JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Gastroprotective Effect of Red Pigments in Black Chokeberry Fruit (*Aronia melanocarpa* Elliot) on Acute Gastric Hemorrhagic Lesions in Rats

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It has been reported that the fruits and leaves of berries such as the blackberry, raspberry, and strawberry contain a high level of scavenging activity for chemically generated active oxygen species. This study investigated the antioxidative activities of black chokeberry fruit (*Aronia melanocarpa* Elliot) both in vitro and in vivo using the DPPH stable radical and rats with ethanol-induced gastric injury, respectively. The red pigment fraction of the black chokeberry contained three main components, one of which was identified as cyanidin 3-*O*-β-glucoside by HPLC analysis and ^{1}H NMR. The black chokeberry red pigment fraction scavenged >44% of DPPH radicals at a concentration of 25 μg/mL compared to the control solution. The black chokeberry extract and its hydrolysate administrated at 2 g/kg of body weight each had nearly the same protective effect as quercetin administrated at 100 mg/kg of body weight in suppressing the area of gastric mucosal damage caused by the subsequent application of ethanol to <30% compared to the control group. The black chokeberry red pigment fraction had a similarly significant protective effect on gastric mucosa in a dose-dependent manner when administered at 30−300 mg/kg of body weight, and the administration of 30 mg/kg of body weight could suppress ethanol-induced gastric mucosal damage by ~50% (ID_{50} = 30 mg/kg of body weight).

KEYWORDS: Black chokeberry; anthocyanin; acute gastric hemorrhagic lesions; antioxidant

INTRODUCTION

Polyphenol compounds are widely distributed in the plant kingdom, especially in fruits and vegetables, and their antioxidative properties have been reported in a great number of research works (*1*). Anthocyanin pigments are used as a colorant in foods. Therefore, those pigments are ingested from colored food stuffs as well as fruits and vegetables and are expected to play a role as dietary antioxidants in the prevention of oxidative damage from active oxygen species in living systems (*2*, *3*). Only a few studies concerning their antioxidative effects and behavior after ingestion, including absorption and metabolism, have been reported recently, mostly because of their instability to heat, acidity, oxidation, and other conditions (*4*, *5*).

It has been reported that the fruits and leaves of berries such as the blackberry, raspberry, and strawberry contain a high level of scavenging activity for chemically generated active oxygen species (*6*). The fruits of the black chokeberry (*Aronia melanocarpa* Elliot), a shrub of the Roseceae family native to North America and Russia, have a dark purple peel and contain high levels of polyphenol compounds (*7*). This paper deals with the antioxidative activities of black chokeberry fruit both in vitro and in vivo using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical and rats with ethanol-induced gastric damage, respectively.

MATERIALS AND METHODS

Chemicals. Quercetin was purchased from Wako Pure Chemical Industries Co. Ltd. (Tokyo, Japan). All other reagents and chemicals were commercially available extrapure grade products.

Preparation of Black Chokeberry Fruit Extract and Red Pigment Fraction. Black chokeberry (*A. melanocarpa* Elliot) fruit (75 g) grown at the Hokkaido Agricultural Experimental Station was extracted with a 9-fold volume of methanol containing 0.1% hydrochloric acid for 3 days at 4 °C. The filtrate of the extract was concentrated in vacuo, giving 7.5 g of dried crude extract. The dried extract was hydrolyzed with 1 N hydrochloric acid for 60 min and concentrated in vacuo. The hydrolysate was dissolved in water and loaded on a Dowex 50W-X2 (H^+, 1L) column, which was washed with water/methanol (1:1). The

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10.1021/jf034818q CCC: $27.50
Published on Web 03/27/2004

red pigment fraction was eluted with 0.1% hydrochloric acid in methanol from the column and concentrated in vacuo (3.7 g). The effluent and washed fraction (nonabsorbed substances) from the Dowex 50W-X2 (H^+) were combined and extracted with ethyl acetate. The ethyl acetate layer, which was expected to contain polyphenol compounds with antioxidative activity, was concentrated in vacuo (0.2 g). The red pigment fraction was analyzed on HPLC with Wakosil C18 (200 × 4.6 mm, 4 μm) monitoring the absorption at 525 nm. Solvent A, 1.5 mol of formic acid/L of water, and solvent B, 1.5 mol of formic acid/L of methanol, followed a linear gradient from 30 to 60% solvent B in 40 min with a flow rate of 0.8 mL/min at 30 °C. ^{1}H NMR spectra were measured with a Bruker AMX-500 spectrometer (500 MHz), and chemical shifts are expressed relative to the residual signals of 1% TFA-*d* in MeOH-d_4.

Radical Scavenging Activity. The radical scavenging activity of the test samples was measured according to the method of Uchiyama et al. with DPPH stable radicals (*8*, *9*). The reaction mixture (final volume = 5 mL) consisted of 1 mL of 0.1 mol of DPPH/L of ethanol, 2 mL of test sample dissolved at various concentrations in ethanol, and 2 mL of 0.1 mol of acetate buffer/L (pH 5.5). After incubation for 30 min at 25 °C, the remaining DPPH was determined by measurement of the absorption of the reaction mixture at 517 nm.

Animals. Totals of 18 and 36 rats were used in experiments 1 and 2, respectively. Seven-week-old male Wistar rats (LC, Shizuoka, Japan) were individually housed in stainless steel wire-mesh cages ~22 °C with a 12 h light cycle. The rats were allowed free access to tap water and a semipurified diet. All animals were fed the control diet composed of casein (250 g/kg of diet), mineral mixture (AIN 76G-MX, 35), vitamin mixture (AIN 76G-VX, 10), choline chloride (2.7), corn oil (50), and sucrose (652.3) for 7 days before the start of the experiment.

Induction of Gastric Lesions. Experiment 1 was performed to examine the antiulcerative effect of crude extracts of black chokeberry and its hydrolysate. Twenty-four rats were divided into four groups of six rats each, and groups were allowed ad libitum access to the control diet for 7 days followed by fasting for 48 h. The first group was a control and administered 10% polyethylene glycol 400 (10 mL/kg) by direct intubation. The second group was administered black chokeberry fruit extract dissolved in 10% polyethylene glycol (2 g/kg of body weight/10 mL). The hydrolysate of black chokeberry fruit extract was given to the third group. The fourth group was a positive control group and administered quercetin (100 mg/kg) dissolved in 10% polyethylene glycol. Experiment 2 was carried out to examine the antiulcerative effect of red pigment fraction and ethyl acetate extract of nonabsorbed substances described above. Thirty rats were divided into five groups of six rats each. The experimental procedure was the same as that of experiment 1. The first group was a control. The second to fourth groups were administered each of the red pigment fractions (30, 100, and 300 mg/kg of body weight, respectively) dissolved in 10% polyethylene glycol (10 mL/kg of body weight). The fifth group was administered ethyl acetate extract of nonabsorbed substances (100 mg/kg) dissolved in 10% polyethylene glycol. Thirty minutes after each administration, 99.5% ethanol (10 mL/kg of body weight) was administered also by direct stomach intubation. Rats were killed by exsanguination under anesthesia (Nembutal, 5 mg/100 g of body weight) 1 h after the ethanol administration, and the stomach was removed and the contents were drained. The stomach content was completely recovered by washing with 10 mL of isotonic saline. The contents and washing were combined and centrifuged (3500 rpm for 10 min). The pH value of the supernatant was measured. The excised stomach was fixed with 10 mL of 3.6% formalin solution and incised along the greater curvature. The area of the gastric ulcer was measured using NIH image and Photoshop software.

The study protocol was approved by the Hokkaido University Animal Use Committee, and animals were maintained under the guidelines for the care and use of laboratory animals at Hokkaido University.

Statistical Analysis. All data were analyzed by ANOVA and tested by Duncan's multiple-range test to determine whether differences between means were significant ($P < 0.05$). Values represent means ± SEM ($n = 6$), unless otherwise specified.

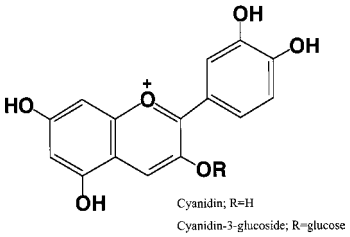

Figure 1. Structure of cyanidin glycosides.

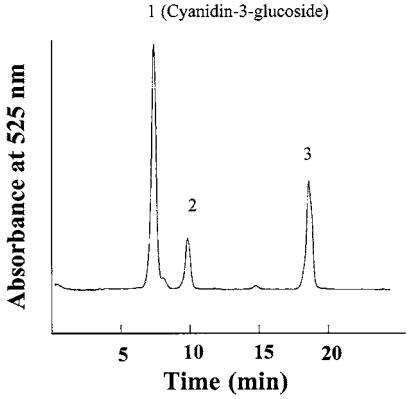

Figure 2. HPLC profile of the red pigment fraction of black chokeberry. The red pigment fraction gave three major peaks on HPLC with Wakosil C18 (200 × 4.6 mm, 4 μm) monitoring the absorption at 525 nm. Solvent A was 1.5 mol of formic acid/L of water and solvent B was 1.5 mol of formic acid/L of methanol, under a linear gradient from 30 to 60% solvent B in 40 min with flow rate of 0.8 mL/min at 30 °C.

RESULTS

Composition of Red Pigments in Black Chokeberry Fruit. The red pigment fraction gave three major peaks (at 525 nm) on HPLC, and one of them (major three peaks on HPLC of the red pigment fraction) had the same retention time as that of cyanidin 3-glucoside. The ^{1}H NMR spectrum of the fraction confirmed that the aglycon of the red pigments in black chokeberry fruit was cyanidin (**Figures 1** and **2**). ^{1}H NMR in 1% TFA-*d* in MeOH-d_4: 6.62 (d, 1H, $J = 1.96$ Hz), 6.87 (d, 1H, $J = 1.23$ Hz), 7.00 (d, 1H, $J = 8.61$ Hz), 8.11 (d, 1H, $J = 2.21$ Hz), 8.23 (dd, 1H, $J = 2.22, 8.73$ Hz), 8.58 (s, 1H).

Radical Scavenging Activity of the Black Chokeberry Red Pigment Fraction. The black chokeberry red pigment fraction could scavenge >44% of DPPH radicals at a concentration of 25 μg/mL compared to the control solution (**Figure 3**).

Protective Effect of the Black Chokeberry Extract and Its Red Pigment Fraction on Gastric Injury in Rats. The black chokeberry extract, its hydrolysate, and quercetin showed significant protective effects on gastric mucosal injury when they were given to rats before the administration of ethanol (**Figure 4**). The black chokeberry extract and its hydrolysate administered at 2 g/kg of body weight each had nearly the same protective effect as quercetin administered at 100 mg/kg of body weight in suppressing the area of gastric mucosal damage caused by the subsequent application of ethanol to <30% compared to the control group (**Figure 5**).

The black chokeberry red pigment fraction had a similarly significant protective effect on the gastric mucosa in a dose-dependent manner when administered at 30−300 mg/kg of body weight (**Figure 6**). Administration of 30 mg/kg of body weight could suppress the gastric mucosal damage caused by the subsequent application of ethanol by ~50% ($ID_{50} = 30$ mg/kg of body weight). The ID_{50} of the effluent fraction from the Dowex 50W-X2 (H^+) was 100 mg/kg of body weight.

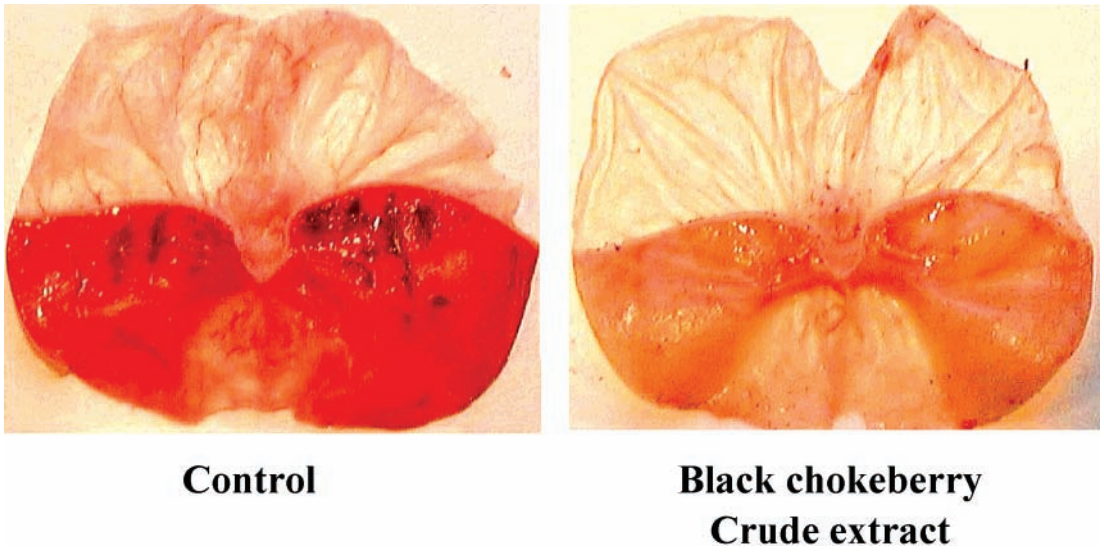

Figure 3. Scavenging effects of the black chokeberry red pigments on DPPH radicals. The reaction mixture containing DPPH in ethanol and sample was incubated at 25 °C for 30 min, and remaining DPPH was measured at 517 nm.

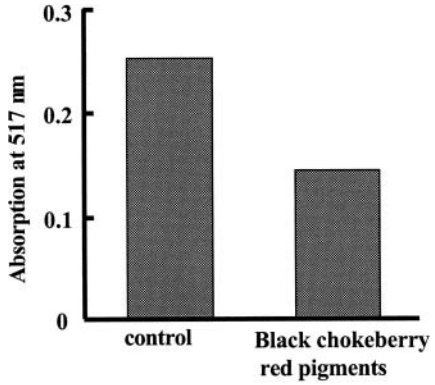

Figure 4. Protection effect of black chokeberry crude extract and its hydrolysate on gastric damage induced by ethanol in rats.

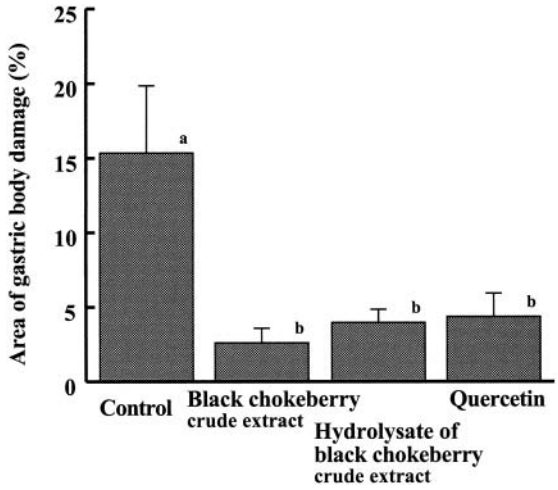

Figure 5. Prevention of black chokeberry crude extract and hydrolysate of black chokeberry crude extract on gastric damage induced by ethanol in rats. Each sample (2 g/kg of body weight of black chokeberry crude extract and hydrolysate of black chokeberry crude extract, 100 mg/kg of body weight of quercetin) was administered orally 30 min before oral administration of ethanol (10 mL/kg of body weight). Rats were killed 1 h after the administration of ethanol. Each sample shows the mean ± SEM for six animals (significant difference from the control at $P < 0.05$).

It was shown that the red pigment fraction did not inhibit gastric acid secretion, because the intragastric pH of rats administered the red pigment fraction was lowered in a dose-dependent manner (**Table 1**).

DISCUSSION

It was shown in this study that the red pigment fraction of black chokeberry fruit has a potent antioxidative activity in both in vitro and in vivo systems. The red pigment fraction contained three main components, which were identified as cyanidin derivatives (*10*−*12*). The red pigment fraction composed of cyanidin derivatives was an effective scavenger of DPPH radical. Polyphenol compounds are well-known as effective antioxidants. It was observed that 2 g of the black chokeberry fruit extract per kilogram of body weight and 300 mg of its red pigment fraction had nearly the same antiulcerative activities (**Figures 4**−**6**). Because the antiulcerative activities of the extract and its red pigment fraction were determined in separate experiments, these data cannot be compared directly. However, it was suggested that nearly all of the antiulcerative activity of the extract of the black chokeberry fruit could be attributed to its red pigment fraction composed of cyanidin derivatives. The ethyl acetate extract of the effluent from the Dowex 50W-X2 (H^+), which was expected to contain polyphenol compounds, was small, and its antiulcerative effect was less than one-third of that of the red pigment fraction. Although the components of the ethyl acetate extract were not analyzed, polyphenol compounds other than anthocyanins in the black chokeberry fruit cannot be expected to play a significant role in antiulcerative activity. In this study, gastric mucosal injury was induced by the intubation of ethanol into the stomach of rats, which is known to cause the contraction and the extension of blood vessels in the stomach wall and induce the production of active oxygen species (*13*, *14*). The active oxygen thus formed induces the ulcerative damage and hemorrhaging in the stomach lining. It seems likely that one of the mechanisms by which the extract of the black chokeberry fruit suppresses the development of gastric mucosal damage is the scavenging of the active oxygen by its red pigment fraction.

It has been reported that one of the antiulcerative mechanisms is the suppression of gastric acid secretion (*15*). However, the black chokeberry red pigment fraction does not contribute to the suppression of gastric acid secretion.

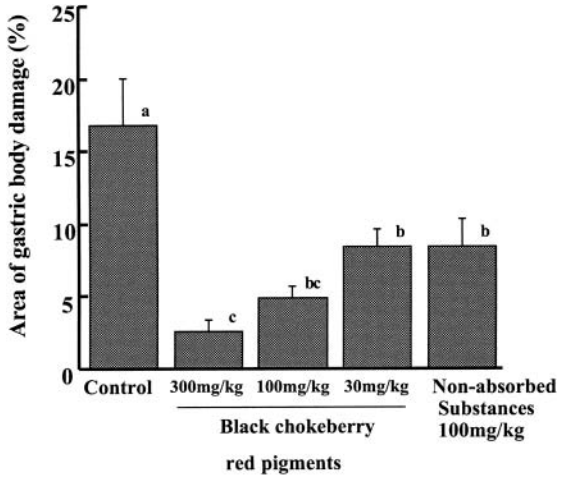

Figure 6. Prevention of gastric damage induced by ethanol in rats with black chokeberry. Each sample (30, 100, or 300 mg of black chokeberry red pigment/kg of body weight and 100 mg of nonabsorbed substances/kg of body weight) was administered orally 30 min before oral administration of ethanol (10 mL/kg of body weight). Rats were killed 1 h after the administration of ethanol, and the stomach was removed to collect the contents. Each value is the mean ± SEM for six animals (significant difference from the control at $P < 0.05$).

Table 1. Effect of Black Chokeberry Red Pigments Given Orally on Gastric Acid Secretion in Rats[a]

sample	pH
control	6.73 ± 0.23 a
red pigment, 30 mg/kg	6.46 ± 0.32 a
red pigment, 100 mg/kg	5.82 ± 0.62 a
red pigment, 300 mg/kg	3.84 ± 0.07 b
nonabsorbed substance, 100 mg/kg	6.07 ± 0.50 a

[a] Each sample (30, 100, or 300 mg of black chokeberry red pigment/kg of body weight and 100 mg of nonabsorbed substances/kg of body weight) was administered orally 30 min before oral administration of ethanol (10 mL/kg of body weight). Rats were killed 1 h after the administration of ethanol, and the stomach was removed to collect the contents. Each sample is the mean ± SEM for six animals (significant difference from the control at $P < 0.05$).

It is possible that the antioxidative activity of the black chokeberry fruit in vivo is not attributable to one mechanism but is expressed by a complex series of mechanisms. It is necessary to elucidate the mechanism of the suppression of gastric mucosal damage by black chokeberry fruit in further detail.

ACKNOWLEDGMENT

We are grateful to Dr. Gensho Ishii, the Hokkaido Agricultural Experimental Station (Sapporo, Japan), and Tsuneo Tanaka, the Hokkaido Food Processing Research Center (Ebestu, Japan), for supplying the black chokeberry fruit.

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Received for review July 23, 2003. Revised manuscript received February 10, 2004. Accepted February 13, 2004.

JF034818Q