Anthocyanin-rich black currant (Ribes nigrum L.) extract affords chemoprevention against diethylnitrosamine-induced hepatocellular carcinogenesis in rats

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Abstract

Anthocyanins are known to possess potent anticarcinogenic properties against several cancers thus demonstrating potential for cancer prevention. Black currant (Ribes nigrum L., Grossulariaceae) fruits have a high anthocyanin content. This “superfruit” is known to possess various pharmacological effects including alleviation of chronic oxidative stress and inflammation. In contrast to a large volume of literature on the health benefits of black currant, limited evidence on antitumor effects of black currant exists with virtually no data on the prevention of experimental carcinogenesis. In the current study, we have investigated the chemopreventive effects of an anthocyanin-rich black currant skin extract (BCSE) utilizing our well-characterized model of rat liver carcinogenesis. Initiation of hepatocarcinogenesis was done by intraperitoneal injection of diethylnitrosamine (DENA) followed by promotion with phenobarbital. The rats were exposed to dietary BCSE for 4 weeks prior to initiation, and the treatment was continued for 22 consecutive weeks. BCSE dose-dependently decreased the incidence, total number, multiplicity, size and volume of preneoplastic hepatic nodules. The antihepatocarcinogenic effect of BCSE was confirmed by histopathological examination of liver sections. Immunohistochemical analysis of proliferating cell nuclear antigen and DNA fragmentation revealed BCSE-mediated inhibition of abnormal cell proliferation and induction of apoptosis at the translational level. These results along with a safety profile of BCSE encourage the development of black currant bioactive constituents as chemopreventive agents for human liver cancer.

Keywords: Black currant; Diethylnitrosamine; Hepatocarcinogenesis; Chemoprevention; Cell proliferation; Apoptosis

1. Introduction

An impressive number of epidemiological studies suggest a reduced risk of cancer at various sites with regular intake of a diet rich in fruits and vegetables [1–3]. Berries represent the most widely consumed fruits in the human diet. Accumulating evidence suggests that edible small and soft-fleshed berry fruits may have an enormous potential for cancer prevention [4–6]. Although berries contain macro- and micronutrients including fibers, minerals and vitamins, the large number of bioactive phytochemicals including phenolic compounds present in these fruits have been considered responsible for the various health benefits including cancer prevention [7–11]. One such important group of berry phytochemicals is represented by the anthocyanins, a major cluster of water-soluble pigments belonging to the flavonoid class. Anthocyanins exist abundantly in the plant kingdom and confer the blue, red, violet and purple colors to the fruits and vegetables including berries, grapes, apples, corn and purple cabbage [12]. These natural constituents exist primarily as glycosides or acylglycosides of their corresponding aglycones which are known as anthocyanidins. It has been estimated that the average daily intake of anthocyanins in the United States population is between 180 and 215 mg [13]. Recent studies provide convincing evidence that anthocyanins possess potent anticarcinogenic properties against several cancers thus demonstrating their potential for cancer prevention [14,15].

Black currant (Ribes nigrum L., Grossulariaceae) fruits contain high amounts of anthocyanins (250 mg/100 g fresh fruit) [16]. Black currant, a shrubby tree, is known to originate from Northern Asia as well as Europe and is widely cultivated in the cooler regions of Europe and New Zealand. In the United States, black currant farming was banned in early 1900s as it was considered to be the vector of white pine blister rust disease. With the lifting of the ban in the state of New York in 2003, black currant is now being grown in the several regions of the east and west coast. Black currant fruits and leaves have been used in both Asian as well as European traditional medicine for treatment of a variety of
Boivin et al. [31] compared the antitumor potential of the extract acts through cytostatic inhibition of cell growth via the WAF1 pathway. Wu et al. [30] confirmed the antiproliferative effect of inhibited proliferation of HT29 colon cancer and MCF-7 breast carcinoma cells. Olsson et al. [29] demonstrated that 0.25 or 0.5% black currant extract exist few reports on the anticarcinogenic activity of black currant. Clinical study conducted by Lyall et al. [28] has confirmed that black currant juice exhibited carcinoma, MDA-MB-231 mammary cancer and PC-3 prostate adenocarcinoma, Caco-2 colorectal adenocarcinoma, MCF-7 mammary carcinoma, and enzymatically digested CAPS afforded better antitumor effect than native CAPS against Ehrlich carcinoma in mice [33]. Recently, we have prepared an aqueous extract from black currant fruit skin which is generally considered as a low-value byproduct of black currant juice production. The chemical composition of the extract has been characterized utilizing high-performance liquid chromatographic (HPLC) and spectrophotometric techniques. This black currant skin extract is rich in anthocyanins with cyanidin-3-O-rutinoside as the predominant anthocyanin [34]. Utilizing an in vitro tumor model employing HepG2 human liver cancer cells, we have further demonstrated that this anthocyanin-rich fraction possesses a potent antitumor effect against hepatocellular carcinoma (HCC) [34]. The objective of the present study was to investigate the chemopreventive potential of the extract against a chemically-induced and clinically relevant rodent model of liver cancer. Our results show, for the first time, that black currant affords a striking chemopreventive effect against diethylnitrosamine (DENA)-initiated and phenobarbital (PB)-promoted hepatocellular carcinogenesis in rats.

2. Materials and methods

2.1. Experimental animals and diet

Pathogen-free male Sprague-Dawley rats, initially weighing 50–74 g, were obtained from Harlan Laboratories (Indianapolis, IN, USA) and housed in the Comparative Medicine Unit at the Northeastern Ohio Universities Colleges of Medicine and Pharmacy (NEOUCOM), which is accredited by the American Association for the Accreditation of Laboratory Animal Care. The animals were acclimatized to standard laboratory conditions including 22±2°C temperature, 30–50% relative humidity, and a 12:12-h dark-light cycle in solid bottom polycarbonate cages (three animals/cage) and provided with drinking water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee at NEOUCOM.

2.2. Preparation of black currant skin extract

Ripe black currant fruits were collected from cultivated plants in the region of Csíkkarcfalva, Romania. The aqueous extract of the skin of black currant was prepared to exert cytotoxicity against Ehrlich ascites tumor cells [32]. Oral administration of black currant juice or CAPS to Ehrlich carcinoma-bearing mice significantly abrogated the growth of solid tumor [32]. It has been shown that CAPS contains immunostimulatory activity [32] and enzymatically digested CAPS afforded better antitumor effect than native CAPS against Ehrlich carcinoma in mice [33].
acclimatization period of 1 week with standard basal diet, rats were randomly divided
reverse-phase HPLC analysis of the extract revealed the presence of
was stored under controlled humidity at room temperature protected from light. The
communication [34]. The final product was a dark pink-colored fine powder, which
difference in body weight gain was observed among various rat groups.
diethylnitrosamine (DENA)-induced hepatocarcinogenesis in rats. No significant
were fasted overnight prior to sacrifice. The rationale for using this specific protocol
withdrawn from the drinking water, and the food did not contain BCSE. The animals
monitored every day, and the body weight of animals were recorded every 2 weeks. All
volume of peanut oil. Following a 2-week recovery period, the promoter PB (Sigma-
200 mg/kg body weight (mixed with peanut oil). Animals belonging to group A
has been calculated that the BCSE dose for group E (BCSE control) was 500 mg/kg body
0.625% w/w) for Groups D and E, respectively. Based upon actual food consumption, it
representation of the experimental protocol is provided as Fig. 2. Following an
phosphate-buffered saline (pH 7.4) to flush out any remaining blood, blotted dry on a
intramuscular injection of 90 mg/kg body weight ketamine (Fort Dodge Animal
1037
Fig. 3. Effect of black currant skin extract (BCSE) on body weight gain during
diethylnitrosamine (DENA)-induced hepatocarcinogenesis in rats. No significant
difference in body weight gain was observed among various rat groups.
and subsequently spray-dried as per the procedure described in our previous communication [34]. The final product was a dark pink-colored fine powder, which was stored under controlled humidity at room temperature protected from light. The reverse-phase HPLC analysis of the extract revealed the presence of ∼1.2% total anthocyanin and ∼0.3% cyanidin-3-O-rutinoside (Fig. 1) [34].
2.3. Experimental diet for chemopreventive studies
The black currant skin extract (henceforth referred to as BCSE) was mixed with pulzerized rat feed using a food processor in the dark. The freshly prepared feed was used for the chemopreventive study. Opaque food containers (Lab Products, Inc., Seaford, DE, USA) were used, and the food was replenished every second day.
2.4. Experimental protocol
The potential chemopreventive role of dietary BCSE was investigated using a well-established and our previously published DENA-initiated and PB-promoted two-stage hepatocarcinogenesis model in rats [35] with a slight modification. A schematic representation of the experimental protocol is provided as Fig. 2. Following an acclimatization period of 1 week with standard basal diet, rats were randomly divided into five groups with 6–12 animals in each group based on a power analysis. Two groups (Groups A and B) were maintained on the basal diet, whereas the remaining three groups (Groups C, D and E) had free access to the basal diet supplemented with BCSE at 1.2 g BCSE/kg food (i.e., 0.125% w/w) for Group C and 6.0 g BCSE/kg food (i.e., 0.625% w/w) for Groups D and E, respectively. Based upon actual food consumption, it has been calculated that the BCSE dose for group E (BCSE control) was 500 mg/kg body weight, whereas the dose for groups C and D were 100 and 500 mg/kg body weight respectively. These doses (100 and 500 mg/kg body weight/day) were selected based on previously reported study in rats [36]. Following 4 weeks of this dietary regimen, hepatocarcinogenesis was initiated in all animals belonging to Groups B, C and D by a single intraperitoneal DENA (Sigma-Aldrich, St. Louis, MO, USA) injection at a dose of 200 mg/kg body weight (mixed with peanut oil). Animals belonging to group A (normal group) and group E (BCSE control) were similarly injected with an equal volume of peanut oil. Following a 2-week recovery period, the promoter PB (Sigma-Aldrich) was incorporated into the drinking water of above three groups (Groups B, C and D) at the concentration of 0.05% for 18 successive weeks. Feeding of rats with BCSE-supplemented food in Groups C, D and E was continued throughout the entire experimental period. Food and water intake as well as behavioral changes were monitored every day, and the body weight of animals were recorded every 2 weeks. All animals were sacrificed after 18 weeks following DENA or vehicle injection, i.e., at 22 weeks following commencement of the study. During the last 4 days, PB was withdrawn from the drinking water, and the food did not contain BCSE. The animals were fasted overnight prior to sacrifice. The rationale for using this specific protocol relates to identifying agents that intervene in initiation and progression of cancer [35].
2.5. Morphology and morphometry of hepatocyte nodules
Rats from each group were anesthetized between 0900 and 1100 h by intramuscular injection of 90 mg/kg body weight ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA) and 10 mg/kg body weight xylazine (Ben Venue Laboratories, Bedford, OH, USA). Following perfusion of the liver through the portal vein using heparinized saline, the liver was quickly excised, rinsed with ice-cold phosphate-buffered saline (pH 7.4) to flush out any remaining blood, blotted dry on a paper towel, weighed and photographed. Each liver was examined macroscopically on the surface as well as in 3-mm cross-sections for gross visible hepatocyte nodules. The grayish-white nodules were easily identified from the surrounding reddish-brown non-nodular liver tissue. The nodules (approximated spheres) were measured in two perpendicular planes with a vernier caliper to the nearest mm to obtain an average diameter of each nodule. The nodules were counted and categorized into three groups (i.e., ≥3, <3–1 and <1 mm) according to their respective diameters. Estimates of nodular volume were determined as described recently [35].
2.6. Histopathological studies
Representative liver specimens (4–5 mm thick) were collected from several lobes, immediately immersed in 4% paraformaldehyde (Ted Pella, Redding, CA, USA), and stored at 4°C. The liver samples were embedded in embedding medium (Tissue-Tek, Sakura Finetek USA, Torrence, CA, USA), freeze-cut serial sections (10 μm thick) were prepared using a cryostat (Leica CM1850, Leica Microsystems, Nussloch, Germany) and placed on poly-L-lysine-coated slides (VWR International, West Chester, PA, USA). The slides were stored at −80°C. Serial sections were stained with hematoxylin and eosin (H&E) according to our published method [37]. Specific hepaticcellular lesions were recognized by light microscopy following established criteria [38]. The histological slides were coded so that the particular dietary treatment was unknown to the individual performing the analysis.
2.7. Cell proliferation assay and localization of Bcl-2 and Bax by immunohistochemistry
Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) as a cell proliferation marker and protein expression of the apoptosis inducer Bax and the apoptosis repressor Bcl-2 were carried out as previously described [35]. In brief, hepatic sections were incubated for 10 min at 80°C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Following a 5-min wash with phosphate-buffered saline (PBS), the endogenous peroxidases were blocked by 1% H₂O₂ in PBS for 5 min. The sections were washed with PBS and blocked by treating with PBS containing 5% normal goat serum for 1 h. The slides were washed and then incubated overnight with primary antibodies obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA (1:500 for PCNA and 1:50 for Bcl-2 and Bax) at 4°C in a humidified chamber. After washing with PBS, the sections were incubated with a biotinylated secondary antibody (1:200 dilution) for 30 min at 37°C using the ABC staining system (Santa Cruz). The chromogenic reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride solution for 3–5 min. The PCNA sections were counterstained with 0.1% fast green (Sigma-Aldrich), whereas hematoxylin was used to counterstain Bcl-2 and Bax slides. Negative control sections were processed similarly but with the omission of the primary antibodies. All sections were viewed under a light microscope and 1,000 hepatocytes were analyzed per animal. The PCNA labeling index (LI) was estimated by counting the number of PCNA-positive hepatocytes per total number of counted hepatocytes × 100.
2.8. Assay of apoptosis
The detection of apoptotic cells in frozen liver sections were carried out by using TdT-FragEL DNA fragmentation detection assay kit (EMD Bioscience, San Diego, CA, USA) following the manufacturer’s protocol as previously reported [35]. The apoptotic cells with the labeled DNA fragments were identified as a dark-brown stain over the nuclei when viewed under a light microscope. The apoptotic index (AI) was expressed as the number of positively stained cells per 100 hepatocytes based on a count of 1,000 cells per animal.
2.9. Expression of results, data analysis and statistical significance
Statistical analysis of the data on hepatocyte nodule incidence was carried out by Fisher’s Exact probability test. All other data are expressed as mean±S.D. One-way analysis of variance was used to estimate overall significance followed by post hoc

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Relative liver weight</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal</td>
<td>12</td>
<td>417±39 a</td>
<td>13.9±1.6</td>
<td>3.18±0.32</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>B. DENA control</td>
<td>12</td>
<td>430±41</td>
<td>18.0±2.0 a</td>
<td>4.17±0.14</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>C. BCSE (100 mg/kg body wt)+DENA</td>
<td>12</td>
<td>461±54</td>
<td>19.0±4.2</td>
<td>4.15±0.45</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>D. BCSE (500 mg/kg body wt)+DENA</td>
<td>12</td>
<td>415±27</td>
<td>17.2±2.4</td>
<td>4.15±0.53</td>
<td></td>
</tr>
<tr>
<td>E. BCSE (500 mg/kg body wt) control</td>
<td>8</td>
<td>416±27</td>
<td>13.5±1.7</td>
<td>3.23±0.31</td>
<td></td>
</tr>
</tbody>
</table>

a Values are presented as means±S.D.
* P<0.05 compared with group A.
** P<0.01 compared with group A.
analyses using the Student-Neuman-Keuls test. A value of $P$ less than .05 was required for results to be considered statistically significant. A commercial software program (SigmaPlot, version 11.0, Systat Software, San Jose, CA, USA) was used for statistical analyses and representation of data.

3. Results

3.1. Food and water intakes

During the entire study period, we did not observe any differences in food or water intake among the various experimental groups. Food and water intakes were 8.2–8.5 g/100 g body weight/day and 8.0–8.9 ml/100 g body weight/day, respectively, for all groups.

3.2. Mortality

There were no treatment-related deaths of rats from any group before the termination of the study, i.e., 22 weeks.

3.3. Body, liver and relative liver weights

Average body weights of different animal groups at various time-points are shown in Fig. 3. No statistical differences were noticed between the growth rates of any of the treatment and normal groups. Final body weight, liver weight and relative liver weight of various groups that were sacrificed following 22 week of the study are presented in Table 1. There was a slight decrease in the final body weight of rats receiving DENA (Group B) as compared to the normal group (Group A), but this result was not statistically significant. BCSE treatment (100 or 500 mg/kg body weight) maintained the body weights of rats in Groups C, D and E as compared to Group A, suggesting that BCSE had no adverse effect on the growth of the rats. The average liver weight of DENA control group (Group B) was significantly ($P < .05$) increased as compared to that of normal group (Group A). BCSE did not alter the liver weights in Groups C and D as compared to Group B. The relative liver weight of Group B was found to be significantly ($P < .001$) higher than that of Group A. There was no significant difference in relative liver weights of all DENA-treated animals in the presence or absence of BCSE. BCSE at a dose of 500 mg/kg body weight (Group E) did not influence the liver weights as compared to normal group (Group A).

3.4. Effects of BCSE on DENA-initiated nodule growth

While there were no visible hepatocyte nodules in the livers of normal group (Group A) as well as BCSE control (Group E), macroscopic nodules arose from the livers of the various DENA-injected groups (Fig. 4). Table 2 summarizes nodule incidence, total number of nodules and average number of nodules/nodule-bearing liver (nodule multiplicity) of DENA-initiated groups with or without BCSE treatment. Dietary BCSE at a dose of 100 mg/kg reduced the nodule incidence in Group C compared to the DENA control (Group B), but the result was not statistically significant. A significantly ($P < .05$) reduced incidence of nodules was observed in the group that received BCSE at a dose of 500 mg/kg body weight (Group D) as compared to Group B. Although the total number of nodules was found to be less in two BCSE-treated groups (Groups C and D) as compared to Group B, the results were mostly prominent for Group D. Similarly, the nodule multiplicity was found to be smaller in Groups C and D than Group B, but a statistically significant ($P < .05$) result was only observed in Group D.

Table 3 presents the data on the size distribution of visible nodules, mean nodular volume and nodular volume as a percentage of liver volume of the experimental groups. BCSE treatment at a dose of 500 mg/kg body weights (Group D) significantly ($P < .001$) reduced the appearance of nodules of more than 3 mm and appeared to increase the development of other nodules when compared with Group B. However, the latter results did not reach statistical significance. Mean nodular volume was found to be inhibited in two BCSE-treated groups.
Animals from normal (Group A) and black currant (500 mg/kg body wt) control group (Group E) did not show any visible hepatocyte nodule.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats with nodules/ total rats</th>
<th>Nodule incidence (%)</th>
<th>Total no. of nodules</th>
<th>Average no. of nodules/ nodule-bearing liver (nodele multiplicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. DENA control</td>
<td>9/10</td>
<td>90</td>
<td>192</td>
<td>21.3±4.9*</td>
</tr>
<tr>
<td>C. BCSE (100 mg/kg body wt)+DENA</td>
<td>9/12</td>
<td>75</td>
<td>170</td>
<td>18.9±4.4</td>
</tr>
<tr>
<td>D. BCSE (500 mg/kg body wt)+DENA</td>
<td>5/12</td>
<td>42</td>
<td>71</td>
<td>14.2±3.3*</td>
</tr>
</tbody>
</table>

*Values are presented as means±S.D.

Effect of black currant treatment on the development of macroscopic hepatocyte nodules induced by DENA in rats

Animals from normal (Group A) and black currant (500 mg/kg body wt) control group (Group E) did not show any visible hepatocyte nodule.

3.5. Effect of BCSE on hepatic histopathology

Histopathological findings on liver sections from various experimental groups of animals are illustrated in Fig. 5. The hepatic sections from normal animals (Group A) revealed normal liver parenchyma with the typical architecture characterized by granulated cytoplasm, central vein and small uniform nuclei (Fig. 5A). Rats injected with DENA alone (Group B) showed abnormal architecture with the presence of irregular-shaped cytoplasm and enlarged and hyperchromatic nuclei (Fig. 5B and C). A large number of abnormal hepatocytes were observed which were binucleated and contained irregular lipid droplets. In addition, extensive vacuolation was noticed in the cytoplasm surrounding the nucleus with masses of eosinophilic material. Treatment of rats with BCSE at a dose of 100 mg/kg body weight (Group C) improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to Group B (Fig. 5D). The cellular architecture of liver sections from rats that received BCSE at 500 mg/kg (Group D) was comparable with that of the normal animals. The hepatocytes from this group had a compact cytoplasm. Furthermore, the liver cells were mostly mononucleated with regular sized nuclei (Fig. 5E). Normal rats fed with BCSE at 500 mg/kg body weight (Group E) showed normal hepatocellular characteristics (Fig. 5F) which indicate the absence of hepatotoxicity of chronic BCSE treatment.

3.6. Effect of BCSE on hepatic cell proliferation

To determine whether BCSE affected cell proliferation during DENA-initiated hepatocarcinogenesis, the expression of PCNA was studied by immunohistochemistry in liver sections from the experimental groups. Representative photomicrographs of PCNA immunohistochemical staining are depicted as Fig. 6A. While the liver sections from normal rats (Group A) showed a limited immunostaining of PCNA (Fig. 6A-a), similar sections from the DENA control group (Group B) exhibited a large quantity of PCNA-positive cells (Fig. 6A-b), indicating vigorous and abnormal cell proliferation during DENA hepatocarcinogenesis. On the other hand, sections from BCSE-treated rats (Groups C and D) indicated a reduced PCNA expression compared to DENA control (Figs. 6A-c and A-d, respectively), suggesting the antiproliferative potential of black currant. Liver sections from BCSE control group (Group E) showed near absence of PCNA immunoreactivity (data not shown). As shown in Fig. 6B, the mean PCNA LI was significantly higher (P<0.001) in DENA-initiated rats (Group B) than that of normal animals (Group A). Compared with DENA control (Group B), the two BCSE-treated groups (Groups C and D) presented smaller hepatic PCNA LI. Nevertheless, a statistically significant (P<0.001) result was only observed in BCSE at 500 mg/kg body weight plus DENA group (Group D) compared to DENA alone (Group B). There was no difference in PCNA LI between the BCSE control and the normal group.

3.7. Effect of BCSE on apoptosis

In the present study, TdT-FragEL DNA fragmentation detection assay was utilized to evaluate the extent of apoptosis in hepatic sections from various groups of rats. The chromogen-generated brown stain was considered as an indication of cells undergoing apoptotic death. While the detection of any positive staining was extremely infrequent for normal (Group A) as well as DENA control (Group B) (Fig. 7A-a and A-b, respectively), we observed numerous brown stain overlapping the condensed chromatin of apoptotic bodies in BCSE-supplemented groups (Fig. 7A-c and A-d), especially the group treated with 500 mg extract/kg body weight. The detection of apoptotic cells in BCSE control (Group E) was very infrequent (data not shown). Fig. 7B illustrates the AI of liver sections from different groups of animals. We did not notice any significant difference in AIs between normal (Group A) and carcinogen control (Group B). On the contrary, there was a significant (P<0.001) increase in apoptosis expression as evidenced from higher AIs in the experimental group that received BCSE at a dose of 500 mg/kg body weight (Group D) when compared to DENA control (Group B). Interestingly, BCSE treatment in normal rats (Group E) did not influence the AI when comparison was made with untreated normal group (Group A).

3.8. Effect of BCSE on expression of Bax and Bcl-2 proteins

The expression of the apoptosis-related proteins, namely Bax and Bcl-2, in hepatic section was studied by immunohistochemistry. We could not detect Bax and Bcl-2 in the livers of normal (Group A) and BCSE (500 mg/kg body weight) control (Group E) animals.

Table 3

Effect black currant treatment on the size distribution and growth of hepatocyte nodules induced by DENA in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>Nodules relative to size (% of total no.)</th>
<th>Mean nodular volume* (cm³)</th>
<th>Nodular volume/liver volumeb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥3 mm‡</td>
<td>3–1 mm‡</td>
<td>1 mm‡</td>
</tr>
<tr>
<td>B. DENA control</td>
<td>9</td>
<td>42±9*</td>
<td>32±8</td>
<td>26±7</td>
</tr>
<tr>
<td>C. BCSE (100 mg/kg body wt)+DENA</td>
<td>9</td>
<td>38±15†</td>
<td>33±8</td>
<td>29±13</td>
</tr>
<tr>
<td>D. BCSE (500 mg/kg body wt)+DENA</td>
<td>5</td>
<td>21±6*</td>
<td>40±10</td>
<td>39±12</td>
</tr>
</tbody>
</table>

*Values are presented as means±S.D.

bP<0.05 compared with group B.

In the present study, TdT-FragEL DNA fragmentation detection assay was utilized to evaluate the extent of apoptosis in hepatic sections from various groups of rats. The chromogen-generated brown stain was considered as an indication of cells undergoing apoptotic death. While the detection of any positive staining was extremely infrequent for normal (Group A) as well as DENA control (Group B) (Fig. 7A-a and A-b, respectively), we observed numerous brown stain overlapping the condensed chromatin of apoptotic bodies in BCSE-supplemented groups (Fig. 7A-c and A-d), especially the group treated with 500 mg extract/kg body weight. The detection of apoptotic cells in BCSE control (Group E) was very infrequent (data not shown). Fig. 7B illustrates the AI of liver sections from different groups of animals. We did not notice any significant difference in AIs between normal (Group A) and carcinogen control (Group B). On the contrary, there was a significant (P<0.001) increase in apoptosis expression as evidenced from higher AIs in the experimental group that received BCSE at a dose of 500 mg/kg body weight (Group D) when compared to DENA control (Group B). Interestingly, BCSE treatment in normal rats (Group E) did not influence the AI when comparison was made with untreated normal group (Group A).

3.8. Effect of BCSE on expression of Bax and Bcl-2 proteins

The expression of the apoptosis-related proteins, namely Bax and Bcl-2, in hepatic section was studied by immunohistochemistry. We could not detect Bax and Bcl-2 in the livers of normal (Group A) and BCSE (500 mg/kg body weight) control (Group E) animals.
Interestingly, immunostaining of these two proteins was detected predominantly in the cytoplasm of hepatocytes from all DENA-treated animals (Fig. 8). Nevertheless, the frequency of Bax-positive cells was extremely low in DENA control (Group B) animals (Fig. 8A-a and B). BCSE, on the other hand, dose-dependently increased the expression of Bax (Fig. 8A-b,c and B) with a significant (\( P < 0.001 \)) result at the higher dose level. Liver sections from DENA control exhibited high frequency of Bcl-2-positive cells (Fig. 8A-d and C) which was reduced by BCSE (Fig. 8A-e,f and C). Nevertheless, a significant (\( P < 0.001 \)) result was achieved with the highest dose of BCSE. BCSE dose-dependently increased the Bax/Bcl-2 ratio in all DENA-initiated rats (Groups C and D) compared to DENA control (Group B). However, the most prominent result (more than ninefold increase, \( P < 0.001 \); Fig. 8D) with Bax/Bcl-2 ratio was achieved in the group that received the highest BCSE dose (Group D) as compared to DENA control (Group B).

### 4. Discussion

Berries are considered to be an essential component of the Western diet, and these small fruits are also used in several other cuisines across the globe. While well known in Europe and several other parts of the world, black currants are gaining widespread popularity in North America for significant health-promoting effects. In contrast to a large volume of information on the health benefits of black currant, limited experimental evidence on antitumor effects of black currant exists with virtually no data on the prevention of chemically induced tumorigenesis in vivo. Our group has recently reported that an anthocyanin-rich extract from black currant skin exhibits significant cytotoxicity against HepG2 liver cancer cells [34]. In the current study, we have investigated the inhibitory effects of the same extract against experimentally induced hepatocarcinogenesis in rats.

Experimental liver cancer in rodents induced by DENA, an environmental and dietary hepatocarcinogen [39,40], has been considered as one of the best characterized experimental models of HCC, allowing the screening of potential anticancer compounds on various phases of neoplastic transformation and development [41–43]. DENA-induced preneoplastic foci and preneoplastic and neoplastic nodule formation in rodents closely mimics HCC development in humans [44–46]. Recently, a cross-species comparison of gene expression patterns has established that DENA-induced liver tumors in rodents closely resemble a subclass of human HCC [47], which allows to extrapolate potential chemopreventive effects of a candidate agent in clinical setting.

Fig. 5. Histopathological profiles of representative liver tissues from various experimental animals. (A) Normal untreated rat liver (Group A) showing normal cellular architecture (H&E; original magnification ×250). (B) and (C) DENA control (Group B) showing areas of aberrant hepatocellular phenotype with variation in nuclear size, hyperchromatism, and irregular sinusoids (H&E; original magnification ×250). (D) liver section from BCSE (100 mg/kg body weight) + DENA (Group C) showing moderate improvement of hepatic histopathology over group B (H&E; original magnification ×250). (E) section from BCSE (500 mg/kg body weight) + DENA (Group D) showing hepatocytes maintaining near-normal architecture (H&E; original magnification ×250). (F) section from BCSE (500 mg/kg body weight) control group (Group E) demonstrating characteristics of normal liver (H&E; original magnification ×250). CV, central vein; BC, binucleated cell; and HN, hyperchromatic nuclei.
In the present study, we have investigated the preventive effect of an anthocyanin-rich BCSE on the appearance of early hepatic preneoplastic events, utilizing a two-stage model of hepatocarcinogenesis initiated with DENA and promoted by PB. The results of our study clearly indicate a beneficial effect of dietary BCSE on chemically-induced rat liver tumorigenesis. To our knowledge, this is the first experimental evidence of the chemopreventive activity of black currant. Under our experimental conditions, addition of BCSE in the diet of DENA-exposed rats resulted in fewer animals developing visible hepatocyte nodules and smaller nodule multiplicity compared to DENA administration. Another conspicuous finding of this study was the BCSE-mediated reduction in the development of nodules more than 3 mm in size with a concomitant attenuation of nodular volume. The putative preneoplastic nodules have been characterized by hepatocyte phenotype when elevated hyperplasia, and alterations of enzymatic markers and critical genes for cell proliferation were evident. Although it has been observed that not all the hepatocyte nodules become cancerous during the lifespan of animals, several experimental findings support the concept that the nodules are precursors of hepatic cancer [48,49]. Moreover, a large body of experience in both experimental and human disease provides a correlation between the number and size of nodular hyperplasia and HCC [50,51]. In the background of these studies, suppression of nodule growth and acceleration of nodule regression by BCSE as
observed in the present study could be viewed as vital steps for cancer chemoprevention. These are the findings of critical importance when one considers the fact that persistent nodules are easily recognizable and have a low tendency to regress spontaneously.

During the entire term of the study, the food and water intake as well as the mean body weight among various rat groups did not differ significantly suggesting a similar nutritional status of the experimental animals. The attenuation of tumor growth by dietary restriction is a well-known concept that has gained renewed interest [52]. It has been observed that nutritional deprivation causing body weight loss may also be associated with reduction in tumor volume [53]. The ability of dietary and caloric restriction in decreasing tumor incidence and arresting tumor growth have been established in chemically-induced as well as spontaneous tumors in rodents [54–57]. Furthermore, a strong dietary restriction inhibits the ability of PB to enhance DENA-induced focal lesion growth in mice [58]. As the rats in this study grew equally irrespective of dietary condition, it can be concluded that the observed inhibitory effects of BCSE on nodule growth is not associated with an impairment of nutritional status of the carcinogen-exposed animals.

Fig. 7. Immunohistochemical analysis of DNA fragmentation in apoptotic cells in hepatic tissues belong to various experimental rat groups. (A) Representative photomicrographs of hepatic sections used for apoptotic study. Immunohistochemistry was carried out using TdT-FragEL kit as described in the Materials and methods section. Brown-stained cells (indicated by arrows) are undergoing apoptosis. (a) Immunostaining of rat liver from normal (Group A). (b) DENA control (Group B) liver showing almost absence of apoptotic cells. (c) BCSE (100 mg/kg body weight) + DENA (Group C). (d) BCSE (500 mg/kg body weight) + DENA (Group D) exhibiting positive brown-stained nuclei (original magnification ×100). (B) Quantification of apoptotic index of hepatic tissue of rats subjected to experimental regimen. One thousand hepatocytes were counted per animal and the results were based on 4 animals per group. Each bar represents the mean±S.D. (n=4 livers). *P<.001 and as compared to DENA control.
Cell proliferation plays a fundamental role in multistage carcinogenesis and is also believed to be involved in the pathogenesis of HCC [59,60]. Additionally, the rate of cell proliferation is considered to be a well-established marker for cancer risk development [61]. Hence, the discovery of agents that can affect abnormal proliferation of liver cells is of immense importance in chemoprevention of hepatocellular carcinogenesis. The content and expression of PCNA, a 36-kDa nuclear protein, have been linked to the late G1 as well as early S-phase of the cell cycle [62]. One common approach to study the proliferative status of transformed cells is detection of PCNA by immunohistochemical techniques [63]. In conjunction with histopathological characteristics, overexpression of PCNA is a reliable marker for evaluating tumor grade or stages of tumor differentiation and assessment of tumor progression as well as early detection and prognostic of HCC [64]. To investigate the mechanism by which BCSE provides chemoprevention of hepatocarcinogenesis, we have examined the extent of cell proliferation in hepatic tissue during DENA-initiated tumorigenesis in the presence or absence of BCSE. Our immunohistochemical analysis displays intense immunostaining of PCNA in the preneoplastic liver tissue from DENA-initiated animals when compared to normal controls. Another interesting finding of our study is the fact that hyperbasophilic cells of the preneoplastic lesions exhibit intense staining for PCNA compared to that of the non-basophilic area of the same tissue section. This indicates that the proliferative preneoplastic lesions with rapidly proliferating cells may constitute primary PCNA-positive focal expression zones. The higher PCNA LI in DENA control rats indicates elevated number of regenerating hepatocytes as well as the severity of hepatic damage. The focal expression of PCNA in areas of high proliferative activity, as observed here, represents an early event in the pathogenesis of DENA-initiated hepatic neoplasia and confirms the observations from other laboratories [65–67]. BCSE treatment remarkably decreased the number of PCNA-positive cells, indicating its ability to suppress abnormal proliferation of initiated hepatocytes through an antiproliferative activity. Our present in vivo data are in line with previous in vitro antiproliferative effects of BCSE against HepG2 [34]. BCSE also exhibited a similar antiproliferative activity against a wide variety of tumor cells [29–31].

Apoptosis or programmed cell death represents a form of physiological cell death which is characterized by a dedicated cellular mechanism involving cellular morphological alterations, chromatin

Fig. 8. Alteration of the protein expression of Bax and Bcl-2 by BCSE in DENA-induced hepatocarcinogenesis. (A) Immunohistochemical findings of Bax and Bcl-2 proteins in the hepatic tissue of several groups of rats. Immunohistochemistry was carried out as described in the Materials and methods section. Arrow heads indicate immuno-positive cells. Bax expression is shown for DENA control (Group B) (a), BCSE (100 mg/kg body weight, Group C) (b) and BCSE (500 mg/kg body weight, Group D) (c). Bcl-2 expression for various groups: DENA control (Group B) (d), BCSE (100 mg/kg body weight, Group C) (e) and BCSE (500 mg/kg body weight, Group D) (f) (original magnification ×100). Quantitative analysis of Bax-immunopositive cells (B), Bcl-2-immunopositive cells (C) and Bax/Bcl-2 ratio (D). Each bar represents the mean±S.E. (n=4 livers). *P<0.001 and as compared to DENA control.
condensation, formation of apoptotic bodies and DNA cleavage [68]. Prior studies have established that onset of HCC is attributable to the loss of control of normal apoptosis and the disturbance of equilibrium between cell proliferation and apoptosis [69,70]. Inhibition of apoptosis facilitates survival of initiated hepatocytes with genotoxic lesions and plays a crucial role in tumor promotion [71,72]. Apoptosis has been postulated as a fundamental mechanism of various chemotherapeutic as well as chemopreventive agents. The detection of DNA fragmentation is routinely accepted as a reliable apoptosis indicator. This approach can be used concurrently with morphologic observations or antibody staining of secondary biomarkers [73]. In the present study, apoptosis during DENA-initiated and PB-promoted hepatocarcinogenesis has been evaluated using immunohistochemical techniques. In line with our earlier observations [35] as well as with reports from other laboratories [66,74], the frequency of apoptotic cells was extremely low in the carcinogen control group. Our results reveal BCSE-mediated induction of apoptosis in hepatic sections of rats subjected to DENA/PB regimen. This is the first study to demonstrate an apoptosis-inducing property of black currant constituents in an experimental in vivo tumor model.

In order to further elucidate the mechanisms involved in BCSE-mediated induction of apoptosis during hepatocarcinogenesis, we have studied the effect of BCSE on expression of proteins belong to the Bcl-2 family. Members of the Bcl-2 family of proteins are considered as cardinal regulators of apoptotic pathways. Several genetic alterations observed in HCC are known to trigger an imbalance between pro- and anti-apoptotic members of the Bcl-2 family [75]. These proteins are also considered valuable targets for anticancer therapy [76]. While Bcl-2 functions as a suppressor of apoptosis leading to the survival of neoplastic cells, ectopic expression of other Bcl-2 family proteins, such as Bax, induces mitochondrial apoptosis with apoptosis-related morphological changes, caspase activation, and subsequent substrate proteolysis [77]. An elevated Bax/Bcl-2 ratio is a reliable index of overall cell propensity to undergo apoptosis. In our present study, an increase in Bax and decrease in Bcl-2 expression were associated with BCSE treatment of DENA hepatocarcinogenesis. Consequently, there has been a significant improvement in the ratio of Bax/Bcl-2 in BCSE-treated groups compared to DENA controls, suggesting the involvement of the members of the Bcl-2 family in induction of apoptosis by BCSE. This is the first evidence that anthocyanins from black currant are able to induce apoptosis, possibly via regulation of Bcl-2 family proteins. Our results are concordant with a recent study that shows that an anthocyanin-rich extract from the Korean vine plant meoru (Vitis coignetiae, Pulliat) induces apoptosis in human hepatoma Hep3B cells with a parallel reduction in the expression of several antiapoptotic proteins including Bcl-2 and an increase in the ratio of Bax/Bcl-2 [78].

The black currant skin is known to contain four major anthocyanins, namely cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside [79]. We have also identified cyanidin-3-O-rutinoside as one of the major anthocyanins present in the extract used in this study [34]. Previous studies have shown that cyanidin-3-O-rutinoside is a highly powerful antioxidant [80] that exhibits potent antitumor effects on leukemic cells through the mitochondrial apoptotic pathway [81,82]. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, derived from black raspberry, have been found to inhibit the growth of esophageal tumor cells with induction of apoptosis [83]. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, extracted from mulberry, exhibit inhibitory effects on the migration and invasion of human lung carcinoma cells [84]. These two anthocyanins have also been reported to possess nuclear factor-κappa B- and activator protein-1-inhibitory activities which have been linked to the chemopreventive effect of black raspberry against chemically-induced esophageal tumors in rats [85]. Based upon these studies, it is highly likely that cyanidin-3-O-rutinoside as well as other anthocyanins may account, at least in part, for the chemopreventive action of black currant against rat liver tumorigenesis. Nevertheless, this does not exclude the possibility of other bioactive constituents present in the black currant skin to contribute to the beneficial effects of the extract used in the current investigation. Several lines of evidence convincingly demonstrate that various plant phytochemicals from dietary sources, including cranberry, raspberry, pomegranate and green tea, may exert better cancer chemopreventive activities when used in combination rather than in single pure form [83,86–88]. Thus, it is tempting to speculate that the observed chemopreventive effects of black currant may be due to the synergistic effect among the various phytochemicals present in the extract. However, additional studies are needed to confirm this hypothesis.

A food-based approach targeting the key molecules and pathways involved in the pathogenesis of malignancy could be the most practical application of chemopreventive agents in several high-risk populations. The development of human HCC has been estimated to take between 30 and 50 years [89]. Hence, chemopreventive agents like the black currant bioactive constituents may be used in healthy populations as life-long interventions to reduce the risk of developing HCC without any toxic manifestations. In this study, normal animals exposed to the highest dose (500 mg/kg body weight) of BCSE did not show evidence of toxicity following a chronic (22 weeks) exposure period, as indicated by the unaltered food and water intakes, growth curve, liver weight and hepatic histopathological indices. The absence of toxicity of BCSE in a mammalian species should be viewed favorably regarding long-term safety of ingestion of black currant constituents for achieving chemoprevention in the human population.

In summary, the results of our present investigation demonstrate, for the first time, that an anthocyanin-rich extract from black currant exerts a striking chemopreventive effect against experimentally-induced in vivo hepatocarcinogenesis in rats. The dose-responsive chemopreventive properties of BCSE have been reflected in its ability to abrogate the development of preneoplastic hepatic nodule formation. Our study also demonstrates that inhibition of cell proliferation and induction of apoptosis may be, at least in part, the underlying mechanisms related to the liver tumor inhibition by BCSE. Furthermore, the BCSE-mediated proapoptotic signal in experimental hepatocarcinogenesis may be propagated through an up-regulation of Bax and a down-modulation of Bcl-2 expression at the translational level. Further studies are warranted to identify and isolate the major bioactive constituents present in BCSE and to delineate their related and additional mechanisms of action responsible for inhibition of hepatic tumorigenesis. Nevertheless, the data presented here clearly encourage the development of black currant phytochemicals for chemoprevention of human liver cancer.

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