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CURATIVE EFFECT OF *EUPHORBIA NERIIFOLIA* AND ITS BIOACTIVE CONSTITUENTS ON HEPATOCARCINOMA INDUCED BY N-NITROSODIETHYLAMINE IN ALBINO MICE

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ABSTRACT : Hepatoprotective efficacy of *Euphorbia neriifolia* (EN) leaves and an isolated flavonoid (ENF) was investigated against N-Nitrosodiethylamine (DENA)-induced hepatic carcinogenicity. Experimental mice were pretreated with 150 and 400 mg/kg body wt of EN, 0.5% and 1% mg/kg body wt of butylated hydroxylanisole (BHA) as a standard antioxidant and 50 mg/kg body wt of ENF for 21 days prior to the administration of a single dose of 50 mg/kg body wt of DENA. Levels of liver markers (AST, ALT & ALP), xenobiotic metabolic enzymes (Cyt P450 and Cyt b5), lipid peroxidation (LPO), antioxidants (SOD, CAT, GST and GSH) and other biochemical parameters TP and TC were measured to determine the hepatic-carcinogenicity caused by DENA. DENA administration significantly ($p<0.001$) decreased the body weight and increased the tissue weight. Activities of liver markers, antioxidants and TP content were significantly decreased ($p<0.001$), while Cyt P450, Cyt b5, LPO and TC levels were significantly ($p<0.001$) increased after DENA administration as compared with the normal control group ($p<0.001$). Pretreatment with EN and ENF counteracted DENA-induced oxidative stress (LPO) and exerted its preventive effects by restoring the levels of liver markers, antioxidants and other biochemical parameters and xenobiotic enzymes in liver tissue. In conclusion, study showed significant anti-carcinogenic potential of the *E. neriifolia* extract and ENF against DENA.

Key words : *Euphorbia neriifolia*, N-Nitrosodiethylamine, flavonoid, hepatocarcinoma, xenobiotic enzymes, butylated hydroxylanisole.

INTRODUCTION

Hepatocellular carcinoma is considered fifth most common ubiquitous deadliest liver cancer representing up to 90% of all cases and one of the most common lethal pathology worldwide with poor diagnosis. N-Nitrosodiethylamine (DENA) is an N-nitroso alkyl compound, categorized as potent environmental hepatic carcinogen among the nitrosamines is considered to be a human carcinogen based on evidences of carcinogenicity in experimental animals (Sharma *et al*, 2012a; Sharma and Pracheta, 2013). It is found in drinking water, food stuffs (milk and meat products), tobacco products, alcoholic beverages, cosmetics, agricultural chemicals, and in few varieties of vegetables (Sharma and Pracheta, 2013). Chemoprevention by using modern medicine and synthetic antioxidants are well-documented but they are suspected to exhibit some toxic effects such as carcinogenicity. Therefore, development of more effective antioxidants of natural origin that have significant scavenging properties, less toxic and inherently safer than synthetic antioxidants is the utmost demand to prevent or retard the hepatocarcinogenesis.

Application of *Euphorbia neriifolia* (Euphorbiaceae), for medicinal purposes can be dated back to antiquity. *E. neriifolia* (leaves, stem and latex) contains wide range of active ingredients (Pracheta *et al*, 2011a; Pracheta *et al*, 2011b; Sharma *et al*, 2011a; Sharma and Janmeda, 2013; Sharma and Pracheta, 2013; Sharma and Janmeda, 2017), which are useful in abdominal troubles, bronchitis, delirium, enlargement of spleen, fever, inflammation, leucoderma, loss of consciousness, piles, tumors and ulcers (Sharma *et al*, 2011). Earlier, reports described the antioxidant, free radical scavenging (Pracheta *et al*, 2011b; Sharma and Pracheta, 2013) haematological, biochemical (Janmeda *et al*, 2011; Pracheta *et al*, 2011c; Sharma *et al*, 2011b; Sharma *et al*, 2012b) as well as analgesic (Sharma *et al*, 2012c) activities of *E. neriifolia* leaves. In view of former reports, the aim of present study is to evaluate the efficacy of hydro-ethanolic extract of *E. neriifolia* (EN) leaves and an isolated flavonoid [2-(3,4-dihydroxy-5-methoxy-phenyl)-3,5-dihydroxy-6,7-dimethoxychromen-4-one] designated as ENF against DENA-induced hepatocarcinogenesis in mice.

MATERIALS AND METHODS

Chemicals and reagents

N-Nitrosodiethylamine ($C_4H_{10}N_2O$) was purchased from Sigma Chemical Co. St. Louis, MO, USA. All other chemicals used were of analytical grade and purchased from reliable firms (SRL, MERCK, SIGMA, SUYOG and Qualigens).

Preparation of extract

Euphorbia neriifolia leaves were collected from medicinal garden of Banasthali University, Banasthali (Rajasthan, India; Latitude N-26°24'14.8414"; Longitude E-73°52'9.7194") in the month of September 2009 and taxonomically identified by botanist. A voucher specimen was deposited in the herbarium (No.: BVH-780141A) of the Department of Bioscience and Biotechnology, Banasthali University. Shade-dried leaves were powdered then soxhlet extracted with 70% (v/v) ethanol and concentrated to dryness under reduced pressure at 60 ± 1ºC. After drying in hot air oven (40-45°C), the sample was put in an air tight container and stored in refrigerator at 5°C (Sharma and Pracheta, 2013; Sharma and Janmeda, 2017). The sample was designated as hydro-ethanolic extract of EN leaves and was used whenever required to assess pharmacological activity.

Extraction and isolation of flavonoids

Dried leaves of *E. neriifolia* (250 g) were extracted with sequential extraction method by using soxhlet apparatus as described earlier. The chromatographic techniques (TLC and HPTLC) used to detect the presence of flavonoid in all extracts as reported previously by using standard mobile phases (Sharma and Janmeda, 2013; Sharma and Pracheta, 2013; Sharma and Janmeda, 2017) n-Butanol: acetic acid: water (BAW, 4: 1: 5; 2: 2: 6). The isolated flavonoid was characterized by using spectroscopic techniques (NMR and LC-MS) as 2-(3,4-dihydroxy-5-methoxy-phenyl)-3,5-dihydroxy-6,7-dimethoxychromen-4-one and designed as *E. neriifolia* flavonoid (ENF) to conduct experiments.

Experimental animals care and monitoring

Healthy male Swiss albino mice (*Mus musculus* L.) procured from C.C.S. Haryana Agricultural University, Hissar (Haryana, India) were housed under standard laboratory conditions of temperature (22±3ºC), relative humidity (50 ± 15%) and photoperiod (12:12 h L:D cycle). Animals had free access to standard food pellet diet (Hindustan Lever Ltd., India) and tap water *ad libitum* (Sharma and Pracheta, 2013). The experiments 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, dated: 23.01.2010).

Treatment regimen

Total 72 mice were randomized after 2 weeks of acclimatization, into 12 groups of 6 mice each. The groups for each parameter were as follows: Group I (normal control, NC), Group II (carcinogen control, CC, received distilled water for 21 days prior to a single dose of DENA, 50 mg/kg body wt, p.o), Groups III and IV (*E. neriifolia* low dose, ENL 150 mg/kg body wt/day and *E. neriifolia* high dose, ENH 400 mg/kg body wt/day, p.o for 21 days), Groups V and VI (BHA low dose, BHAL 0.5% and BHA high dose, BHAH 1% mg/kg body wt/day, p.o for 21 days, dissolved in 0.5% acetone, standard treated group) and Group VII (ENF 50 mg/kg body wt/day; p.o, for 21 days, dissolved in distilled H_2O). The groups VIII-XII were first pre-treated with EN, BHA (low and high dose) and ENF for 21 days and on day 22 DENA was administered and left for 10 days. Body weight was measured at regular intervals during experimental period of 31 days. The dose for DENA (Sigma-N0258-Material Safety Data Sheet, 2003), EN (Pracheta *et al*, 2011c), BHA (Sharma and Pracheta, 2013) and ENF were selected on the basis of LD_{50} calculated in our own laboratory and other published reports (Janmeda *et al*, 2011; Pracheta *et al*, 2011; Sharma *et al*, 2011b; Sharma *et al*, 2012b; Sharma and Pracheta, 2013). After completion of treatment period, the animals of all groups were euthanized by cervical dislocation. Liver lobules were excised immediately, washed, cleaned and rinsed with ice cold normal saline solution (0.9% NaCl), pH 7.4, until bleached of all the blood and blotted dry on filter paper sheets to remove blood. The wet weight of the organ was noted only after drying the tissue. Tissue homogenate was prepared in ice-cold 0.1 M sodium phosphate buffer (pH-7.4) at 1-4ºC (10% homogenate w/v). Homogenate was centrifuged at 10,000 rpm for 15 min at 4ºC and the supernatant was kept frozen (-80ºC) until assayed.

Estimation of liver markers

Liver markers include aspartate aminotransferase (AST), alanine aminotransferase (ALT; Reitman and Frenkel, 1979) and alkaline phosphatase (ALP; Sadashivam and Manickam, 2004).

Biochemical parameters

Total protein content (TPC; Lowry *et al*, 1951) and total cholesterol (TC; Zak, 1977) levels were also estimated in liver homogenate.

Determination of xenobiotic enzymes

Phase I xenobiotic enzymes i.e. cytochrome P450 and cytochrome b5 contents were assayed in the liver homogenate by the method of Omura and Sato (1964) using an absorption coefficient of 91 and 185 cm^2/mmol.

Cellular metabolic parameters

Lipid peroxidation in tissue was determined by measuring the accumulation of thiobarbituric acid-reactive substance and expressed as malondialdehyde (MDA) content (Ohkawa *et al*, 1979). Liver metabolic enzymatic antioxidant assays superoxide dismutase (SOD; Marklund and Marklund, 1974), catalase (CAT; Abei, 1983), glutathione-S-transferase (GST; Habig *et al*, 1974) and non enzymatic antioxidant (GSH; Jollow *et al*, 1974) were also performed.

Statistical analysis

Experimental results were expressed as mean ± SEM of three replicates. The data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post-hoc multiple comparison test by fixing significant values as $p<0.001$, $p<0.01$ and $p<0.005$ using the S.P.S.S. (version 16.0) program.

RESULTS AND DISCUSSION

In the present study, 21 days treatment of hydro-ethanolic extract & isolated flavonoid of *E. neriifolia* leaves were found quite effective in removing the liver carcinogenicity induced by DENA as explained below by using different experiments.

Gross general observations, morphological and behavioral changes

No visible side effects (respiratory distress, abnormal locomotion, convulsion and catalepsy) were observed in NC group I whereas, carcinogen control (CC) group II showed visible side effects, which included respiratory distress, abnormal locomotion, tremor and convulsion, muscular numbness of the hind and fore legs catalepsy, stretching, swollen body and increased urination respectively. *E. neriifolia* (group III and IV) and ENF (group VII) did not cause any apparent clinical signs such as survivability or any gross visible changes attributable to carcinogenicity in the liver of mice except that the animals were crunching the mouth and becoming healthy day by day. All animals survived in good condition until their scheduled sacrifices. Pre-treatment of EN extract (group VIII and IX) and ENF (group XII) before intoxicated with DENA restore normal condition to a certain extent. Animals treated with BHA (group V and VI) showed visible side effects included intestine swelling and stretching body and these symptoms persisted till the end of the study period. Based on morphology, EN (at both doses) and ENF effectively protected the liver from carcinogenic effects of DENA in dose dependent manner.

Effect on organ weight

Liver weight was increased significantly ($p<0.01$) in CC group II as compared to NC group I (Table 1). The EN, BHA (group III, IV, V and VI) and ENF (group VII) insignificantly caused alterations in absolute liver weight in comparison to NC. Pre-treatment with ENL, ENH ($p<0.05$) and ENF prior to intoxication with DENA markedly reduced the organ weight as compared to the CC (group II), which suggesting the protective effect of extract and isolated flavonoid on carcinogen exposure. Similarly, pre-treatment with BHA (at both doses) prior to intoxication with DENA insignificantly reduced the organ weight as compared to CC (group II). BHA was less effective and did not modulate the organ weight towards normal (Table 1) in comparison to EN extract and ENF and this may be attributed due to the beneficial effect of EN extract and ENF on body weight. In previous study we found that body weight was significantly ($p<0.01$) decreases after DENA administration whereas EN (at both doses) and ENF treatment caused dose dependent increase in body weight, as compared to control animals (Sharma and Pracheta, 2013).

Liver is the main site of DENA metabolism. ROS are continuously generated *in vivo* as a result of oxidative stress and carcinogenicity caused by DENA administration that damaged the biological systems by injuring tissues, altering biochemical compounds, causing chromosomal instability, eroding cell membranes and mutation, which are involved in all steps of carcinogenesis, *i.e.* initiation, promotion and progression (Janmeda *et al*, 2011; Sharma *et al*, 2011b). The pathological changes induced by DENA were monitored by determining the levels of various biochemical and oxidative stress liver markers (Pracheta *et al*, 2011c; Sharma *et al*, 2012b).

Effect on liver markers

DENA administration significantly ($p<0.001$) decrease the AST, ALT and ALP levels, as compared to NC animals (group I). Liver markers were decreased in tissue is mainly due to the excessive generation of ROS in liver that leads to cell rupture and leakage of these enzymes from the liver cytosol into the blood stream which gives an indication of hepatotoxic effect of DENA that accompanied by tendency of apoptosis (Sharma *et al*, 2012b). DENA (50 mg/kg body weight) caused severe distortion of cyto-architecture of liver tissue and induced carcinogenicity was confirmed by histological examination (Sharma and Janmeda, 2014).

The AST, ALT and ALP levels were significantly ($p<0.001$, $p<0.01$) increased by administration of EN (group III and IV), BHA (group V and VI) and ENF (group VII), as compared with NC group. Pre-treatment with EN, BHA (at both doses; group VIII to XI) and ENF (group XII) prior to DENA administration significantly ($p<0.001$; $p<0.01$) increased the levels of liver markers, as compared with CC (group II; Table 1). Administration of EN (group VIII and IX) and ENF (group XII) before administration of DENA restored the liver markers towards normal and preserve the structural integrity of the organs from the toxic effects of DENA (Sharma and Pracheta, 2013; Sharma and Janmeda, 2014). Such reverse in liver marker enzyme activities might be attributed to the ability of EN and ENF to inhibit CYP 2E1 activity, presumably by serving as a competitive inhibitor, leading to a decrease in the formation and bio-activation of these nitrosamines. Due to its ability to reduce free radical-induced oxidative damage in the liver, EN extract has been shown to decrease liver enzymes in tissue and prevent liver damage of mice (Pracheta *et al*, 2011c).

Effect on biochemical parameter

DENA administration significantly ($p<0.001$) decreased the TP content in comparison to NC group. TP content was significantly ($p<0.001$) increased in plant extract (ENH and ENL), BHA (at both dose) and ENF (group III to VII), as compared to NC group. Administration of EN, BHA (at both doses) and ENF before intoxicated with DENA significantly ($p<0.001$; $p<0.05$) improved the TP level in hepatic tissue, as compared to CC group (Table 2). The decreased TP level in CC animals was significantly revert by pretreatment of EN (at both doses) and ENF, indicated hepatoprotective activity, as stimulation of protein synthesis accelerates the regeneration process and production of liver tissue (Humes *et al*, 1989).

Total cholesterol (TC) content in DENA administered animals was significantly ($p<0.001$) increased in comparison to NC group. This might be due to the inhibition of bile acids synthesis from cholesterol, which is synthesized in liver (Bertolotti *et al*, 2003). A significant decrease ($p<0.001$; $p<0.05$) in TC content was observed in group III to VII, when compared with NC group. Pre-treatments with EN, BHA (at both doses; group VIII and IX) and ENF (group XII) before intoxication with DENA significantly ($p<0.001$) decreased the TC content, as compared with CC group II (Table 2).

Effect on xenobiotic enzymes (Cyt P450 and Cyt b5)

Cytochrome P450 and b5 levels showed a significant ($P<0.001$) increase in their levels in CC group II, as compared to NC (group I). DENA is reported to undergo metabolic activation by cytochrome P450 enzymes to form reactive electrophiles which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Archer, 1989). Cyt P450 and b5 activity was significantly altered by ENL ($p<0.05$), ENH ($p<0.001$) and ENF ($p<0.001$; $p<0.05$), while BHA (at both doses) insignificantly increased their levels as compared to NC (group I). Pre-administration of EN (group VIII and IX), BHA (group X and XI) and ENF (group XII) before intoxicated with DENA (carcinogen control) significantly ($p<0.001$; $p<0.01$) increased the levels of cytochrome in hepatic tissue as compared to CC group II (Table 2). These findings suggested that pre-treatment with EN and ENF acted as bifunctional inducer or "blocking agent", as it induced Cyt P450 and b5 that furnished the balance of xenobiotic metabolism towards detoxification and augmented the sequential reduction of xenobiotic substrates preparing them for phase-II metabolism.

Effects on cellular metabolic parameters

Lipid peroxidation (LPO) is an important marker of oxidative deterioration of polyunsaturated lipids that cause per-oxidative tissue damage, toxicity and carcinogenicity induced by DENA. Free radicals react with lipids cause peroxidation, which may lead to the formation of several toxic products (Sharma and Pracheta, 2013). These products attack demonstrate high reactivity with proteins and DNA, thereby inducing mutagenicity and carcinogenicity (Banakar *et al*, 2004).

In the present study, DENA administration significantly ($p<0.001$) increased the malondialdehyde level in hepatic tissue as compared to NC group I, indicates that DENA administration increases the generation of ROS (Pracheta *et al*, 2011c). EN extract, (group III, and IV; $p<0.001$), BHA (group V and VI; $p<0.01$) and ENF (group VII; $p<0.001$) significantly decreased the MDA level as compared to NC mice (group I; Table 3). Compared with CC group the TBA-reactive product (MDA) was significantly ($p<0.001$; $p<0.01$) decreased by EN, BHA and ENF before intoxicated with DENA (group VIII to XII). Protective effect of EN (group VIII and IX) & ENF (group XII) against DENA might be due to inhibitory action of EN and ENF on tumor cell proliferation due to their increased anti-oxidative capabilities. ENH (group IX) treatment was found to be more effective in comparison to ENF (group XII) and

Table 1 : Effect of EN, BHA (at both doses) and ENF on absolute organ weight, relative organ weight and on liver biomarkers (AST, ALT, ALP) against DENA-induced hepatic-carcinoma in mice.

Groups	Absolute organ wt	Relative organ wt (%)	AST(IU/L)	ALT(IU/L)	ALP (µmole PNPmin^{-1}g^{-1})
NC (I)	1.73±0.03	5.4	51.42 ± 0.11	49.91 ± 0.06	71.36 ± 0.08
CC (II)	2.02±0.02^{b}	10.87	21.35 ± 0.21 ^{a}	19.54 ± 0.16 ^{a}	33.47 ± 0.22 ^{a}
ENL (III)	1.74±0.02^{c}	5.14	53.33 ± 0.15 ^{aa}	54.55 ± 0.22 ^{aa}	74.12 ± 0.25 ^{aa}
ENH (IV)	1.70±0.03^{b}	4.88	57.61 ± 0.23 ^{aa}	59.09 ± 0.30 ^{aa}	83.43 ± 0.28 ^{aa}
BHAL (V)	1.80±0.05	7.33	52.33 ± 0.25 ^{aa}	51.56 ± 0.24 ^{ba}	71.80 ± 0.20 ^{aa}
BHAH (VI)	1.83±0.04	8.25	50.76 ± 0.17 ^{aa}	52.79 ± 0.22 ^{aa}	72.78 ± 0.24 ^{aa}
ENF (VII)	1.72±0.04^{b}	4.98	55.32 ± 0.22 ^{aa}	56.31 ± 0.25 ^{aa}	76.64 ± 0.18 ^{aa}
ENL+CC (VIII)	1.84±0.05	6.49	41.17 ± 0.23 ^{aa}	41.21 ± 0.27 ^{aa}	52.64 ± 0.21 ^{aa}
ENH+CC (IX)	1.76±0.06^{c}	5.56	48.47 ± 0.31 ^{aa}	46.18 ± 0.26 ^{aa}	64.12 ± 0.28 ^{aa}
BHAL+CC (X)	1.95±0.07	9.6	31.45 ± 0.26 ^{aa}	26.09 ± 0.19 ^{ba}	43.41 ± 0.21 ^{aa}
BHAH+CC (XI)	1.92±0.08	8.18	35.38^{ab} ± 0.16	34.55 ± 0.20 ^{aa}	46.43 ± 0.27 ^{ab}
ENF+CC (XII)	1.81±0.04	6.0	43.73^{aa} ± 0.27	42.79 ± 0.23 ^{aa}	56.51 ± 0.19 ^{aa}

Significance change is calculated as ^{a}p<0.001 compared to NC group; ^{b}p<0.01 compared to NC group; ^{c}p<0.05 compared to NC group and ^{a}p<0.001 compared to CC mice; ^{b}p<0.01 compared to CC mice; ^{c}p<0.05 compared to CC mice. NC: Normal control group; CC: Cancer/ carcinogenic control group (DENA); ENL: EN low dose; ENH: EN high dose; BHAL: BHA low dose; BHAH: BHA high dose; ENF: *E. neriifolia* flavonoid. [Values are represent mean ± SEM (n=6)].

Table 2 : Effect of EN, BHA (at both doses) and ENF on liver TP, TC and on xenobiotic enzymes (phase I) against DENA-induced carcinogenicity in mice.

Groups	TP	TC	Cytochrome P450	Cytochrome b5
NC (I)	8.30 ± 0.08	92.82 ± 0.04	3.31 ± 0.23	2.94 ± 0.20
CC (II)	3.67 ± 0.19^{a}	142.26 ± 0.46^{a}	16.65 ± 0.19^{a}	11.02 ± 0.33 ^{a}
ENL (III)	9.75 ± 0.10^{aa}	84.16 ± 0.22^{aa}	3.97 ± 0.21^{ca}	3.15 ± 0.26^{a}
ENH (IV)	10.27 ± 0.09^{aa}	69.09 ± 0.24^{aa}	5.12 ± 0.27^{aa}	3.85 ± 0.22^{aa}
BHAL (V)	8.69 ± 0.12^{aa}	92.13 ± 0.27^{ca}	3.76 ± 0.25^{a}	3.05 ± 0.21^{a}
BHAH (VI)	9.14 ± 0.11^{aa}	89.82 ± 0.32^{ab}	4.36 ± 0.19^{aa}	11.02 ± 0.33^{aa}
ENF (VII)	10.02 ± 0.07^{aa}	78.27 ± 0.19^{aa}	4.83 ± 0.21^{aa}	3.62 ± 0.16^{ca}
ENL+CC (VIII)	7.63 ± 0.15^{aa}	112.28 ± 0.16^{aa}	10.32 ± 0.23^{aa}	9.75 ± 0.24^{aa}
ENH+CC (IX)	7.94 ± 0.08^{ba}	98.67 ± 0.23^{aa}	5.72 ± 0.14^{aa}	5.18 ± 0.23^{aa}
BHAL+CC (X)	6.58 ± 0.18^{ac}	126.09 ± 0.33^{aa}	14.17 ± 0.21^{ab}	10.53 ± 0.17^{aa}
BHAH+CC (XI)	7.07 ± 0.21^{aa}	122.21 ± 0.24^{aa}	9.56 ± 0.34^{aa}	8.91 ± 0.19^{aa}
ENF+CC (XII)	7.82 ± 0.06^{aa}	108.64 ± 0.21^{aa}	7.42 ± 0.27^{aa}	7.01 ± 0.14^{aa}

Significance change is calculated as ^{a}p<0.001 compared to NC group; ^{b}p<0.01 compared to NC group; ^{c}p<0.05 compared to NC group and ^{a}p<0.001 compared to CC mice; ^{b}p<0.01 compared to CC mice; ^{c}p<0.05 compared to CC mice. NC = Normal control group; CC = Cancer/ carcinogenic control group (DENA); TP: g/ml; TC: mg/g; Cytochrome P450 and b5: nmole/mg. Other abbreviations are same as in Table 1. [Values are represent mean ± SEM (n=6)].

BHA (group X and XI). This protective effect might be due to the presence of wide range of polyphenols and active ingredients in EN extract (Pracheta *et al*, 2011a; Pracheta *et al*, 2011b). ENF also reduced the peroxidation level, possibly due to antioxidant properties of flavonoids, which protect tissues against oxygen free radicals and may help in the prevention of cancer and chronic inflammation.

DENA significantly (p<0.001) decreased the SOD, CAT, GST, and GSH levels, as compared to NC group. Decreased level of SOD and CAT in CC group indicated the toxic effects of ROS produced by DENA, which increased scavenging of free radicals and superoxides results in enhanced sensitivity to free radical-induced cellular damage (Rajesh *et al*, 2012). EN, BHA (group III to group VI) and ENF treatment significantly increased the SOD, CAT, GST activities (p<0.001) and GSH content (p<0.01; p<0.05), in comparison to NC group I. Pre-treatment with ENH, ENL, BHAL, BHAH and ENF before intoxication with DENA significantly (p<0.001; p<0.01; p<0.05) increased the SOD, CAT, GST and GSH level, as compared to CC group (Table 3). ENH and ENF showed protective mechanism in response to ROS and significantly restored the antioxidant molecules suggesting

Table 3 : Effect of HEEN, BHA and ENF on enzymatic antioxidant profile in DENA induced hepatic carcinogenicity in mice.

Groups	LPO	SOD	CAT	GST	GSH
NC (I)	92.46 ± 0.08	3.40 ± 0.08	4.48 ± 0.09	222.16 ± 0.11	2.10 ± 0.16
CC (II)	152.69 ± 0.07[a]	0.87 ± 0.17[a]	1.12 ± 0.18[a]	144.62 ± 0.38[a]	1.52 ± 0.25[a]
ENL (III)	62.36 ± 0.08[ab]	4.84 ± 0.08[aa]	6.18 ± 0.10[aa]	238.70 ± 0.08[aa]	2.34 ± 0.09[ca]
ENH (IV)	51.13 ± 0.08[aa]	5.64 ± 0.09[aa]	6.97 ± 0.11[aa]	241.01 ± 0.52[aa]	2.48 ± 0.07[aa]
BHAL (V)	81.63 ± 0.15[ba]	3.73 ± 0.15[aa]	4.93 ± 0.10[aa]	226.75 ± 0.13[ba]	2.19 ± 0.15[ba]
BHAH (VI)	70.07 ± 0.11[ba]	4.09 ± 0.21[aa]	5.77 ± 0.19[ab]	231.59 ± 0.42[aa]	2.26 ± 0.27[a]
ENF (VII)	54.53 ± 0.07[aa]	5.32 ± 0.06[aa]	6.65 ± 0.07[aa]	239.32 ± 0.08[aa]	2.43 ± 0.13[ba]
ENL+CC (VIII)	109.56 ± 0.06[aa]	2.63 ± 0.09[aa]	3.52 ± 0.09[aa]	198.17 ± 0.16[ba]	1.94 ± 0.23[a]
ENH+CC (IX)	101.71 ± 0.07[aa]	3.11 ± 0.11[ba]	4.07 ± 0.08[aa]	218.45 ± 0.37[aa]	2.05 ± 0.32[a]
BHAL+CC (X)	130.18 ± 0.12[ab]	1.39 ± 0.07[ab]	2.15 ± 0.09[aa]	180.28 ± 0.27[ab]	1.81 ± 0.34[cc]
BHAH+CC (XI)	122.60 ± 0.18[aa]	2.09 ± 0.18[aa]	2.64 ± 0.09[aa]	186.20 ± 0.18[ab]	1.88 ± 0.37[cb]
ENF+CC (XII)	104.47 ± 0.05[aa]	2.91 ± 0.07[aa]	3.82 ± 0.06[aa]	211.46 ± 0.12[aa]	2.00 ± 0.21[a]

Significance change is calculated as [a]p<0.001 compared to NC group; [b]p<0.01 compared to NC group; [c]p<0.05 compared to NC group and [a]p<0.001 compared to CC mice; [b]p<0.01 compared to CC mice; [c]p<0.05 compared to CC mice. SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-Transferase. LPO: n mole MDA/g; SOD: U/ml; CAT: µmoles of H_2O_2/ min/ mg protein; GST: µ mole CDNB min-1mg-1 protein; GSH: µ mole/g. Other abbreviations are same as in Table 1. [Values are represent mean ± SEM (n=6)].

that the EN extract and ENF possess antioxidant properties and protects tissue against DENA induced oxidative stress. EN and ENF treatments might have changed the tissue redox system by scavenging the free radicals and improving the antioxidant status in the liver during DENA hepatic disorders. Our results are in line with the earlier reports (Janmeda *et al*, 2011; Sharma and Pracheta, 2013).

Results showed a dose-dependent study, i.e. higher dose of plant extract (400 mg/kg body weight) and ENF (50 mg/kg b.wt.) showed pronounced effect in amelioration of drug metabolizing enzyme in hepatic tissues followed by ENH>ENF>ENL>BHAH>BHAL.

The possible mechanism(s) of protection offered by EN against DENA-induced hepatic carcinogenicity attributed due to the presence of higher contents of phytoconstituents such as flavonoids, alkaloids, saponins, triterpenoids and other known active ingredients (Pracheta *et al*, 2011a; Pracheta *et al*, 2011b). These active ingredients provide maximum conjugation with free radical species, thus reducing the number of free radicals available as well as oxidative stress-related diseases such as cancer of major organ like liver (Janmeda *et al*, 2011; Sharma and Janmeda, 2013b). Active ingredients have been known to cause inhibition of tumorigenesis either by preventing the formation of active carcinogen from their precursors or by suppressing the expression of neoplacia (Nakamura *et al*, 2000).

In conclusion, the present study demonstrated the *in vivo* protective effect of hydroethanolic extract of *E. neriifolia* leaves and ENF against DENA-induced hepatocarcinoma. Given that there is no effective treatment measure for hepatocarcinoma, our findings may suggests a potential use of EN and ENF for hepatocellular carcinoma chemo-prevention.

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