RESEARCH COMMUNICATION

Chemopreventive Effect of Hydroethanolic Extract of *Euphorbia neriifolia* Leaves Against DENA-Induced Renal Carcinogenesis in Mice

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Abstract

The present study was conducted to investigate the chemopreventive effects of hydro-ethanolic extract of *Euphorbia neriifolia* (EN) on N-nitrosodiethylamine (DENA) induced renal cancer in male Swiss albino mice. Animals were pretreated with EN extract (150 and 400 mg/kg body weight; p.o) and butylated hydroxyanisole (BHA) as a standard (0.5% and 1% BHA p.o) both for two week prior to the administration of single dose of DENA (50 mg/kg body weight; p.o). Various *in vivo* antioxidant biochemical parameters like lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT) were evaluated to determine the reno-protective and antioxidant activity of EN. DENA increased oxidative stress through increase in LPO and decrease in antioxidant enzymes (SOD, and CAT). The EN extract significantly restored the antioxidant enzyme level in the kidney and exhibited significant dose dependant protective effect against DENA induced nephrotoxicity, which can be mainly attributed to the antioxidant property of the extract. This study rationalized the ethno-medicinal use of EN for protection against renal cancer.

Keywords: *Euphorbia neriifolia* - renal cancer - N-nitrosodiethylamine - antioxidant enzymes - BHA

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Introduction

Kidney cancer is the third most common malignancy of the genitourinary system account for 2 to 3 % of all cancers in men worldwide, with 130,000 new cases and 63,000 deaths from the disease occurring annually (Ferlay et al., 2004). Certain toxic chemicals and medicines can cause renal damage, are recognized as a toxicological problem.

N- Nitrosodiethylamine (DENA) is the most important environmental carcinogen among the nitrosamines (Thirunavukkarasu and Sakthisekaran, 2001). DENA has been suggested to cause oxidative stress and cellular injury due to involvement of free radicals (Noguchi et al., 2000). Lipid peroxidation and associated membrane damage are key features of DENA- induced carcinogenesis (Anis et al., 2001). The antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase protect membrane and cytosolic components against damage caused by free radicals during carcinogenesis (Banakar et al., 2004). There are reports that oxygen free radicals and related lipid peroxides play a key role in the protection against exogenous oxidative stress and pathogenesis of age-related chronic degenerative diseases (Marklund and Marklund, 1974). Thus, antioxidants are expected to decrease the vulnerability of the kidney to oxidative challenges. Considerable experimental evidence has contributed to support a key role of reactive oxygen species (ROS) in the numerous mechanisms of seemingly unrelated nephropathies (Rodrigo and Rivera, 2002). There are two basic categories of antioxidant namely synthetic and natural ones. Although synthetic antioxidant like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and tertiary butyl-hydroquinone (TBHQ) are known to ameliorate oxidative damages but they are suspected to have some toxic effects such as carcinogenicity (Ghafar et al., 2010). 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 is desired (Vadlapudi and Naidu, 2010).

Thus, antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals. Medicinal properties of plants have also been investigated in the light of recent scientific developments through out the world, due to their potent pharmacological activities, low toxicity and economic viability, when compared with synthetic drugs (Vadlapudi and Naidu, 2010).

Euphorbia neriifolia (Euphorbiaceae) commonly known as "sehund or siju" in Hindi and "thohar" in Rajasthan, is found throughout the Deccan Peninsula

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of India and grows luxuriously around the dry, hilly, rocky areas of North, Central and South India. Ayurveda describes the plant as bitter, pungent, laxative, carminative, improves appetite and useful in abdominal troubles, bronchitis, tumors, loss of consciousness, delirium, leucoderma, piles, inflammation, enlargement of spleen, anaemia, ulcers and fever(Anonymous, 1952; Sharma et al., 2011).

In spite of its various medicinal uses, no systematic studies in the literature regarding its pharmacological effect on use of EN leaves extract for renal protective activity has been reported. Therefore, the aim of this current investigation was to evaluate the chemopreventive activity of the hydro-ethanolic extract of leaves of *Euphorbia neriifolia Linn* against DENA-induced renal cancer in mice.

Materials and Methods

Chemicals and reagents

DENA was purchased from Sigma Chemical Co., USA. All other materials and chemicals used in the study were of analytical reagent grade and of highest quality available, and are purchased from reliable firms and institutes (SRL, MERCK, RANBAXY, HIMEDIA, SIGMA and SUYOG). Standard kits for LPO, SOD and CAT were obtained from Cayman Chemicals, USA.

Experimental plant

Euphorbia neriifolia leaves were collected from Pharmacological garden of Banasthali University, Banasthali, India, in the month of September 2009. The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra, Banasthali Vidyapith, Banasthali, Tonk district.

Preparation of hydro-ethanolic crude extract

Freshly collected *Euphorbia neriifolia* leaves were air dried in shade and coarse powder (500g) was defatted in 1.5 L of ethanol (70% v/v) using soxhlet apparatus. The extracted mixture was evaporated at 40°C, using a hot air oven (Mvtex, India) and kept in desiccator for two days. The yield of the extract was 20% w/w of the powdered plant material. Dried extract was collected and stored at 5°C in air tight container. The residue was designated as hydro-ethanolic extract and used to assess nephro-protective and antioxidant activity.

Experimental animals

Male Swiss Albino mice (*Mus musculus*) weighing 15-30 g were obtained from Haryana Agricultural University, Hissar (India) for experimental purpose. The animals were acclimatized for a month prior to experiment. The Institutional Animal Ethical Committee approved the animal studies. All experiments were conducted on adult male albino mice weighed 25-35g (3-4 months old). Colony bred adult male albino mice were maintained under standard laboratory conditions at a temperature of 22 ± 3ºC, relative humidity of 50±5 % and photoperiod of 12h (12h-dark and 12h-light cycle). The mice were housed in polypropylene cages. In order to avoid diurnal variation all the experiments were carried out at the same time of the day. Animals lead free access to standard food pellet diet (Hindustan Lever Limited: metal contents in parts per million dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and drinking water *ad libitum* throughout the study. Essential cleanliness and, to the best extent, sterile condition were also adopted according to SPF facilities.

Treatment regimen

Adult Swiss albino male mice divided into ten groups of 10 mice each were treated by oral gavage. Treatment consisted of pretreatment phase of EN in distilled water followed by the second phase in which the animals were given 50 mg/kg DENA on day 15. The animals were then euthanized 4 days after DENA administration. The groups were as follows-

a) Group 1: served as control (normal untreated mice), and received 1ml distilled water daily by oral gavage

b) Group 2: received pretreatment with distilled water for 14 days prior to a single dose of DENA (50 mg/kg body weight: p.o) served as DENA control group.

c) Groups 3 and 4 were administered with hydro-ethanolic extract of leaves of EN (150 and 400 mg/kg body weight: p.o) daily for 14 days, served as EN treated control group.

d) Group 5: received BHA (0.5 % mg/kg body weight: p.o) daily for 14 days, dissolved in 0.5% acetone and served as standard treated control group.

e) Group 6: received 1% BHA (p.o) daily for 14 days, dissolved in 0.5% acetone and served as standard treated control group.

f) Group 7 and 8 were treated with hydro-ethanolic extract of leaves of EN (150 and 400 mg/kg body weight; p.o) daily for 14 days, before being intoxicated with DENA (50 mg/kg body weight; p.o, once) dissolved in 0.9% normal saline.

g) Groups 9 and 10: received BHA (0.5 % and 1% mg/kg body weight: p.o) daily for 14 days, before being intoxicated with DENA (50 mg/kg body weight; p.o, once) dissolved in 0.9% normal saline.

The dose for DENA, standard antioxidant, and plant were decided and selected on the basis of LD50 calculated in the laboratory and on the basis of other published reports (Bharali et al., 2003; Sigma-N0258- Material Safety Data Sheet, 2003;Bigonia and Rana, 2009).

Nephroprotective activity

After 19 days of duration the mice were fasted overnight and then sacrificed under light ether anesthesia. The animal kidneys were dissected out and washed immediately with ice cold saline to remove blood and the wet weight was noted and then stored at -80°C for various biochemical assays, and histological studies. Half of each kidney was processed for biochemical analysis and the other half was used for histological examination. The enzyme levels were assayed using standard Cayman assay kits from USA.

Preparation of renal homogenate

Kidney homogenate was prepared in cold 50 mM potassium phosphate buffer (pH 7.4), for LPO but for

SOD and CAT 1mM EDTA was added in it, using Remi homogenizer. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15 min at 4°C using a Remi cooling centrifuge and the supernatant was used for the estimation of LPO, SOD, and CAT.

Renal oxidative parameters

a) Estimation of lipid peroxidation (LPO): Cayman's Lipid hydroperoxide Assay Kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions (Mihaljevic et al., 1996). In brief, 0.5 ml of sample was treated with 1 ml of chloroform and centrifuged at 1500g for 5 min. The supernatent was collected and used for assay. The chloroform-methanol solvent (degassed, 2:1, 0.45 ml) was added to 0.5 ml of supernatent followed by addition of 50ul chromagen and incubation for 5 min. Lipid hydroperoxide standards (50 μM ethanolic solution of 13-hydroperoxy octadecadienoic acid) were prepared at different concentrations (0-5 nmol) in chloroform-methanol solvent. The absorbance was measured at 500 nm against reference blank (chloroform-methanol solvent). The LPO is expressed as μM/ gm of protein.

b) Superoxide dismutase (SOD): The tissue SOD activity was assayed following the procedure of Cayman's Superoxide Dismutase Kit, which utilizes a tetrazolium salt for detection of superoxide radicals (Marklund and Marklund 1980). Kidney homogenate (10 μl) was taken, and 200 μl of dilute radical detector (24μm NBT) were added. The reaction was initiated by adding 20 μl of xanthine oxidase (50 μl in 1.95 ml 50 mM Tris-HCL, pH 8.0) to all the wells. The control was simultaneously run without renal homogenate. SOD standard (bovine erythocyte SOD) were prepared at different concentrations (0-0.25 U/ml) in 50 mM Tris-HCL, pH 8.0. The plate was incubated for 20 min and the absorbance was measured at 440-460 nm using plate reader. The enzyme activity is expressed as unit ml-1 and 1 unit of enzyme is defined as amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

c) Catalase (CAT): Catalase activity in the kidney was assayed following the procedure of Cayman Catalase Assay Kit, which utilizes the peroxidatic function of CAT for determination of enzyme activity (Johansson et al., 1988; Wheeler et al., 1990). Kidney homogenate (20 μl) was taken with 30 μl of methanol and 100 μl of assay buffer (100 mM potassium phosphate, pH 7.0) in two wells. Formaldehyde standards were prepared by addition of 20 μl formaldehyde (4.25 mM) at different concentrations instead of samples. The reaction was initiated by the addition of 20 μl of H_2O_2 (30 mM). Blank without kidney homogenate was prepared with 100 μl of phosphate buffer and 20 μl of H_2O_2. The plate was incubated on shaker for 20 min. The reaction was terminated by adding 30 μl of potassium hydroxide (10 M) to each well and then 30 μl of purpled chromogen was added to each well. This was followed by incubation for 10 min and addition of 10 μl potassium periodate to each well. The decrease in optical density due to decomposition of H_2O_2 was measured at the end of 5 min against the blank at 540 nm using plate reader. One unit of activity is equal to the mol of H_2O_2 degraded min-1 protein at 25° C. The specific activity expressed in terms of units per mg of proteins.

Statistical Analysis

The experimental results obtained are expressed as the mean ± standard deviation (SD) of three replicates. The data was subjected to one way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test by fixing P values as p<0.05 and p<0.001 using the SPSS 16.0 (Statistical program for Social Sciences) program.

Results

Effect on lipid peroxidation and anti-oxidant enzyme levels

Results of the study clearly indicate that the leaves of EN possess renal-protective activity. Table 1 illustrate the effect of hydro-ethanolic extract of EN and BHA on lipid peroxidation and antioxidant-enzymes (i.e. SOD and CAT) in control and treated groups against DENA- induced renal toxicity in male mice.

In vivo lipid peroxidation

Effect of EN extract and BHA (at both doses) on the level of LPO in kidney of control and treated mice is shown in Figure 1. LPO content in the kidney homogenate was significantly increased (p<0.001) in DENA treated group when compared to control group. Oral administration of hydro-ethanolic extract of EN at both doses (150 and 400 mg/kg body weight) and BHA (0.5% and 1%) significantly

Table1. Effect of Hydroethanolic Extract of the Leaves of *Euphorbia neriifolia* in the Levels of LPO and the Activities of SOD and CAT Against DENA-induced Renal Toxicity in Mice

Groups	Treatments mg/kg body wt	LPO (uM/mg protein)	CAT (nmol/min/ml)	SOD (U/ml)
Control	-	82.2±1.07^{a}	3.44±0.005^{a}	1.82±0.003
DENA	50	98.7±1.24$^{*/a}$	2.25±0.004$^{*/a}$	0.97±0.004
EN	150	67.1±0.93$^{*/a}$	4.05±0.001$^{*/a}$	2.81±0.007$^{*/a}$
	400	63.4±0.89$^{*/a}$	5.08±0.005$^{*/a}$	3.41±0.007$^{*/a}$
BHA	0.5%	60.4±1.12$^{*/a}$	3.83±0.044^{a}	2.43±0.004$^{*/a}$
	1%	57.3±1.02$^{*/a}$	3.97±0.046$^{**/a}$	2.67±0.005$^{**/a}$
EN + DENA	150 + 50	86.3±1.10$^{*/a}$	3.03±0.007$^{*/a}$	1.48±0.004
EN + DENA	400 + 50	84.1±0.87$^{/a}$	3.28±0.008^{*a}	1.65±0.006
BHA+ DENA	0.5% + 50	83.2±0.75$^{**/a}$	2.69±0.003$^{*/a}$	1.34±0.003
	1% + 50	82.3±1.09$^{**/a}$	2.87±0.052$^{*/a}$	1.42±0.009

Values are expressed as mean ± SD; *p<0.001, **p<0.05 vs. control group; ^{a}p<0.001, ^{b}p<0.05 vs. treated group (DENA)

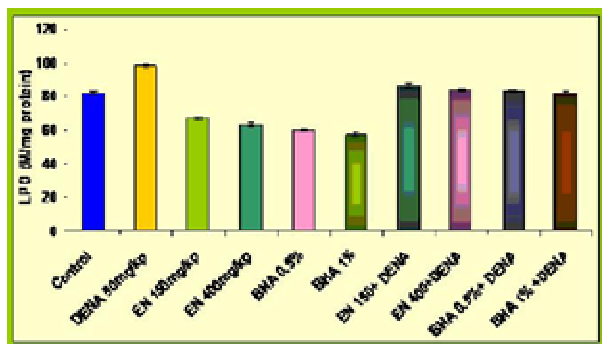

Figure 1. DENA Induced Changes in Renal Lipid Peroxidation and Their Responses to Administration of Hydro-ethanolic Extract of EN in Male Mice.

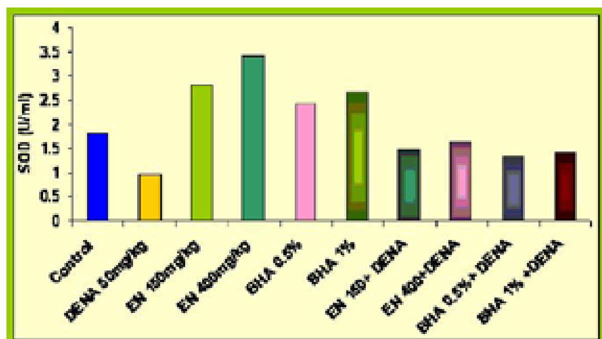

Figure 2. Effect of Administration of EN Extract and BHA on SOD Against DENA-induced Renal Toxicity in Mice

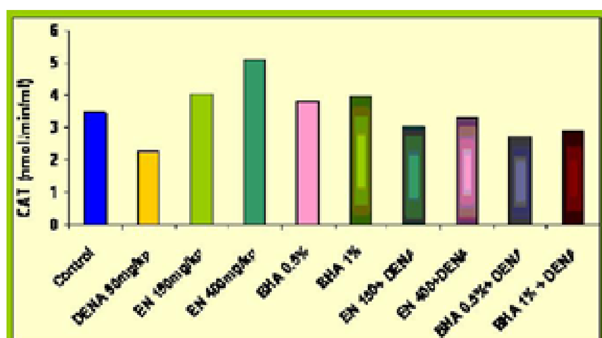

Figure 3. Effect of Administration of EN Extract and BHA on CAT Against DENA-induced Renal Toxicity in Mice.

decreased (p<0.001) the LPO level in comparison to control group.

Intake of EN extract and BHA (at low and high dose) along with DENA significantly (p< 0.001; p<0.05) improved the LPO level. In comparison to DENA treated group pre administration of hydro-ethanolic extract of EN, BHA (at low and high dose) and combination groups significantly (p<0.001) alleviated the LPO level.

Estimation of SOD

Figure 2 depicts the effect of EN extract and BHA on SOD against DENA-induced renal toxicity in mice. SOD activity was insignificantly decreased (p>0.001) in DENA treated group when compared to control group. Oral administration of hydro-ethanolic extract of EN (150 and 400 mg/kg body weight) and BHA (0.5% and 1%) significantly increased (p< 0.001; p<0.05) the SOD activity in comparison to normal animals. SOD activity was recovered insignificantly (p>0.001) by plant extract (low and high dose) and BHA (both doses) along with DENA in male mice. In comparison to DENA treated group pre administration of hydro-ethanolic extract of EN and BHA (at both doses) showed significant (p<0.001) elevation in SOD activity.

Estimation of CAT

Treatment with hydro-ethanolic extract of EN to normal mice for 14 days induced a dose dependent increase in the level of catalase in the kidney tissue. Figure 3 depicts the effect of EN extract and BHA on CAT against DENA-induced renal toxicity in mice. The results showed significant decrease (p<0.001) of catalase activity in DENA treated group, when compared to control group. Oral administration of hydro-ethanolic extract of EN at both doses and BHA (1%) significantly increased (p<0.001; p<0.05) the CAT activity, whereas BHA (0.5%) insignificantly increased the CAT activity in comparison to control group. CAT activity was also recovered significantly (p<0.001) by plant extract and BHA (low and high dose) along with DENA in male mice in comparison to untreated group. When compared to DENA treated group pre administration of hydro-ethanolic extract of EN, BHA (at both doses) and combination groups showed significant (p<0.001) elevation in CAT activity.

Behavioral and toxic effects

In the toxicity study, no morality occurred within 24h with the different doses of plant extract tested. The hydro-ethanolic extract of the leaves of EN was found to be non-toxic up to the dose of 800 mg/kg (Pracheta et al., 2011c). The nephro-protective effect offered by EN 400 mg/kg was found to be greater than that of 150 mg/kg treatment.

Discussion

Search for new chemopreventive and antitumor agents that are more effective and less toxic than existing agents has kindled great interest in phytochemicals. Phytochemicals possess good antioxidant activities and has been reported to exhibit multiple biological effects including anti-inflammatory, antitumor activities. Presence of phenolics like saponins, flavonoids and tannins in the extract of EN acts as primary antioxidants or free radical scavengers (Pracheta et al., 2011a, b). We have reported the chemoprotective activity of hydro-ethanolic extract of Euphorbia neriifolia Linn. leaves against DENA-induced liver carcinogenesis in mice. EN extract restored SOD and CAT enzyme levels in the liver (Pracheta et al., 2011c).

The reactive oxygen species formed may cause cellular and subcellular damage by peroxidation of membrane lipids, by denaturing cellular proteins and by breaking DNA strands, disrupting cellular functions (Maxwell, 1995). Aerobic cells are endowed with extensive antioxidant defense mechanisms including both low molecular weight scavengers, which counteract the damaging effects of toxic oxygen species (Halliwell and Gutteridge, 1989; Mukerjee, 2003) and endogenous antioxidant enzyme such as superoxide dismutase which converts the superoxide free radical anion to hydrogen

peroxide. Catalase is capable of scavenging the hydrogen peroxide radical, which is formed during various biochemical and metabolic reactions. The glutathione is involved in many important cellular functions, ranging from the control of Physicochemical properties of cellular proteins and peptides to the detoxification of free radicals (Meister, 1983). However, when the balance between these species and antioxidants is altered, state of oxidative stress results possibly leading to permanent cellular damage.

The antioxidant prevention against renal diseases has been poorly analyzed. The renoprotective effect of polyphenols is thought to be mainly due to their large array of biological actions, such as free radical-scavenging, metal chelation and enzyme modulation abilities (Pietta et al., 1998). All these compounds (Flavonols, Flavonids and steroids etc.) are likely responsible for an enhancement of the antioxidant capacity of plasma in humans (Duthie et al., 1998; Durak et al., 1999), thereby modulating the systemic antioxidant defense system. Oxidative stress mediates a wide range of renal impairments, ranging from acute renal failure (Paller et al., 1998; Baliga et al., 1999; Shah, 2001), rhabdomyolysis (Vanholder et al., 2000), obstructive nephropathy (Klahr, 2001), hyperlipidemia (Wanner et al., 1997; Sakatsume et al., 2001) and glomerular damage (Kitamura and Ishikawa, 1999) to chronic renal failure and hemodialysis and associated inflammation (Handelman et al., 2001).

Renal disorders remain as one of the serious health problems. DENA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis (Gey, 1993). However we do not have satisfactory nephro-protective drugs in allopathic medical practice for serious renal disorders. Herbal drugs play a role in the management of various renal disorders in addition to other natural healing processes of the kidney.

E. *neriifolia* treatment in the present study showed an extremely significant rise in SOD and CAT along with significant decrease in renal lipid peroxidation, which signifies the reported and well defined antioxidant activity on kidney of the treated animals. LPO is regarded as one of the basic mechanisms of cellular damage caused by free radicals.

Free radicals react with lipids causing peroxidation, resulting in the release of products such as melondialdehyde, hydrogen peroxide, and hydroxyl radicals. An increase in lipid peroxides indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function, and cell death (Pomplla et al., 1991). Lipid peroxidation plays an important role in carcinogenesis (Banakar et al., 2004) and may lead to the formation of several toxic products, such as malondialdehyd (MDE), F2- isoprostanes and 4-hydroxynonenal. Thus, increased levels of malondialdehyde and F2- isoprostanes, two products of lipid peroxidation, have been reported in various clinical settings associated with renal damage (Martín-Mateo et al., 1999), although most of these studies have been performed in rats or mice.

Oxidative stress may alter the structure and function of the glomerulus because of the effect of ROS on mesangial and endothelial cells (Klahr, 1997). The glomerulus is considerably more sensitive to oxidative injuries than other nephron segments. The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major reactive oxygen species that function in concert to induce LPO (lipid peroxidation) of cell membrane lipids. The toxic peroxidative products cause wide spread cellular injury (Fridovich, 1986). The present study showed the depletion in the lipid peroxidation as observed by significant decrease in the thiobarbituric acid reactive substances (TBARS) level of the kidney in the treated groups. SOD metabolizes the superoxide radical anion. It is an effective defense of the cell against endogenous and exogenous generation of superoxide (Brawn and Fridovich, 1980). The ROS scavenging activity of SOD is effective only when it is followed by the action of CAT and GPX, because of the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which is more toxic than oxygen derived free radicals and required to be scavenged further by CAT and GPX (Blake et al., 1987). The administration of hydroethanolic extract of 400 mg/kg B/W significantly increased the level of catalase and SOD in kidney. This shows the antioxidant nature of the extract.

Although, the possible mechanism(s) of its protection against DENA induced nephrotoxicity was not studied in the current study, it is possible that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with nephroprotective properties to mediate their protection via antioxidant and/or free radical scavenging activities due to the high concentration of flavonoids and alkaloids they contain (Miller and Rice-Evans, 1997; Adeneye and Benebo, 2008; Paliwal et al., 2011). Equally, saponins present in EN have been reported to protect liver and kidneys toxicity (Bigonia et al., 2009). In addition, EN has been reported to contain flavonoids, alkaloids, saponins and other active phytocomponents (Pracheta et al., 2011a). Generally, results for the kidney have shown fewer changes in antioxidant activity compared to liver (Pracheta et al., 2011c).

Summing these facts, it is plausible for the alkaloid, flavonoid and saponin components of EN to be responsible for the observed biological effects. These could constitute areas of future research. Again, the nephroprotection offered by the extract could be due to the presence of any of the phyto-principles contained in it.

In conclusion, It can be concluded that, the extract of the leaves of *Euphorbia neriifolia* offers protective effect against DENA-induced renal-toxicity in experimental mice. The antioxidant action of hydro-ethanolic extract of EN may be attributed to the presence of known flavonol, flavonoids and other active ingredients, which provides maximum conjugation with free radical species, thus reducing the number of free radicals available as well as oxidative stress-related diseases of major organ such as kidney. Further investigation could be done on the isolation and identification of antioxidative components in *Euphorbia neriifolia*.

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