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Anthocyanin-Rich Black Currant Extract Suppresses the Growth of Human Hepatocellular Carcinoma Cells

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Dietary antioxidants, such as anthocyanins, are helpful in the prevention and control of various diseases by counteracting the imbalance of oxidative and antioxidative factors in the living systems. Black currant (*Ribes nigrum* L.*,* Grossulariaceae) is known to contain high amounts of anthocyanins (250 mg/100 g fresh fruit). Black currant fruits have been used in Asian and European traditional medicine for the treatment of a variety of diseases. Black currant extract has recently been found to be the second most effective amongst nine different berry extracts studied for their free radical scavenging activity. Constituents present in black currant juice have been found to exert a number of health-promoting effects, including immunomodulatory, antimicrobial and antiinflammatory actions, inhibition of low-density lipoprotein, and reduction of cardiovascular diseases. Although antioxidant and antiinflammatory effects of black currant juice could be of value in preventing and treating oxidative stress- and inflammation-driven cancers, no experimental evidence is available to now. The objective of the present study was to evaluate the potential antiproliferative effects of black currant fruit skin extract against $HepG_2$ human liver cancer cells. The aqueous extract yielded an anthocyanin-rich fraction with cyanidin-3-*O*-rutinoside as one of the major anthocyanins. This fraction exhibited a potent cytotoxic effect on $HepG_2$ cells and this effect was more pronounced than that of delphinidin and cyanidin, two major aglycones of anthocyanins present in black currant. Our results indicate, for the first time, that black currant skin containing an anthocyanin-rich fraction inhibits the proliferation of liver cancer cells, possibly due to additive as well as synergistic effects. This product could be useful in the prevention and treatment of human hepatocellular carcinoma.

Keywords: Black currant, *Ribus nigrum*, anthocyanin, delphinidin, cyanidin, cyanidin-3-*O*-rutinoside, cytotoxicity, $HepG_2$, hepatocellular carcinoma.

Epidemiological studies over the past few years have suggested that regular intake of fruits and vegetables may significantly reduce the risk of age-related chronic illnesses in certain populations [1a]. Dietary antioxidants, including anthocyanins, play a vital role in the prevention and control of several major chronic illnesses, such as arthritis, atherosclerosis, diabetes, cardiovascular ailments, Alzheimer's disease, and Parkinson's disease, as well as cancer by counteracting the imbalance of oxidative and antioxidative factors in the living systems [1b-1d]. Anthocyanins, a predominant group of water-soluble pigments belonging to the flavonoid class, are present in various plants, contributing to most of the blue, red, violet and purple colors [2a]. It has been estimated that the average daily intake of anthocyanins in the United States population is 180–215 mg [2b]. Edible berry fruits such as blueberry, strawberry, black and red raspberry, as well as black and red currants represent an abundant source of structurally diverse anthocyanins in quantitative as well as qualitative terms [2c,2d]. Black currant (*Ribes nigrum* L.*,* Grossulariaceae) fruits are known to contain high amounts of anthocyanins (250 mg/100 g fresh fruit) [2e]. Black currant fruits and leaves have been used in both Asian as well as European traditional medicine for the treatment of a variety of diseases including inflammatory disorders [3a,3b]. Black currant extract has been found to be the second most effective

antioxidant amongst nine different berry extracts studied for their free radical scavenging activity [3c]. Black currants have been recently termed as "superfruits" as they are believed to possess a number of health benefits including alleviation of chronic oxidative stress-related ailments [3d]. Constituents present in black currant have been found to exert a variety of health-promoting effects, including immunomodulatory, antimicrobial and antiinflammatory actions, inhibition of low-density lipoprotein, as well as reduction of cardiovascular diseases [4a-4e]. Black currant juice and extract have also been found to suppress the proliferation of breast, prostate, stomach, intestine and colon cancer cells *in vitro* [5a-5c] and significantly inhibit the growth of Ehrlich carcinoma *in vivo* [5d,5e]. In a recent clinical study, it has been found that an anthocyanin-rich black currant extract exhibited antioxidant, antiinflammatory and immunostimulatory properties [5f].

Berries including black currants are used to prepare juice, wines, jams, jellies, ice cream, and cake toppings, as well as other food products. The solid residue that remains following the extraction of black currant juice results in the production of a byproduct, known as pomace. The pomace of black currant can be regarded as a good source of phenolic antioxidants, including anthocyanins and flavonols, which predominantly contribute to the high radical scavenging property of this press residue [6a,6b]. The skin of black currant fruit represents a considerable portion of the pomace. Although this dark-colored fraction is likely to contain anthocyanins and other constituents with important biological activities, data on characterization of the phytochemicals exclusively from the skin of black currant are very limited. Likewise, the anticancer potential of the constituents of black currant skin has not been investigated until now. In view of this, the aim of the current study was to prepare and characterize an extract from black currant skin. As black currant anthocyanins possess potent antioxidant and antiinflammatory properties [2c,4e,5f,6c,7a], an anthocyanin-rich extract from the skin of this berry fruit has been tested for its efficacy in treating oxidative stress- and inflammation-driven hepatocellular carcinoma (HCC) using an *in vitro* cell culture model.

In the present study, the skin of *R. nigrum* fruit was extracted with water. Following evaporation and spray-drying, a dark pink-colored solid material was obtained. This extract was used for *in vitro* experiments. The chemical composition of the extract was characterized by total anthocyanin content, which was determined by spectrophotometry and high performance liquid chromatograpy (HPLC). Moreover, the content of cyanidin-3-*O*-rutinoside, as one of the major anthocyanins of *R. nigrum* skin has also been quantified. The reverse phase-HPLC determination resulted in 1.15 ± 0.05% total anthocyanin and 0.29 ± 0.06% cyanidin-3-*O*-rutinoside. In accordance with these data, the total anthocyanin content of the black currant extract was measured by spectrophotometry as 1.10 ± 0.05%, expressed as cyanidin-3-*O*-rutinoside chloride.

Our data are in accordance with previous studies that have indicated that anthocyanins (anthocyanidin glycosides) dominate in black currant fruits with the concentrations ranging from 0.17 to 0.36% (fresh weight) depending on specific cultivars [6c,7b]. The four major anthocyanins previously found in black currant fruit extracts are cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside, which collectively represent 94-98% of the total anthocyanin content depending on the cultivar [2c-2e]. Moreover, the aforementioned four anthocyanins have also been detected by HPLC in extracts prepared from black currant pomace and residues, accounting for approximately 90% of total anthocyanin content [6a]. Additionally, acidic hydrolysis following extraction yielded two anthocyanidins, namely delphinidin and cyanidin [6a].

In order to evaluate the antitumor effects of anthocyanin-rich black currant skin extract on HCC, we treated $HepG_2$ human liver cancer cells with varying concentrations of this extract. The cytotoxic potential of the extract was investigated by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. As illustrated in Figure 1A, addition of the extract to the cell culture medium exhibited a striking cell killing effect in a concentration-dependent manner, with an estimated IC_{50} value (half maximal inhibitory concentration) of 1.35 mg/mL. A statistically significant ($P<0.05$) result was obtained at an extract concentration of 1.5 mg/mL or above. Delphinidin and cyanidin were also found to suppress the growth of $HepG_2$ cells in our experimental conditions (Figure 1B). Although both anthocyanidins exhibited cytotoxicity against $HepG_2$ cells, delphinidin (IC_{50} = 240 μM) appears to be more potent than cyanidin (IC_{50} = 360 μM).

Our results are in agreement with previous studies showing cytotoxicity of anthocyanins on various cancer cells, including those from hepatic origin. For example, a procyanidin-rich fraction from grapes significantly reduced the viability of $HepG_2$ cells [7c]. In another study, an aqueous extract from the Korean vine plant meoru (*Vitis coignetiae,* Pulliat), containing diglucosides of cyanidin and delphinidin as major constituents, exhibited antiproliferative, antiinvasive and apoptotic effects on human hepatoma Hep3B cells

[7d]. Cyanidin and delphinidin, two anthocyanin aglycones, have been found to exert strong growth inhibitory effects against $HepG_2$ and to a lesser extent against Hep3B cells [7e]. Recently, Feng and coworkers [7f,7g] have reported that cyanidin-3-*O*-rutinoside, extracted and purified from black raspberry (*Rubus occidentalis* L.), had selective cytotoxicity against HL-60 human leukemic cells, but not on SMMC7721, HCCLM3 and MHCC97L liver cancer cells. The same group has also shown that cyanidin-3-*O*-rutinoside and delphinidin (the latter extracted from bilberry) caused growth retardation of HCC cells by macroautophagy [7g]. Cyanidin-3-*O*-rutinoside (extracted from mulberry, *Morus alba* L.) exerted a dose-dependent inhibitory effect on the migration and invasion of highly metastatic A549 human lung carcinoma cells [7h]. Cyanidin-3-*O*-rutinoside has also been identified as one of the major bioactive constituents of black raspberry [7i,8], with multiple anticarcinogenic effects, as reviewed by Wang and Stoner [9a].

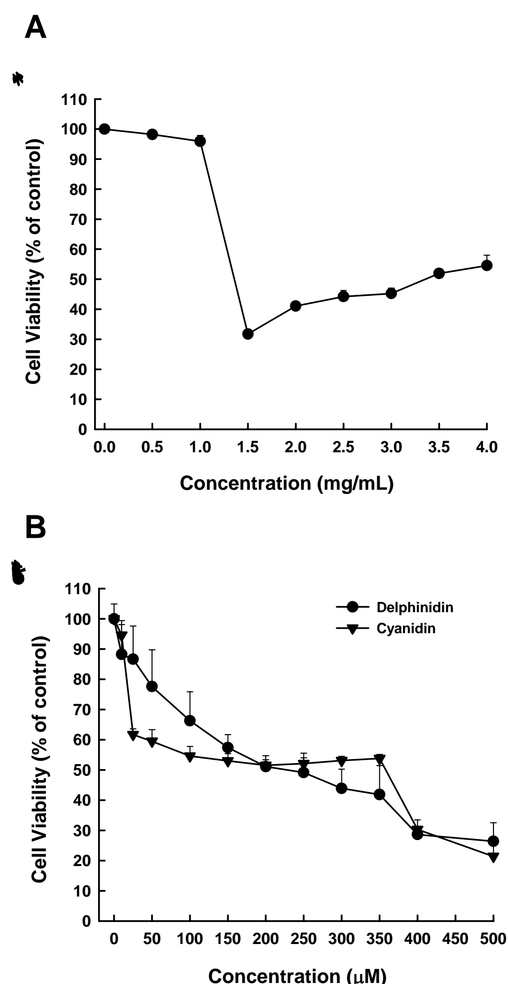

Figure 1: Effects of black currant skin extract (A), delphinidin and cyanidin (B) on the proliferation of $HepG_2$ cells. Cells were plated into 96-well dishes (4×10^3 cells/well) 24 h prior to the addition to the extract of either delphinidin or cyanidin at various concentrations. Following 24 h incubation, the cell proliferation was determined by MTT assay. Results are presented as mean±SE based on quadruplicate determinations in three independent experiments. Statistical analysis performed by one-way analysis of variance followed by Student-Newman-Keuls test. *$P<0.05$ and •$P<0.05$ as compared with corresponding control.

The underlying mechanisms of the antitumor effects of black currant skin extract on $HepG_2$ cells, as observed in the current study, remain to be elucidated. We have identified cyanidin-3-*O*-rutinoside as one of the major anthocyanins in the skin fraction that supports previous studies, as mentioned above. Delphinidin and cyanidin have also been reported to be present in black currant [2c]. Additionally, other bioactive phytochemicals, such as phenolic acids, proanthocyanidins and other flavonoids may also be present in black currant skin extract. All these constituents are known to possess *"pleiotropic"* biochemical and pharmacological effects, including antioxidant, antiinflammatory, immunostimulatory, anti-apoptotic, cell cycle arrest-inducing, anti-invasive and anti-angiogenic properties through modulation of multiple signal transduction pathways [9b,9c]. All these could contribute to the observed cytotoxicity of the extract tested in our study. Our experimental results indicate that a complex mixture of phytochemicals present in black currant skin extract is more effective in inhibiting the growth of HCC cells than the individual constituents evaluated. This effect may be due to either the presence of other active compounds or through additive or synergistic effects. Our results are in agreement with a previous study showing a better antiinflammatory effect of a total flavonoid-rich black currant extract than its two major components [10]. Accumulating evidence suggests that several plant phytochemicals from diverse dietary sources, including cranberry, raspberry, pomegranate and green tea, may be more effective anticancer agents when used in combination rather than in single pure form [11-14]. It is also plausible that over enrichment or purification may result in the loss of pharmacological activities and hence therapeutic benefits of plant extracts [13].

In conclusion, skin of black currant fruit should not be considered as low-value waste as it contains an anthocyanin-rich fraction with potent antitumor activity against $HepG_2$ human liver cancer cells. The cytotoxic effects of black currant skin extract could be due to the presence of diverse bioactive phytochemicals rather than a single constituent. A better understanding of the observed inhibitory effects of black currant skin extract on the proliferatation of liver cancer cells would benefit the development of this product for the prevention and treatment of HCC.

Experimental

Plant material: Ripe black currant (*Ribes nigrum* L. Grossulariaceae) fruits were gathered in July 2008 from

cultivated plants in the region of Csíkkarcfalva (Romania). A voucher specimen (No. 770) has been deposited in the herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Preparation of the extract: The collected fresh fruits were crushed with a fruit-grinder and after 48 h standing the juice was pressed. Because of the high pectin content, the fruit mass was stirred several times during the standing with the aim of facilitating effective squeezing. The pressed material, containing seeds and skin, was dried at room temperature, and the seed and skin of the fruits were separated by sifting. The dried skin was extracted with a 5-fold volume of water for 24 h at room temperature. After squeezing the plant material, the extract was then filtered and evaporated under reduced pressure to one-tenth of its volume.

Spray drying of the extract: The aqueous extract of black currant skin was spray-dried, with continuous mechanical stirring, using a Büchi B-191 Laboratory Spray-dryer (Büchi Co., Flawil, Switzerland) with a standard 0.7 mm nozzle. The particles were separated in the cyclone, with a high separation and recovery rate. Spray-drying was carried out under the following conditions: 10 L/min air flow, 5 bar pressure, and 3 mL/min pump flow rate. The inlet temperature was set to 110°C, and the outlet temperature varied in the range 80 ± 5°C. The final product was a dark pink colored fine powder, which was stored under controlled humidity at room temperature.

Reagents and solutions: Cyanidin-3-*O*-rutinoside chloride (keracyanin chloride CDX-00011325-005) was purchased from LGC Standard GmbH (Wesel, Germany). Chromatographic grade acetonitrile was purchased from Merck (Darmstadt, Germany) and water plus for HPLC from Carlo Erba (Rodano, Italy). The other chemicals were of analytical reagent grade and obtained from local firms. The extraction and all aqueous solutions were made up with deionized water. Formic acid (98-100%) was purchased from Molar Chemicals Kft (Budapest, Hungary).

Preparation of standard and sample solutions: Stock solution of cyanidin-3-*O*-rutinoside chloride was prepared by dissolving accurately weighted portions of the standards in 10% aqueous formic acid. The stock solution was diluted to make 1, 10, 20, 50, 70 and 100 μg/mL concentrations, and the calibration curve was determined with these concentrations. The spray-dried black currant extract was dissolved in 10% aqueous formic acid to yield 1% concentration. The same standard and test solutions were used for HPLC determination and spectrophotometry.

HPLC conditions: The HPLC analyses were performed on a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a SPD 10A/10 solvent delivery unit, SCL-10A system controller and SPD-10A UV-VIS detector. The samples were separated on LiChrospher 10 RP-18 (4 x 250 mm, 10 μm particle size; Merck) and the column temperature was maintained at 25°C. The analysis was performed using a gradient solvent system, consisting of solvent A: formic acid/H_2O 1:9 (v/v), and solvent B: acetonitrile (AcCN). Elution profile was: 0-0.5 min 1% B (v/v); 0.5-1 min linear gradient from 1 to 7% B (v/v); 1-4 min, linear gradient from 7-14% B (v/v); 4-9.5 min linear gradient from 14-20% B (v/v); 9.5-10.5 min linear gradient from 20-60% B (v/v); 10.5-14 min, linear gradient from 60-90% B (v/v); 14-18 min linear gradient from 90-100% B (v/v); 18-19 min column wash with 100% B. Post time: 11 min with 1% B (v/v). Detection was made at 520 nm. The flow rate throughout the chromatographic analysis was 1.00 mL/min, and the total run time was 30 min. The injection volume was 10 μL. The HPLC determination was carried out using cyanidin-3-*O*-rutinoside chloride as external standard.

Spectrophotometric determination: Total anthocyanin content was determined on a HELIOS Beta single-beam UV-VIS spectrophotometer (Thermo Spectronic) at 520 nm. Aqueous formic acid (10%) was used as the blank solution. The calibration and sample analyses were carried out in triplicate.

Cell culture: $HepG_2$ cells were purchased from American Tissue Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin and grown at 37°C under a humidified atmosphere containing 5% CO_2 in a humidified incubator. Cells were cultured at preconfluent densities by the use of 0.25% tryptin-EDTA solution (Millipore, Phillipsburg, NJ).

Cell proliferation assay: Cell proliferation was determined by the ability of $HepG_2$ cells to cleave tetrazolium salt to formazan. In brief, $HepG_2$ cells were seeded onto 96-well plates at a density of $4x10^3$ cells/well in 100 μL of the aforementioned medium. After 24 h of culture, the medium was removed carefully and replaced with 100 μL fresh medium, or medium with various concentrations of either black currant extract or pure compounds (delphinidin, cyanidin) for an additional 24 h. At the end of this time-point, 50 μL of MTT (5 mg/mL) was added to the wells containing 100 μL of media and cells were incubated for 4 h. Subsequently, the culture medium was removed

and the formazan crystals (produced by metabolically active cells) were dissolved by the addition of 100 μL of DMSO and 15 μL of glycine. The absorbance was measured at 570 nm with a SpectraMax 340 PC microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). All experiments were performed at least 3 times with 4 samples for each concentration.

Statistical analysis: Data are presented as an average value ± the standard error (SE) of the mean. The one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test was used for statistical analysis. A value of P less than 0.05 was considered as statistically significant.

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