

Chemoprotective activity of hydro-ethanolic extract of *Euphorbia neriifolia* Linn leaves against DENA-induced liver carcinogenesis in mice

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Abstract

Liver cancer is one of the leading causes of cancer death worldwide. This idea has prompted to evaluate the hepatoprotective properties of hydro-ethanolic extract of *Euphorbia neriifolia* (EN) Linn against N-Nitrosodiethylamine (DENA) induced liver cancer in mice. Male mice were pre-administered with EN extract (150 and 400 mg/kg body weight; p.o.) and standard (0.5% BHA) prior to single dose of DENA (50 mg/kg body weight; p.o.). Various *in vivo* biochemical parameters like lipid peroxidation, superoxide dismutase and catalase were evaluated to determine the hepatoprotective and antioxidant activity of EN. DENA significantly increased LPO and decreased the endogenous antioxidant enzymes (SOD and CAT). The EN extract significantly restored the antioxidant enzyme level in the liver and exhibited significant dose dependant protective effect against DENA induced liver toxicity, which can be mainly attributed to the antioxidant property of the extract. This study rationalized the ethno-medicinal use of the EN for curing liver cancer.

Keywords: *Euphorbia neriifolia* Linn; hepatoprotective; N-Nitrosodiethylamine; lipid peroxidation; BHA; albino mice.

Introduction

Cancer is one of the most dreaded diseases of the 20th century and spreading further with continuance and increasing incidence in 21st century. By 2030, it has been estimated that more than 12 million people could die from this disease every year (Seffrin, 2008). Hepatocarcinoma is the fifth most common cancer in the world (representing up to 83%) and the majority of patients with liver cancer will die within one year (Shaarawy *et al.*, 2009). Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver toxicity (Farazi *et al.*, 2006; Jemal *et al.*, 2007). It is the most important and major detoxifying organ in vertebrate body, which involves intense metabolic activities. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. Certain toxic chemicals and medicines can cause liver damage, which has been recognized as a toxicological problem. Most of the hepatotoxic chemicals damage liver cells mainly

by inducing lipid peroxidation and other oxidative damages (Ilango and Chitra, 2009).

N-Nitrosodiethylamine (DENA) is the most important environmental carcinogen among the nitrosamines described as an effective hepatotoxin and hepatotoxic in experimental animals (Jose *et al.*, 1998; Thirunavukkarasu and Sakthisekaran, 2001). Foodstuffs such as cheese, soybeans, smoked, salted and dried fish, cured meat, alcoholic beverages and a few varieties of vegetables are the principal sources of DENA (Tricker *et al.*, 1991; Liao *et al.*, 2001; Bansal *et al.*, 2005). Metabolism of certain therapeutic drugs is also reported to produce N-nitrosodiethylamine (Akintonwa, 1985). Oxidative stress is considered as critical mechanism contributing to DENA-induced hepatotoxicity, and the use of antioxidant agents reduced liver damage (Vitaglione *et al.*, 2004). 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). Nakae *et al.* (1997) reported that administration of DENA resulted in lipid peroxidation (LPO) and enhanced chemi-luminescence in liver preneoplastic nodules, indicating formation of activated oxygen species.

Reactive oxygen species (ROS) have been implicated in the patho-physiology of various clinical disorders, including ischemia, reperfusion injury, atherosclerosis, acute hypertension, hemorrhagic shock, diabetes mellitus and cancer (Hemnani *et al.*, 1998). Chemoprevention involving the use of synthetic or natural products to inhibit or reverse the carcinogenic process is an effective approach to control cancer (Metlin, 1997).

In spite of tremendous advances in modern medicine, there are not many effective drugs available that stimulate liver function, offer protection to the liver from damage or help to regenerate hepatic cells (Chattopadhyay, 2003). In the absence of reliable liver-protective drugs in modern medicine, there are a number of medicinal preparations in ayurveda recommended for the treatment of various ailments, including hepatopathy (Vadivu *et al.*, 2008) that have been in use for centuries because of their effectiveness fewer side effects and relatively low cost. 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 Linn. (Euphorbiaceae) commonly known as "Sehund or thohar" in Hindi, is found throughout the Deccan Peninsula 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). *E. neriifolia* hydro-ethanolic extract was found to contain alkaloids, saponin, tannins, flavonoids and cardiac glycosides on preliminary phytochemical analysis (Pracheta *et al.*, 2011).

In spite of its various medicinal uses, no systematic studies in the literature regarding the pharmacological effect of use of EN leaves extract for liver protective activity have been reported. Therefore, the aim of this investigation was to evaluate the chemopreventive activity of the hydro-ethanolic extract of leaves of *Euphorbia neriifolia* Linn against DENA-induced hepatic 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 were 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 airtight container. The residue was designated as hydro-alcoholic extract and used to assess hepatoprotective 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 when

they weighed 25-35g (3-4 months old). Colony bred adult male albino mice were maintained under standard laboratory conditions at a temperature of $22 \pm 3^\circ\text{C}$, relative humidity of $50 \pm 5\%$ 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 had 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 adopted according to SPF facilities.

Acute toxicity studies

Acute oral toxicity was performed as per OECD-423 guidelines (Ecobichon, 1997). The albino mice were fasted overnight provided only water, after which the hydro-ethanolic extract of the leaves of EN was administered by gastric intubation to the relevant animals at the single dose of 50 mg/kg body weight. The animals were then observed for 14 days. However, mortality was not observed, the procedure was repeated for further higher doses such as 50, 100, 150, 200, 300, 400, 800, 1600 mg/kg body weight. Mortality was not noticed up to 400 mg/kg, whereas, the LD_{50} of the extract was found to be 1600 mg/kg body weight. Toxic symptoms for which the animals were observed for 72 h include behavioral changes, locomotion, convulsions and mortality.

Study design

Adult Swiss albino male mice divided into eight 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:

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

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.

Group 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.

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.

Group 6 and 7 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.

Group 8: received BHA (0.5 % 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 LD_{50} calculated in the laboratory and on the basis of other published reports (Bharali *et al.*, 2003; Bigonia and Rana, 2009; Sigma-N0258, Material Safety Data Sheet, 2003).

Hepatic oxidative stress parameters

After 19 days of duration, the mice were fasted overnight and then sacrificed under light ether anesthesia. Liver lobules were dissected out, washed immediately with ice-cold saline to remove blood, and the wet weight noted and then stored at -80°C for various biochemical assays, and histological studies. Half of each liver 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 liver homogenate

Liver 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.

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 supernatant was collected and used for assay. The chloroform-methanol solvent (degassed, 2:1, 0.45 ml) was added to 0.5 ml of supernatant 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 $\mu\text{M/gm}$ of protein.

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). Liver homogenate (10 μl) was taken, and 200 μl of dilute radical detector (24 μM 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 liver homogenate. SOD standard (bovine erythrocyte 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.

Catalase (CAT)

Catalase activity in the liver 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). Liver 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 liver 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 \pm 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 $P < 0.001$ using the SPSS 16.0 (Statistical Program for Social Sciences) program.

Results

The hydro-ethanolic extract of the leaves of EN was found to be non-toxic up to the dose of 800 mg/kg. The hepatoprotective effect offered by EN 400 mg/kg was found to be greater than that of 150 mg/kg treatment.

Effect on lipid peroxidation and anti-oxidant enzyme levels

Table 1 illustrates the effect of hydro-ethanolic extract of EN and BHA on lipid peroxidation and antioxidant-enzymes (SOD and CAT) in control and treated groups against DENA-induced hepatotoxicity in male mice. The antioxidant enzyme levels in normal control group were observed to be significantly higher than DENA control group. LPO content in the liver homogenate was significantly increased ($p < 0.01$) whereas the SOD and CAT antioxidant activity were significantly decreased ($p < 0.001$) in DENA treated group when compared to normal control group. Oral administration of hydro-ethanolic extract of EN (150 and 400 mg/kg body weight) and BHA (0.5 %) insignificantly decreased ($p > 0.01$) the LPO level and significantly increased ($p < 0.001$) the SOD and CAT activity in comparison to control group.

In comparison to DENA treated group, preadministration of hydro-ethanolic extract of EN at low and high dose and BHA showed significant ($p < 0.001$) elevation in SOD and CAT activity. Intake of EN extract at low and high dose along with DENA insignificantly ($p > 0.01$) improved LPO, whereas BHA significantly ($p < 0.01$) decreased the LPO level. SOD and CAT activity were also recovered significantly ($p < 0.001$) by plant extract (low and high dose) and BHA in male mice. Figure 1 shows the level of LPO in liver of control and treated mice. Figure 2 and 3 depicts the effect of EN extract and BHA on SOD and CAT against DENA-induced liver toxicity in mice.

Table 1: Effect of hydro-ethanolic extract of the leaves of *Euphorbia neriifolia* in the levels of LPO and the activities of SOD and CAT against DENA-induced hepatotoxicity in mice.

Groups	Treatments mg/kg	LPO (uM/mg protein)	CAT (nmol/min/ml)	SOD (U/ml)
Control	-	101.80±0.005 ^b	3.25±0.005 ^a	1.9±0.003 ^a
DENA	50	138.80±0.007 ^{**}	1.89±0.002 ^{*/a}	0.84±0.003 [*]
EN	150	98.72±0.184 ^b	5.21±0.008 ^{*/a}	4.71±0.002 ^{*/a}
	400	84.32±0.008 ^a	6.32±0.010 ^{*/a}	5.39±0.004 ^{*/a}
BHA	0.5%	82.28±0.67 ^a	3.47±0.009 ^{*/a}	4.3±0.003 ^{*/a}
EN + DENA	150 + 50	123.40±0.055	3.15±0.005 ^{**/a}	1.58±0.002
EN + DENA	400 + 50	110.04±0.009	3.19±0.002 ^a	1.71±0.003 ^a
BHA+ DENA	0.5% + 50	105.92±0.074 ^b	3.11±0.002 ^{*/a}	1.24±0.001

Values are expressed as mean ± SD (n=6).

*p<0.001, **p<0.01 vs. control group

^ap<0.001, ^bp<0.01 vs. treated group (DENA)

Discussion

The essential nature of any cancer in humans or in animals continues to challenge many scientists and practitioners interested in the biology, prevention and therapy of this disease. Therefore, the 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. The presence of phenolics like saponins, flavonoids and tannins in the extract of EN act as primary antioxidants or free radical scavengers.

Liver diseases remain as one of the serious health problems. Liver is the commonest site affected during the toxic manifestation of many drugs. N-nitrosodiethylamine (DENA) is a major environmental hepatocarcinogen. Since liver is the main site of DENA metabolism, the production of ROS in the liver may be responsible for its carcinogenic effects (Bansal *et al.*, 2005). 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 liver protective drugs in allopathic medical practice for serious liver disorders. Herbal drugs play a role in the

management of various liver disorders in addition to other natural healing processes of the liver (Subramoniam *et al.*, 1998).

E. neriifolia treatment in the present study showed an extremely significant rise in SOD along with catalase. The extract also showed extreme significant decrease in liver lipid peroxidation, which signifies the reported and well-defined antioxidant activity on liver of the treated animals. These observations are in confirmation with Bigonia and Rana (2006). 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 malondialdehyde, 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 malondialdehyde (MDE) and 4-hydroxynonenal. These products can attack cellular targets including DNA, thereby inducing mutagenicity and carcinogenicity (de Zwart *et al.*, 1999). Recently, the increase in lipid peroxidation was reported during DENA-induced hepatocarcinogenesis (Jeyabal *et al.*, 2005).

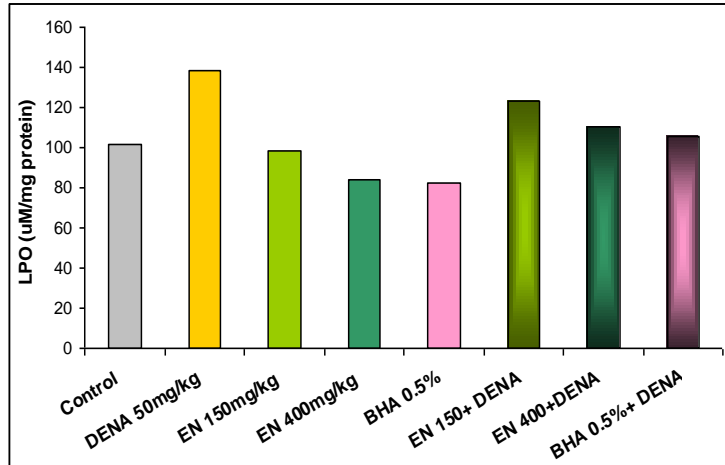


Figure 1: DENA-induced changes in hepatic lipid peroxidation and their responses to administration of hydro-ethanolic extract of EN in male mice.

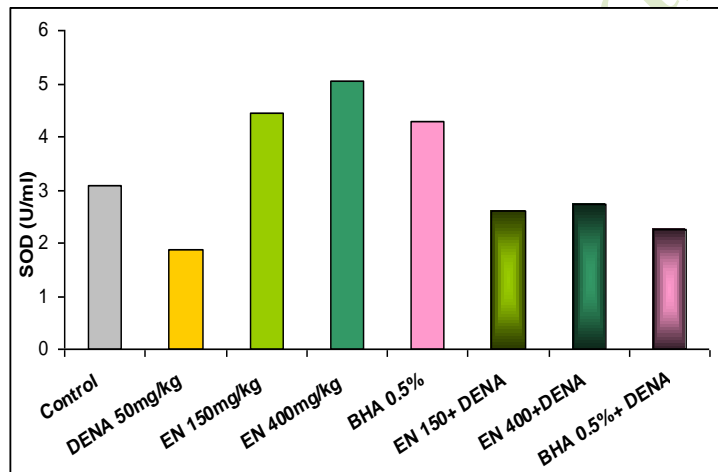


Figure 2: Effect of administration of EN extract and BHA on SOD against DENA-induced liver toxicity in mice.

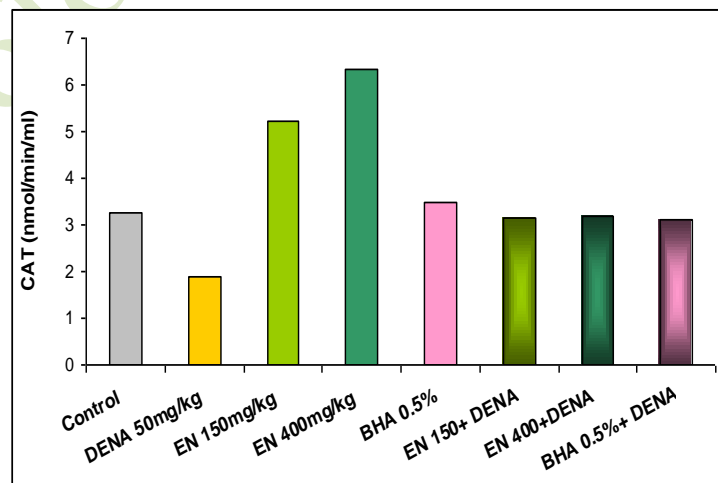


Figure 3: Effect of administration of EN extract and BHA on CAT against DENA-induced liver toxicity in mice.

Our data showed that DENA profoundly increased the level of lipid peroxidation. Free radicals, mostly ROS, cause cellular injury, the consequences of which are often exhibited and measured as lipid peroxidation (Spiteller, 1996). Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids to form radical intermediates that cause oxidative damage.

Free radical scavenging enzymes such as superoxide dismutase (SOD) protect the biological systems from oxidative stress. SOD and CAT provide the first defense against oxygen toxicity by catalyzing the dismutation of superoxide anion to hydrogen peroxide and decomposition of hydrogen peroxide to water and molecular oxygen. Earlier reports showed the decreased activities of SOD and CAT in hepatoma (Corrocher *et al.*, 1986). The current study showed a significant decrease in SOD and CAT activity in mice treated with DENA. On the other hand, there was a significant increase in SOD and CAT activities in groups treated with plant extract. Decreased activities of SOD and CAT in DENA-treated mice could be due to overutilization of these non-enzymatic and enzymatic antioxidants to scavenge the products of lipid peroxidation. Tumor cells have been reported to sequester essential antioxidants from the circulation, in order to meet the demands of the growing tumor (Buzby *et al.*, 1980).

Although, the possible mechanism(s) of its protection against DENA-induced hepatotoxicity is not studied in the current study, but may be assumed that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with hepatoprotective 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). In addition, EN has been reported to contain flavonoids, alkaloids, saponins and other active phyto-components (Pracheta *et al.*, 2011a, b). Summing these facts, it is plausible for the alkaloid, flavonoid and saponin components of EN to be responsible for the observed biological effects. Again, the hepatic-protection offered by the extract could be due to the presence of any of the phyto-principles contained in it.

Conclusion

It may be inferred from the present study that the hepatoprotective activities of the hydro-ethanolic extract of *Euphorbia nerifolia* Linn. leaves in DENA-induced hepatotoxicity may involve its antioxidant or oxidative free radical scavenging activities by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants (SOD and CAT). The mechanism of action is yet to be investigated but may be due to the antioxidant effects of saponins, flavonoids and free radical scavenging properties found to be present in the leaves. This plant has immense potential and have broad spectrum of activity on several ailments. The global changing scenario is showing a tendency towards use of nontoxic plant products having good traditional medicinal background. This plant can be used safely for longer duration as a cheap source of active therapeutics for alleviation of commonly occurring ailments by the poor and under privileged people of India. Also, the results from this study have confirmed the rationale for the folkloric use of the EN in the treatment of cancer-related hepatic disorders. Further studies are in progress in our laboratory to evaluate the potential of EN extracts in counteracting DENA-induced nephrotoxic effects which would suggest the plausible clinical applications of EN extracts.

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