Effect of blueberry pretreatment on diethylnitrosamine-induced oxidative stress and liver injury in rats

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ABSTRACT
Diethylnitrosamine (DEN) treatment increases the generation of reactive oxygen species (ROS), apoptosis, necrosis and proliferation in the liver. Blueberries (BB; Vaccinium corymbosum L.) contain polyphenols and other active components and have high antioxidant capacities. We investigated the effect of BB pretreatment on DEN-induced liver injury and oxidative and nitrosative stress in male rats. Rats were fed with 5% and 10% BB containing diet for six weeks and DEN (200 mg/kg; i.p.) was applied two days before the end of this period. Liver function tests were determined in serum and histopathological evaluation was performed in the liver tissue. Apoptosis-related proteins, Bax and B cell lymphoma-2 (Bcl-2) and proliferating cell nuclear antigen (PCNA) expressions were also examined. Oxidative and nitrosative stress were evaluated in the liver by measuring thiobarbituric acid reactive substances, diene conjugate, protein carbonyl and nitrotyrosine levels, and glutathione levels and glutathione peroxidase, superoxide dismutase and glutathione transferase (GST) activities. Pretreatment with high dose of BB reduced apoptotic, necrotic and proliferative changes in the liver induced by DEN. Dietary BB also decreased hepatic lipid peroxidation, protein oxidation and nitrotyrosine levels together with increased GST activity. In conclusion, BB may have an inhibiting effect on acute liver injury by reducing apoptosis, necrosis, proliferation, oxidative and nitrosative stress in DEN-treated rats.

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1. Introduction

N-diethylnitrosamine (DEN) is one of the most important hepatotoxins and hepatocarcinogens. N-nitrosamines originate mainly from protein containing foods and are also form from nitrate precursor, which are abundant in leafy and root vegetables. Under the acidic condition of stomach, the amines present in food are activated by reaction with nitrate, leading to N-nitrosamines (Sadik et al., 2008; Janani et al., 2010; Amin et al., 2011; Jayakumar et al., 2012).

Several chemicals such as DEN induce hepatocyte injury and promote liver cancer in rodents. DEN affects the initiation stage of carcinogenesis together with enhanced cell proliferation accompanied by hepatocellular necrosis (Maeda et al., 2005; Naugler et al., 2007; Glauert et al., 2010; Qiu et al., 2011).
DEN administration induces genetically altered hepatocytes during initiation (Vásquez-Garzón et al., 2012). Since DEN does not itself exert carcinogenicity, it needs to be bioactivated by cytochrome P450 enzymes (CYP), especially CYP2E1 in the liver, resulting in DNA-adducts that form through an alkylatation mechanism (Verna et al., 1996; Kang et al., 2007; He et al., 2012). DNA damage/mutations in critical genes, chromosomal aberrations and micronuclei may occur in the liver (Glauert et al., 2010; Janani et al., 2010; Amin et al., 2011; Jayakumar et al., 2012; He et al., 2012). The bioactivation process has been considered a crucial step to initiate the process of carcinogenesis (Kang et al., 2007; Sadik et al., 2008). Moreover, it has been suggested that DEN, besides being metabolized to reactive electrophiles, cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Amin et al., 2011; Ghosh et al., 2012; He et al., 2012; Jayakumar et al., 2012). Based on these observations, the potential roles of several antioxidants on oxidative stress-induced tissue damage by wide range of carcinogens including DEN have been investigated (Ramakrishnan et al., 2006; Bishayee et al., 2010; Glauert et al., 2010; Janani et al., 2010; Amin et al., 2011; Ghosh et al., 2012; He et al., 2012; Jayakumar et al., 2012).

Blueberries (BB; Vaccinium corymbosum L.) are the fruits having high antioxidant capacities. Their antioxidant value largely derived from polyphenols such as anthocyanins, proanthocyanidins, flavonols (predominantly quer cetin derivatives) and phenolic acids (caffeic, chlorogenic, p-coumaric and ferulic acid) (Castrejón et al., 2008). It has been suggested that BB is useful in aging, inflammation, diabetes mellitus, hepatic, cardiac and neuronal disorders and cancers. Its protective effects are related to powerful antioxidant actions of constituents in BB (Neto, 2007; Zafra-Stone et al., 2007).

In our study, we wanted to investigate whether or not BB pretreatment may have protective effect on oxidative and nitrosative stress and liver injury induced by DEN. For this reason, a single dose of DEN (200 mg/kg) was administered to rats and liver function tests in serum, apoptosis, necrosis, proliferation, oxidative and nitrosative stress parameters in the liver were evaluated.

2. Materials and methods

2.1. Chemicals

DEN and other chemicals were supplied from Sigma–Aldrich (St. Louis, Missouri, USA).

2.2. Animals and experimental design

Male Wistar rats weighing 200–250 g were used in the study. They were obtained from the Experimental Medical Research Institute of Istanbul University. Rats were housed in a light-and temperature-controlled room on a 12/12-h light/dark cycle. The animals allowed free access to food and water and were kept in wire-bottomed stainless steel cages. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of the Istanbul University.

Rats randomly divided into six groups (n = 6, each) as follows; (a) control group: animals were fed with commercial rat chow, (b) BB1 group: rats were fed with 5% BB containing diet for six weeks. (c) BB2 group: rats were fed with 10% BB containing diet for six weeks, (d) DEN group: they were fed with control diet for six weeks and DEN (200 mg/kg; i.p.) was applied two days before the end of this period. (e) DEN+BB1 group: they were fed with 5% BB containing diet for six weeks and DEN (200 mg/kg; i.p.) was applied two days before the end of this period. (f) DEN+BB2 group: they were fed with 10% BB containing diet for six weeks and DEN (200 mg/kg; i.p.) was applied two days before the end of this period.

2.3. The preparation of BB containing diets

Fresh Northern highbush “Patriot” BB (V. corymbosum L.) were donated by Gedik Flora (Kartal-Istanbul). BB was collected manually in the first ten days of July-2011. They were stored at −35 °C until use and BB containing diets were prepared as previously reported (Coban et al., 2013a,b). According to this, BB homogenized for 3 min using a blender. BB homogenates were mixed with powdered rat chow by using a mixer for 15 min. Then, this mixture was dried and prepared as a pellet chow containing 5 and 10% BB (w/w) by Barbaros Denizeri AS (Istanbul). The BB containing diets were made by replacing 5 and 10% sucrose in the control diet with 5 and 10% BB.

2.4. Total phenolic and total flavonoids content assay in fresh BB

A part of fresh BB homogenates was diluted by distilled water to determine total phenolic and flavonoids content. Total phenolic compounds were determined with the Folin–Ciocalteu reagent and expressed as mg gallic acid equivalent per 100 g fresh BB (Liu et al., 2010). The total flavonoid levels were measured with aluminum chloride colorimetric method. The results are presented as mg quercetin equivalents per 100 g fresh BB (Yin et al., 2008). Total soluble phenolic compounds and total flavonoid levels were detected as 260 mg gallic acid equivalents and 105 mg catechin equivalents per 100 g fresh BB, respectively.

2.5. Blood and tissue samples

All rats were sacrificed by taking blood via cardiac puncture under sodium thiopental anesthesia (50 mg/kg, i.p.) two days after the DEN injection. Blood was collected in dry tubes by cardiac puncture. The livers were rapidly removed, washed in 0.9% NaCl and kept in ice. The materials were stored at −80 °C until they were analyzed.

2.6. Determinations in serum

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and γ-glutamyl transferase (GGT) measurements were performed on Cobas Integra 800 autoanalyzer (Roche Diagnostics, Mannheim, Germany).
2.7. Determination of hepatic lipid peroxides

Liver tissue was homogenized in ice-cold 0.15 M KCl (10%, w/v). Lipid peroxidation was assessed by two different methods in the tissue homogenates. First, the levels of thiobarbituric acid reactive substances (TBARS) were measured the method of Ohkawa et al. (1979). The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard. Results were expressed as mmol/g tissue. Second, diene conjugate (DC) levels were determined in tissue lipid extracts at 233 nm spectrophotometrically and calculated using a molar extinction coefficient of 2.52 × 10³ M⁻¹ cm⁻¹ (Buege and Aust, 1976). Lipids were extracted with chloroform:methanol (2:1) (Folch et al., 1956). Results were expressed as μmol/g tissue.

2.8. Determination of hepatic protein carbonyl (PC) and nitrotyrosine (NT) levels

The oxidative protein damage was measured by the quantification of carbonyl groups based on spectrophotometric detection of the reaction with 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl groups (PC) to form protein hydrazones. PC levels were calculated from the maximum absorbance (360 nm) using a molar absorption coefficient of 22,000 M⁻¹ cm⁻¹. The results were expressed as nmol carbonyl per mg protein (Reznick and Packer, 1986).

3-Nitrotyrosine (NT) was measured as a marker of nitrosative injury and peroxynitrite formation in the supernatants using an OxiSelect Nitrotyrosine competitive ELISA kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. For this reason, liver, heart and brain samples (10%, w/v) were homogenized in a solution containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, aprotinin, leupeptin and soy bean trypsin inhibitor (1 μg/mL each) at 0–4 °C using a polytron homogenizer and homogenates were centrifuged at 12,000 × g at 4 °C for 5 min. Total protein concentration in the supernatants was measured using bichinchoninic acid (Smith et al., 1985).

2.9. Determination of hepatic non-enzymatic and enzymatic antioxidants

Glutathione (GSH) levels were measured in the homogenates with 5,5-dithiobis-(2-nitrobenzoate) at 412 nm (Beutler et al., 1979). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and glutathione transferase (GST) activities were determined in postmitochondrial fraction of the tissues, which were separated by sequential centrifugation. In brief, tissue homogenates were centrifuged at 600 × g for 10 min at 4 °C to remove crude fractions. Then, supernatants were centrifuged at 10,000 × g for 20 min to obtain the postmitochondrial fraction. SOD activity was assayed by its ability to...
increase the effect of riboflavin-sensitized photooxidation of o-dianisidine (Myroie et al., 1986). GSH-Px activity was measured using cumene hydroperoxide as substrate (Lawrence and Burk, 1976). GST activity was determined 1-chloro-2,4-dinitrobenzene as substrate (Habig and Jacoby, 1981). Protein levels were also measured in postmitochondrial fractions (Smith et al., 1985).

2.10. Histopathological analysis

Liver tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histological studies.

2.11. Immunohistochemistry for Bax, B cell lymphoma-2 (Bcl-2) and proliferating cell nuclear antigen (PCNA)

Apoptosis-related proteins, proapoptotic Bax and antiapoptotic Bcl-2 expressions were determined in the liver. Hepatic PCNA expressions were also examined to evaluate proliferation. For this reason, all specimens were fixed in 10% buffered formalin. Paraffin blocks prepared from routinely processed specimens were cut into 5-mm slices and deparaffinized. Bax mouse monoclonal IgG2b (1:100 dilution; Santa Cruz Biotecnoiy), Bcl-2 alpha Ab-1 (100/D5) MS-123-R7 (Neomarkers) and PCNA (Santa Cruz Biotecnoiy) were used to perform the antigen retrieval. After this process, biotinylated secondary antibody (goat anti-mouse IgG-HRP; Santa Cruz Biotecnoiy), streptavidin peroxidase and substrate-chromogen (AEC) solution were applied, respectively. Nuclear staining was performed with hematoxylin. Staining intensity was defined as a percentage and given a score ranging from 1 to 3: 5–30% (+), 100-60% (+++) and >60% (+++).

2.12. Statistical analysis

The results were expressed as mean ± SD. Experimental groups were compared using Kruskal–Wallis variance analysis test followed post hoc Mann–Whitney U-test.

3. Results

Serum ALT, AST, LDH and GGT activities resulted in 100-, 49-, 4.5- and 4.5-fold increases in DEN group as compared to controls (Fig. 1A–D). BB1 and BB2 pretreatments alone did not alter these enzyme activities in serum of rats. Although BB1 pretreatment did not change high activities of ALT, AST, LDH and GGT in serum of DEN-treated rats, these activities tended to decrease, but not significantly, except LDH activities due to BB2 pretreatment.

There were no changes in hepatic TBARS, DC, PC and NT levels due to BB1 and BB2 pretreatments alone (Fig. 2A–D).
Significant increases in these parameters were detected due to DEN treatment as compared to controls. Both BB1 and BB2 pretreatments decreased high TBARS, DC, PC and NT levels in the liver of DEN-treated rats.

BB1 and BB2 pretreatments alone did not alter hepatic GSH levels and SOD, GSH-Px and GST activities (Fig. 3A–D). Hepatic GSH levels were observed to increase due to DEN. Although SOD activity did not change, GSH-Px and GST activities decreased in DEN group as compared to controls. GSH levels, SOD and GSH-Px activities in the liver of DEN-treated rats were observed not to change by BB1 and BB2 pretreatments. Only, liver GST activity increased due to BB2 pretreatment in DEN-treated rats.

Normal liver structure was observed in control (Fig. 4A), BB1 (Fig. 4B) and BB2 (Fig. 4C) groups histopathologically. Histopathologic evaluation of rats in DEN group (Fig. 4D) showed parenchyma necrosis, neutrophils, lymphocytes and plasma cells infiltrations, distinct edema and extravasate erythrocytes around the central vein, midzonal and portal areas. Vacuolar degeneration in parenchymal cells and extensive vacuolation/swelling in cytoplasm were noticed. Blood vessels with thrombus were determined at the periphery.

There was no difference in histopathological findings in DEN + BB1 group (Fig. 4E) as compared to DEN group. However, DEN + BB2 group (Fig. 4F) showed less liver cells damage than DEN group. Portal areas were intact in many sections and parenchymal necrosis was also displayed less than DEN group.

Hepatic Bax and Bcl-2 expressions remained unchanged due to BB1 and BB2 pretreatments alone (data not shown). Hepatic Bax expression was strongest in rats in the DEN-treated rats (Fig. 5B). Although there was no change in hepatic Bax expression in DEN + BB1 group (Fig. 5C), decreases in Bax expression was observed in DEN + BB2 group (Fig. 5D) as compared to DEN group. DEN treatment also caused slight increases in hepatic Bcl-2 expression (Fig. 5B), but there were no change among DEN, DEN + BB1 (Fig. 5C) and DEN + BB2 (Fig. 5D).

BB1 and BB2 pretreatments alone did not alter hepatic PCNA expression (data not shown). DEN caused remarkable increases in PCNA expression as compared to controls. PCNA expression remain unchanged in DEN + BB1 group, but this expression decreased in DEN + BB2 group as compared to DEN group (Fig. 7A–D and Table 1).

4. Discussion

DEN-induced HCC is a widely used experimental model. In initiation-promotion protocols, an initiator such as DEN is used along with a proliferative stimulus like phenobarbital.
Another method is to use a necrogenic dose of an initiator in the absence of other proliferative stimuli (Glauert et al., 2010). The necrogenic dose of DEN causes hepatocyte death and stimulates compensatory proliferation in the liver (Maeda et al., 2005; Naugler et al., 2007; Glauert et al., 2010; Qiu et al., 2011). The death of DEN-exposed hepatocytes was found to activate adjacent Kupffer cells to produce hepatomitogens that promote compensatory proliferation of surviving hepatocytes. Compensatory proliferation which is a response triggered by hepatocyte death appears to have a critical role in DEN-induced hepatocarcinogenesis (Maeda et al., 2005; Naugler et al., 2007; Glauert et al., 2010; Qiu et al., 2011).

In the literature, there have been some reports about the effect of a single dose of DEN on hepatic injury in rats (Bansal et al., 2000, 2005; Sayed-Ahmed et al., 2010; Metwally et al., 2011) and mice (Maeda et al., 2005; Naugler et al., 2007; Qiu et al., 2011; He et al., 2012) together with compensatory proliferation (Maeda et al., 2005; Naugler et al., 2007; Qiu et al., 2011). However, data obtained show some different results according to the applied DEN dose and investigation time used in the experiments. In this study, a LD<sub>50</sub> dose of DEN (200 mg/kg) was used. This dose was shown to initiate carcinogenesis and to produce necrosis in the liver (Bansal et al., 2000; Sayed-Ahmed et al., 2010). The experiments were performed 48 h after DEN treatment, because DEN-induced liver damage was reported to

Fig. 4 – Histopathological appearance of liver of rats. 5% BB (BB1) and 10% BB (BB2) were supplemented in the diet for six weeks before and two days after a single dose of DEN (200 mg/kg; i.p.). Liver tissues were obtained two days after the DEN injection. Normal liver histology was seen in control (A), BB1 (B), and BB2 (C). There were foci of parenchymal necrosis in the central portal and midzonal areas and inflammatory cell inflammation and distinct edema in the DEN (D) and DEN + BB1 (E) groups. Same changes were mildly observed in DEN + BB2 (F) (H&E 200 ×). (DEN: N-diethylnitrosamine; BB1: 5% and BB2: 10% blueberry containing diet).
be evident at this time (Bansal et al., 2000; Sayed-Ahmed et al., 2010). In our study, significant increases in serum ALT, AST, LDH and GGT activities as well as hepatic necrotic changes and elevations in PCNA expression, an indicator of increased proliferation, in the liver were observed.

Apoptosis also leads to cell death and differs from necrosis by distinct morphological and biochemical features. Oxidative and nitrosative stress play an important role in induction of apoptosis as observed in necrosis. There are two classes of regulatory proteins in the Bcl-2 family which have opposite effects on apoptosis: the proapoptotic members (Bax, Bcl-X<sub>l</sub>) promote programmed cell death whereas the antiapoptotic members (Bcl-2, Bcl-X<sub>i</sub>) protect cells against apoptosis. Therefore, the ratio of Bax/Bcl-2 is accepted to be a parameter of apoptotic cell death (Orrenius et al., 2007). In the current study, hepatic Bax expression showed to be remarkably increased in DEN-treated rats. Bcl-2 expression was also observed to be increased slightly. These results indicate that apoptosis is also involved in DEN-induced liver injury as previously reported (Qiu et al., 2011; Naugler et al., 2007).

There have been some reports about hepatic oxidative stress in rats (Bansal et al., 2000, 2005; Sayed-Ahmed et al., 2010; Metwally et al., 2011) and mice (He et al., 2012) following acute DEN administration. In the current study, we observed increases in hepatic TBARS, DC, FC and NT levels and decreases in GSH-Px and GST activities due to DEN treatment. These findings agree with the results of previous studies in which a similar DEN dose and application time were used (Bansal et al., 2000; Sayed-Ahmed et al., 2010).

BB contains polyphenols, flavonoids and other active components and its powerful antioxidant actions may be due to free radical scavenger properties (Neto, 2007; Zafra-Stone et al., 2007). BB consumption was reported to be useful in oxidative stress related conditions (Neto, 2007; Zafra-Stone et al., 2007). Some investigators have studied the effect of BB extracts on hepatic functions. Osman et al. (2007) have

Table 1 - Staining intensity of Bax and antiapoptotic B cell lymphoma-2 (Bcl-2) and proliferating cell nuclear antigen (PCNA) in the liver of diethylnitrosamine (DEN)-treated rats with and without blueberry (BB) pretreatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bax</th>
<th>Bcl-2</th>
<th>PCNA</th>
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<tbody>
<tr>
<td>Control</td>
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<td>+</td>
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<td>BB1</td>
<td>+</td>
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<td>BB2</td>
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<tr>
<td>DEN</td>
<td>++</td>
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<tr>
<td>DEN + BB1</td>
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<tr>
<td>DEN + BB2</td>
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BB1 and BB2: 5% BB and 10% BB containing diet, respectively.
reported that BB exert protective effects on endotoxin- and \( \text{d}- \)galactosamine-induced acute liver injury. These authors have found that BB reduced serum transaminase activities, hepatic myeloperoxidase activities and proinflammatory cytokine levels such as tumor necrosis factor-\( \alpha \) and interleukin-\( \beta \). Although malondialdehyde levels did not alter, GSH levels increased due to BB treatment. We previously reported that BB supplementation reduced oxidative stress and liver injury in hypercholesterolemic guinea pigs (Çoban et al., 2013a) and \( \text{d}- \)galactose-treated rats (Çoban et al., 2013b) by acting as an antioxidant. Wang et al. (2010b) have reported that BB has preventive and protective effects on carbon tetrachloride-induced hepatic fibrosis by reducing hepatocyte injury and lipid peroxidation. In addition, it has been reported that BB induced expressions of \( \text{NF-E2-related factor 2 (Nrf2)} \), heme oxygenase-1 (HO-1) and NADPH quinone oxidoreductase 1 (Nqo1), which can protect hepatocytes from oxidative stress (Wang et al., 2010a, 2010b). Sadik et al. (2008) have investigated the effect of BB on chronic DEN-induced hepatocarcinogenesis. DEN (10 mg/kg; p.o., 5 times per wk for 15 weeks) administration caused significant increases in alpha-fetoprotein (AFP), a tumor associated-fetal protein and histopathological changes indicating hepatocarcinogenesis. 4% freeze dried BB containing diet for 15 wk ameliorated the histopathological changes and decreased serum AFP levels. According to this, authors have proposed that BB may be useful in the prevention of DEN-induced hepatocarcinogenesis. Yi et al. (2006) have also found that phenolic compounds in BB could inhibit HepG2 liver cancer cell population growth.

There is no study in the literature investigating the effect of dietary BB on liver injury and oxidative stress which is produced by DEN in rats. Therefore, in this study, we wanted to investigate whether BB may have inhibiting effect on DEN-induced hepatic necrosis, apoptosis, proliferation and oxidative stress. The individual phenolic compounds in BB have strong antioxidant activities, but the antioxidant activities of the combination of phenolics may be better than the individual phenolics. Therefore, in the current study, whole fresh BB supplemented diets were used and their concentrations were chosen according to previous studies (Osman et al., 2007; Çoban et al., 2013a, 2013b). The daily consumption of BB in 5% and 10% BB containing diets is roughly equivalent to 0.75 and 1.5 g fresh BB/per rat, respectively. According to our results, BB1 pretreatment did not change high activities of ALT, AST, LDH and GGT in serum of DEN-treated rats. However, serum enzyme activities tended to decrease, but not significantly, except LDH activities due to BB2 pretreatment. Although there were no marked differences in hepatic histopathological findings between DEN and DEN + BB1 groups, the extent of necrotic lesions in the liver were fewer in DEN + BB2 group. Similarly, in the current study, increases in apoptosis and proliferation in DEN-treated rats observed to decrease due to BB2 pretreatment, but not BB1. However, both BB1 and BB2 pretreatments decreased high

Fig. 6 – The effects of blueberry (BB) pretreatments on antisapoptic B cell lymphoma-2 (Bcl-2) in the liver of diethylnitrosamine (DEN)-treated rats. 5% BB (BB1) and 10% BB (BB2) were supplemented in the diet for six weeks before and two days after a single dose of DEN (200 mg/kg; i.p.). Liver tissues were obtained two days after the DEN injection. Groups: (A) control, (B) DEN, (C) DEN + BB1 and (E) DEN + BB2 (200 \( \times \)).
TBARS, DC, PC and NT levels in the liver of DEN-treated rats. GSH levels, SOD and GSH-Px activities in the liver of DEN-treated rats were observed not to change by BB1 and BB2 pretreatments. Only, liver GST activity increased due to BB2 pretreatment in DEN-treated rats. These results show that BB has powerful antioxidant actions which may be due to free radical scavenger properties of polyphenols and flavonoids and other active components.

In conclusion, BB may have an inhibiting effect on acute liver injury by reducing apoptosis, necrosis, proliferation, oxidative and nitrosative stress in DEN-treated rats.

**Conflict of interest statement**

None declared.

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**References**


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**Fig. 7** – The effects of blueberry (BB) pretreatments on proliferating cell nuclear antigen (PCNA) expression in the liver of diethylnitrosamine (DEN)-treated rats. 5% BB (BB1) and 10% BB (BB2) were supplemented in the diet for six weeks before and two days after a single dose of DEN (200 mg/kg; i.p.). Liver tissues were obtained two days after the DEN injection. Groups: (A) Control, (B) DEN, (C) DEN + BB1 and (D) DEN + BB2 (200×).


