



EVALUATION OF ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF *TERMINALIA BELLIRICA* AGAINST N-NITROSODIETHYLAMINE (DIETHYLNITROSAMINE)-INDUCED LIVER CANCER IN MALE WISTAR ALBINO RATS

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Abstract

Cancer remains a leading global cause of mortality, with hepatocellular carcinoma (HCC) presenting as a highly aggressive tumor often linked to chronic liver disease and chemical carcinogens such as N-nitrosodiethylamine (DEN). DEN induces intense oxidative stress, lipid peroxidation (LPO), and the depletion of endogenous antioxidant defenses. This study investigates the antioxidant and chemopreventive efficacy of the ethanolic extract of *Terminalia bellirica* (EETB) on DEN-induced liver cancer in male Wistar albino rats. Rats were divided into four groups (n = 6): a normal control, a DEN-induced cancer group, a DEN + EETB treatment group (300 mg/kg body weight orally for 30 days), and an EETB-only control group. Biochemical evaluations revealed that DEN intoxication significantly elevated basal and stimulated lipid peroxidation (TBARS) while drastically depleting enzymic (SOD, CAT, GPx) and nonenzymic (GSH, Vitamin C, Vitamin E) antioxidants in both serum and liver tissues (P < 0.001). Oral administration of EETB successfully reversed elevated TBARS levels and significantly restored both enzymic and nonenzymic antioxidant profiles toward baseline homeostatic concentrations. These findings demonstrate that EETB effectively combats DEN-induced oxidative stress and exhibits robust therapeutic and chemopreventive potential against hepatocellular carcinoma.

Keywords: Terminalia bellirica, Hepatocellular carcinoma, N nitrosodiethylamine (DEN), Lipid peroxidation

Introduction

Cancer remains one of the most devastating and feared diseases globally, serving as a principal cause of mortality [1]. Hepatocellular carcinoma (HCC) is recognized as one of the most aggressive solid tumors worldwide, frequently developing against a backdrop of chronic liver disease, cirrhosis, and chemical carcinogen exposure [2]. N-nitrosodiethylamine (DEN) is a well-established environmental and experimental hepatocarcinogen found in processed meats, tobacco smoke, and groundwater [3]. DEN undergoes metabolic activation via cytochrome P450 enzymes to yield reactive electrophilic intermediates, which provoke intense oxidative stress, cellular damage, mutagenesis, and carcinogenesis [4].

Oxidative stress stems from an imbalance between the excessive generation of reactive oxygen species (ROS) and endogenous antioxidant defense mechanisms [5]. Unchecked ROS cause lipid peroxidation (LPO) of polyunsaturated fatty acids within cell membranes, disturbing membrane fluidity and triggering cell death [6]. Although endogenous enzymes—such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)—alongside nonenzymic molecules (reduced glutathione, vitamins C and E) form a protective shield, they become severely depleted during chemical carcinogenesis [7].

Terminalia bellirica (Gaertn.) Roxb., belonging to the family Combretaceae, is a large deciduous tree traditionally valued in Ayurvedic medicine (notably as a core component of Triphala) for managing liver disorders, inflammation, and metabolic afflictions [8]. Rich in active phytochemicals like tannins, ellagic acid, and gallic acid, *Terminalia bellirica* exhibits profound antioxidant, hepatoprotective, and anti-tumor properties [9]. Despite its traditional utility, comprehensive scientific documentation evaluating the restorative role of its ethanolic extract on LPO and antioxidant status during DEN-induced hepatocarcinogenesis is vital [10]. Therefore, this study investigates the antioxidant and therapeutic efficacy of the ethanolic extract of *Terminalia bellirica* (EETB) on DEN-induced liver cancer in male Wistar albino rats [11].

Methodology

Plant source

The plant *Terminalia bellirica* was collected during July 2007 in Chennai, Tamil Nadu, India. The plant was authenticated by botanist, Captain Srinivasa Murthi Drug Research Institute for Ayurveda and Siddha, Chennai, Tamil Nadu, India.

Plant Extraction

Fresh fruits of *Terminalia bellirica* were shade-dried, coarsely powdered, and macerated with 95% ethanol for 10 days. The filtrate was concentrated under reduced pressure at 55°C using a rotary evaporator to obtain a semi-solid residue, yielding approximately 13% w/w, which was stored in a refrigerator until further biological evaluation.

Experimental Animals and Design

Healthy male Wistar albino rats (100 ± 20 g) were housed under standard environmental conditions with a 12 $\pm$ 1-hour light/dark cycle and fed standard laboratory chow and water ad libitum. Animal handling protocols complied strictly with national institutional guidelines for animal experimentation.

The rats were randomly allocated into four groups of six animals each (n = 6):

- □ **Group I (Control):** Received normal saline (0.9%) daily.
- □ **Group II (DEN-induced Cancer):** Administered a single intraperitoneal injection of DEN (200mg/kg body weight) in saline. Two weeks' post-injection, phenobarbital (PB) at 0.05% was incorporated into their diet for 14 successive weeks to promote liver tumor development.
- □ **Group III (DEN + EETB Treatment):** Cancer-bearing animals treated orally with EETB (300 mg/kg body weight) daily for 30 days.
- □ **Group IV (EETB Control):** Healthy animals treated exclusively with EETB (300 mg/kg body weight) orally for 30 days.

Biochemical Assays

Following the experimental regimen, animals were sacrificed, blood was drawn for serum separation, and liver tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4).

- □ **Lipid Peroxidation (LPO):** Estimated by quantifying thiobarbituric acid reactive substances (TBARS) under basal, H₂O₂-induced, ascorbic acid-induced, and FeSO₄-induced conditions.
- □ **Enzymic Antioxidants:** Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were measured using established spectrophotometric protocols.
- □ **Nonenzymic Antioxidants:** Reduced glutathione (GSH), ascorbic acid (Vitamin C), and α -tocopherol (Vitamin E) levels were determined in tissue and serum homogenates.

Animals

Male adult albino rats of Wister strain weighing between 100 ± 20 g were procured from "Tamil Nadu Veterinary and Animal Sciences University" Chennai, India. The animal house was well ventilated, and the animals had 12 ± 1 h day and night rhythm throughout the experimental period. The animals were housed in large spacious polypropylene cages, and they were given food and water ad libitum. During the course of the experiments, the temperature remained between 27°C and 37°C. Guidelines for breeding and Experiments on Animals, 1998 defined by the Ministry of Social Justice and Empowerment of India was followed (IAEC/CSMDRIA/04/2007)

Statistical analysis

Values are expressed as mean $\pm$ standard deviation. The results were statistically evaluated using one-way analysis of variance by SPSS 10.0 (Minitab: Turkey's multiple comparison method, Brandon Court unit, E1-E2, Coventry, United Kingdom) student version followed by Turkey's multiple comparison method to compare means of different groups. The mean difference is significant at the 0.05 levels

Results

Lipid Peroxidation Levels

The levels of LPO (expressed as nmoles of TBARS formed/mg protein/min) showed a highly significant elevation ($P < 0.001$) under basal and stimulated conditions in the liver tissues of DEN-induced cancer-bearing rats (Group II) compared to normal controls (Group I). Oral administration of EETB to cancer-bearing animals (Group III) successfully and significantly reversed these elevated LPO markers back

toward near-normal values ($P < 0.01$). Group IV animals treated solely with EETB showed no significant deviation from normal controls.

Table 1: Levels of Lipid Peroxidation in Liver of Control and Experimental Animals

Parameters (n moles of TBARS formed/mg protein/min)	Group I (Control)	Group II (DEN)	Group III (DEN + EETB)	Group IV (EETB Control)
Basal	0.72 $\pm$ 0.04	2.95 $\pm$ 0.18a*	1.82 $\pm$ 0.15b*	0.74 $\pm$ 0.05a, NS
H ₂ O ₂ induced	0.95 $\pm$ 0.12	3.10 $\pm$ 0.21a*	1.94 $\pm$ 0.11b*	0.98 $\pm$ 0.10a, NS
Ascorbic acid induced	1.85 $\pm$ 0.09	2.62 $\pm$ 0.14a*	1.76 $\pm$ 0.12b*	1.88 $\pm$ 0.11a, NS
FeSO ₄ - induced	0.98 $\pm$ 0.11	2.35 $\pm$ 0.19a*	1.52 $\pm$ 0.14b*	1.01 $\pm$ 0.07a, NS

Values represent mean $\pm$ SD of six animals. aGroup II, IV compared with Group I; bGroup III compared with Group II; * $P < 0.001$, NS: Not significant.

Enzymic and Nonenzymic Antioxidant Status

In DEN-intoxicated rats (Group II), the systemic and hepatic profiles demonstrated a severe, marked depletion ($P < 0.001$) of key enzymic antioxidants (SOD, CAT, GPx) and nonenzymic constituents (GSH, Vitamin C, Vitamin E) compared to Group I. Treatment with the ethanolic extract of *Terminalia bellirica* in Group III led to a striking, statistically significant restoration ($P < 0.001$) of both enzymic and nonenzymic antioxidant parameters toward baseline homeostatic concentrations in both serum and liver tissues.

Table 2: Effect of EETB Extract on Enzymic and Nonenzymic Antioxidants in Serum

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + EETB)	Group IV (EETB Control)
SOD (U/mg protein or mg/dL)	6.80 $\pm$ 0.52	3.95 $\pm$ 0.15a*	12.10 $\pm$ 0.64b*	14.85 $\pm$ 0.72a, NS
CAT (μ mol H ₂ O ₂ /min/mg protein)	56.20 $\pm$ 1.25	31.40 $\pm$ 1.05a*	58.30 $\pm$ 1.10b*	68.90 $\pm$ 3.80a, NS
GPx (nmol oxidized GSH/min/mg protein)	5.72 $\pm$ 0.15	3.12 $\pm$ 0.14a*	9.15 $\pm$ 0.48b*	12.05 $\pm$ 0.45a, NS
GSH (μ mol/g wet tissue)	7.45 $\pm$ 0.11	4.10 $\pm$ 0.07a*	9.20 $\pm$ 0.55b*	12.40 $\pm$ 0.38a, NS
Vitamin C (mg/g wet tissue)	3.65 $\pm$ 0.07	2.30 $\pm$ 0.05a*	3.40 $\pm$ 0.22b*	3.55 $\pm$ 0.28a, NS
Vitamin E (mg/g wet tissue)	3.90 $\pm$ 0.05	2.42 $\pm$ 0.06a*	4.75 $\pm$ 0.18b*	5.30 $\pm$ 0.12a, NS

Values represent mean $\pm$ SD of six animals. *P < 0.001

Table 3: Effect of EETB Extract on Enzymic and Nonenzymic Antioxidants in Liver

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + EETB)	Group IV (EETB Control)
SOD	17.40 $\pm$ 1.40	6.95 $\pm$ 0.55a*	16.10 $\pm$ 0.85b*	17.15 $\pm$ 0.65a,NS
CAT	70.10 $\pm$ 1.60	42.50 $\pm$ 1.20a*	68.20 $\pm$ 0.40b*	55.40 $\pm$ 4.80a,NS
GPx	13.20 $\pm$ 0.50	5.90 $\pm$ 0.52a*	11.80 $\pm$ 0.45b*	11.10 $\pm$ 0.35a,NS
GSH	13.80 $\pm$ 0.85	5.60 $\pm$ 0.60a*	12.50 $\pm$ 0.40b*	11.60 $\pm$ 0.25a,NS
Vitamin C	3.70 $\pm$ 0.28	1.42 $\pm$ 0.18a*	3.50 $\pm$ 0.12b*	3.58 $\pm$ 0.07a,NS
Vitamin E	5.45 $\pm$ 0.45	3.15 $\pm$ 0.20a*	4.90 $\pm$ 0.15b*	3.70 $\pm$ 0.10 a,NS

Values represent mean $\pm$ SD of six animals. P < 0.001

Discussion

The experimental induction of hepatocellular carcinoma (HCC) via N-nitrosodiethylamine (DEN) represents a well-established and reliable model for evaluating the genesis and chemoprevention of liver cancer [2]. DEN undergoes metabolic activation mediated by hepatic cytochrome P450 enzymes, generating reactive electrophilic intermediates and reactive oxygen species (ROS) that provoke severe oxidative stress [3]. This excess of free radicals targets polyunsaturated fatty acids (PUFAs) within cellular and organelle membranes, initiating lipid peroxidation (LPO), impairing membrane fluidity, and causing macromolecular injury that culminates in cellular necrosis and carcinogenesis [4]. The markedly elevated levels of thiobarbituric acid reactive substances (TBARS) observed under basal and induced conditions in Group II animals corroborate these extensive toxic and peroxidative mechanisms [5]. Furthermore, the concurrent exhaustion of primary endogenous enzymic defenses—including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)—alongside nonenzymic antioxidants like reduced glutathione (GSH), Vitamin C, and Vitamin E, highlights the profound systemic and hepatic redox imbalance caused by DEN [6].

When evaluated against contemporary scientific literature, the outcomes of the present investigation align closely with recent findings regarding plant-derived polyphenols and tannins in cancer chemoprevention [7]. For instance, recent studies evaluating the hepatoprotective and anti-carcinogenic properties of plant extracts emphasize that natural antioxidants play a pivotal role in intercepting free radical chains and upregulating cellular defense cascades [8]. Similarly, recent investigations into *Terminalia* species have demonstrated that hydrolyzable tannins, ellagic acid, and gallic acid act as robust primary antioxidants capable of mitigating chemical-induced oxidative injury [9]. Compared to investigations focusing solely on single isolated compounds—such as purified ellagic acid or isolated flavonoids—the present study utilizing the crude ethanolic extract of *Terminalia bellirica* (EETB) captures a synergistic multi-targeted pharmacological profile [10]. Whole-extract administration provides a diverse array of bioactive constituents that concurrently scavenge ROS, chelate transition metal ions, and modulate phase-II detoxifying enzymes [11].

Despite these encouraging outcomes, several limitations are inherent in this and comparable preclinical screening studies [12]. Many previous investigations, as well as the current experimental model, rely heavily on whole-organ homogenates and systemic serum markers, which capture a global tissue overview but lack single-cell spatial resolution regarding specific pathways like ferroptosis or targeted apoptotic signaling [13]. Furthermore, while in vivo rodent models successfully mirror human multi-step hepatocarcinogenesis, biochemical responses in rodents can occasionally exhibit higher metabolic clearance rates or distinct pharmacokinetic profiles compared to human clinical scenarios [14]. Another distinct limitation of utilizing crude ethanolic extracts lies in batch-to-batch phytochemical variability influenced by geographical, climatic, and harvesting conditions, which can introduce fluctuations in active constituent concentrations unless standardized rigorously through advanced chromatography [15]. The distinct advantages of the present study center on its comprehensive evaluation of both enzymic and nonenzymic parameters across multiple compartments (both hepatic tissue and serum) alongside a multi-condition assessment of lipid peroxidation (basal, H₂O₂-induced, ascorbic acid-induced, and FeSO₄-induced) [16]. By demonstrating that oral administration of EETB at 300 mg/kg body weight successfully reverses elevated TBARS back toward baseline while concurrently restoring depleted SOD, CAT, GPx, GSH, and vitamins C and E, this study bridges traditional Ayurvedic knowledge with rigorous quantitative pharmacology [17, 19]. Future investigative directions must address current methodological limitations by integrating molecular expression profiling—such as tracking Nrf2/HO-1 nuclear translocation and inflammatory cytokine downregulation—to fully elucidate the precise cellular mechanisms governing *Terminalia bellirica*-mediated protection against hepatocellular carcinoma [18, 20].

Conclusion

The administration of the ethanolic extract of *Terminalia bellirica* (EETB) effectively combats DEN-induced oxidative stress in rat liver cancer models. EETB successfully suppresses heightened lipid peroxidation while restoring enzymic and nonenzymic antioxidant defense systems toward normal baseline levels, demonstrating a robust therapeutic and chemopreventive potential against hepatocellular carcinoma.

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