Research Article

The Potential Chemopreventive Efficacy of β-Ionone against Diethylnitrosamine-induced Hepatocarcinogenesis in Male Wistar Rats

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ABSTRACT

Oxidative stress plays a crucial role in hepatocellular carcinoma (HCC) pathogenesis. HCC was associated with increased lipid peroxidation (LPO) resulting in disturbance of the equilibrium between the production of reactive oxygen species (ROS) and endogenous enzymatic and non-enzymatic antioxidants that exacerbated by exhaustion of these endogenous defense mechanisms. β-ionone (βI) has been shown to have specific anticancer properties. Our objective was to investigate the potential chemopreventive impact of βI on diethylnitrosamine (DENA)-induced hepatocarcinogenesis in male Wistar rats and its safety profile on hematopoietic system. HCC was induced by administrating 0.01% DENA (100 mg/L) through free access of drinking water for 8 successive weeks. The effects of βI (160 mg/kg/day), administered by oral gavage for 16 weeks, were evaluated by morphological, morphometrical, biochemical, lipid profile, hematological and histopathological analysis. Comparing with DENA-induced HCC, cotreatment with βI significantly decreased the incidence, total number and multiplicity of visible hepatocyte nodules. Likewise, mean nodular volume and nodular volume as percentage of liver volume was significantly diminished. The elevated serum liver toxicity and tumor biomarkers also significantly reduced. Interestingly, there was a significant improvement in lipid profile and hematological parameters. Moreover, antioxidant enzymes activities were significantly increased and the level of LPO was significantly reduced. This was further confirmed by a marked alleviation of histopathological changes in DENA-induced HCC group. Conclusively, βI may be a relevant candidate as a chemo preventive agent opening the door for further testing on human HCC.

Keywords: β-ionone, Hepatocarcinogenesis, Diethylnitrosamine, Oxidative stress, α-fetoprotein.

INTRODUCTION

The most common primary malignant tumor of the liver is hepatocellular carcinoma (HCC) and it is considered to be the world's third leading cause of all cancer-related deaths and the world's fifth most common cancer. HCC's incidence rate was calculated to be about 626,000 cases each year and nearly the same number of these cases dies each year[1]. HCC is associated with a high rate of recurrence after curative resection. Furthermore, drug resistance and adverse side effects as well as toxicity to normal cells remained the major obstacles of chemotherapy agents in controlled clinical trials [2]. Thus, the development of novel therapeutics for the management of HCC is particularly needed.

An effective chemo preventive agent will interfere early in the carcinogenesis process in order to eliminate premalignant cells before they become malignant or protect normal cells from transformation [3]. Among chemo preventive agents, β-ionone (βI), a cyclic isoprenoid, exhibits wide biological activities, including antimutagenic, antifungal and anticarcinogenic against various neoplasms[4, 5]; nonetheless, in vivo antitumorigenic effects against HCC are required for further study.

Diethyl nitrosamine (DENA) is a powerful hepatocarcinogenic agent of the family of N-nitroso compounds found in cigarette, cured and fried meals, water, cheddar cheese and a variety of alcoholic beverages[6]. DENA is known to cause instability in the DNA repair/replication system's nuclear enzymes. Moreover, DENA metabolism is associated with its reactive ethyl radical metabolite, which in turn interacts with DNA, creating various DNA adducts that cause mutational changes that lead to cancer[7].

Oxidative stress caused by the animal's prolonged exposure to these free radicals leads to carcinogenesis through several mechanisms including damage of protein, lipid membranes, carbohydrates and DNA, intracellular signaling changes, and even gene expression changes. At the same time, these oxidative changes facilitate abnormal cell growth and carcinogenesis [6, 7]. Therefore, in order to maintain cell health it is important to find a specific scavenger to efficiently and effectively reduce multiple ROS.

The intent of this study is to explain the potential chemo preventive effect of βI on hepatocarcinogenesis induced by DENA and its safety profile on hematopoietic system.

MATERIALS AND METHODS

Chemicals

β-ionone and DENA were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). DENA was administered every day to rats in drinking water (100 mg/L) as a freshly prepared solution and administered in bottles covered by

dark aluminum foil. Other chemicals and reagents with high-analytical quality were used.

Experimental Animals

For this study, adult male Wistar albino rats (190±10 g) were selected randomly. This study's experimental procedure was performed in compliance with animal testing standards and approved by *the Faculty of Pharmacy's Research Ethics Committee, Beni-Suef University, Egypt.* Rats were housed in stainless steel cages under controlled environmental conditions (25 °C-30 °C ambient temperature and 45%-55% relative humidity with an alternating 12 h light/dark cycle) and allowed free access to standard diet pellets and to drink DENA or tap water *ad libitum*. Before the beginning of the experiment, animals were required to acclimatize in the laboratory for one week. Food was withheld for 3-4 hours before animals were sacrificed. Samples were obtained under anesthesia from each animal and every effort was made to alleviate pain.

Experimental Design

Forty rats were randomly divided as follows into four groups (10 rats each):

Group 1: Animals in this group served as a normal control fed with standard diet, pure drinking water and a vehicle (corn oil) alone at 0.1 ml/100 g body weight.

Group 2: Animals were treated with βI alone(160 mg/kg body weight, dissolved in corn oil (0.1 ml/100 g body weight) administered orally every day for 16 successive weeks [4]; to assess, if any, the cytotoxicity of βI.

Group 3: Animals were provided with 0.01% DENA (100 mg/L) through free access of drinking water for 8 successive weeks followed by another 8 successive weeks free of DENA to induce HCC by the 16th week according to the method explained by Fathyet al. (2017)and confirmed by pathological investigation[8].

Group 4: Animals were cotreated with βI, as mentioned previously in group 2, from the beginning of 0.01% DENA administration and continued for 16 weeks.

The rats were fasted overnight after the experimental period of 16 weeks. Blood samples were obtained from the retro-orbital venous plexus through puncture under diethyl ether anesthesia. For the hematological study, a portion (0.5 ml) of the blood sample was collected in ethylenediamine tetraacitic acid (EDTA) tubes and the remainder was left to clot at room temperature for 15 min. Sera were separated by centrifugation at 2500xg at 20 °C for 15 min, and the clear sera was removed and held frozen at -20 °C for use in biochemical analysis. After collecting blood samples, the animals were sacrificed by cervical decapitation and the livers were separated, dissected quickly and washed thoroughly with isotonic ice-cold saline, dried on filter paper and weighed immediately and photographed. Then, a small section of each liver was then preserved in Davidson's fixative solution for histopathological examination, whereas a large section of each liver was homogenized in (0.15 M) KCl. Under cooling at 1000xg the homogenates were centrifuged for 20 min and the supernatants were then aliquoted and preserved at -20 ° C to be used.

Morphological and morphometrical evaluation of hepatocyte nodules (HNs) development

Each liver was examined macroscopically on the surface as well as in 3-mm cross-sections for visible HNs. The HNs were grayish-white color and easily identified from the surrounding reddish-brown liver tissue by their sharp demarcation. The nodules were counted and divided into three categories according to their respective diameters (namely, ≥3, <3 to >1 and ≤1 mm). The nodular volume (V) were determined as previously revealed, using the following equation formula: $V = \frac{4}{3}\pi.r^3$, where r is the half of the average diameter (mm)[9].

Serum Liver Toxicity Biomarkers

The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) [10], alkaline phosphatase (ALP) [11], total protein [12], albumin [13], and total bilirubin [14] were calculated by the manufacturer's instructions using colorimetric assay kits of operation from Bio-Diagnostic Co., Egypt. The globulin was determined by subtracting the albumin from the total protein for each sample.

Serum Tumor Biomarkers

Serum γ-glutamyl transferase (γGT) level was determined kinetically (Elitech Diagnostic Co., France)[15]. Serum α-fetoprotein (AFP) (LifeSpanBioSciences, Inc., Seattle, Washington, USA) level was determined by ELISA technique in accordance with the instructions given by the manufacturer with the AFP test kit.

Lipid Profile

The serum lipid profile levels of triglycerides (TG) [16], total cholesterol (TC) [17] and high density lipoprotein cholesterol (HDL-C) [18]have been measured using kits from Bio-Diagnostic Co., Egypt. Low density lipoprotein cholesterol (LDL-C) was calculated according to the formula of Friedewald and very low density lipoprotein (VLDL) = (Triglycerides/5) [19].

Hematological Parameters

The counts of red blood cells (RBCs), platelets, total and differential white blood cells (WBCs), percentage of hematocrit (Hct) and concentration of hemoglobin (Hb) were measured using a CBC analyzer *(Sino thinker. sk9000, USA).*

Hepatic Oxidative Stress Parameters

Estimation of lipid peroxidation (LPO)

Lipid peroxidation product (TBARS) was estimated by the method of Uchiyama and Mihara (1978)[20]. TBARS content in nmol/g tissue was measured and expressed. The difference in absorption was spectrophotometrically

measured at 535 and 520 nm and used in each sample to determine the TBARS content.

Estimation of superoxide dismutase (SOD) and catalase (CAT) activities

Superoxide dismutase was assessed in tissue by the method of Marklund and Marklund (1974), where pyrogallol auto-oxidized rapidly in aqueous solution to yield a yellow color that could be read at 430 nm[21]. One enzyme activity unit was defined as the amount of SOD needed to inhibit pyrogallol auto-oxidation at pH 7.8 at 25 ° C by 50 %. The activity of SOD is expressed as protein of U/mg.

Catalase was calculated in tissue according to the method described by Claiborne (1985)[22].Due to the decomposition of hydrogen peroxide by the action of CAT, the assay depends on the reduction in absorbance at 240 nm. In terms of U/mg protein, catalase activity was determined. The following equation was used to measure one unit of CAT activity: $F = \frac{2.303}{T} \log \frac{A1}{A2}$, Where: 2.303 = First order reaction rate constant. T= Time interval in min., A1= Absorbance at time 1 and A2= Absorbance at time 2. CAT activity (U/mg protein) = F/ mg protein present in sample.

Estimation of reduced glutathione (GSH) content, glutathione peroxidase (GP_X) and glutathione-S-transferase (GST) activities

The reduced GSH content was calculated using the described method by Beutler (1963)[23]. The resulting yellow color was measured at 412 nm within 5 min. spectrophotometrically. The content of GSH in mg /g tissue was expressed.

The method of Paglia and Valentine (1967) was used with some modifications to test GP_X activity. The GP_X activity unit was expressed as the sample quantity that will catalyze 1 µmole GSH to glutathione disulfide in 1 min[24].

The activity of GST was determined by the method of Mannervik and Guthenberg (1981) with some modifications. In a total volume of 2 ml, the reaction mixture consisted of 0.1 M phosphate buffer (pH 7.4), 4 mM GSH, 4 mM 1-chloro-2-dinitrobenzene (CDNB) and 10% tissue homogenate. The change in absorbance at 25 °C was recorded at 430 nm and the activity of the enzyme was calculated using a molar extinction coefficient of $9.6*10^3$ $M^{-1}cm^{-1}$ as nmol CDNB conjugate formed /min /mg protein[25].

Histopathological Examination

After fixing the liver specimens in Davidson's fixative solution for 24 h, the tissues were washed with running tap water, followed by an ascending grade of ethanol (30%, 50%, 70%, 90%, and absolute) for dehydration. Specimens were then washed in xylene and deposited for 24 h in a hot oven at a temperature of 56 °C in paraffin. Paraffin wax tissue blocks were prepared using a sledge microtome to divide into 4 µm thick sections. For histopathological analysis under the electric light microscope *(LEICA DM 2500)*, the obtained tissue sections (minimally three rats in each group) were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin (H&E) to determine histopathological changes.

Statistical Analysis

Statistical analysis was carried out using the Social Science Statistical Package (SPSS) software (version 22.0). Data are expressed as mean ± standard mean error (S.E.M). Data statistical assessment was performed using One-Way Analysis of Variances (ANOVA), with the least significant difference (LSD) post-hoc check for multiple comparisons. Statistically significant was considered to be at a $p<0.05$.

RESULTS

Effect of βI-cotreated rats on body, liver and relative liver weights in DENA-administered rats.

Initial and Final bodyweights, liver weight and relative liver weight of different groups are illustrated in Table 1. There was a significant decrease ($p<0.05$) in the final body weight of rats receiving DENA as compared to the normal control group. Treatment with βI alone or βI-cotreated rats retained normal rat body weights as compared to normal control group, indicating that βI had virtually no adverse effect on the rats' growth response. The average liver weight of DENA-induced HCC was significantly increased ($p<0.05$) as compared to that of control group. βI-cotreated rats significantly decreased ($p<0.05$) the relative liver weights as compared to DENA group.

Table 1: Effect of βI-cotreated rats on body, liver and relative liver weights in DENA-administered rats.

Groups	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Relative liver weight (g liver/100 g body)
Control	175.00±4.00	383.00±4.72@	11.57±0.32@	3.02±0.12@
βI alone	183.33±17.63	420.00±15.27@	14.63±1.10@	3.47±0.14@
DENA	174.67±8.41	256.33±12.02*	24.50±5.90*	9.80±1.80*
DENA+βI	180.40±5.04	310.60±13.26@	15.44±1.66@	4.48±0.38@

All results were expressed as means ± standard error of mean (S.E.M), *Significantly different from normal group at $p<0.05$, @ Significantly different from DENA group at $p<0.05$. Means with the same superscript symbol do not significantly different at $p<0.05$. Diethylnitrosamine, DENA; β-ionone, βI.

Effect of βI-cotreated rats on morphological and morphometrical evaluation of the development of hepatocyte nodules in DENA-administered rats.

Fig. 1 and Table 2. show the incidence, total number per group, average number per nodule-bearing liver, the size distribution of visible HNs, mean nodular volume and nodular volume as a percentage of liver volume. There were no visible hepatocyte nodules in the livers of normal control group, however the administration of DENA significantly increased *(p<0.05)* the incidence of HNs. The administration of βI as compared to the corresponding DENA group resulted in a significantly lower incidence of nodules *(p<0.05)*. The total number of nodules per group was smaller in rats treated with βI than in DENA-induced HCC. Likewise, the average number of nodules per nodule-bearing liver (multiplicity) was smaller. In addition, βI-cotreatment significantly reduced *(p<0.05)* the appearance of nodules of greater than 3mm and appeared to increase the development of nodules of smaller than 1mm in size when compared with DENA group. However, the later result did not attain statistical significance. Mean nodular volume was found to be significantly diminished *(p<0.05)* in βI-cotreated group as compared to DENA group. Similarly, there is a significant decrease *(p<0.05)* in nodular volume as a percentage of liver volume in βI-cotreated group.

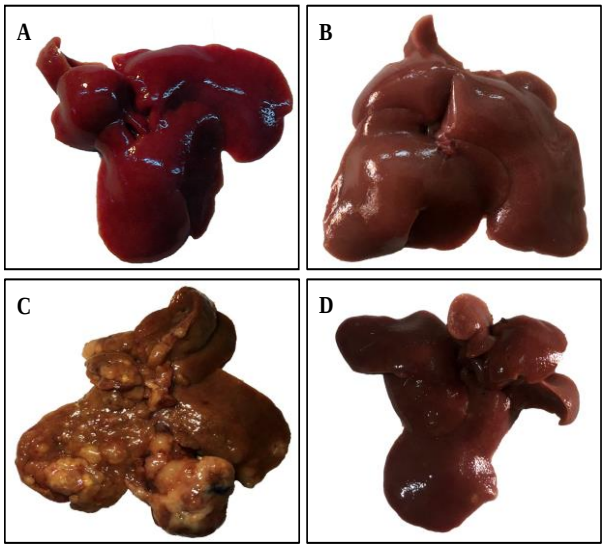

Figure 1: Effect of βI-cotreated rats on morphological evaluation of the development of hepatocyte nodules in DENA-administered rats. Representative three livers were taken from each group: **(A):** Normal control group: Showing normal hepatic picture with no visible HNs; **(B):** βI control group: Showing normal hepatic picture with no visible HNs; **(C):** DENA-induced HCC group: Showing numerous visible HNs with various sizes; **(D):** βI-cotreated group: showing little visible HNs.

Effect of βI-cotreated on Liver Toxicity Biomarkers in DENA-administered Rats

The levels of liver toxicity biomarkers are shown in Fig. 2 . The group administered by DENA exhibited a significant elevation *(p<0.05)* in ALT, AST, ALP, total bilirubin, total protein, and globulin, accompanied by a significant decrease *(p<0.05)* in albumin and albumin/globulin ratio compared to their respective control group values. The βI-cotreated rats, on the other hand, significantly reduced the elevated liver toxicity biomarkers and substantially restored albumin and albumin /globulin ratio to near normal values relative to DENA-induced rats at *p<0.05.*

Effect of βI-cotreated on HCC Tumor Biomarkers in DENA-administered Rats

The DENA-administered groups recorded a significant increase (*p<0.05)* in serum levels of γGT and AFP when compared to their control group values. In contrast with HCC carrying rats, the βI-cotreated rats significantly decreased (*p<0.05)* serum γGT and AFP levels (Table 3).

Effect of βI-cotreated on Lipid Profile and Risk Ratios in DENA-administered Rats

The DENA-administered group saw a significant increase *(p<0.05)* in the TG, TC, LDL-C, VLDL, TG/HDL-C, TC/HDL-C and LDL-C/HDL-C risk ratios and a significant decrease *(p<0.05)* in HDL-C compared to their respective control group values. Nonetheless, a surprising lipid profile finding was observed in the group received βI where TG, TC, LDL-C, VLDL, the risk ratios for TG /HDL-C, TC /HDL-C and LDL-C/HDL-C decreased significantly *(p<0.05)* while HDL-C increased significantly *(p<0.05)* compared to HCC carrying rats (Table 3).

Effect of βI-cotreated on Hematological Parameters in DENA-administered Rats

The DENA group showed a significant reduction *(p<0.05)* in the count of RBCs, concentration of Hb, Hct percentage, count of platelets, count of WBC and lymphocyte percentage. On the other hand, the proportion of neutrophils and monocytes provided by the DENA group increased significantly *(p<0.05)* relative to the corresponding control group values. Interestingly, the effect of βI-cotreatment on hematological parameters showed a significant increase *(p<0.05)* in the count of RBC, Hb concentration, Hct percentage, count of platelets and count of WBC, while the proportion of lymphocytes was not significantly higher compared to HCC bearers. Conversely, the percentage of neutrophils decreased significantly *(p<0.05)*, while the percentage of monocytes in βI-cotreated rats decreased significantly compared to HCC carrying rats (Table 4).

Table 2: Effect of βI-cotreated rats on morphometrical evaluation of the development of hepatocyte nodules in DENA-administered rats.

Groups	No. of rats with nodules/ total rats	Nodule incidence (%)	Total no. of nodules	Average no. of nodules/nodule-bearing liver (nodule multiplicity)	Nodules relative to size (% of total no.)			Mean nodular volume [a] (cm^3)	Nodular volume/liver volume [b] (%)
					≥3mm	<3 to >1mm	≤1mm		
Control	0/10	0	0	0	0	0	0	0	0
βI alone	0/10	0	0	0	0	0	0	0	0
DENA	10/10	100*	450*	45.00±4.00*	27.90±1.37*	14.46±0.17*	57.64±1.54*	0.25±0.04*	1.06±0.09*
DENA+ βI	8/10	80@	119@	14.87±0.85@	8.10±1.22@	22.03±7.79@	69.86±7.18@	0.07±0.03@	0.56±0.07@

All results were expressed as means ± standard error of mean (S.E.M), [a] Individual volume of nodule was calculated from two perpendicular diameters on each nodule, [b] One gram of liver was considered to occupy $1cm^3$ for this calculation,*Significantly different from normal group at $p<0.05$, @ Significantly different from DENA group at $p<0.05$. Means with the same superscript symbol do not significantly different at $p<0.05$. Diethyl nitrosamine, DENA; β-ionone, βI.

Table 3: Effect of βI-cotreated Rats on Tumor Biomarker, Lipid Profile and Risk Ratios in DENA-administered Rats.

Groups	γGT (IU/l)	AFP (ng/ml)	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	VLDL (mg/dl)	TG/HDL-C risk ratio (mg/dl)	TC/HDL-C risk ratio (mg/dl)	LDL-C/HDL-C risk ratio (mg/dl)
Control	11.30±0.40@	2.20±0.06@	84.00±1.00@	65.49±1.88@	36.855±0.55@	56.62±1.26@	16.80±0.20@	1.43±0.02@	1.16±0.06@	0.63±0.01@
βI alone	13.30±0.58@	1.70±0.17@	81.50±3.17@	64.13±2.31@	42.10±3.30@	57.54±0.58@	16.30±0.63@	1.41±0.07@	1.15±0.05@	0.70±0.08@
DENA	126.50±1.15*	19.50±0.58*	166.52±6.32*	210.04±10.33*	170.57±6.34*	26.51±2.01*	33.30±1.26*	6.27±0.47*	8.59±1.00*	6.64±0.60*
βI+DENA	33.5±1.73@	2.5±0.23@	93.49±2.45@	127.01±4.49@	76.66±2.27@	48.31±2.62@	18.70±0.49@	1.85±0.16@	2.67±0.17@	1.65±0.18@

All results were expressed as means ± standard error of mean (S.E.M), *significantly different from normal group at $p<0.05$, @ Significantly different from DENA group at $p<0.05$. Means with the same superscript symbol do not significantly different at $p<0.05$. Diethylnitrosamine, DENA; β-ionone, βI;γGT, γ-glutamyl transferase; AFP, α-fetoprotein; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein.

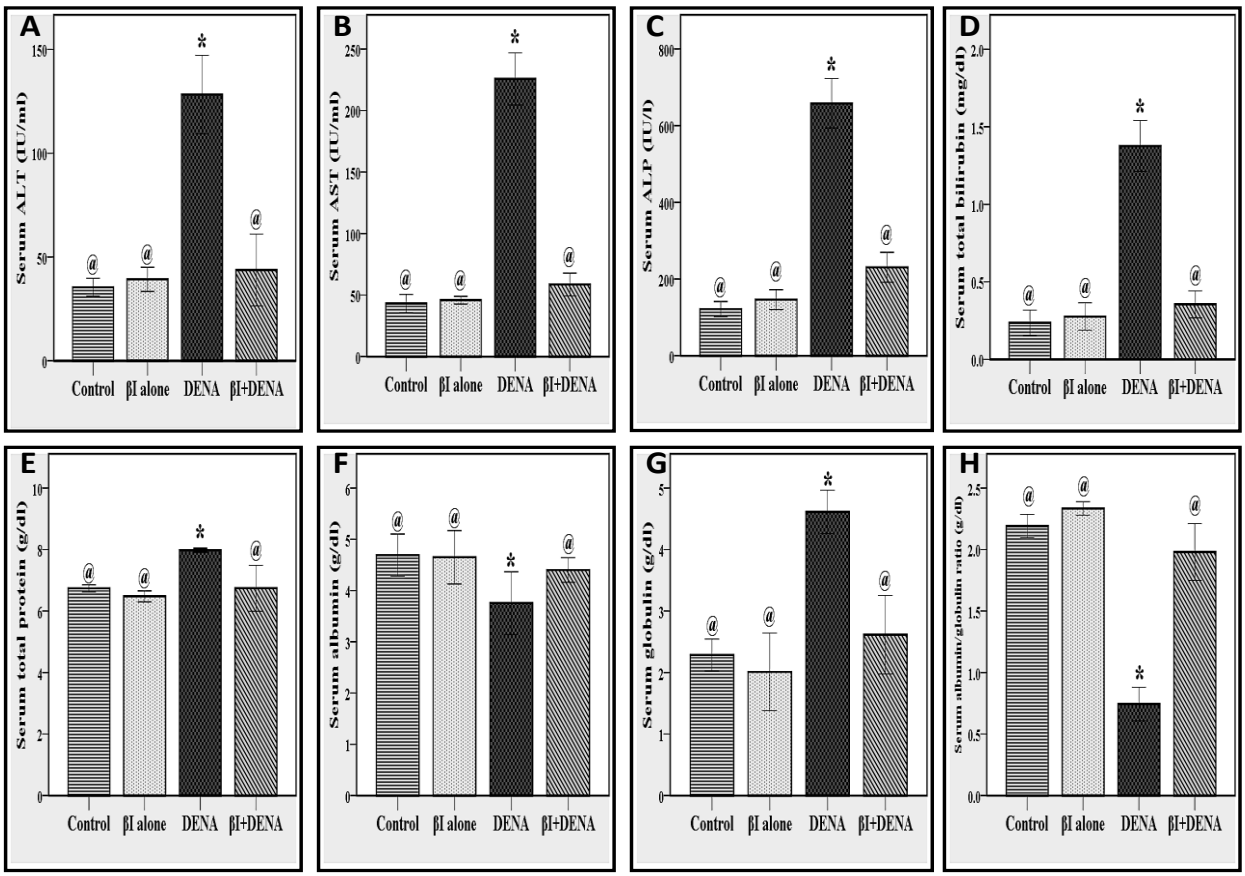

Figure 2: Effect of βI-cotreated Rats on Liver Toxicity Biomarkers in DENA-administered Rats.**(A):** Serum ALT activity of normal control, βI-alone and DENA with or without βI-cotreatment; **(B):** Serum AST activity of normal control, βI-alone and DENA with or without βI-cotreatment; **(C):** Serum ALP activity of normal control, βI-alone and DENA with or without βI-cotreatment; **(D):** Serum total bilirubin of normal control, βI-alone and DENA with or without βI-cotreatment; **(E):** Serum total protein of normal control, βI-alone and DENA with or without βI-cotreatment; **(F):** Serum albumin of normal control, βI-alone and DENA with or without βI-cotreatment. **(G):** Serum globulins of normal control, βI-alone and DENA with or without βI-cotreatment. **(H):** Serum albumin/globulins ratio of normal control, βI-alone and DENA with or without βI-cotreatment. All results were expressed as means ± standard error of mean (S.E.M), *Significantly different from normal group at *p<0.05*, @ Significantly different from DENA group at *p<0.05.* Means with the same superscript symbol do not significantly different at *p<0.05*. Diethylnitrosamine, DENA; β-ionone, βI; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Table 4: Effect of βI-cotreated Rats on Hematological Parameters in DENA-administered Rats.

Groups	RBCs count (X 10^6/mm^3)	Hb concentration (g/dl)	Hct (%))	Platelets count (X 10^3/mm^3)	WBCs count (X 10^3/mm^3)	Differential WBCs count		
						Lymphocyte (%)	Neutrophils (%)	Monocytes (%)
Control	9.34±0.24@	16.35±0.09@	51.05±2.63@	841.00±2.00@	6.15±1.07@	76.75±0.03@	13.35±0.03@	9.9±0.06@
βI alone	9.40±0.33@	16.30±0.63@	48.70±0.87@	757.00±3.00@	14.30±0.30@	77.65±2.63@	12.40±2.02@	9.95±0.61@
DENA	6.29±0.22*	9.55±0.03*	29.45±0.03*	459.00±1.00*	21.90±1.10*	52.30±0.35*	33.55±0.03*	14.15±0.37*
βI+DENA	9.22±0.16@	15.73±0.33@	45.00±1.33@	672.00±19.81@	10.55±0.78@	68.87±5.47	11.90±0.35@	12.07±0.93

All results were expressed as means ± standard error of mean (S.E.M), *Significantly different from normal group at *p<0.05*, @ Significantly different from DENA group at *p<0.05*. Means with the same superscript symbol do not significantly different at *p<0.05*. Diethylnitrosamine, DENA; β-ionone, βI; RBCs, red blood cells; Hb, hemoglobin; Hct, hematocrit; WBCs, white blood cells.

Effect of βI-cotreated Rats on Hepatic LPO Level in DENA-administered Rats

In the HCC bearing group, there was a significant increase *(p<0.05)* in LPO compared to their respective control group. Although TBARS level in animals treated with βI-cotreated rats significantly reduced *(p<0.05)* compared to HCC-bearing rats (Table 5).

Effect of βI-cotreated Rats on SOD and CAT activities in DENA-administered Rats

The SOD and CAT activities in the DENA-administered group showed a significant reduction *(p<0.05)* relative to normal control group. Specific SOD and CAT activities in the βI-cotreated group was significantly elevated *(p<0.05)* compared to HCC carrying rats (Table 5).

Effect of βI-cotreated Rats on Reduced GSH Content, GP_X and GST Activities in DENA-administered Rats

The reduced GSH content was significantly declined *(p<0.05)* in DENA group compared to control group. While, βI-cotreated group significantly restored *(p<0.05)* its content compared to HCC bearing rats (Table 5).

In contrast, DENA-administered group revealed a significant decrease *(p<0.05)* in GP_X and GST activities relative to their control group values. βI-cotreated animals displayed a significant induction *(p<0.05)* of GP_X and GST activities compared to HCC bearing rats (Table 6).

Effect of βI-cotreated Rats on Histopathological Examination of Liver Sections in DENA-Administered Rats

The findings of βI-cotreatment in HCC-induced rats on liver histopathological features are illustrated in Fig. 3. Essentially, histopathological examinations affirm the findings of serum enzyme and tumor marker assays. The normal control animals showed typical hepatic architecture (Fig. 3; A). βI alone displayed typical hepatic lobular architecture, suggesting the non-toxic nature of βI (Fig. 3; B). In contrast, the liver sections of rats exposed to DENA displayed extremely varied patterns of histological appearance; the neoplastic cells develop in nests and thick cords divided by thin walled sinusoids. Often consistent with disturbance of normal liver architecture is the typical trabecular pattern of HCC. There are also numerous mitotic figures (regeneration), pleomorphism, malignant hepatocytes with moderate to marked nuclear anaplasia and hydropic degeneration (Fig. 3; C1 and C2). Treatment of rats with βI showed a small number of cells and hepatocytes that were neoplastically transformed and maintained near normal architecture. Liver cells also displayed karomegaly, pyknotic nuclei and binucleated cells that were substantially less numerous than those in the DENA group (Fig. 3; D).

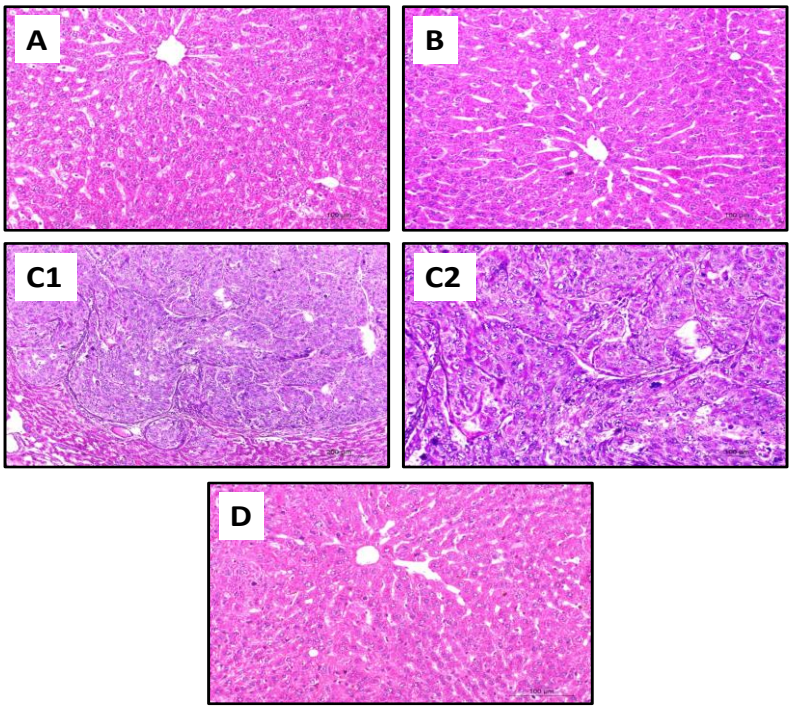

Figure 3: Effect of βI-cotreated Rats on Histopathological Examination of Liver Sections Stained with (H&E) in DENA-administered Rats.**(A):** Normal control group: Showing normal hepatic architecture. Scale bare =100µM; **(B):** β-ionone control group: Showing normal hepatic architecture. Scale bare =100µM; **(C1):** DENA-induced HCC group: Showing extremely variegated histological appearance patterns; the tumor cells are growing in nests and thick cords that are separated from one another by thin walled sinusoids. Scale bare =200µM; **(C2):** DENA-induced HCC group: Showing numerous mitotic figures, pleomorphism, and malignant hepatocytes with moderate to marked nuclear anaplasia and hydropic degeneration. Scale bare =100µM; **(D):** βI-cotreated group: showing hepatocytes maintaining near-normal architecture. Scale bare =100µM.

Table 5: Effect of βI-cotreated Rats on Hepatic Oxidative Stress Parameters in DENA-administered Rats.

Groups	TBARS (nmol/g protein) level	CAT (U/mg protein) activity	SOD (U/mg protein) activity	GSH (mg/g protein) content	GPx (nmol/min/mg protein) activity	GST (nmol/min/mg protein) activity
Control	25.31±0.94@	20.79±0.93@	51.48±1.02@	1.95±0.05@	318.05±16.60@	368.75±9.02@
βI alone	25.00±0.95@	20.02±0.22@	47.16±0.94@	2.22±0.07@	334.90±27.64@	377.60±3.64@
DENA	99.43±4.06*	8.46±0.62*	22.49±2.11*	0.86±0.04*	74.00±7.65*	201.56±9.92*
βI+DENA	45.67±1.90@	18.05±0.73@	45.85±1.90@	1.72±0.13@	267.34±19.15@	300.00±3.61@

All results were expressed as means ± standard error of mean (S.E.M), *significantly different from normal group at *p<0.05*, @ Significantly different from DENA group at *p<0.05*. Means with the same superscript symbol do not significantly different at *p<0.05*. Diethylnitrosamine, DENA; β-ionone, βI; TBARS, thiobarbituric acid reactive substances; CAT, catalase; SOD, superoxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; GST, glutathione-s-transferase.

Effect of βI Treatment Alone

Interestingly, no significant changes in either enzymatic or non-enzymatic behavior compared to normal control group were observed when βI (160 mg/kg body weight) was given to normal rats. Likewise, the histological findings, lipid profile and hematological parameters demonstrating the safety profile and the non-toxic nature of βI (Fig. 3; B).

DISCUSSION

Due to limited treatment options, poor treatment effectiveness, adverse side effects and drug resistance, a chemopreventive approach to the treatment of HCC remained elusive and strongly needed to be justified. Experimentally induced HCC in rats, in particular by chemical carcinogen (DENA), is similar to human HCC [2], allowing the screening of possible anticancer compounds in different phases of neoplasia growth.

Natural antioxidants may inhibit the generation of ROS and hence reduce the associated oxidative stress intracellular. Recent focus on isoprenoid-rich diets has strong antioxidant capacity and βI is one such isoprenoid whose potential chemopreventive effect has been documented [26]. Thus, the chemopreventive effect of βI on chemically induced HCC in rats was investigated in this study.

In the present investigation, the relative liver weight of βI-cotreated rats was significantly reduced due to the propensity of βI animals to sustain and restore their body weights faster, suggesting better resistance to DENA-induced tumor growth and the smaller number of HNs in their livers. Similar results have been previously described for rats fed βI for 7 weeks and subjected to resistant hepatocyte (RH) model [27]. Another striking result from this study was βI-mediated reduction of nodular volume along with nodular volume as a percentage of liver volume.

In general, hepatocellular damage caused by DENA is associated with disruption of the metabolism of the liver cells and membrane instability and thereafter induces distinctive changes in serum enzymes levels [6]. In the present study, there was a significant elevation in the serum levels of AST, ALT, ALP, total bilirubin and globulin while albumin concentration significantly decreased. In consistent with the present study, a recent study stated that increased levels of AST and ALT, ALP, total bilirubin and globulin are predictors of DENA-induced liver damage. Furthermore, the rise in ALP represents biliary flow pathological changes and the total bilirubin discharge indicates a non-specific alteration in the integrity and/or permeability of the plasma membrane and the reduction in albumin production shows a decline in liver function[8].Conversely, cotreatment with βI reduced the enhanced level of these enzymes. It is suggested that βI helps to regenerate parenchymal cells in the liver and thus protect the membrane integrity by decreasing the enzymes leakage. In addition, βI was shown to inhibit microsomal enzymes in the liver of rats and was suggested to be able to antagonize the effects of procarcinogens [27]. Therefore, βI defensive actions suggested being linked to inhibition of the DENA metabolism.

In addition, DENA-induced HCC increased γGT level. This is in accordance with many previous studies that have shown an increase in γGT levels during HCC development, which may indicate the specific tumor burden [28]. While, βI-cotreated animals showed a significant decrease in the level of γGT compared to tumor-bearing animals, this might be due to a reduction in the tumor burden. This result is in harmony with Asokkumar et al. (2012)[26].

The α-fetoprotein is a well-known tumor marker for HCC diagnosis. Rat exposure to certain carcinogens, such as DENA increases the circulating AFP level which is attributable to tumor secretion [1]. In the current study, DENA-induced rats showed a significant increase in the level of AFP while AFP level in βI-cotreated rats was found to be reduced.

Excess lipids accumulation in the liver speeds up the development of HCC. Our results have indicated an

elevation in the lipid profile and its risk ratios in the DENA group. This is consistent with a previous studies reported that animals submitted to DENA characterized by increased lipid profile with increased cholesterol in the hepatic nodule [8, 27]. Compared to the DENA group, in contrast, βI-cotreated rats significantly deceased TG, TC, LDL-C, VLDL and risk ratios as well as significantly increased HDL-C. A proposed mechanism for inhibiting the tumor development by βI is therefore based on their ability to post-transcriptionally inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, thereby reducing cholesterol synthesis and mevalonate intermediates such as farnesyl and geranylgeranyl pyrophosphates necessary for the protein farnesylation and geranylgeranylation of certain proto-oncogenes [27].

In the present study, DENA also caused a dramatic reduction in the hematological parameters. These findings were consistent with the study results by Fathy *et al*. (2017) who stated that RBCs, Hb, Hct, platelets, total and differential WBCs decreased by DENA administration. This might be due to the disruption of the hematopoietic system or increased permeability of the cell membrane, resulted in osmotic swelling and erythrocyte hemolysis[8]. It might also be due to increased destruction of mature cells or increased plasma volume, or a decreased oxygen hemoglobin affinity that induced hypoxia through reduced transport of O_2 from the lungs to the blood and decreased release of O_2 from oxyhemoglobin to the tissues [29]. Interestingly, the administration of βI alone on the hematopoietic cells was found to have a non-toxic effect indicating its safety profile. Moreover, βI-cotreated rats attenuated the detrimental effects of DENA on hematological parameters that could be due to increased antioxidant enzymes resulting in reduced oxidative stress on hematopoietic cells production or maturation systems.

Lipid peroxidation is a useful oxidative stress marker owing to its association with ROS overproduction when DENA is metabolized by cytochrome-P [6]. Surprisingly, we noticed that rats co-treated with βI effectively reversed DENA-induced LPO, indicating that mitigating this oxidative event is crucial to prevent the HCC development. This is in conformity with a previous study showed that the chemo preventive role of βI against DMBA induced mammary carcinogenesis related to its antioxidant activity [30].

With respect to the non-enzymatic antioxidant, GSH has been essential to preserve the normal reduced cell state and to ameliorate the oxidative stress deleterious effects [31]. In the current study, DENA depleted the reduced GSH content. This is similar to the study of Fathy *et al*. (2017) who stated that DENA, an electrophilic carcinogen, can interact with GSH's large nucleophilic pool diminishing macromolecules and carcinogenic interactions, this allows accumulation of ROS and subsequent hepatocarcinogenesis[8]. On the other hand, a substantial elevation of hepatic GSH content was observed in βI-cotreated rats relative to DENA-induced rats. This is compatible with a previous study by Liu *et al.* (2009) which showed that the non-enzymatic antioxidant GSH in the rats treated with βI was increased[28]. It may therefore be due to the concept of reducing DNA-carcinogenic interaction by this isoprenoid, and then avoiding desirable carcinogenic conditions.

Concerning the enzymatic antioxidants, as the first line of defense against superoxide radicals, SOD dismutates two superoxide radicals to H_2O_2 and O_2, while CAT and GPx support antioxidant enzymes as CAT reduces H_2O_2 to H_2O, resulting in free radicals being detoxified and GPx eliminates lipid hydroperoxides by using two GSH molecules, thereby reducing the amount of biomolecules with destructive properties. Similarly, GSH conjugation by GST is an important reaction to detoxify electrophiles by means of which antioxidant drugs could be used as anticarcinogens [31]. Therefore, there is still an effective antioxidant defense system in the liver; it can be overwhelmed under oxidative stress conditions. In the present study, reducing the activities of these endogenous antioxidant enzymes might be caused by increasing radical production during the metabolism of DENA. In contrast, βI considerably increased the activities of these antioxidant enzymes. This is in line with a previous studyof Asokkumar *et al.* (2012) who reported that βI antioxidant capacity mediated through elevation of SOD, CAT, GPx and GST activities in benzo(a)pyrene-induced lung cancer[26].

The histopathological examination of the liver section of DENA group revealed the characteristic trabecular pattern of HCC, numerous mitotic figures, pleomorphism, hydropic degeneration and malignant hepatocytes with moderate to marked nuclear anaplasia. This is consistent with those described in a recent study which reported that untreated HCC group displayed the fibrosis-extended portal area and divided the hepatic parenchyma into dysplastic lobules with prominent nucleoli degenerated hepatocytes[1]. On the other hand, βI-cotreated group demonstrated a noticeable improvement in the architecture of the liver tissue due to the reversal of the negative effects of DENA-induced HCC.

CONCLUSION

In conclusion, our findings highlight the potential modulatory effect of βI against DENA-induced hepatocarcinogenesis, its amenable effect on hematopoietic system and its primary mechanism of chemoprevention, at least partly, attributed to the oxidative stress attenuation. Furthermore, in order to confirm the underlying mechanisms, additional studies concerning other signaling pathways are required. Nevertheless, the present findings suggest the possible utility of βI as a chemopreventive agent against human HCC as an antioxidant supplement and/or adjunct anticancer therapy.

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