeks leads to a drastic weight loss and a marked elevation in the

levels of serum AST, ALT and GGT. Increased serum transaminases and GGT indicated liver malfunction. The increase in the activities of AST, ALT and ALP in serum of toxic control rats might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream [17]. Treatment with MEWF increased the activities of serum enzymes indicating the curative effect of the extract. Treatment with MEWF at two different doses lowered the activities of these enzymes. The extract 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 [18]. 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 along with some non-enzyme antioxidants like GSH, GST 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 [19].

Treatment with MEWF increased the hepatic GSH content 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 [20]. GR is also essential for the maintenance of GSH levels in vivo. This evidently shows the antioxidant property of the extract against oxygen free radicals. Hepatic damage induced by NDEA administration is associated with cellular necrosis and depletion in the tissue GSH, GST and GR activities.

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. PCNA present in cell nucleus is directly involved in DNA replication. It has been found that the positive expression of PCNA was 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 [21]. The results indicated that the administration of MEWF could remarkably decrease the PCNA in HCC bearing tissue, suggesting the efficacy in suppressing the malignant proliferation of hepatocytes.

Cyclin D1 is also an important cancer marker. Cyclin D1 is the regulatory subunit of a holoenzyme that promotes progression through the G1-S phase of cell cycle. Amplification or overexpression of Cyclin D1 plays an important role in the development of a subset of human tumorigenesis and cellular metastasis [22]. In the present study MEWF treatment dose dependently inhibit the expression of cyclin D1 also in NDEA induced HCC tissue.

Preliminary phytochemical analysis revealed the presence of saponins (steroids and terpenes), phenolics, alkaloids, flavanoids, tannins etc in the methanolic extract of *Woodfordia fruticosa*. The compounds reported from the dried flowers of *Woodfordia fruticosa* are β -sitosterol, kaempferol, ellagic acid, octacosanol, meso-inositol, quercetin, woodfordins A, B, C, D and oenotherin A and B [23]. In our previous studies LCMS analysis of MEWF revealed the presence of octacosanol, malonic acid, oxaloacetic acid, octanoic acid, isocaryophyllene, confertin, Quercetin methyl ether, ellagic acid, ursolic acid, stigmaterol, hdroxy methyl flavan etc. Confertin showed antiproliferative effect on DLA cell line [24]. Quercetin methyl ether suppresses proliferation of mouse epidermal JB6P+ cells by targeting ERKs [25]. It showed hepatoprotective effect on copper induced oxidative damage in hepatocytes and it also have an in vitro anti inflammatory effect [26]. 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 [27]. Stigmaterol (β sitosterol) is a component reported as a hepatoprotective agent [28]. So the mentioned components in single or in combination with other components present in the extract might be responsible for the anticancer activity of the extract.

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. The antioxidant and anticancer properties of this plant can be attributed to these identified classes of phytochemicals. 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 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.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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