Antihepatotoxic activity of *Euphorbia nerifolia* extract against *N*-nitrosodiethylamine-induced hepatocarcinogenesis in mice

Veena Sharma¹, Pracheta Janneda¹, Ritu Paliwal¹, Shatruhan Sharma²
1. Department of Bioscience and Biotechnology, Banasthali University, Banasthali 304022, Rajasthan, India
2. Maa Anandmayi Institute, Jaipur 302012, Rajasthan, India

**OBJECTIVE:** To scrutinize the protective role of hydroethanolic extract of *Euphorbia nerifolia* leaves (HEEN) against *N*-nitrosodiethylamine (DENA)-induced hepatocarcinogenesis in male Swiss albino mice.

**METHODS:** Experimental mice were pretreated with 150 and 400 mg/kg of HEEN, or 0.5% and 1% of butylated hydroxyanisole (BHA) as a standard for 14 d prior to the administration of a single dose of 50 mg/kg of DENA. Levels of xenobiotic metabolizing enzymes such as cytochrome (Cyt) P450 and Cyt b5, activities of reduced glutathione (GSH), glutathione-S-transferase (GST), aspartate aminotransaminase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP), and total protein and cholesterol content in the liver tissues were measured to determine the hepatotoxicity caused by DENA.

**RESULTS:** The levels of Cyt P450 and Cyt b5 were significantly increased, and GST and GSH were significantly depleted after DENA treatment ($P<0.01$). The activities of AST, ALT and ALP, and the total protein content were also significantly dropped off ($P<0.01$). The total cholesterol level was markedly increased by DENA as compared with the normal group ($P<0.01$). However, the pre-supplementation of HEEN showed a remarkable amelioration of these abnormalities, and the levels of the antioxidant enzymes in the liver were significantly restored, which exhibited the dose-dependent protective effect against DENA-induced hepatotoxicity.

**CONCLUSION:** HEEN exerts its chemopreventive effects by alleviating the xenobiotic enzymes and enhancing the levels of antioxidants and biochemical assays in DENA-induced carcinogenesis by reducing the formation of free radicals.

**KEYWORDS:** *Euphorbia*; diethylnitrosamine; xenobiotic; oxidative stress; butylated hydroxyanisole; mice
Hepatocarcinoma is the fifth most common cancer in the world (representing up to 83%) and the majority of patients with liver cancer die within one year[1]. Viral hepatitis infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, medicines, and air and water pollutants are the major risk factors of liver toxicity and can cause liver damage, which has been recognized as a toxicological problem[2].

N-nitrosodiethyamine (C₄H₈N₂O₂, DENA) is an N-nitro alkyl compounds present in environment and food chain. Different types of foodstuffs, alcoholic beverages and a few varieties of vegetables are the principal sources of DENA[3]. It is the most important environmental hepatocarcinogen that has been used as an initiating agent in some phase II (initiation and promotion) protocols for hepatocarcinogenic studies. It is metabolized to reactive the electrophilic reactants that alter the structure of DNA and form alkyl DNA adducts[4,5].

Reactive oxygen species (ROSs) have been implicated in the pathophysiology of various clinical disorders. Chemoprevention involving the use of synthetic antioxidants is known to ameliorate oxidative damages, but they are suspected to have some toxic effects such as carcinogenicity[6]. 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.

_Euphorbia neriifolia_ (Euphorbiaceae) commonly known as “Schund or Siju” in Hindi and “milk hedge” in English, 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, improving appetite and useful in abdominal troubles, bronchitis, tumours, loss of consciousness, delirium, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers and fever[7].

In spite of its various medicinal uses, a few systematic studies in the literature regarding the pharmacological effects of the extract of _E. neriifolia_ leaves on hepatocarcinoma have been reported. Therefore, the aim of the present investigation was to validate the inhibitory and protective properties of hydroethanolic extract of _Euphorbia neriifolia_ leaves (HEEN) against DENA-induced hepatocarcinogenesis in mice.

1 Materials and methods

1.1 Drugs and chemicals All chemicals and materials used in the study were of analytical reagent grade and purchased from SRL, Merck, Ranbaxy, Himedia, Qualigens and Suyog, India. DENA was purchased from Sigma Chemical Co., USA.

1.2 Preparation of plant extract _E. neriifolia_ leaves were collected from the medicinal garden of Banasthali University, Banasthali, India, October, 2009, and were identified taxonomically at Krishi Vigyan Kendra, Banasthali University, Banasthali, India. The leaves were air-dried in shade and the coarse powder (50 g) was placed in the Soxhlet thimble with 70% ethanol (volume ratio) in a 250 mL flat bottom flask using Soxhlet apparatus. The extracted mixture was concentrated at (50±1) °C in a vacuum rotatory evaporator and then freeze-dried at a low temperature at 5 °C (yield 20% of extract mixture, weight ratio) in an air-tight container. The residue was designated as hydroethanolic extract and used to assess the hepatoprotective activity.

1.3 Experimental animals Male Swiss albino mice (Mus musculus) weighing approximately 15 to 30 g were procured from Chaudhary Charan Singh Haryana Agricultural University, Hisar (Haryana, India). The animals were acclimatized for a month prior to experiment. All experiments were conducted on healthy adult male Swiss albino mice weighing 20 to 30 g. Animals were maintained under standard laboratory conditions at (22±3) °C, relative humidity of 50%±5% and photoperiod of 12 h (12 to 12 h dark-light cycle). They were housed in polypropylene cages throughout the experiment and were provided with standard food pellet (Hindustan Lever Ltd.) and drinking water.
ad libitum. Experiments and protocols described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Banasthali University, Rajasthan (CPCSEA Reg. No: IAEC/814 dt. 23/01/2010).

1.4 Treatment regime Adult Swiss albino mice were divided into six groups with six in each. The groups were as follows: group 1 served as control group; group 2 received distilled water for 14 d prior to a single dose of 50 mg/kg of DENA, and served as DENA-treated control group; groups 3 and 4 respectively received 150 and 400 mg/kg of HEEN for 14 d prior to being intoxicated with 50 mg/kg of DENA dissolved in 0.9% normal saline; groups 5 and 6 respectively received 0.5% and 1% of BHA for 14 d prior to being intoxicated with 50 mg/kg of DENA dissolved in 0.9% normal saline.

The doses of DENA, standard antioxidant, and plant extract were decided and selected on the basis of lethal dose (LD50) calculated in the laboratory and on the basis of other published reports[6,8,9,10]. The standard oro gastric cannula was used for oral administration. After 19 d, the mice were fasted overnight and then sacrificed under light ether anesthesia, and the liver lobules were dissected out for various oxidative stress and biochemical assays. Hepatic tissue was homogenized in 0.1 mol/L ice-cold sodium phosphate buffer (pH 7.4) at 1 to 4 °C (10% homogenate, weight/volume) using Potter Elvehjem homogenizer and centrifuged at 11,800 × g for 15 min at 4 °C in a cooling centrifuge.

1.5 Determination of xenobiotic metabolizing enzymes
Cytchrome (Cyt) P450 and Cyt b5 content was assayed in the microsomal suspension by the method of Omura and Sato[15], using an absorption coefficient of 91 and 185 cm²/mmol, respectively.

1.6 Cellular metabolite enzyme estimation
The enzyme levels were assayed using standard protocols. Liver metabolic enzyme antioxidant, namely, glutathione-S-transferase (GST), was estimated by the method of Habig et al[11] and the activity of non-enzymatic antioxidant, namely, reduced glutathione (GSH) was measured by the method of Jollow et al[23].

1.7 Biochemical analysis of the marker enzymes of liver damage
Biochemical analysis of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels was assayed by the method of Reitman and Frankel[15] and alkaline phosphatase (ALP) level was determined according to the protocol described in laboratory practical manual[14]. Total protein content of various samples was estimated by the method of Lowry et al[15] using bovine serum albumin as a standard. Total cholesterol level was estimated using cholesterol as standard by the method of Zak[16].

1.8 Statistical analysis
The data were expressed as mean ± standard error of mean of three replicates. The data were subjected to one-way analysis of variance and the differences between samples were determined by Tukey’s multiple comparison test using the computer-based program (Prism, Graph pad). When $P<0.01$, it was considered to have significant statistical difference.

2 Results

2.1 Effects of HEEN and BHA on xenobiotic metabolizing enzymes
Cyt P450 and Cyt b5 showed a significant escalation which was above their basal levels in their content in the carcinogenic control group as compared with the normal control group ($P < 0.01$). As compared with DENA-treated group, the enzyme levels were significantly restored and improved in the groups pretreated with 150 and 400 mg/kg of HEEN, and 0.5% and 1% of BHA before the DENA challenge ($P < 0.01$). Results revealed that HEEN exhibited a dose-dependent activity. BHA showed less significant effect as compared with HEEN at both doses. See Table 1.

| Table 1 Effects of HEEN and BHA on xenobiotic metabolizing enzymes against DENA-induced hepatotoxicity |
|---------------------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Group                                                                 | $n$                       | Cytochrome P450 (nmol/mg) | Cytochrome b5 (nmol/mg) |
| Control                                                              | 6                        | 2.22±0.14                  | 1.78±0.21                  |
| DENA (50 mg/kg)                                                       | 6                        | 8.36±0.32**                | 6.14±0.26**                |
| HEEN (150 mg/kg) plus DENA (50 mg/kg)                                 | 6                        | 5.51±0.18△△               | 4.78±0.17△△               |
| HEEN (400 mg/kg) plus DENA (50 mg/kg)                                 | 6                        | 3.96±0.18△△               | 3.01±0.19△△               |
| BHA (0.5%) plus DENA (50 mg/kg)                                       | 6                        | 7.24±0.40△△               | 5.21±0.25△△               |
| BHA (1%) plus DENA (50 mg/kg)                                         | 6                        | 5.93±0.26△△               | 4.53±0.31△△               |

** $P<0.01$, vs control group; △△ $P<0.01$, vs DENA group.

DENA: N-nitrosodiethylamine; HEEN: hydro-ethanolic extract of Euphorbia neriifolia; BHA: butylated hydroxyanisole.

2.2 Effects of HEEN and BHA on DENA-induced hepatic oxidative stress and toxicity
The activities of GST and GSH were significantly decreased in the liver of DENA-treated mice, as compared with control group mice ($P<0.01$). The activity of GST was significantly recovered and restored in the groups treated with 150 and 400 mg/kg of HEEN, and 0.5% and 1% BHA prior to being intoxicated with DENA, as compared with DENA group ($P<0.01$). And the GSH level was significantly elevated after the pretreatment with HEEN and BHA at both doses before the DENA challenge ($P<0.01$). See Table 2.
Table 2 Effects of HEEN on enzymatic and non-enzymatic antioxidant profile in DENA-induced hepatotoxicity in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GST (nmol/g) (min * mg protein)</th>
<th>GSH (mmol CDN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>175.11 ± 0.11</td>
<td>1.77 ± 0.10</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>104.23 ± 0.26</td>
<td>1.17 ± 0.13</td>
</tr>
<tr>
<td>HEEN (150 mg/kg) plus</td>
<td>6</td>
<td>158.37 ± 0.10</td>
<td>1.59 ± 0.13</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>171.61 ± 0.07</td>
<td>1.71 ± 0.10</td>
</tr>
<tr>
<td>HEEN (400 mg/kg) plus</td>
<td>6</td>
<td>131.47 ± 0.15</td>
<td>1.36 ± 0.09</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>151.12 ± 0.12</td>
<td>1.56 ± 0.15</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group; ** ** P < 0.01, vs DENA group.

DENAFN: N-nitrosodiethylamine; HEEN: hydro-ethanolic extract of *Euphorbia neriifolia*; BHA: butylated hydroxyanisole; GST: glutathione-S-transferase; GSH: reduced glutathione.

2.3 Effects of HEEN and BHA on hepatic biochemical marker enzymes altered by DENA. The activities of AST, ALT and ALP and total protein content was significantly decreased in the DENA-treated mice, and the total cholesterol level of the DENA-treated mice was significantly elevated, as compared to the normal control group (P < 0.01). However, in groups treated with 150 and 400 mg/kg of HEEN and 0.5% and 1% of BHA for 14 d prior to being treated with DENA, the activities of AST, ALT and ALP and total protein content were significantly elevated as compared to those of the DENA group (P < 0.01). The levels of total cholesterol were significantly dropped off in the mice treated with 150 and 400 mg/kg of HEEN, and 0.5% and 1% of BHA before the treatment with DENA, when compared with the DENA-treated mice (P < 0.01). See Table 3.

Table 3 Effects of HEEN and BHA on liver marker enzymes against DENA-induced hepatic toxicity in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (μmol PNP/ (min * g))</th>
<th>Total protein (g/mL)</th>
<th>Total cholesterol (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>43.22 ± 0.08</td>
<td>57.31 ± 0.06</td>
<td>37.73 ± 0.10</td>
<td>5.45 ± 0.07</td>
<td>61.32 ± 0.07</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>31.71 ± 0.15</td>
<td>43.21 ± 0.22</td>
<td>11.03 ± 0.14</td>
<td>1.87 ± 0.23</td>
<td>102.11 ± 0.21</td>
</tr>
<tr>
<td>HEEN (150 mg/kg) plus</td>
<td>6</td>
<td>35.53 ± 0.05</td>
<td>44.37 ± 0.05</td>
<td>29.64 ± 0.13</td>
<td>4.01 ± 0.17</td>
<td>78.26 ± 0.09</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>41.21 ± 0.03</td>
<td>54.32 ± 0.06</td>
<td>34.33 ± 0.08</td>
<td>4.85 ± 0.15</td>
<td>66.31 ± 0.12</td>
</tr>
<tr>
<td>HEEN (400 mg/kg) plus</td>
<td>6</td>
<td>21.23 ± 0.08</td>
<td>33.53 ± 0.03</td>
<td>19.46 ± 0.04</td>
<td>3.21 ± 0.22</td>
<td>91.56 ± 0.07</td>
</tr>
<tr>
<td>DENA (50 mg/kg)</td>
<td>6</td>
<td>27.42 ± 0.08</td>
<td>38.76 ± 0.06</td>
<td>22.32 ± 0.11</td>
<td>3.78 ± 0.21</td>
<td>87.35 ± 0.12</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group; ** ** P < 0.01, vs DENA group.


3 Discussion

Pragmatic results accomplished the objective of current investigation by modulating the enzyme levels against DENA intoxication. The plant *E. neriifolia* was found quite effective in removing the toxicity induced by DENA.

The production of phase I enzymes is measured as a cancer risk factor. Cyt P450 is a heme-thiolate and found in greatest concentration in smooth endoplasmic reticulum of hepatocellular. Another membrane-bound low-spin heme protein is Cyt b5, which acts as an electron-transfer mediator in several redox pathway, and interacts in vivo with a number of redox partners. DENA exerts its toxic effect after bioactivation by biotransformation enzymes. The hydroxylation reaction for DENA leads to consequent production of ethyl diazonium salts as well as dinitrogen. Since this alkylation is quite simple and rapid, it was inferred that its alkylation represents the local level of bio-activation capacity because the diazonium ion formed is too reactive to be transported to other organs in sufficient amount. This activation leads to DNA adduct formation lucially, which can result in mutation and ultimately lead to damage in the form of micronucleus formation. Observed results showed that HEEN pretreatment significantly improved the levels of the measured components of Cyt P450 system (Cyt P450 and Cyt b5). Thus, the HEEN by virtue of its action as inducer of Cyt P450 may be speculative of acting as blocking agent.

GSH is the major cytosolic thiol compound which plays important cellular functions ranging from the control of physicochemical properties of cellular proteins, levels of active forms of β-carotene, ascorbic acid and α-tocopherol, and peptides to the detoxification of hydrogen peroxide, lipid peroxides and free radicals. However, when the balance between these species and antioxidants is altered, the state of oxidative stress possibly results in permanent cellular damage. Administration of DENA caused depletion of GSH, which may be responsible for the increased lipid peroxidation. Pretreatment with HEEN and BHA increased the GSH content in the liver as compared to the animals treated with DENA alone, and could prevent DENA-induced hepatocarcinogenesis in mice. This may be due to
the decreased expression of these antioxidants during hepatocellular damage.

Our study shows that mice treated with HEEN and BHA prior to DENA administration improved the activity of GST. This improvement may be resulted from changing the tissue redox system by scavenging the free radicals and improving the antioxidant status.

Aminotransferases contribute a group of enzymes that catalyze the inter-conversion of amino acids and α-keto acids by the transfer of amino groups. These are liver-specific enzymes, and are considered to be very sensitive and reliable indices for necessary hepatotoxic effects of various compounds. ALP is a membrane-bound glycoprotein enzyme with a high concentration in sinusoids and endothelium.[25]

It is known that N-nitroso compounds act as strong carcinogens in various mammals including primates[26]. The drop off of these enzyme levels in tissue could potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage and lead to a marked elevation in the levels of these enzymes including AST, ALT and ALP, which is indicative of hepatocellular damage in serum.[5,21]

In this study, we also demonstrated that the oral administration of DENA to mice escorted to a marked decline in the levels of AST, ALT and ALP which is indicative of toxic effect of DENA in the hepatic tissue. Administration of HEEN to DENA-treated mice showed the restoration of AST, ALT and ALP activities. Such reverse in liver marker enzyme activities could be attributed to the ability of HEEN to inhibit the hepatic P450 IIE1 activity, presumably by serving as a competitive inhibitor, leading to a decrease in the formation or bioactivation of these nitrosamines. We have already reported that administration of E. nerifolia significantly restored the activity of liver and kidney oxidative stress markers such as lipid peroxidation, superoxide dismutase and catalase in DENA-induced mice.[5,8]

Decrease in total protein level was observed in the liver of the DENA-treated mice. At both dose levels (150 and 400 mg/kg body weight), the reduction of the enzyme levels in a dose-dependant manner and the increase of total protein level by HEEN indicate the hepatoprotective activity as stimulation of protein synthesis accelerating the regeneration process and the protection of liver cells. We also demonstrated that the total cholesterol level was augmented after intake of DENA and the bile acids synthesis inhibition was reversed. This is due to the inhibition of bile acids synthesis from cholesterol, which is synthesized in liver, leading to elevation in cholesterol level.

The possible mechanism of hepatocarcinoma protection against DENA-induced hepatotoxicity by HEEN may be due to the presence of higher content of phytoconstituents such as flavonoids, alkaloids, saponins, triterpenoids and other known active ingredients[1,15,26], and is mediated through antioxidant action or free radical scavenging activities 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 liver[27]. Antioxidants have the capacity to scavenge the free radicals directly or to interfere with the generation of free radical events which results in the inhibition of neoplastic process. Okahwa et al.[28] reported that free radicals play an important role in the complex course of multistep carcinogens. Antioxidant enzymes are altered during the carcinogenesis or after tumor formation. All these compounds such as flavonoids, flavonoids and steroids are likely responsible for an enhancement of the antioxidant capacity of plasma in humans[25], thereby modulating the systemic antioxidant defense system. Equally, saponins present in E. nerifolia have been reported to protect liver and renal from toxicity[19]. Another approach of chemoprevention is the elevation of antioxidant defense that can combat the oxidative stress produced by ROS, which often leads to mutation and cancer.

E. nerifolia contains a wide range of active ingredients and restores the enzymes which indicates the therapeutic implication of E. nerifolia to patients and scientific community. It 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 in India.

4 Conclusion

E. nerifolia could mediate its anticarcinogenic activity by modulating the activities of AST, ALT and ALP, total protein content and total cholesterol level, and could increase GSH and GSH-dependent enzymes. The global changing scenario is showing a tendency towards the uses of plant products having good traditional medicinal background. But the major limitation with E. nerifolia is that it is not dietary edible plant, thus the isolation and purification of active ingredient from this plant constitutes future research. Further studies are in progress in our laboratory to evaluate the potential of E. nerifolia ingredients in counteracting DENA-induced oxidative stress, cytotoxic effects and carcinogenicity which would suggest the plausible clinical applications of it.

5 Acknowledgements

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6 Competing interests

The authors declare that they have no competing interests.
REFERENCES


目的：通过 N-亚硝基二乙胺 (N-nitrosodimethylamine, DENA) 诱导白化小鼠肝癌，研究金刚薯水乙醇提取物抗小鼠肝毒性的作用。

方法：在给予实验小鼠口服 50 mg/kg 的 DENA 两周前，先予 150 或 400 mg/kg 金刚薯水乙醇提取物或浓度为 0.5％或 1％的苯基羟基苯胺（butylated hydroxyanisole, BHA）连续灌胃。测定各组小鼠肝脏内细胞色素 (cytochrome, Cyt) P450 及 b5，还原型谷胱甘肽 (reduced glutathione, GSH)、谷胱甘肽转移酶 (glutathione-S-transferase, GST) 以及肝组织天冬氨酸氨基转移酶 (aspartate aminotransaminase, AST)、丙氨酸氨基转移酶 (alanine aminotransaminase, ALT)、碱性磷酸酶 (alkaline phosphatase, ALP) 活性及总胆固醇和白蛋白含量。

结果：口服 50 mg/kg 的 DENA 可显著降低 Cyt P450、Cyt b5 及 GSH、GST 的浓度 (P＜0.01)，肝组织 AST、ALT、ALP 活性和总蛋白含量也显著降低 (P＜0.01)，但总胆固醇含量显著增加。经 150 或 400 mg/kg 金刚薯水乙醇提取物预处理 14 d 的实验小鼠，其由 DENA 诱发的各项组织生化指标异常发生显著逆转 (P＜0.01)。

结论：金刚薯水乙醇提取物可通过提高细胞抗氧化活性、减少自由基的生成来减少致癌物 DENA 造成的肝脏损伤。

关键词：大鼠属；二乙基亚硝胺；异生物质；氧化应激反应；苯基羟基苯胺；小鼠