Preventive Effect of blueberries on Diethylnitrosamine-Induced Hepatocarcinogenesis in Rats

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Abstract

Nitrosamine compounds are known hepatic carcinogens. This study was designed to study the efficacy of dietary supplementation with blueberries (BB) on diethylnitrosamine (DEN)-initiated hepatocarcinogenesis in male wistar rats. Rats were divided into three groups. The first group served as normal control group, the second group received DEN at a dose of 10 mg/kg body weight five times a week for 15 weeks. The third one received DEN as in DEN-treated group simultaneously with 4% BB-supplemented diet. The results showed that BB caused significant decrease in the elevated serum levels of α-fetoprotein (AFP), homocysteine (Hcy) along with levels of glutathione(GSH), deoxyribonucleic acid (DNA), ribonucleic acid (RNA)and activity of glutathione reductase (GR) in liver. Normalization of elevated 2-macroglobulin (2M) and total antioxidant capacity (TAC) levels in serum, hepatic Glutathione-S-transferase (GST), glutathione peroxidase (GPx) activities and liver weight was achieved whereas body weight was significantly decreased. Moreover, no significant change was observed in elevated relative liver weight, hepatic glucose-6-P-dehydrogenase (G6PD), lactate dehydrogenase (LDH) along with serum aminotransferases, alkaline phosphatase (ALP) and γ -glutamyltransferase (γ-GT) activities. Significant increase in reduced hepatic activity of xanthine oxidase (XO) was achieved and histopathological damage was minimized in BB-treated group. It is suggested that BB suppress DEN-induced hepatocarcinogenesis and could be developed as a promising chemoprotective natural supplement for liver cancer.

Keywords: blueberries (BB), diethylnitrosamine, hepatocarcinogenesis, rats, α2-macroglobulin, α -fetoprotein, homocysteine, glutathione.

1. INTRODUCTION:

Diethylnitrosamine (DEN) is an established hepatocarcinogen in rats (Hussein et al., 2015). DEN has been reported to induce hepatocarcinogenesis by alteration of the DNA structure, formation of alkyl DNA adducts and induction of chromosomal aberrations and micronuclei in the liver (Llovet et al., 2013).

Diethylnitrosamine (DEN), a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in deoxyribonucleic acid (DNA) repair/replication and is normally used as a carcinogen to induce liver cancer in animal models (Kanda et al., 2015). DEN has been shown to be metabolized to its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis (Anis et al., 2001; Chakraborty et al., 2007). Experimental, clinical and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer. Diethylnitrosamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals (Tell, et al., 2013; Paula Santos, et al., 2014). Nitrate and nitrite are added to meat and fish for the purpose of preservation, as colour fixatives and as flavouring. Ingestion of nitrite and nitrate can result in the endogenous formation of nitroso compounds, particularly in the presence of nitrosatable precursors, such as primary amines, in the acidic condition of the stomach (Abe et al., 2012). In addition, exposure of man to preformed nitrosamines occurs due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals (Wedge et al., 2001). In humans, average intake of nitrosamines from food is approximately 1 mg/day (Thirunavukkarasu and Sakthisekaran, 2003). Recent approach of chemoprevention serves as an attractive alternative to control malignancy (Galle, et al., 2014). Epidemiologic and clinical studies have shown that dietary intakes of blueberries (BB) may have potential to reduce various types of cancers (Wang et al., 2013; Coban et al., 2014), including liver cancer (Kumar, 2015). Blueberries, nature's only 'blue' food, are a rich source of polyphenols, potent antioxidants that include phenolic acids, tannins, flavonols and anthocyanins. The main anthocyanidins present are delphinidin, cyanidin, petunidin, peonidin and malvidin. Yi et al. (2006) have found that phenolic compounds in blueberries could inhibit HepG2 liver cancer cell population growth and induce apoptosis. Moreover, blueberry constituents are likely to act by mechanisms that counteract oxidative stress, decrease inflammation, and modulate macromolecular interactions and expression of genes associated with disease processes (Ilknur, et al., 2016). Several biochemical markers have been suggested for biomonitoring the actions of anticancer agents. Serum α-fetoprotein (AFP) is a useful tumour marker for the detection and monitoring of liver cancer development although the false negative rate with AFP level alone may be as high as 40% for patients with small size tumour (Qin and Tang, 2002; Yao et al., 2006). Recently, α2-macro-globulin (α2M), a homotetrameric major acute-phase glycolprotein has been suggested as a novel cytochemical marker characterizing preneoplastic and neoplastic rat liver lesions negative for hitherto established cytochemical markers (Chatterjee and Mitra.,2015). It is only recently that homocysteine (Hcy) has been implicated in increased cancer susceptibility and development. The present study aims to carry out a systematic investigation of the protective influences of blueberries against DEN-induced hepatocarcinogenesis by analyzing serum α-fetoprotein, α2-macroglobulin and homocysteine levels along with the non-enzymatic and enzymatic anti-oxidants as biochemical end points of chemoprevention

2. MATERIAL AND METHODS:

Chemicals

Blueberries are obtained from local market. Whole blueberries (500g) were lyophilized (freeze dried) for 72h using a Vickers accelerated freeze drier, pilot plant model. HPLC analysis revealed that 1 g of freeze-dried blueberries contained 22 mg of anthocyanins (Liu et al., 2010). Freeze-dried blueberries were kept at -20° C. Blueberries were mixed with diet daily and presented fresh to rats. Diethylnitrosamine and other chemicals were supplied from (purchased) from Sigma-Aldrich (St. Louis, Missouri, USA)

Experimental design

Male Wistar rats weighing (150 − 160 g) were obtained from the animal house of the Faculty of Medicine (Al-Azhar), Dommita Al-Azhar University, Egypt and were used after a 1-week acclimatization period. Animals were divided into three groups (each group was eight rats). The first group (untreated control) received neither DEN nor BB. The second group (DEN) received DEN intragastrically at a dose of 10 mg/kg 5 times a week for 15 weeks (Karimov et al., 2003). The third group (DEN + BB) received DEN as mentioned in second group along with dietary supplementation of blueberries at a dose of 4 g/100g diet (Ilknur et al., 2016).

Tissue sampling and biochemical assays

At the end of the experimental period, the body weight of each rat was taken before sacrifice. Blood was collected and serum was separated for the estimation of AFP, α2M, Hcy and total antioxidant capacity (TAC) levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and ϒ- glutamyltransferase (ϒ-GT) activities. The abdominal cavity of rats was dissected immediately after decapitation and the liver was rapidly removed, washed by ice-cold saline, weighed and blotted dry. A portion of the liver was homogenized in ice-cold saline using Potter— Elvejhem glass homogenizer and the homogenate was divided into three portions. A portion of the homogenate was mixed with 0.1M Tris buffer pH 8.1, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and centrifuged at 105000 xg at 4° C for 40 min using Dupont Sorvall ultracentrifuge (USA). The resulting cytosolic fraction was used for the determination of glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), glucose-6-P dehydrogenase (G6PD), lactate dehydrogenase (LDH) and xanthine oxidase (XO) activities. Another portion was mixed with sucrose/Tris buffer, pH 7.5, for subsequent estimation of DNA and ribonucleic acid (RNA) levels. The remaining portion was precipitated with 7.5% 5-sulfosalicylic acid, centrifuged at 600xg for 15 min and the resulting protein free supernatant obtained was used for the estimation of GSH level. Finally, portion of the liver from all the animals was formalin-fixed and paraffin-embedded for subsequent histopathological examinations. Serum level of AFP was estimated using ELISA kit supplied from Anogen (Mississauga, Ontario, Canada). Serum α2M was determined using radialimmuno diffusion kit (Biocientifica SA, Buenos Aires-Argentina), whereas serum Hcy level was measured using Axis

Homocysteine EIA kit (Axis-Shield AS, Germany). Serum ALT and AST were assayed using commercially available kits provided by ProDia international, Germany, according to the method of Reitman and Frankel (1957). Serum ALP was estimated using kit supplied by Biolabo SA, France according to method described by Kind and King (1954) whereas serum Υ-GT was measured by the method of Orlowski and Meister (1965) using kit supplied by Scalvo Diagnostics, Italy. TAC was measured in serum by the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain,1996) which measures the ferric reducing ability of antioxidants in serum. Measurement of liver cytosolic enzyme activities of GST (Habig et al., 1974), GR (Long and Carson, 1961), GPx (Paglia and Valentine, 1967), G6PD (Kornberg and Horecker, 1955) and XO (Prajda and Weber, 1975) were carried out. LDH activity was measured using commercially available kit provided by Stanbio, San Antonio, TX, USA, according to the method of Buhl and Jackson (1978). GSH was determined according to the method of Beutler et al. (1963). Hepatic levels of DNA and RNA were estimated according to method of Burton (1956) and Blobel and Potter (1968), respectively as modified by Giles and Myers (1965). The protein content was measured by the method of Lowry et al. (1951).

Statistical analysis

Data were expressed as the mean ± S.E.M. Means were compared by one − way analysis of variance (ANOVA) followed by Tukey- Kramer test, which was used to identify differences between groups. A value of P< 0.05 was accepted as significant

3. RESULTS:

General findings Significant increase was observed in serum levels of AFP, α2M, Hcy along with activities of ALT, AST, ALP and Υ-GT in the DEN group as compared with the control group. No significant change was observed in body weight in the DEN-treated group whereas increased liver

weight and relative liver weight was shown as compared with the control group. BB supplementation resulted in normalization of serum α2M and significant decrease of AFP and Hcy levels whereas no significant change in activities of ALT, AST, ALP and Υ-GT was observed as compared with DEN group. Significant decrease in body weight was detected in BB-group as compared with either control or DEN-group. Blueberries supplementation was able to restore liver weight whereas no significant change was obtained in relative tissue weight as compared with DEN-group as shown in Table (1).

Table1. Effect of blueberries on DEN-induced alteration in serum AFP, α2M, Hcy, liver function tests, body weight, liver weight and relative liver weight

parameter	control	DEN	DEN+BB
AFP (ng/ml)	0.4 ±0.03	0.96 ±0.06 [a]	0.64±0.036 [ab]
α2M(mg/dl)	132.9 ± 3.6	225.7 ± 5.3[a]	135.7 ± 2 [b]
Hcy(μmol/l)	4.2±0.26	9.6±0.6 [a]	6.4±0.25 [ab]
ALT(U/l)	80 ± 6.9	123.4 ± 6.2[a]	118.3 ± 8.5[a]
AST(U/l)	90.8 ± 4.4	129.7 ± 9.9[a]	112.4 ± 8.7[a]
ALP(U/l)	79.4 ± 3.1	121 ± 0.4[a]	113.9 ± 2.9[a]
ɤ-GT(U/l)	1 ±0.03	5.3 ±0.4[a]	5.9 ±1.1[a]
Body weight(g)	257.9 ± 11	237 ± 7.2	176.4 ± 10.3[ab]
Liver weight(g)	5.8 ±0.08	7.3 ± 0.4[a]	4.7 ±0.44[b]
Relative liver weight (g %)	2.4± 0.06	2.95 ± 0.11 [a]	3 ± 0.21 [a]

Values represent mean ± S.E.M for eight rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at P < 0.05.

Table 2. Effect of blueberries on DEN-induced alteration in antioxidant status.

parameter	control	DEN	DEN + BB
GST(U/mg protein)	415.6 ± 20.6	847.3 ± 68.7[a]	546 ± 59.2[b]
GR(U/mg protein)	131 ±7.1	255.7 ± 15.8[a]	194.1± 4.1[ab]
GPx(U/mg protein)	278.2 ± 29.9	436.4 ± 16.2[a]	335.4 ± 17.4[b]
G6PD(mU/mg protein)	16.2 ± 1.1	44.9 ± 6.1[a]	61 ± 10.9[a]
GSH(mg/g)	2 ± 0.1	3.3± 0.1[a]	2.7 ± 0.16[ab]
TAC(μmol/l)	702.9 ± 13.3	1182 ± 87.8[a]	675.7 ± 56.8[b]

Values represent mean ± S.E.M for eight rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at P < 0.05.

Antioxidants status

As shown in Table (2), significant increase was observed in the hepatic activities of GST, GR, GPx and G6PD along with level of hepatic GSH, serum TAC in DEN- treated group. Blueberries supplementation caused normalization of hepatic GST and GPx activities and serum TAC level. Significant decrease was observed in the elevated hepatic GSH level and GR activity where as no significant change was observed in the hepatic activity of G6PD as compared with the DEN-treated group. Hepatic levels of DNA, RNA and activities of XO and LDH As shown in Table (3), significant increase was observed in levels of DNA and RNA and activity of LDH whereas significant decrease was observed in XO activity in liver as compared

with the control group. Blueberries supplementation caused significant decrease in hepatic levels of DNA and RNA and increase in XO activity whereas no change was detected in LDH activity.

Table 3. Effect of blueberries on DEN-induced alteration in hepatic levels of DNA and RNA along with activities of LDH and XO.

parameter	control	DEN	DEN + BB
DNA(mg/g)	1.77 ± 0.03	2.14 ± 0.06^a	1.4 ± 0.13 [ab]
RNA(mg/g)	1.3 ± 0.07	2.1 ± 0.1^a	1.7 ± 0.07 [ab]
LDH(U/mg protein)	2 ± 0.09	2.56 ± 0.08^a	2.6 ± 0.19^a
XO(U/mg protein)	6.3 ± 0.3	4.3 ± 0.1 [a]	5.2 ± 0.19 [ab]

Values represent mean ± S.E.M for eight rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at $P < 0.05$.

Histopathological findings

Histopathological examination of liver sections from control animals revealed normal architecture. DEN group showed appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis. Restoration of most of the normal hepatocytes architecture with regular dark nuclei and dysplasia was shown in BB group, Figure 1(a-b-c).
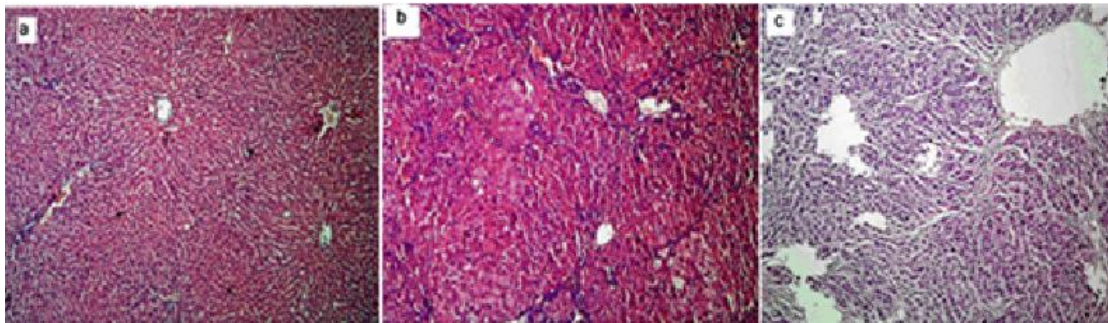
Figure 1. Light photomicrographs of rat liver: (a) control group; (b)DEN-group; (c)DEN+BB-treated group

4. DISCUSSION:

It is well known that liver cancer is one of the most important cancers in the world, resulting in more than 1 million patients and over 260,000 deaths per year (Akita et al., 2014). Therefore, the chemoprevention and treatment of liver cancer is very important. AFP, α2M, Hcy, ALT, AST, ALP, ϒ-GT, liver weight and relative liver weight are valuable references, widely used in animal studies to diagnose and observe the development of hepatocarcinogenesis (Zhang et al., 2013). In the present study, the values of previously mentioned parameters showed sharp increase in DEN-group as compared with that of the normal control group (Table1). AFP, a tumour-associated fetal protein, has long been employed as a serum fetal tumour marker to monitor disease progression (Abelev, 1971; Liu et al., 2006). The observed

significant increase of serum α2-macroglobulin in DEN-induced rats is in harmony with Sukata et al. (2004) who stated that α2M might be tightly linked to the rat hepatocarcinogenesis from the initial stage to tumour progression even in conditions, which are undetectable, by established cytochemical markers such as placental glutathione-S-transferase (GST-P) and γ-GT-positive lesions. Sukata et al. (2004) also confirmed that the observed increases in serum α2M concentrations during hepatocarcinogenesis and in animals, bearing hepatic tumours was not a result of secretion by the host liver of α2M as an acute-phase reactant in response to inflamematory injury. α 2M functions as a carrier protein and regulator for various growth factors and cytokines such as transforming growth factor-β (known to be involved in the onset of hepatocyte apoptosis (James, 1990). Furthermore, α2M partially counteracts the inhibitory effects of transforming growth factor-β on proliferation of neoplastic hepatocytes, suggesting that under some conditions, α2M can promote hepatocarcinogenesis by perturbing transforming growth factor-β-induced apoptosis (Kishida et al., 2016).

Increased serum Hcy and hepatic DNA and RNA levels along with decreased XO activity seem to be interrelated in the present study. Animal and human studies have increasingly demonstrated associations between folate deficiency, serum Hcy elevations, and a variety of cancers. The observed increase in serum Hcy in this study is suggested to reflect inhibition of homocysteine metabolism due to folate deficiency reported in other studies(Eichholzer et al., 2001; Davis and Uthus, 2004). Folate is important for normal DNA synthesis, repair, and converting homocysteine to methionine (Davis and Uthus, 2004). Therefore, increased demand of folate is postulated to be a result of increased hepatic levels of DNA and RNA (Table 3) and might indicate increased DNA and RNA synthesis and proliferation of cancer cells in response to growth stimulation. Metabolically active and proliferating tissues require large amounts of purine nucleotides for transmission of metabolic energy and synthesis of nucleic acids.

Xanthine oxidoreductase (XOR) catalyzes the final reactions of the purine catabolic pathway, oxidizing hypoxanthine to xanthine, and xanthine to uric acid. The decrease in XO activity which depresses the ability to degrade purines was thus reported to provide purines which can be recycled for nucleic acid biosynthesis in the salvage pathway (Linder et al., 2005). Progressive decrease of XOR activity has been shown in all hepatomas irrespective of growth rate or differentiation as compared with the corresponding normal tissues (Prajda and Weber, 1975). The observed decrease in liver cytosolic XO activity shown in Table (3) may be attributed to down-regulation of xanthine oxidoreductase which might include loss of heterozygosity at chromosome 2p where XOR is located, decreased gene promoter activity, increased XOR mRNA degradation, or posttranslational changes (O'Connell et al.,1998; Linder et al., 2005).

Results of the present study revealed significant increase in liver cytosolic GST activity in DEN-received group. This increase in hepatic GST activity may be attributed to the fact that induction of GSTs, phase II enzymes that detoxify certain carcinogens, is regarded as a potential mechanism of blockade of the early stages of carcinogenesis. Sato (1989) has found that, of the preneoplastic marker enzymes, rat placental form of glutathione-S-transferase is markedly and specifically inducible in preneoplastic foci. Recently, immunohistochemical studies have revealed significant increase in the number of hepatocellular enzyme altered foci, which stained positive for GST-P (Kumar, (2015)).

The significant increase of hepatic GSH level observed in the present study is in harmony with Marinho et al. (1997) who also reported that increased GSH concentration in DEN-treated rat liver is probably due to the simultaneous observed two-fold increase in the activities of Υ-GT and Υ-glutamylcysteine synthetase (Υ-GCS), two main enzymes involved in GSH formation. The ubiquity of elevated Υ-GT levels in many rodent and human hepatic and extrahepatic carcinomas (Yadav and Bhatnagar., 2007) have led to the hypothesis that Υ-GT provides a growth advantage to focal cells during carcinogenesis. A plausible hypothesis is that the advantage may be due to the role of Υ-GT in the transport of GSH constituents, leading to increase in cellular GSH. The latter is required for proliferation and resistance (Komlosh et al., 2002). Υ-GT is reported to act by circumventing the rate-limiting Υ-GCS and its feedback inhibition by GSH and may thus participate in promotion of growth. Previous studies have shown that an increased GSH level was associated with an early proliferative response and was essential for the cell to enter the S phase. The requirement for increased GSH or thiols prior to DNA synthesis may be related to the fact that proliferating cells require increased amounts of pentoses and thiols. DNA synthesis depends absolutely on the formation of pentoses and on their conversion into deoxyribose by ribonucleotide reductase. The activity of this rate-limiting enzyme in DNA synthesis requires reduced glutaredoxin or thioredoxin, which are maintained by GSH. An increase in the cellular GSH content may change the thiol-redox status of the cell that activates genes essential for G1 to S transition (Li et al., 2015).

GR mediates the reduction of oxidized glutathione (GSSG) to GSH. This reduction reaction requires NADPH, which is supplied by the enzyme G6PD in the pentose phosphate pathway. G6PD is significantly increased in DEN-treated group, which increases the availability of NADPH, which in turn enhances the activity of GR shown in Table 2. The activity of GPx, which is another constituent of GSH redox cycle, also showed a significant increase in DEN-treated rats as reported previously (Sarkar et al., 1995). All these events eventually lead to increase hepatic level of GSH observed in the present study. Increased serum TAC level in DEN- group (Table 2) might be regarded as an adaptive response to oxidative stress imposed by DEN. TAC depicts the synergistic interaction among different antioxidants present in plasma, thus providing an insight into the delicate in vivo balance between oxidants and non-enzymatic antioxidants (Ozcelik, et al., 2014). The elevated hepatic activity of G6PD and LDH in this study may be related to enhanced glucose metabolism. It was discovered in the 1920s that cancer cells constitutively

up regulate glucose metabolism (Warburg, 1930). Thus, cancer cells tend to synthesize ATP mainly through 'glycolysis', a metabolic state that is linked to high glucose uptake and local acidification owing to lactate production. Gatenby and Gillies (2004) and Zu and Guppy (2004) have reported that when glycolysis prevails, pyruvate is reduced to lactate in order to reoxi-dize NADH to NAD that is required for sustained glycolysis. Increased glucose breakdown provides building blocks for the synthesis of nucleotides via the pentose phosphate pathway. In addition, local acidification of the tumour microenvironment may facilitate tumour invasion (Weber, 2001; Kroemer, 2006). Glycolytic enzymes are induced by oncogenes (Plas and Thompson, 2005) or by the hypoxia-inducible transcription factor (Carew and Huang, 2002; King et al., 2006) or a dysfunctional tricarboxylic acid cycle owing to loss of function of mitochondrial tumour suppressor genes (Eng et al., 2003; Gottlieb and Tomlinson, 2005). In recent years, considerable attention has been paid to anthocyanins due to their abilities to inhibit oxidative stress and cell proliferation (Joseph et al., 2007, Chatterjee and Mitra,. 2015).

In current study, BB was able to restore serum α2M and to reduce AFP and Hcy levels significantly. The antioxidant potential of blueberries was clarified in the present study from its modifying effect of GSH and its related enzymes. Blueberries supplementation was able to restore hepatic activities of GST and GPx along with serum TAC level. Significant decrease was also observed in hepatic GR and GSH level. The regulations of apoptosis and phase II enzymes such as GST are other potential mechanisms through which flavonoids such as anthocyanins may prevent cancer as reported previously (Gong , et al., 2014). The results of Srivastava et al. (2007) indicated that apoptosis was confirmed in colon cancer cell line (HT-29 cells) when treated with anthocyanins from blueberries at 50 - 150 µg/ml with consequent decrease of GST activity rather than induce it. Blueberries and other berries, notably raspberries and strawberries, contain a potent anticarcinogenic compound called ellagic acid.

Polyphenols exert antiproliferative and an antiangiogenic effects that are particularly pronounced in tumour cells (Ray, 2005; Larrosa et al., 2006). Interestingly, adding blueberries, red wine and other sources of polyphenols to the diet is thought to be one reason for the greater slenderness of the French compared with other Europeans. Therefore, it is not surprising that our study showed significant decrease in body weight in BB-group and that this decrease counteracted any significant change in relative tissue weight despite of the normalization of liver weight caused by blueberries. The decrease in body weight in BB-supplemented group may be attributed at least partly to the slowing down of digestion by inhibiting enzymes such as amylase, protease and lipase activities at levels, which could affect carbohydrates, protein, and fat digestion and absorption (McDougall and Stewart, 2005).

In this study, BB was observed to decrease levels of DNA and RNA and to increase activity of XO in liver. Pterostilbene, an active constituent of blueberries, was found to induce apoptosis through activating the caspase cascade via the mitochondrial and Fas/FasL pathway, growth arrest DNA damage-inducible gene (GADD) expression, and by modifying cell cycle progress and changes in several cycle-regulating

proteins (Imamoto et al., 2014). In another study, it was demonstrated that anthocyanin's contribute to the inhibition of tumourigenesis by blocking activation of the mitogen-activated protein kinase (MAPK) pathway. These findings provide the first molecular basis for the anticarcinogenic action of anthocyanidins (Shen et al., 2014). The data provided so far by the current study and from other laboratories warrants further investigation into the chemopreventive and chemotherapeutic effects of blueberries in clinical studies. In sum, current results indicate that dietary intake of blueberries high in antioxidant activity may be an important component of a healthy living strategy designed to increase our resistance to liver cancer.

Acknowledgments

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