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Original Article

Chemopreventive Role of *Euphorbia neriifolia* (Linn) and its Isolated Flavonoid Against N-Nitrosodiethylamine-induced Renal Histopathological Damage in Male Mice

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ABSTRACT

Aims: This study is an attempt to evaluate the tissue protective efficacy of isolated flavonoid and hydro-ethanolic extract of *Euphorbia neriifolia* (HEEN) leaves against N-nitrosodiethylamine (DENA) induced renal carcinoma. **Materials and Methods:** Carcinogenicity was induced in Albino mice by oral administration of DENA (50 mg/kg body weight). The HEEN (150 and 400 mg/kg body weight), Butylated hydroxyanisole (BHA; 0.5 and 1%), and *Euphorbia neriifolia flavonoid* (ENF; 50 mg/kg body weight) were evaluated for their possible tissue carcinogenesis protective potential. **Results:** DENA treated animals showed alterations in normal renal histo-architecture, which comprised of necrosis (N) and vacuolization of the cells. On the other hand, the mice treated with *Euphorbia neriifolia* lower (ENL) and higher (ENH) dose and ENF before intoxicated with DENA showed that the renal cells were normal (Day 31). Whereas, BHA higher (BHAH) and lower (BHAL) dose failed to diminish the abnormalities caused by DENA. **Conclusions:** The findings of the present study Suggested that ENH and ENF showed highest renal-protective activity among all the pretreatments. The results could also be expressed in the order of ENH > ENF > ENL > BHAH > BHAL.

Key words: *Euphorbia neriifolia*, flavonoid, kidney, necrosis, N-nitrosodiethylamine

INTRODUCTION

Nitrosamine compounds are known carcinogens. In the metabolism of nitrosamines, such as N-nitrosodiethylamine (DENA), there is evidence of the formation of reactive oxygen species (ROS) that results oxidative stress or cellular injury, which may be one of the factors in the etiology of some human cancer.[1] Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year.[2] Globally, kidney cancer is the 3rd most common malignancy of the genitourinary system that account for 2-3% of all cancers in men and 1-2% in women, with 130,000 new cases and 63,000 deaths from the disease occurring annually.[3] In modern medicine, beneficial effects of synthetic drugs are well-documented, however, these are suspected to have some toxic effects such as carcinogenicity and hence are no longer in use.[4] Chemotherapy of cancer is also not found to be safe because of the side-effects of the drugs on the healthy tissues.[2] Therefore, research for the determination, development, and utilization of more effective antioxidants of natural origin that have significant scavenging properties and are less toxic and inherently safer than synthetic antioxidants are desired.

Many studies have been carried out using edible, non-edible plants and vegetables to treat and reduce the severity of reno-carcinogenesis[5-7] but these studies did not report any histological changes during carcinogenesis. Histo-pathology,

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the microscopic study of diseased tissue is an important tool in anatomical pathology, since accurate diagnosis of many diseases usually requires histo-pathological examination of samples. Moreover, no published information is available on the histological study by the use of ENF (2-[3, 4-dihydroxy-5-methoxy-phenyl]-3,5-dihydroxy-6, 7-dimethoxychromen-4-one) and HEEN against renal carcinogenesis. Therefore, we examined its effects in amending the DENA induced renal carcinoma and its chemopreventive efficacy.

MATERIALS AND METHODS

Chemicals

DENA ($C_4H_{10}N_2O$) was purchased from Sigma Chemical Co., USA. All other chemicals used for the study were of analytical grade and were purchased from reliable institutes.

Preparation of HEEN

Euphorbia neriifolia leaves were collected from medicinal garden of Banasthali University, Banasthali and nearby areas of Banasthali (Latitude N-26°24'14.8414"; Longitude E-73°52'9.7194"), in the month of October, 2009 and was taxonomically identified. Shade dried leaves were powdered, soxhlet extracted with 70% (v/v) ethanol and vacuum concentrated to dryness under reduced pressure at 60°C ± 1°C. After drying in hot air oven (40-45°C), it was stored in an air tight container in refrigerator at 5°C. The residue was designated as HEEN.

Isolation of flavonoid from *Euphorbia neriifolia*

Dried leaves of *Euphorbia neriifolia* (500 g) were extracted successively with pet-ether, benzene, chloroform, ethyl acetate and ethanol and finally, macerated with distilled water (non-polar to polar) to get respective extracts. Flavonoid contents and their presence were determined by the method of Harborne,[8] using quercetin as standard. Out of all extracts dark-brown sticky semi-solid ethanolic extract (48.9 g) contained bulk of flavonoids was used for chromatographic separation. The ethanol extract obtained was concentrated and chromatographed on silica plates by using n-butanol: Acetic acid: Water (2:2:6) as mobile phase. As a result three spots were resolved which were nomenclatured as IF_1, IF_2, and IF_3 having an R_f values of 0.60, 0.79 and 0.90 respectively. The R_f of IF_2 was coinciding with standard quercetin R_f value that was found to be 0.79. IF_2 fraction on crystallization, gave solid pale yellow crystal, soluble in water and in organic solvents was designed as ENF. High performance thin layer chromatography (HPTLC) and Infra-red (IR) spectrum of ENF in KBr pellet confirmed the presence of hydroxyl (-OH) group with H-bonded primary alcohol. With the help of ^1H NMR and MS the ENF was characterized as 2-(3, 4-dihydroxy-5-methoxy-phenyl)-3, 5-dihydroxy-6, 7-dimethoxychromen-4-one. ENF was used to assess tissue-protective activity.

Experimental animals

Healthy male Swiss Albino mice (*Mus musculus* L.) procured from Chaudhary Charan Singh Haryana Agricultural University, Hissar (Haryana, India) were housed under standard laboratory conditions of temperature (22°C ± 3°C), relative humidity (50 ± 15%) and photoperiod (12:12 h L: D cycle). Animals lead free access to standard food pellet diet (Hindustan Lever Limited, India) and tap water *ad libitum*. The studies were carried out in accordance with the guidelines given by Committee for the Purpose of Control and Supervision on Experiments on Animals (Reg. No: IAEC/814 date. 23.01.2010).

Treatment regimen

Swiss Albino mice were randomly divided in to 7 groups of six mice each. After 2 week of acclimatization they were administered orally by gavage. Treatment regimen was as follows:

Group I: Normal control,
Group II: Carcinogen control, received distilled water administered for 21 days, and then single dose of DENA (50 mg/kg body weight) was administered on 22 day and then left for 10 days,
Group III: ENL (150 mg/kg body weight/day) and
Group IV: ENH (400 mg/kg body weight/day) administered for 21 days, before being intoxicated with DENA, once, and,
Group V: BHAL (0.5% mg/kg body weight/day) and
Group VI: BHAH (1% mg/kg body weight/day) Administered for 21 days,

Dissolved in 0.5% acetone, before being intoxicated with DENA, once and

Group VII: ENF (50 mg/kg body weight/day) administered for 21 days, dissolved in dH_2O, before being intoxicated with DENA.

Then, the animals were sacrificed on 10 day after DENA administration and the total duration of experiment was of 31 days. The dose for DENA (Sigma-N0258-Material Safety Data Sheet, 2003),[5-7] BHA,[9] ENF,[10] and plant[5-7] were selected on the basis of LD_{50} calculated in our laboratory and other previous published reports.

Autopsy and isolation of tissue

After 31 days of treatment, mice were deprived of food overnight and then on next day they were sacrificed under light chloroform anesthesia. After postpartum kidneys were excised immediately, washed, cleaned, and rinsed in ice-cold normal saline solution (0.9% NaCl, pH 7.4), until bleached

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of all the blood and blotted dry on filter paper sheets to remove blood. Small portion of tissue was fixed in buffered formalin (10%) for histological studies.

Histopathological studies

Kidneys were preserved or fixed in 10% formalin (10% formaldehyde diluted using normal saline) for histopathological evaluation. Fixation was followed by washing the tissue in running water, dehydrated by graded series of alcohol, cleaned in xylene, and embedded in paraffin wax. Paraffin blocks were prepared and sections of about 3-5 μm in thickness were cut on rotatory microtome. Staining was done by haematoxylin and eosin and they were analyses for pathology by light microscopy (Model-Motic).[11]

RESULTS

Percentage of tissue carcinogenesis

The animals were examined for DENA-induced tumors in the Kidney. None of the mice from either control or HEEN groups developed renal tumors, whereas all the mice treated with DENA (100%) showed kidney carcinogenesis [Table 1]. However, pre-treatment with HEEN and ENF before intoxicated with DENA reduced renal tumor incidence to 0-16.66%. These results suggested that HEEN and ENF can prevent DENA-initiated tumor development in the mice kidney tissue.

Histo-pathological/Histological exploration of the renal tissue

Table 2 depicts the histo-pathological features seen in the renal tissue of mice of different groups.

The section through the kidney cortex of Albino mice in control untreated animals displayed normal architecture details of the kidney tissue i.e. a tuft of blood capillaries (the glomerulus), intact Bowman's capsule (BC) lined with thick cubic epithelium, urinary space (US; *) with simple

Table 1: Effect of hydro-ethanolic extract of *Euphorbia neriifolia*, Butylated hydroxyanisole (at both doses) and isolated flavonoid on N-nitrosodiethylamine-induced reno-carcinogenesis in Swiss Albino mice

Groups	No. of animals	No. of tumor bearing animals	% of tumor incidence
NC (I)	6	0	0
CC (II)	6	6	100
ENL+CC (III)	6	1	16.66
ENH+CC (IV)	6	0	0
BHAL+CC (V)	6	3	50
BHAH+CC (VI)	6	2	33.33
ENF+CC (VII)	6	0	0

NC = Normal control, CC = Carcinogen control, ENL = *Euphorbia neriifolia* lower dose, ENH = *Euphorbia neriifolia* higher dose, BHAL = Butylated hydroxyanisole lower dose, BHAH = Butylated hydroxyanisole higher dose, ENF = *Euphorbia neriifolia* flavonoid

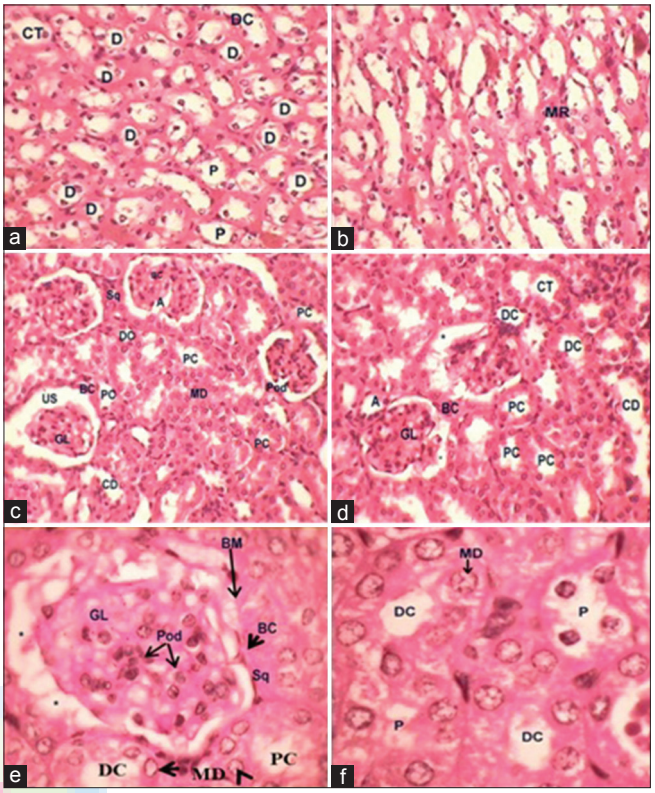


Figure 1a-f: Photomicrograph of longitudinal section of control mice kidney (H and E) showing normal glomeruli with an intact Bowman's capsule, Proximal convoluted tubules, Distal convoluted tubules, Medullary rays, Artioles, Macula densa, Urinary space and thickly squamous epithilium (a-d : ×40 and e-f: ×100)

Table 2: Histopathological features seen in the kidneys of mice of different treatment groups

Histopathological changes	GP I	GP II	GP III	GP IV	GP V	GP VI	GP VII
Glomerular congestion	–	+++	–	–	+	–	–
Tubular casts	–	+++	–	–	–	–	–
Peritubular congestion	–	+++	–	–	+	++	–
Epithelial desquamation	–	+++	–	–	–	+	–
Blood vessel congestion	–	+++	–	–	–	+	–
Inflammatory cells	–	+++	–	–	–	–	–
Interstial edema	–	+++	–	–	++	+	–
Necrosis	–	+++	–	–	+	+++	–
Vacuolated tubules	–	+++	–	–	–	+	–
Cell debris	–	+++	–	–	–	+	–
Hyperplatic glomeruli	–	+++	–	–	–	–	–
Swollen renal tubules	–	+++	–	–	–	+	–
Albuminious material	–	+++	–	–	+	++	–
Atrophied glomeruli	–	+++	–	–	+	++	–
Fibroblasts	–	+++	–	–	–	–	–
Mononuclear cell infiltration	–	+++	–	–	–	+	–
Damage macula densa	–	+++	–	–	–	–	–
Dispersed medullary ray	–	+++	–	–	+	+	–
Dilation in US	–	+++	–	–	+	++	–

– = Absent, +=Present, ++ = More, +++ = Most, GP = Group

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squamous epithelium, medullary rays, artioles, macula densa, proximal and distal tubules (PT and DT) with mononuclear cells in to their epithelium and collecting ducts (CD). Relatively regular distinct lumen was also seen [Figure 1a-f].

As shown in the Figure 2a-i, autopsy revealed remarkable damage in the kidney of persistently cancerous animals group II. DENA renal intoxication was associated with severe glomerular and tubulo-interstitial necrosis (N), which was characterized by hydropic degeneration of the glomerular and tubular cells with complete obliteration of the tubular lumen. The distortion of cyto-architecture of the renal cortical structures, degenerative and atrophic changes (from hydropic degeneration and tubular casts), and renal corpuscles were less identified. Bowman's spaces were also sparsely distributed when compared to normal animals kidney. Hyperplastic glomeruli and swollen lining epithelium of the renal tubules with obliterated lumina was also observed. Peri-glomerular albuminous material deposition with edema and infiltration of inflammatory cells [Figure 2a and e], were observed in association with sharply demarcated sub-capsular areas of N [Figure 2e and h] involving the renal tubules, glomeruli, and even the walls of the blood vessels. Photomicrographs also showed degenerated structures with severe atrophied glomeruli, albuminous material in dilated US, mononuclear cell infiltration, fibroblasts, and perivascular edema [Figure 2a, d and g]. Congested glomeruli, degenerated CD with vacuolated tubules (VT) surround by extensive tubular N were observed in Figure 2b. Degenerated tubules and the tubular lumens were completely obliterated and filled with fluid and casts with mononuclear infiltration and VT were also seen [Figure 2c]. We have clearly noticed the sharp demarcated sub-capsular area of necrosis (SN), inflammatory cells surrounded by edema and severe hydropic glomerular degeneration (HGD) in Figure 2e and i. Swollen renal tubules surrounded by cell debris, N and emersion of large number of inflammatory cells were also observed [Figure 2f]. Figure 2i showed the shrunken glomerulus, nucleated tubules filled with protein cast.

The section through the kidney from Albino mice treated with HEEN at both doses before intoxicated with DENA

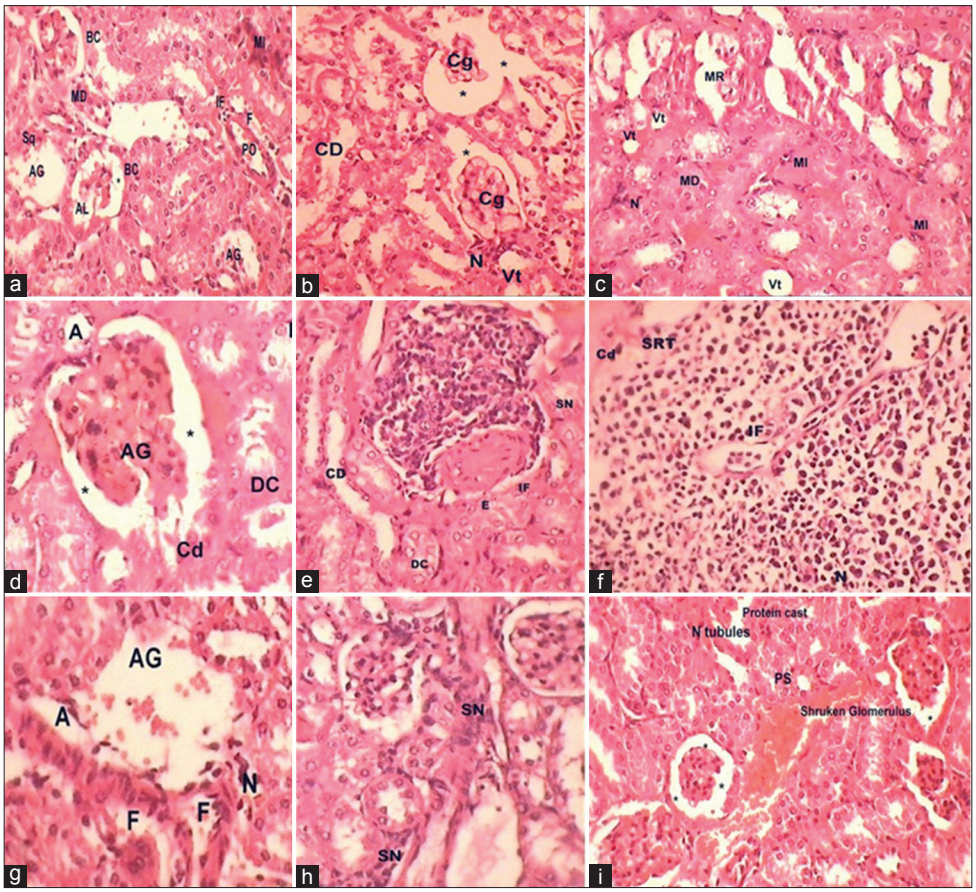


Figure 2a-i: Photomicrographs of longitudinal section of kidney of N-nitrosodiethylamine-intoxicated mice (H and E, ×40) showing severe degenerated structure viz. (a, d-g) Atrophied glomeruli, albuminous material in dilated urinary space, mononuclear cell infiltration, fibroblasts and perivascular edema, (b) Congested glomeruli, degenerated collecting duct with vacuolated tubules (VT) surrounded by extensive tubular necrosis (N), (c) Degenerated tubules and the tubular lumens were completely obliterated and filled with fluid and casts with mononuclear infiltration and VT, (h and e) Sharply demarcated sub-capsular area of necrosis, inflammatory cells surrounded by Edema and severe hydropic glomerular degeneration, (f) Swollen renal tubules surrounded by cell debris and N, (i) shrunken glomerulus and nucleated tubules filled with protein cast

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showed gross efficacy of normal architecture by recovery in BC and low-US and tubular vacuolation [Figure 3a-e]. The disintegration of the tubules and glomeruli were the same as ruptures epithelium layer of BC and vacuolated and congested glomerulus structure with little cloudy swelling were also improved with high-dose of HEEN (Group IV). Pre-treatment with HEEN at low-dose (150 mg/kg body weight/day) before being intoxicated with DENA (Group III) showed normalized glomeruli with tubules. The tubular lumens were slightly filled with fluid and casts and thinning out of the BC [Figure 3a and b], whereas, pre-treatment with HEEN at high-dose (400 mg/kg/day), before intoxicated with DENA (Group IV) showed recovery with thinning out of the BC, normal proximal convoluted tubule (PC) and distal convoluted tubule (DC) and is comparable to control group (Group I) as dipicts in Figure 3c-e.

Figure 4a-d dipicts that the pre-treatment of 0.5% BHA (Group V), before intoxicated with DENA showed mesengial proliferation (MP) with thinning out of the BC. Figure 4e-g represents the effect of 1% BHA pre-treatment, before intoxicated with DENA (Group VI). Higher dose of BHA showed un-normalized tubules, MP with thinning out of the BC. At both doses, there was severe tubular cast deposition interposed with dispersed PC and DC, segregated and congested glomeruli, severe HGD, deposition of albuminious material in US, cell debris, edema along with sharply demarcated SN and fibroblast were also observed.

Group VII exhibited a tendency towards normal morphology as shown in Figure 5a-c. A longitudinal sectional representation of kidney of ENF (50 mg/kg/day) pre-treated mice before intoxicated with DENA showed recovery with little MP with thinning out of the BC. Also shows normalization of glomeruli to some extent. There was little tubular cast deposition interposed with normal PC and DC.

DISCUSSION

Carcinogenesis is a complex and protracted multi-stage process. Certain toxic chemicals and medicines can cause renal damage, are recognized as a toxicological problem.

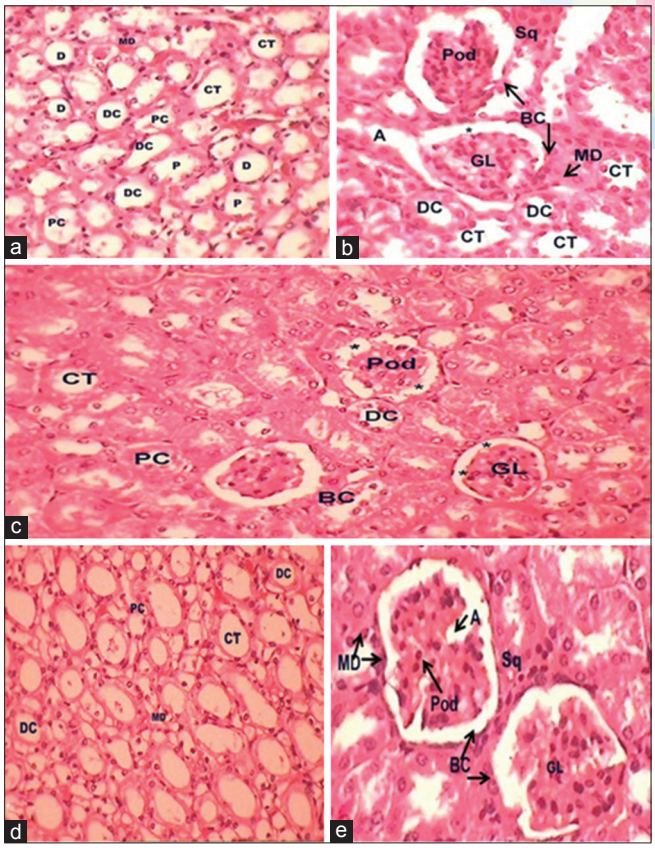


Figure 3a-e: A longitudinal section representation of kidney of group III (a-b) and group IV (c-e) (H and E, ×40) showing recovery

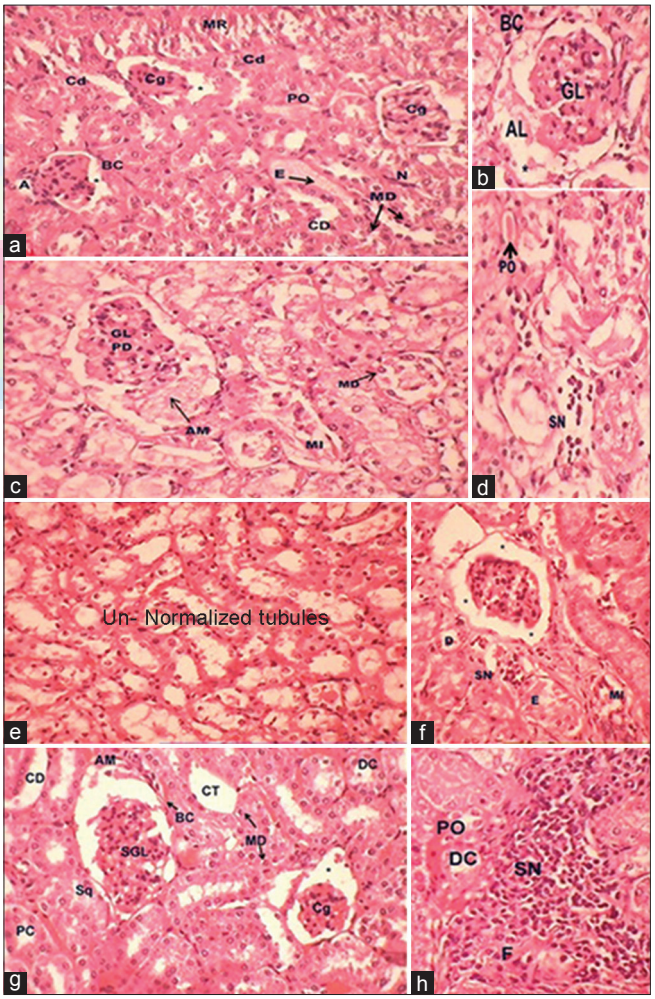


Figure 4a-h: A longitudinal section representation of kidney of group V (a-d) showing mesengial proliferation (MP) with thinning out of the Bowman's capsule (BC). There is severe tubular cast deposition interposed with dispersed proximal convoluted tubule (PC) and distal convoluted tubule (DC); group VI (e-h) showing (H and E, ×40) un-normalized tubules, MP with thinning out of the BC

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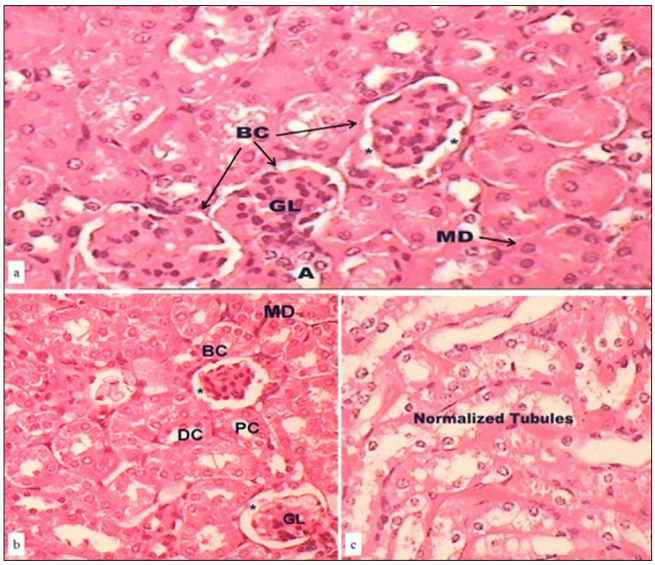


Figure 5 a-c: A longitudinal section representation of kidney of *Euphorbia neriifolia* isolated flavonoid (50 mg/kg body weight/day) pre-treated mice before being intoxicated with DENA N-nitrosodiethylamine (H and E, ×40) showing recovery

Foodstuffs such as milk and meat products, salted and dried fish, alcoholic beverages and a few varieties of vegetables (soybeans) are the principal sources of DENA.[1] Oxidative damage in the tissue occurs when the concentration of ROS generate exceeds the antioxidant capacity of the tissue.[12] Oxidative stress may alter the structure and function of the glomerulus because of the effect of ROS on mesangial and endothelial cells[13] and the toxic peroxidative products cause wide spread cellular injury.[14] Since kidneys are the major organs of drug excretion, the occurrence of nephrotoxicity is of great concern. Nephrotoxicity occurs as a disturbance in renal function due to various adverse drug interactions, inadequate elimination of radioactive contrast materials and chemicals. However, the end point of nephrotoxicity is always cell death so it is important to identify the mechanism in addition to the site of action, in order to formulate a strategy for damage prevention.

In the last few decades, there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and less side-effects. India, being a treasure trove of medicinal plants, contributes much to these.[15] The antioxidants protect membrane and cytosolic components against damage caused by free radicals during carcinogenesis.[16,17] Thus, antioxidants are expected to decrease the vulnerability of the kidney to oxidative challenges.[18]

The results of the current study suggests that the distortion of the cyto-architecture of the kidney might be due to the interference of DENA on the kidney. This distortion could have been associated with functional changes that may have been detrimental to the health status of the animal. Administration of the DENA caused varying degree of cyto-architectural distortion and reduction in the number of renal corpuscle in the treated groups compared with the control group. Degenerative and atrophic changes were observed in the kidneys of mice that received the DENA. This change may represent an early stage in tumorigenesis, whereas the tubular and intra-cystic papillary lesions may represent a later stage. However, daily pre-treatment with HEEN at both dose levels for 21 days before intoxicated with DENA conferred nephro-protection on the DENA renal injured mice. Higher dose of HEEN was found to normalize the changes occurred in tissues after DENA administration and restored a marked recovery in kidneys as evidenced microscopically. It showed predominantly regenerative stage than that of the other treatment group. The epithelial cells of the PC of this group were intact. In this group, the glomerular changes were scanty. However, ENF at the dose of 50 mg/kg body weight was also able to diminish the disorders and injury caused by DENA but to little extent in comparison to ENH. Whereas, BHA at both dose levels failed to diminish the abnormalities caused by DENA.

In this study, *Euphorbia neriifolia* acts as antioxidant agent as this contains wide range of active ingredients viz. flavonoids, alkaloids, saponins and other active phytoconstituents[19,20] and mediate nephro-protection.[21] These bioactive constituents might be responsible to inhibit or to slow-down the severity of cancer. These active ingredients especially, flavonoids[22] are known to possess tissue protective activity as they neutralize free radicals and intermediates of metabolism that are highly reactive since they contain a non-paired electron.[23] These active ingredients also avoid DNA damage, protecting cells from damage by free radicals, bind to free radicals to inactivate or kill them and enhance body's own defense system.[24]

In conclusion, from the overall result of the histopathological examinations, it could be inferred that renal carcinogenesis, which was induced by DENA was effectively inhibited by HEEN and by ENF. Mice treated with ENL, ENH, and ENF before intoxicated with DENA showed that the kidney cells were normal, with very little necrosis (Day 31). The results could also be expressed in the order of ENH > ENF > ENL > BHAH > BHAL. The present findings provide and validate the scientific evidence to the ethno-medicinal therapeutic use of this plant by the tribal people in treating cancer.

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