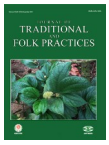
Volume 09 (01 & 02) December 2021, 181-189
ISSN 2278- 5906
http://jtfp.jntbgri.res.in



Journal of Traditional and Folk Practices

Woodfordia fruticosa (L.) Kurz flowers dose dependently down regulates VEGF expression and improves antioxidant status in *N*- nitrosodiethylamine induced hepatocellular carcinoma in wistar rats

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Received: 08 May 2021 Accepted: 30 May 2021

Abstract

The present study was carried out to investigate the dose dependent effect of methanolic extract of *Woodfordia fruticosa* (L.) Kurz flowers on *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma in wistar rats. Male wistar rats were divided into five groups of six rats in each group. Hepatocellular carcinoma was induced by the oral administration of NDEA (0.02%, 2 ml, 5days/weeks) for 20 weeks to all groups except group I and VI. Group I animals was treated as vehicle 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 methanolic extract of *W. fruticosa* (MEWF) 100 mg/kg, b.w and 200 mg/kg, b.w respectively for 28 days. Serum and tissue biochemical analysis, histopathological study and immunohistochemical analysis were done to evaluate the antioxidant and anticancer effect of MEWF. Treatment with MEWF significantly ($p \leq 0.05$) prevented the NDEA induced elevation of serum alpha feto-protein (AFP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin and hepatic malondialdehyde (MDA) levels. When compared to NDEA alone treated group NDEA plus MEWF treated group exhibited significant ($p \leq 0.05$) increase in hepatic GPx and catalase (CAT) activities. Histopathological and immunohistochemical analysis also supported the dose dependent protective effects of MEWF. These results suggest that MEWF has the potential to dose dependently down regulate Monoclonal anti-vascular endothelial growth factor (VEGF) expression and improves antioxidant status in NDEA induced hepatocellular carcinoma.

Keywords: Anticancer, Antioxidant, Hepatocellular carcinoma, Vascular endothelial growth factor

1. Introduction

India has a unique wealth of biota which includes a large number of medicinal and aromatic plants. India has been identified as one of the mega bio-diversity center of the world. Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity.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 interrupts 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. So efforts to develop less toxic drugs that affect only malignant cells and mechanism based approach are necessary in cancer therapy (Sivalokanathan *et al.,* 2006).

Woodfordia fruticosa (L.) Kurz. (syn. *Woodfordia floribunda* Salisb.) belongs to the family Lythraceae, is a much branched beautiful shrub, 1-3 m high. English names of the plant are Fire Flame Bush and Shiranjitea. In Malayalam it is known as *Thathiri*. It is a much used medicinal plant in Ayurvedic and Unani systems of medicines (Chopra *et al.,* 1956; Watt, 1972). The flowers are used in the preparation of Ayurvedic fermented drugs called "*Aristhas*" (hot extraction followed by month-long slow fermentation) and "*Asavas*" (cold percolation followed by month-long slow fermentation) (Atal *et al.,* 1982. 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 (Das *et al.,* 2007; Gunawardana and Jayasuriya, 2019). The dried flowers powder sprinkled over ulcers and wounds to diminish discharge and promote granulation (Khorya and Katrak, 1984). They are also used as tonic in disorders of mucous membranes, hemorrhoids and in derangement of the liver (Chopra *et al.,* 1956; Anonymous, 1988; Mhaskar *et al.,* 2000). Phenolics, particularly hydrolysable tannins and flavonoids were identified as major components of *W. fruticosa* flowers (Das *et al.,* 2007). Our preliminary study reports supported the hepatoprotective effect of the methanolic extract of *W. fruticosa* in experimental animals (Nitha *et al.*, 2012). Thus the present study was undertaken to evaluate the curative effect of *W. fruticosa* flower on NDEA induced hepatocellular carcinoma in experimental rats.

Fig. 1. Habit of *Woodfordia fruticosa* (L.) Kurz

Hepatocellular carcinoma (HCC) is the fifth most common ubiquitous deadliest cancer worldwide with poor diagnosis and accounts for approximately about 500,000 to 1,000,000 new cases per year (Shiota *et al.*, 1999). The liver is an organ of paramount importance and it plays an essential role in drug and xenobiotic metabolism. Hepatitis, 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 dialkylnitrosoamine used to induce liver cancer in animal models. NDEA belongs to the group of N-nitrosamines, causing a wide range of tumors in all animal species and suspected to be health hazards to man (Loeppky, 1999; Pandi-Perumal *et al*., 2006). Administration of NDEA to animals causes cancer in liver and at low incidence in other organs also. NDEA 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 (Singh *et al.*, 2009). It has been shown that the mechanism of action is due to the 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). 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, mutagenicity and carcinogenicity (Archer, 1989). Oxidative stress is considered as a critical mechanism contributing to NDEA induced hepatotoxicity, and the use of antioxidant agents reduced liver damage (Vitaglione *et al.*, 2004).

2. Materials and methods

2.1. Chemicals

N-nitrosodiethylamine (NDEA), silymarin, anti-mouse IgG horse radish 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. Alpha feto-protein (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. All other chemicals were of analytical grade.

2.2. 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 50g of dried powder was soxhlet extracted with 400ml of methanol for 48h. The extract was concentrated under reduced pressure using a rotary evaporator and was kept under refrigeration. The yield of methanolic extract of *W. fruticosa* (MEWF) was 12.5% (w/w). MEWF was suspended in 5% Tween 80 for the present study.

2.3. Animals and diets

Male wistar rats weighing 160-180g 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^{0}C with a 12 h light: 12 h dark cycle. Animal studies were followed according to Institute Animal Ethics Committee regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg. No. B 2442009/4) and conducted humanely.

2.4. Induction of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) was induced by oral administration of 0.02% NDEA (2 ml, 5 days/week) for 20 weeks (Wills *et al.*, 2006). 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 of MEWF (100 mg/kg and 200 mg/kg) were also prepared for oral administration to the animals. It is already reported that the *W. fruticosa* flower extracts do not show any sign of toxicity up to oral dose of 2000 mg/kg (Chandan *et al.*, 2008).

2.5. 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 - NDEA + Silymarin

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

Group V - NDEA + MEWF (200 mg/kg, b.w)

Group VI - MEWF (200 mg/kg) alone

After the intoxication with NDEA for 20 weeks, groups III, IV and V were treated with daily doses of Silymarin and MEWF 100 mg/kg, b.w and 200 mg/kg, b.w respectively for 28 days. In order to nullify the effect of Tween 80, group I was administered with 5% Tween 80 for 28 days.Group VI served as drug control received MEWF (200 mg/kg) alone for the last 28 days. Group II received normal diet and 1 ml of 5% tween 80 daily for 28 days. 48 hour after the last dose of MEWF administration, animals were anesthetized with pentothal sodium and blood was collected from neck blood vessels, followed by neck decapitation.

2.6. Serum enzyme analysis

Blood was collected from neck blood vessels and kept for 30min at 4^{o}C. Serum was separated by centrifugation at 2500 rpm at 4^{o}C for 15 min. Quantifying the serum levels of AFP, ALP, LDH and bilirubin by kinetic method using a standard diagnostic kit. Activities of these serum enzymes were measured by using semi auto analyzer (RMS, India).

2.7. Tissue analysis

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

2.8. 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.

2.9. Biochemical assays

Ten percent of homogenate was prepared in 0.1 M TrisHCl buffer (pH-7.4). The homogenate was centrifuged at 3000 rpm for 20 min at 4^{o}C and the supernatant was used for the estimation of glutathione peroxidase (GPx), catalase (CAT), malondialdehyde (MDA) and total protein.

GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3(Ajith *et al.*, 2007). Tissue CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H_2O_2 (El-Demerdash *et al.*, 2009). The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1'1'3'3' tetramethoxypropane as standard (Hwang *et al.*, 2009). Protein content in the tissue was determined using bovine serum albumin (BSA) as the standard (Lowry *et al.*, 1951).

2.10. Histopathological studies

Small pieces of liver 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 under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam 1000 camera at original magnification of 100×.

2.11. Immunohistochemical analysis

Tissue sections were deparaffinised in three changes of xylene at 60^{0}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 5min 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% H_2O_2 in water for 10min to block endogenous peroxidase activity. Non specific binding was blocked with 3% BSA at in room temperature for 1h and then incubated with VEGF 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 45min. After triplicate washing with PBS, sections were incubated for 30min with streptavidin–HRP complex. Sections were then washed with PBS and incubated for 5-10 min in a solution of diaminobenzidine (6mg/10 mL 50mM Tris–HCl, pH 7.6) containing 0.01% H_2O_2. Counterstaining was performed with Mayer's hematoxylin. Images were taken at original magnification of 100× (Motic AE 21, Germany and Moticam 1000 camera).

2.12. Statistical analysis

Results were expressed as mean ± S.D and all statistical comparisons were made by using 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 and Discussion

3.1. Body weight and liver weight

Body weight of NDEA treated animals declined significantly (p£ 0.05) at the end of the 20th week of exposure when compared with the normal control. After 20 weeks, treatment with MEWF dose dependently reversed the liver weight. MEWF alone treated rats didn't show any significant changes in body weight and liver weight when compared to vehicle control (Table 1).

3.2. Serum analysis

The serum levels of AFP, ALP, LDH 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 MEWFat a dose of 100 and 200mg/kg showed a dose dependent decrease ($p \leq 0.05$) in AFP,ALP, LDH and bilirubin levels (Plate 1). MEWF at a dose of 200 mg/kg, b.w showed better results when compared to the standard drug, silymarin.

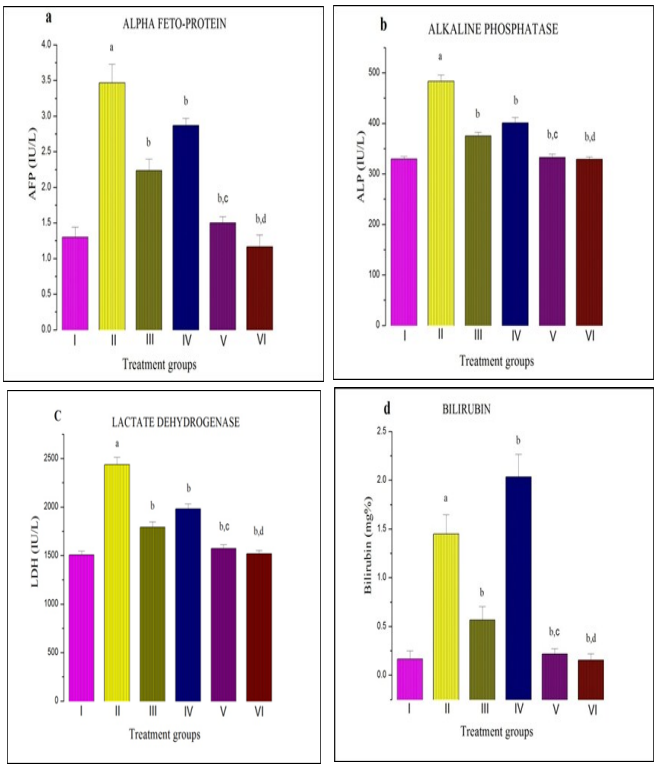

Plate 1. Effects of MEWF on changes in serum enzyme levels of rats post-treated with MEWF. a. Alpha feto-protein; b.Alkalinephosphatase;c. Lactate dehydrogenase; d. Bilirubin

**(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 100 mg/kg, NDEA + MEWF 100 mg/kg, NDEA + MEWF 200 mg/kg and MEWF 200 mg/kg alone were significantly different from NDEA control; [c] NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100 mg/kg treated group; [d] MEWF 200 mg/kg alone treated group non significantly different from normal control*

Table 1. Body weight and liver weight pattern of different groups of rats treated with MEWF

Treatment groups	Body weight (g)		Final Liver weight (g)	Liver to body weight ratio
	Initial	Final		
Normal control	174.3± 4.2	271.5± 4.8	7.2±1.1	0.026
NDEA control	176.1±3.8 [a]	207.6±5.0 [a]	18.5±2.5 [a]	0.089
NDEA + Silymarin (100 mg/kg)	168.7±4.6 [b]	238.4±3.9 [b]	8.6±0.9 [b]	0.036
NDEA + MEWF (100 mg/kg)	172.5±5.3 [b]	220.0±5.5 [b]	11.2±1.3 [b]	0.050
NDEA+ MEWF (200 mg/kg)	164. 3±4.1 [b.c]	254.7±4.5 [b.c]	7.8±0.9 [b.c]	0.030
MEWF alone (200 mg/kg)	169.0±4.9 [b,d]	274.5±4.0 [b,d]	6.8±1.0 [b,d]	0.024

**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 100 mg/kg, NDEA + MEWF 100 mg/kg , NDEA + MEWF 200 mg/kg and MEWF 200 mg/kg alone were significantly different CCl_4 control; [c] NDEA + MEWF 200mg/kg treated group differs significantly from NDEA + MEWF 100 mg/kg treated group; [d] MEWF 200 mg/kg alone treated group non significantly different from normal control.*

3.3. Liver morphology

The morphological variations of rat livers in different experimental groups were shown in Plate 2. NDEA treated rat liver (Plate 2.b) is morphologically different from the liver of normal control group. 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 200 mg/kg MEWF alone for the last 28 days reversed the liver morphology as that of normal rats.

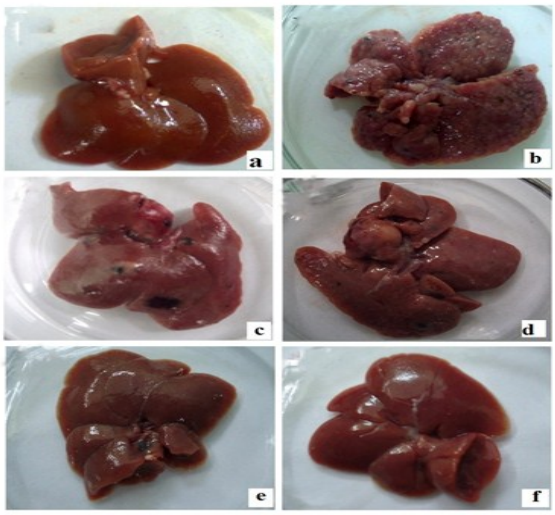

Plate 2. Morphological variations of liver in control and treated rats. a. Normal control; b. NDEA control (0.02%);c. NDEA+ Silymarin (100 mg/kg); d. NDEA+ MEWF (100 mg/k; e. NDEA + MEWF (200 mg/kg); f. MEWF (200 mg/kg) alone

3.4. Tissue biochemical analysis

3.4.1. Estimation of Glutathione Peroxidase (GPx)

Activities of hepatic GPx were significantly ($p \leq 0.05$) lowered in NDEA treated rats (Table 2). MEWF dose dependently restored the decreased GPx activity compared to NDEA alone treated group. When treated with MEWF at a dose of 200 mg/kg showed protection of 97 % and 76.3% for silymarin based on glutathione peroxidase levels.

3.4.2. Estimation of Catalase (CAT)

The CAT activity in liver showed a significant ($p \leq 0.05$) reduction in NDEA intoxicated rats when compared to normal control. MEWF treatment increased the activity of CAT (Table 2). Treatment with MEWF 200 mg/kg exhibited significant increase i.e., 85.7% and silymarin treatment restored the CAT activity by 69.9%.

3.4.3. Estimation of Malondialdehyde (MDA)

A significant ($p \leq 0.05$) elevation of malondialdehyde levels were observed in NDEA intoxicated rats. In silymarin treated groups the percentage of protection was 60.2%. Treatment with MEWF at a dose of 200mg/kg exerted its protection by 95% in 200 mg/kg treated group when compared to NDEA control (Table 2).

Table 2. Activities of GPx, CAT and levels of MDA in liver tissue of control and treated rats

Treatment groups	**GPx** (nmol of GSH oxidized/ min/mg protein)	**CAT** (U/mg protein)	**MDA** (nmol/g tissue)
Normal control	281.2±5.8	47.1±1.5	42.3±1.3
NDEA Control (0.02%)	165.1±4.9 [a]	33.8±1.8 [a]	72.5±2.3 [a]
NDEA + Silymarin (100 mg/kg)	253.8±6.1 [b]	43.1±1.4 [b]	54.3±1.9 [b]
NDEA + MEWF (100 mg/kg)	220.2±3.8 [b]	38.1±1.6 [b]	61.5±1.5 [b]
NDEA + MEWF (200 mg/kg)	278.5±5.2[b,c]	45.2±0.9[b,c]	43.8±0.8[b,c]
MEWF alone	280.6±4.2[b,d]	47.8±1.2[b,d]	44.1±1.2[b,d]

**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 100 mg/kg, NDEA + MEWF 100 mg/kg, NDEA + MEWF 200 mg/kg and MEWF 200 mg/kg alone were significantly different CCl_4 contro;. [c] NDEA + MEWF 200 mg/kg treated group differs significantly from NDEA + MEWF 100 mg/kg treated group; [d] MEWF 200 mg/kg alone treated group non significantly different from normal control.*

3.5. Histopathological studies

The histological analysis of all the experimental groups were depicted in Plate 3. The normal rat liver showed normal architecture (Plate 3.a). Here the cells were 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 (Plate 3.b). Microscopic examination also revealed the trabecular structure of liver tissue, which is a typical feature of hepatocellular carcinoma. Silymarin (100 mg/kg b.w) treated group showed necrotic tissues in certain areas (Plate 3.c). However, in MEWF (100 mg/kg) treated groups some degenerating hepatic cells were detected (Plate 3.d). Treatment with MEWF at a dose of 200 mg/kg b.w, the liver tissue showed almost normal architecture with normal hepatocytes and uniform sinusoids (Plate 3.e). The liver of MEWF alone treated group showed no appreciable changes or histological abnormalities (Plate 3.f).

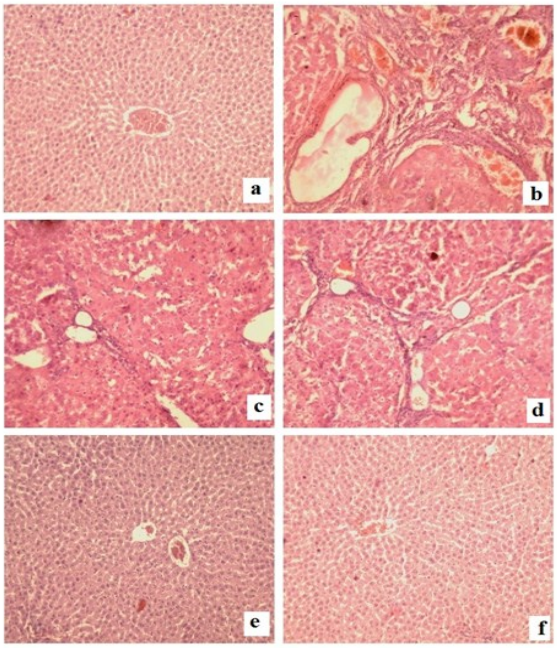

Plate 3.Histopathological changes occurred in rat liver due to post-treatment with MEWF. a. Normal control; b. NDEA control (0.02%); c. NDEA+ Silymarin (100 mg/kg); d. NDEA+ MEWF (100 mg/kg); e. NDEA + MEWF (200 mg/kg); f. MEWF (200mg/kg) alone

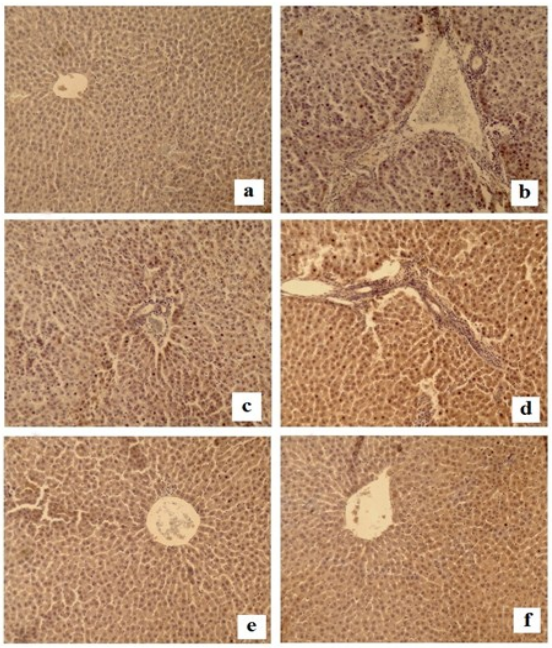

Plate 4. Immunohistochemical localizations of VEGF in control and treated groups. a. Normal control; b. NDEA control (0.02%); c. NDEA+ Silymarin (100 mg/kg); d. NDEA+ MEWF (100 mg/kg); e. NDEA + MEWF (200 mg/kg); f. MEWF (200mg/kg) alone

3.6. Immunohistochemical analysis

Immunohistochemical analysis of the normal rat tissue showed regularly stained nucleus. Immunohistochemical analysis of VEGF demonstrated that hepatocytes located in the periportal areas were the main source of VEGF production. In NDEA intoxicated rats the localization of VEGF around the periportal area was prominent. A significant down regulation of VEGF was spotted in MEWF (200 mg/kg) alone treated groups (Plate 4).

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.

In this study, the NDEA administration to rats for 20 weeks leads to decreased body weight, increased liver weight and a marked elevation in the levels of serum AFP, ALP, LDH and bilirubin. Treatment with MEWF, enhanced the body weight and increase the activities of serum enzymes indicating the curative effect of the extract. The increase in the activities of serum enzymes might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Atef Al-Attar, 2011) and this showed the stress condition of the NDEA treated animals. The extract at a dose of 200 mg/kg produced better results than 100 mg/kg, showed the dose response action of the extract.

The most widely used tumor marker for diagnosis of HCC is α-feto protein (AFP), which is a unique immunomodulatory glycoprotein (65kDa) normally made by the immature liver cells in the fetus (Sell and Beckar, 1978). Its detection during monitoring of HCC treatment is well accepted in patients with increased AFP levels prior to therapy, and is recommended by the European Association for the Study of the Liver (EASL). It has long been recognized that the exposure of rats to certain carcinogens like NDEA increases the circulating AFP levels. This corroborates the results showing a significant rise in levels of AFP obtained in NDEA-treated rats (Sivaramakrishnan *et al.*, 2008) was reduced by MEWF treatment.

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 intracellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants like GSH, GST, GR, GPx and CAT constitute the first line of cellular antioxidant

defense enzymes. When excess free radicals are produced, the equilibrium is lost and consequently the oxidative insult was established (Manna *et al.*, 2007).

The significant ($p \leq 0.05$) restoration of GPx activity due to 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 H_2O_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 H_2O_2. The treatment of MEWF significantly ($p \leq 0.05$) aided to maintain the CAT activity near to normal level in hepatic tissues. This shows 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 GPx and CAT activities.

Lipid peroxidation plays an important role in carcinogenesis (Banakar *et al.*, 2002) 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.*, 2007). In this study, we observed the increased levels of MDA in hepatic tissue of rats intoxicated with NDEA. This was prevented by the treatment with MEWF, which reveals the antioxidant potential of the extract.

Histopathological analysis reveals the variations in cell architecture due to NDEA intoxication. The recovery towards normalization of histological architecture by MEWF post-treatment was almost similar to that of normal control. Indeed there was a remarkable reduction in the characteristic features of HCC such as the formation of necrotic tissues, enlarged nuclei and lymphocyte infiltration. Here the curative effect of MEWF in NDEA treated rats were also evidenced by normal hepatocytes with small emboli of degenerating hepatic cells in experimental groups.

In the present study, immunohistochemical analysis showed the localization of over expressed VEGF around the periportal area in NDEA intoxicated rats. Treatment with MEWF significantly inhibited the over expression of VEGF indicating the inhibitory role of neo-vasculature formation in rats during NDEA administration.

The compounds reported from the dried flowers of *Woodfordia fruticosa* were β-sitosterol, kaempferol, ellagic acid, octacosanol, *meso*-inositol, quercetin, woodfordins A, B, C, D and oenothein A and B (Das *et al.*, 2007). 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, stigmasterol, hrdroxy methyl flavan etc (Nitha *et al*, 2016). Confertin showed antiproliferative effect on DLA cell lines (Thara and Zuhura, 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 a hepatoprotective agent (Al-Qarawi *et al.*, 2004).

4. Conclusion

In conclusion, the results presented in this study indicated the anticancer efficacy of MEWF, cured the chronic effects of hepatocellular carcinoma. HCC induced by *N*-nitrosodiethylamine was effectively cured 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 identified in MEWF have potent anticancer properties. So the components in single or in combination with other components present in the extract might be responsible for the anticancer activity of the extract. This finding suggested a possible basis for the potential use of the flowers of *W. 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.

Acknowledgements

The authors acknowledge the financial support given by Mahatma Gandhi University. The authors are thankful to Dr. M C Joseph, Former Professor, Department of Pathology, Medical College, Kottayam for extending his invaluable servive for the interpretation of the histopathological observations . Authors also express sincere thanks to Dr. Ansil P N and Dr. Prabha S P, SBS, M.G University, Kottayam for their help in this work.

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