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Antiulcer Activity of Anthocyanins from *Rubus coreanus* via Association with Regulation of the Activity of Matrix Metalloproteinase-2

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ABSTRACT: Anthocyanins were extracted from the fruits of *Rubus coreanus*. Whether their antioxidant properties and antiulcer activity in gastric ulceration have been accompanied by the activation of matrix metalloproteainse-2 (MMP-2) was investigated. To assess the effect of anthocyanins on gastric ulcer, the rats were administered with anthocyanins (20, 50, and 80 mg/kg of body weight) before treatment with naproxen (80 mg/kg of body weight) to induce gastric ulceration. Lipid peroxidation and the activities of radical scavenging enzymes such as catalase, superoxide dismutase, and glutathione peroxidase were determined. The MMP-2 level was tested by zymography and Western blot. Anthocyanins of *R. coreanus* exhibit possible antiulcer activity in acute ulcer in a rat model by preventing lipid peroxidation and a significant increase in the activities of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase. Also, anthocyanins induce activation of MMP-2 and attenuate the activity of the proinflammatory molecules, such as tumor necrosis factor-α and interleukin-1β.

KEYWORDS: Anthocyanins, gastric ulcer, matrix metalloproteinase-2, reactive oxygen species

INTRODUCTION

Many nonsteroidal anti-inflammatory drugs (NSAIDs), including naproxen, are widely used clinically.[1] Naproxen is a noncorticosteroid drug with anti-inflammatory, antipyretic, and pain-relieving properties. However, side effects of the drug include erosions, antral ulceration, and petechial bleeding in the stomach mucosa.[2] The gastrointestinal damage involves a number of mechanisms including inhibition of prostaglandin synthesis, generation of reactive oxygen species (ROS), and induction of apoptosis.[3]

Antiulcer medications aim to protect against the various causes of gastric damage. The medications greatly attenuate the formation of gastric lesions induced by ethanol, stress, and NSAIDs and ischemia reperfusion.[4,5] The protective mechanisms by antioxidant materials have been attributed to the scavenging of ROS. Lipid peroxidation and hydroxyl radical damage are attenuated through the protection of gastric mucosa and reactive oxygen intermediate-mediated cytotoxicity.[6]

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that are capable of digesting extracellular matrix (ECM) and basement membrane[7] and are a major factor in gastric ulcer formation. In particular, the gelatinase family consists of pro-MMP-2 (gelatinase A, 72 kDa) and pro-MMP-9 (gelatinase B, 92 kDa), which are the major structural components of the basement membrane. These enzymes are involved in numerous physiological processes including embryogenesis, wound healing, cancer metastasis, angiogenesis, arthritis, and inflammatory diseases. MMP-2 participates in the physiological turnover of the gastric ECM, while MMP-9 is important in the early phase of chronic gastric ulcers.[8]

Rubus coreanus is a deciduous leaf shrub that belongs to the family *Rosaceae*. Its distribution is limited to southeast Asian countries.[9] The fruits of *R. coreanus* are traditionally used as a treatment for spermatorrhea, enuresis, asthma, and allergic diseases.[10] The effect of the fruits of *R. coreanus* may be related with various bioactive phytochemicals such as phenolic acids, organic acids, terpenosides, tannins, and flavonoids.[11] Flavonoids are gastroprotective due to their antioxidant properties.[12] The aim of the present study was to investigate the potential effects of anthocyanins from *R. coreanus* on gastric ulceration in rats by determining their effect on activation of MMP-2 via scavenging of ROS.

MATERIALS AND METHODS

Preparation of Anthocyanins from *R. coreanus*. Ripe *R. coreanus* fruit was hand-harvested in Gochang, Korea, and stored at −20 °C until use. This plant was identified and authenticated by professor Ki-Joong Kim, College of Life Sciences and Biotechnology, Korea University. A voucher specimen (2011-0394) of the plant was deposited at the herbarium of Korea University. Ten grams of the fruit was pulverized and blended with 100 mL of 60% (v/v) methanol containing 0.1% trifluoroacetic acid (TFA) for 24 h. The extract was

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filtered through Whatman filter paper (no. 4), and the solvent was removed under vacuum using an evaporator at 40 °C. For further purification, the methanol extracts was dissolved in 100 mL of purified water and applied to a column packed with Amberlite XAD7 resin.[13,14] The isolated anthocyanins, which were used for subsequent studies, were initially analyzed with an ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA). UPLC separation was achieved using a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size) maintained at 35 °C, with a flow rate of 400 μL/min. Mass spectrometry (MS) was performed using a Waters Q-TOF mass spectrometer. The instrument was operated using an electrospray source in positive ion mode using a scan spectra from 100 to 600 m/z. The ionization source parameters were as follows: capillary voltage, 2.8 kV; source temperature, 100 °C; desolvation gas temperature, 250 °C; and cone voltage, 35 V. To determine the anthocyanin composition of *R. coreanus* quantitatively, high-performance liquid chromatography (HPLC) analysis was pursed with a system (P680, Dionex, United States) equipped with an Xterra analytic column (4.6 mm × 250 mm). The flow rate was 0.8 mL/min, and a linear gradient elution mode was applied with two eluents of 0.1%TFA (eluent A) and acetonitrile:water = 1:1 (v/v) 0.1% TFA (eluent B). The proportion of eluent B increased from 20 to 24% for the first 10 min, then went up to 25% for the next 40 min, and finally reached 40% for the last 10 min. The peaks were detected at 520 nm using a UV−vis detector (UVD170U, Dionex). Each anthocyanin peak was quantified using the standard curves fitted with anthocyanin compounds purified by the method reported previously.[15]

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity. The free radical scavenging activities of the anthocyanins were measured by a DPPH free radical scavenging assay.[16] The method involved the reaction of the antioxidant with the stable DPPH in 95% ethanol. Briefly, a 60 μM DPPH solution was freshly made in 95% ethanol. Anthocyanins (200 μL) were reacted with 3.8 mL of DPPH solution for 30 min. The absorbance at 515 nm was measured against a blank of pure 95% ethanol. The antioxidant activity was calculated as: % DPPH scavenging activity = $[(A_0 - A_t/A_0) \times 100]$, where A_0 = the absorbance of the control and A_t = the absorbance in the presence of anthocyanins.

Reducing Power. The reduction capability of anthocyanins was determined as previously described.[17] The anthocyanins in distilled water (1 mL containing 10, 25, 50, or 100 μg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide $[K_3Fe(CN)_6]$. The reaction mixture was incubated for 20 min at 50 °C. TFA (10%, 2.5 mL) was added to the mixture and centrifuged for 10 min at 1000*g*. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Cell Culture and Assessment of Cellular Oxidative Stress. Human gastric epithelial cell line AGS was maintained in RPMI 1640 (Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum. Cellular oxidative stress was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich, St. Louis, MO) cell-permeable fluorescent probes with fluorescence-activated cell sorting (FACS) analysis. Cells were treated with the particular anthocyanins for the indicated time, the medium was aspirated, and the cells were incubated in warm phosphate-buffered saline (PBS) containing 6 μM H_2DCFDA at 37 °C for 30 min. The PBS containing the probe was removed, and warm medium was added for 10 m in. The cells were collected and resuspended in cold PBS. All samples were analyzed on a BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) with a laser excitation wavelength of 488 nm.

Naproxen-Induced Gastric Ulceration and Preparation of Anthocyanins. Procedures involving animals were in accordance with the Guide for Experimental Animal Research from Institutional Animal Care and Use Committee at Korea University (KLG 08-010). Sprague−Dawley rats (180−220 g) were purchased from Orient Bio (Seoul, Korea). To evaluate the potential protective effects of the anthocyanin extracts, rats were divided into five groups ($n = 5$ per group). Normal rats received only vehicle (distilled water) twice daily for 3 days by oral route. Control rats received 80 mg/kg of naproxen twice daily for 3 days. The experimental groups were pretreated with 20, 50, or 80 mg/kg of anthocyanins twice daily for 3 days, followed by 80 mg/kg of naproxen twice daily for 3 days. The rats were euthanized under deep ether anesthesia 12 h after the final naproxen treatment. Ulceration was judged macroscopically by clear depth of penetration into the gastric mucosal surface.[2]

Measurement of Lipid Peroxidation. Lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) equivalents according to a modified method.[18] Stomach tissues of rats were homogenized with 3.86% perchloric acid and centrifuged for 1 min at 13800 rpm. The supernatant fraction was mixed with 20 mM 2-thiobarbituric acid (TBA, 1:1 v/v) and heated in boiling water for 30 min. The absorbance was determined at 531 nm. The TBA values were expressed as milligrams MDA equivalents per kilogram tissue and were derived from the standard curve. 1,1,3,3-Tetraethoxypropane (TEP) in distilled water was used for the standard curve instead of supernatant.

Measurement of Superoxide Dismutase (SOD) Activity. Stomach tissue SOD activity was measured by using a commercially available kit (Cayman Chemical, Ann Arbor, MI) as previously described.[19] One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Tissues were homogenized and centrifuged at 1500*g* for 5 min at 4 °C. The supernatant was processed for total SOD activity assay according to the protocol provided by the assay kit manufacturer. The absorbance was read at 450 nm. The SOD activity was expressed as U/mg protein. Protein was determined using the Bradford protein assay.

Measurement of Catalase Activity. The catalase activity in tissues of rats was measured using a commercially available kit (Biomedical Research Service Center, Buffalo, NY). Catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) into oxygen and water. The colorimetric catalase assay kit is based on oxidation by H_2O_2 of an azo chromogen with an accompanying increase in optical density at 410−420 nm. The presence of catalase reduces the amount of H_2O_2, inhibiting the oxidation of azo chromogen and the appearance of the yellowish color. The results were expressed as units/mg protein.

Measurement of Glutathione Peroxidase Activity. Glutathione peroxidase activity in the gastric mucosa of rats was determined as previously described,[20] with some modifications. The reaction mixture consisted of glutathione peroxidase assay buffer and the NADPH assay reagent. A sample of the supernatant fluid mixed with the homogenate solution and 50 mM potassium phosphate buffer (pH 7.5) was prepared by centrifugation at 1000*g* for 10 min at 4 °C. Glutathione peroxidase assay buffer (900 μL), 50 μL of NADPH assay reagent, and 50 μL of sample were added to the cuvette and mixed by inversion. The reaction was initiated by adding 10 μL of 30 mM *tert*-butyl hydroperoxide or 80% cumene hydroperoxide, and absorbance was measured at 340 nm. The enzymatic activity was the sum of the activity measured using *tert*-butyl hydroperoxide and cumene hydroperoxide.

Histology. The stomach was exposed following a midline laparotomy and opened with an incision along the greater curvature. It was pinned over a plexiglass platform and clamped with a plexiglass cylinder. Then, these stomach tissues were fixed in 10% neutral formalin and embedded in paraffin, and 7 μm thick sections were prepared and stained with hematoxylin and eosin as previously described.[2]

Tissue Extraction and Gelatin Zymography. Each stomach was washed with PBS (pH 7.4), minced, and incubated for 10 min at 4 °C. After centrifugation at 12000*g* for 15 min, the supernatant was

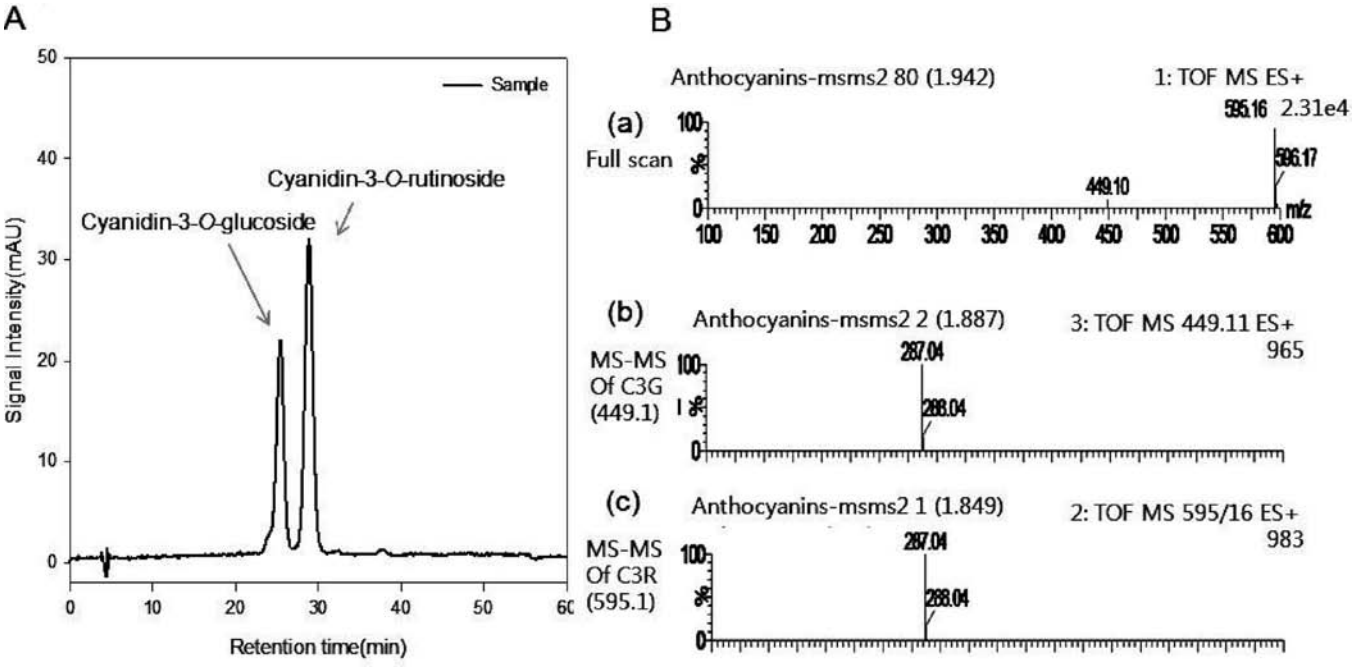

Figure 1. Characterization of anthocyanin compounds in *R. coreanus*. (A) HPLC analysis was used to separate anthocyanins in *R. coreanus* into two major compounds. (B) UPLC QTOF-MS/MS analysis was used to confirm anthocyanins in two major mass fragmentation ions. It consists of cyanidin-3-glucoside (m/z 449.1) and cyanidin-3-rutinoside (m/z 595).

saved. The pellet was then extracted in lysis buffer (10 mM Tris−HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) and centrifuged at 12000*g* for 15 min to obtain extract. For assay of MMP-2 activity, the extracts were processed by 8% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/mL gelatin under nonreducing conditions. The gels were washed in 2.5% Triton X-100 and incubated in TNC buffer (40 mN Tris−HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 0.05% Brij35, 0.02% NaN_3, and 2% dimethylsulfoxide) for 24 h at 37 °C and stained with 0.1% Coomassie blue followed by destaining.[21]

Western Blotting. Proteins were resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk solution in 20 mM Tris−HCl, pH 7.4, 150 mM NaCl, and 0.02 Tween 20 (TBST) for 1 h at room temperature and then incubated at 4 °C in a 1:1000 dilution of MMP-2 antibody in TBST overnight. The membranes were then washed with TBST and incubated with horse radish peroxidase-conjugated secondary antibody, and bands were visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA).

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Total cellular RNA was isolated from ulcer stomach tissues by RNA Iso-plus reagent (TaKaRa Bio, Shiga, Japan). Total RNA was reverse-transcribed into cDNA. PCR amplification of the cDNA was performed using a thermal cycler (Bio-Rad Laboratories, Hercules, CA). The sequences of PCR primers were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense (5′-TGG GGT GAT GCT GGT GCT GAG-3′) and antisense (5′-GTT TTC TCC AGG CGG CAT GTC-3′); MMP-2 sense (5′-ACG GCT TCC TCT GGT GTT-3′) and antisense (5′-CGT AGT TGG TTG TGG TTG C-3′); tumor necrosis factor-α (TNF-α) (5′-CGC CCA GCC TTC CTT ACG GAA C-3′) and antisense (5′-GGC GAT TAC AGT CAC GGC TCC C-3′); interleukin-1β (IL-1β) sense (5′-GCT ACC TAT GTC TTG AAG AGA ACC-3′) and antisense (5′-GAC CAT TGC TGT TTC CTA GG-3′). After amplification, the products were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide.

Table 1. Quantification of Anthocyanin Components in the Purified Extract of *R. coreanus*

anthocyanin components	concn (mg/mL)
cyanidin-3-*O*-glucoside	0.48 ± 0.02
cyanidin-3-*O*-rutinoside	0.72 ± 0.03

Northern Blotting. RNA was electrophoresed on a 1% agarose gel containing 6.3% formaldehyde in buffer, transferred overnight to a nylon membrane (Hybond-N), and cross-linked using an ultraviolet cross-linker. A cDNA fragment of MMP-2 was obtained by RT-PCR from rat stomach tissues. The cDNA probe was labeled with α-^{32}P-dCTP using the Ladderman labeling kit (TaKaRa Bio). The membrane was prehybridized and hybridized at 65 °C in 7% SDS, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in 2× SSC and 0.1% SDS at room temperature, and twice in 2× SSC and 0.1% SDS at 65 °C. The autoradiogram was quantified using a Bioimage BAS 1800 analyzer (Fuji Film, Tokyo, Japan).

Statistical Analysis. Each experiment was repeated at least three times. Data were plotted as means ± standard deviations (SDs). The statistical significance of the assay was evaluated using SPSS software (SPSS, Chicago, IL). A $P < 0.05$ was considered significant.

RESULTS

Isolation of Anthocyanins from *R. coreanus*. Analysis of the anthocyanins with HPLC and UPLC coupled with quantitative time-of-flight-tandem mass spectrometry confirmed the structure. The anthocyanins had two major picks (Figure 1). Then, we performed to confirm these substances by MS/MS. One was cyanidin-3-glucoside (cyanidin-3-glucoside, $C_{21}H_{21}O_{11}$, M + 1), and the other was cyanidin-3-rutinoside (cyanidin-3-rutinoside, $C_{21}H_{21}O_{11}$, M + 1) of individual anthocyanin in the positive mode. The detection of ions at m/z 449 and 595 suggested that

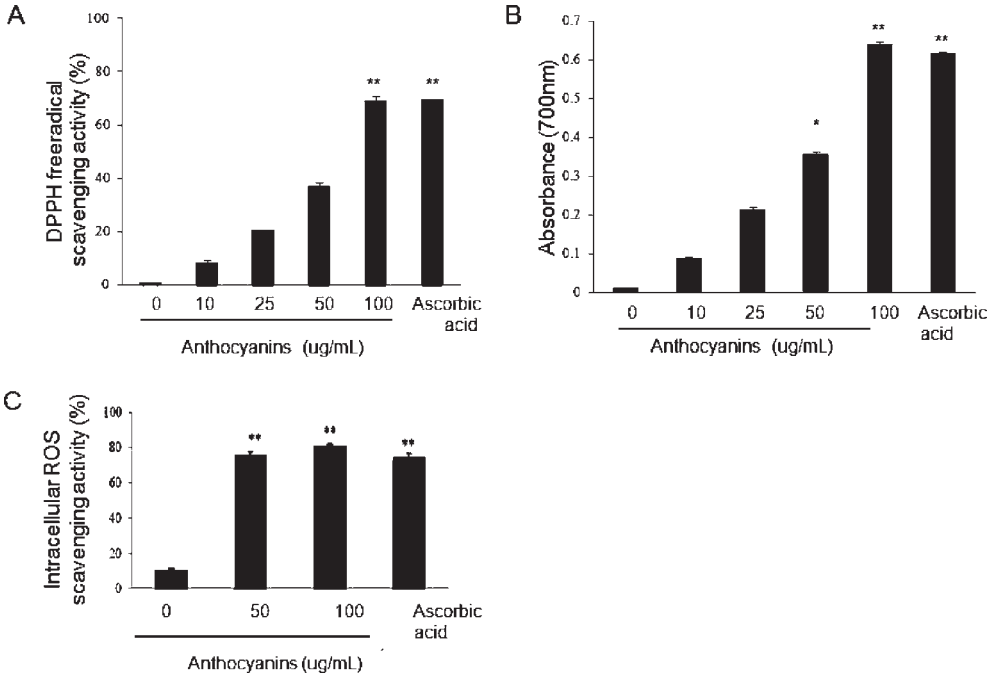

Figure 2. Antioxidant activity of anthocyanins from *R. coreanus*. (A) DPPH free radical scavenging activity of the anthocyanins and ascorbic acid. (B) Reducing power of the anthocyanins and ascorbic acid. (C) Anthocyanins dose-dependent suppression of the generation of intracellular ROS. Results are reported as the means ± SDs ($n = 3$). The statistical significance of the assay was evaluated using the *t* test (*, $P < 0.05$; **, $P < 0.01$ as compared with normal groups).

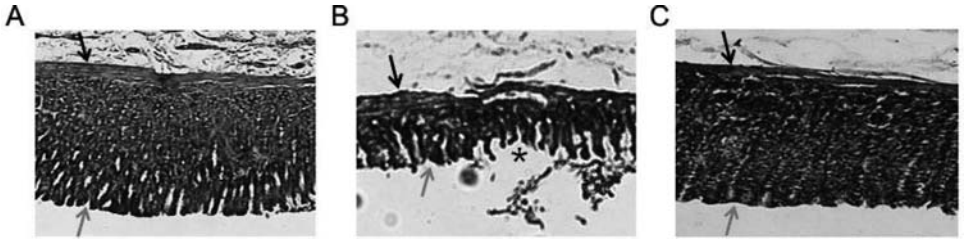

Figure 3. Histological analysis of rat gastric tissue after ulcer induction by naproxen and gastroprotection by anthocaynins from *R. coreanus*. Histological sections were stained with H&E, and photographs were taken at 40× magnification. (A) Normal gastric antrum from vehicle-treated normal rats. (B) Gastric antral ulcer in the naproxen-treated rats. (C) Gastric antrum in the anthocyanin pretreated rats. Gastric mucosa epithelium (↑), muscularis mucosae (↑), and disruption of gastric mucosa (*) are indicated.

the aglycone was cyanidin (m/z 287).[22] From the HPLC analysis, it was clearly shown that the mass ration of cyanidin-3-glucoside and cyanidin-3-rutinoside was 1:1.5 (w/w) in the purified anthocyanin (Table 1).

Antioxidant Activities of the Anthocyanins. In the DPPH free radical scavenging assay, the anthocyanins exhibited activities of 8.46, 20.47, 37.31, and 69.17% at 10, 25, 50, and 100 μg/mL, respectively. The anthocyanins displayed a similar scavenging activity with ascorbic acid (Figure 2A). The reducing power of the anthocyanins was also measured. The reducing power of anthocyanins and ascorbic acid increased in a concentration-dependent manner (Figure 2B). Especially, at 25 and 100 μg/mL, the anthocyanins displayed a higher reducing power than ascorbic acid.

Anthocyanins Reduce Intracellular Oxidative Stress. Having established that the anthocyanins from *R. coreanus* possessed potent radical scavenging and reducing capabilities (Figure 2), the next experiment directly examined the possibility that these anthocyanins might reduce cellular oxidative stress in AGS human gastric adenocarcinoma cells. The anthocyanins significantly down-regulated intracellular oxidative stress in a dose-dependent manner (76.1% at 50 μg/mL and 81.1% at 100 μg/mL; Figure 2C). Especially, these doses produced a greater reduction of intracellular oxidative stress than ascorbic acid.

Antiulcer Effect of the Anthocyanins in Naproxen-Induced Gastric Ulceration in Rats. The effect of the anthocyanins on naproxen-induced gastric antral ulceration in rats was examined. Superficial or deep erosions, bleeding, and antral ulcers were observed in rats receiving 80 mg/kg naproxen for 3 days. However, pretreatment with anthocyanins for 3 days reduced the depth and severity of the naproxen-induced gastric antral ulcers (Figure 3). Evidence of the ulcers was attenuated in stomach tissues pretreated with the anthocyanins, consistent with an anthocyanin-mediated induction of mucosal layer recovery.

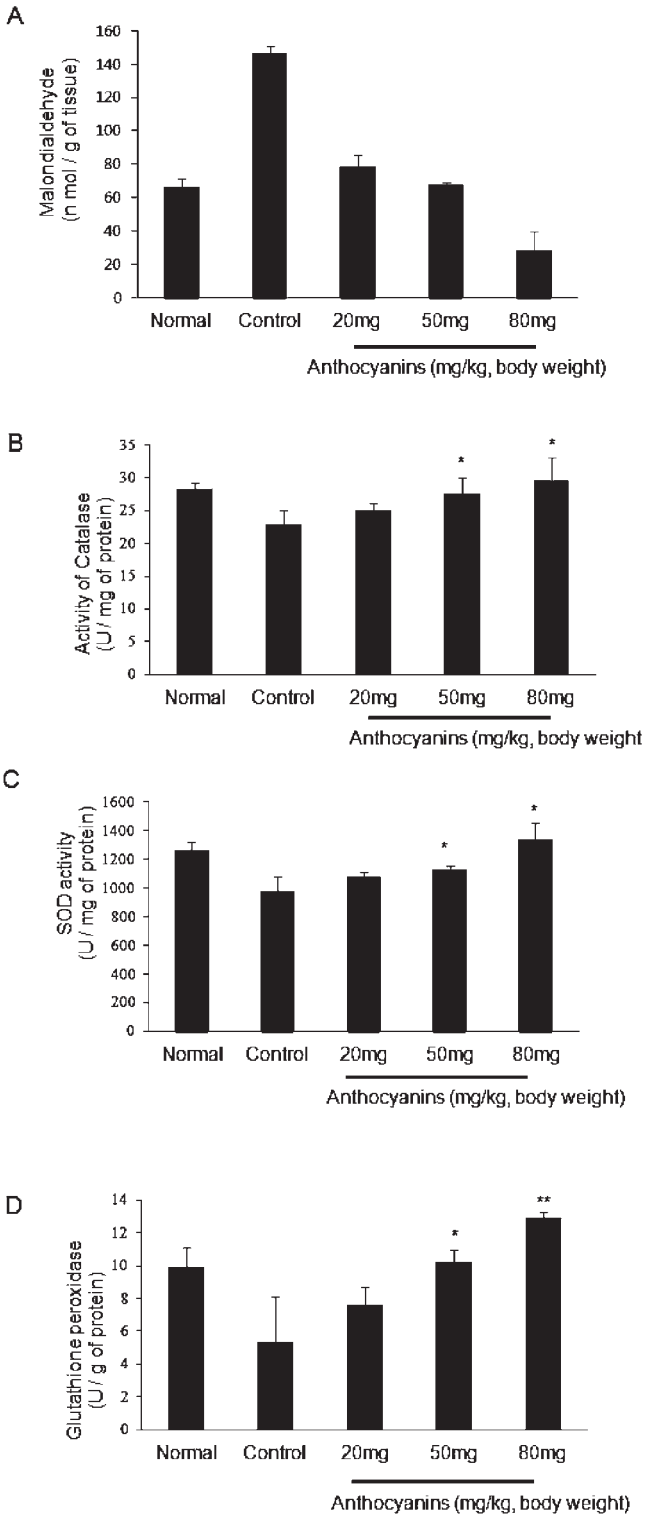

Figure 4. Effect of anthocyanins on naproxen-induced oxidative damage and antioxidant enzyme activity. Rats were treated with anthocyanins (20, 50, and 80 mg/kg twice daily for 3 days) and with 80 mg/kg of naproxen twice daily for 3 days. Control rats received only 80 mg/kg naproxen for 3 days. (A) Lipid peroxidase, (B) catalase, (C) SOD, and (D) glutathione peroxidase. Results are reported as the mean ± SDs (n = 5). The statistical significance of the assay was evaluated using the t test (*, $P < 0.05$; **, $P < 0.01$ as compared with control rats).

Anthocyanins Mediate a Reduction in Oxidative Gastric Damage and Induce Radical Scavenging Enzymes in Naproxen-Induced Ulcer Rats. In rats in which ulceration had been induced (control rats), the concentration of MDA equivalents in the stomach was 146.54 nmol/g of tissue, whereas the concentration of MDA equivalents in the stomach of normal rats remained at 66.27 nmol/g tissue. The increase in the concentration of MDA equivalents was reduced in a dose-dependent manner in the rats pretreated with the *R. coreanus* anthocyanins. Especially, pretreatment with 80 mg/kg of the anthocyanins for 3 days produced a significant reduction of MDA equivalents concentrations in stomach tissues as compared with the ulcerated rats (Figure 4A). The catalase activity in the stomach tissue of rats with induced ulceration was also reduced to 22.83 U/mg of protein, whereas the catalase activity in the stomach tissue of normal rats was 28.35 U/mg of protein. Pretreatment with 80 mg/kg of anthocyanins for 3 days significantly increased catalase activity as compared to that in the ulcerated rats (Figure 4B). The SOD activities in the ulcerated rats were reduced to 980.82 U/mg of protein, whereas the activities of SOD in the normal rats were 1266.02 U/mg of protein. However, oral administration of the anthocyanins (20, 50, and 80 mg/kg body weight) for 3 days increased SOD activity in a dose-dependent manner. Especially, the 80 mg/kg pretreatment significantly increased SOD activity as compared to that in the ulcerated rats (Figure 4C). The activity of glutathione peroxidase in the stomach tissue of ulcerated rats was reduced to 5.33 nmol/min/mg protein, whereas the activity of glutathione peroxidase in the stomach tissue of normal rats was 9.96 nmol/min/mg protein. Pretreatment with 80 mg/kg of anthocyanins for 3 days significantly increased the glutathione peroxidase activity as compared to that in the ulcerated rats (Figure 4D).

Anthocyanins Show an Antiulcer Effect Because of a Modulation of MMP-2. The foregoing data were consistent with the notion that pretreatment with anthocyanins was highly effective in inhibiting naproxen-induced gastric ulcer formation. Appropriately, an experiment was performed to measure MMP-2 activity in gastric tissue extracts from different pretreatment concentrations of anthocyanins (20, 50, and 80 mg/kg body weight) in rats. The anthocyanins dose dependently blocked naproxen-induced gastric ulcer formation and the down-regulation of MMP-2 activity. Naproxen exhibited the opposite effect, reducing the activity of MMP-2. Pretreatment with the anthocyanins caused MMP-2 expression to be marginally up-regulated (Figure 5). To evaluate the relationship between oxidative gastric damage and expression of MMP-2 genes, naproxen-induced ulceration in the absence and presence of pretreatment with the anthocyanins was assessed by Northern blotting. As expected, the anthocyanins that have antioxidant and antiulcer activities inhibited the suppression of MMP-2 expression with naproxen (Figure 6A).

Inhibition of Proinflammatory Cytokine and MMP-2 Gene Expression by Anthocyanins. The expression of MMPs is regulated at different levels by ROS and cytokine-mediated pathways.[23] To investigate the relationship of MMP-2 expression and proinflammatory cytokines in naproxen-induced gastric ulcers, the mRNA expression level was determined by RT-PCR. It shows a decrease in MMP-2 mRNA level in naproxen-treated gastric tissues, while anthocyanins pretreatment induced the expression of MMP-2 (Figure 6B). In the case of proinflammatory cytokines (such as TNF-α and IL-1β), It was indicated that naproxen induced mRNA expression of TNF-α and IL-1β. On the other hand, pretreatment with anthocyanins inhibited increasing mRNA expression (Figure 6C).

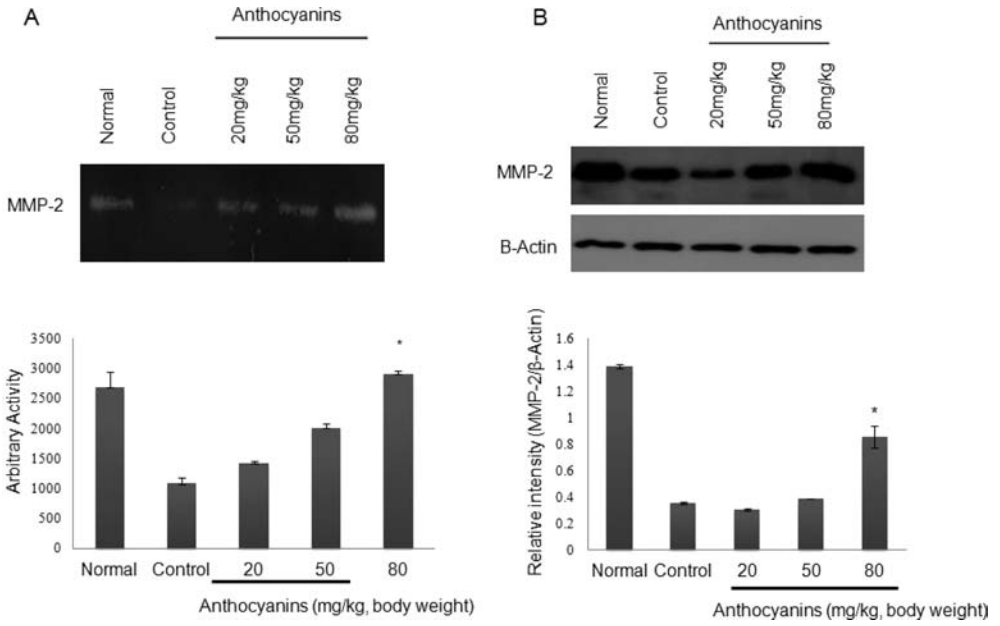

Figure 5. Effect of anhocyanins from *R. coreanus* on MMP-2 during the prevention of gastric ulcer. (A) The MMP-2 profile was monitored through gelatin zymogram loading equal amount of extracts from gastric tissue of each group of rats. (B) Western blotting of MMP-2 and β-actin in control, naproxen treated, and anthocyanins from *R. coreanus* pretreated naproxen-treated rat groups.

DISCUSSION

Various factors are thought to be involved in the formation of NSAIDs-induced gastric ulcers. The mechanisms of the damage and the interactions among the various factors remain poorly understood. The present study provides the evidence of antiulcer effects. Anthocyanins inhibition NSAIDs induced oxidative gastric damage is accompanied by the activation of MMP-2.

Natural pigments have recently a lot of useful functions as potential chemopreventive and chemotherapeutic agents. Among them, we focused on anthocyanins from *R. coreanus*. Anthocyanin is a well-known natural pigment in various materials. It has been widely investigated because of its antioxidant activity. So, we confirmed anthocyanin by HPLC and UPLC-MS/MS analysis (Figure 1), which it composed major two compounds such as cyanidin-3-glucoside and cyanidin-3-rutinoside. Anthocyanins from *R. coreanus* displayed pronounced radical scavenging activity and reducing power and down-regulation of ROS (Figure 2). The death of cells is induced by excessive production of ROS. So, our results might show that removal of ROS by anthocyanins have attenuated on cellular damage.

NSAIDs induce severe gastric mucosal damage by interfering with prostaglandin (PG) synthesis by blocking cyclooxygenase-1 and -2.[24] Inhibition of PG secretion is not the only significant pathogenic factor for mucosal damage.[1,25] Also, NSAIDs (naproxen, indomethacin, and other agents) cause the generation of the ROS that play a crucial role in the mucosal damage.[26,27] Imbalance of ROS may influence gastric damage.[28] Antioxidant substances show antiulcer activity through scavenging of radicals and blockage of apoptotic cell death.[29] Interestingly, the present data indicate that anthocyanins from *R. coreanus* show gastroprotective effect on naproxen-induced gastric antral ulcer by preventing from oxidative damage through removing lipid peroxides and free radicals induced by naproxen and by an increase in the activities of radical scavenging enzymes. Histological observations revealed that anthocyanins could completely recover the damage of gastric mucosal layer.

High levels of mRNA to various MMPs are detected in various human gastric diseases including ulcers and Crohn's disease.[30] The present study investigated the hypothesis that naproxen induces gastric ulceration in association with ROS and MMPs. Naproxen at a dose of 80 mg/kg body weight maximally induced gastric ulceration. Naproxen also increased lipid peroxidation by creating oxidative damage and decreasing the activity of radical scavenging enzymes. Also, the activity of MMP-2 was reduced by naproxen exposure in gastric tissues. These data are entirely consistent with an association between oxidative damage and activity of MMP-2. Moreover, the present study explains the antiulcer activity of anthocyanins from *R. coreanus* and their association with regulation of MMP-2 activity and expression of proinflammatory cytokines through the radical scavenging activity. Especially, MMP-2 is involved in ulceration as well as in ECM remodeling.[24] Presently, during gastroprotection, anthocyanins induced the up-regulation of MMP-2 activity. Figures 5 and 6 show that naproxen induced gastric ulcers; altered activation of MMP-2 was evident in naproxen-treated rats. Otherwise, the protective effect of anthocyanins accelerated the re-expression of MMP-2. Furthermore, the anthocyanins, which displayed antioxidant activity, regulated radical scavenging enzymes. Also, the anthocyanins enhance ECM remodeling via up-regulation of MMP-2 activity. Also, it is possible to modulate ECM homeostasis by blocking oxidative damage.[23]

The current data confirm that MMP-2 gene expression was inhibited during gastric ulceration by naproxen. However, pretreatment with the anthocyanins induced the expression of the gene encoding MMP-2 and the anthocyanins blockage of oxidative stress. Contrary to this, proinflammatory cytokine genes were up-regulated by naproxen and down-regulated by the anthocyanins. Gastric ulcers generated by naproxen secreted proinflammatory cytokines but repressed MMP-2 gene expression. Overall, this influenced gastric damage through the accumulated

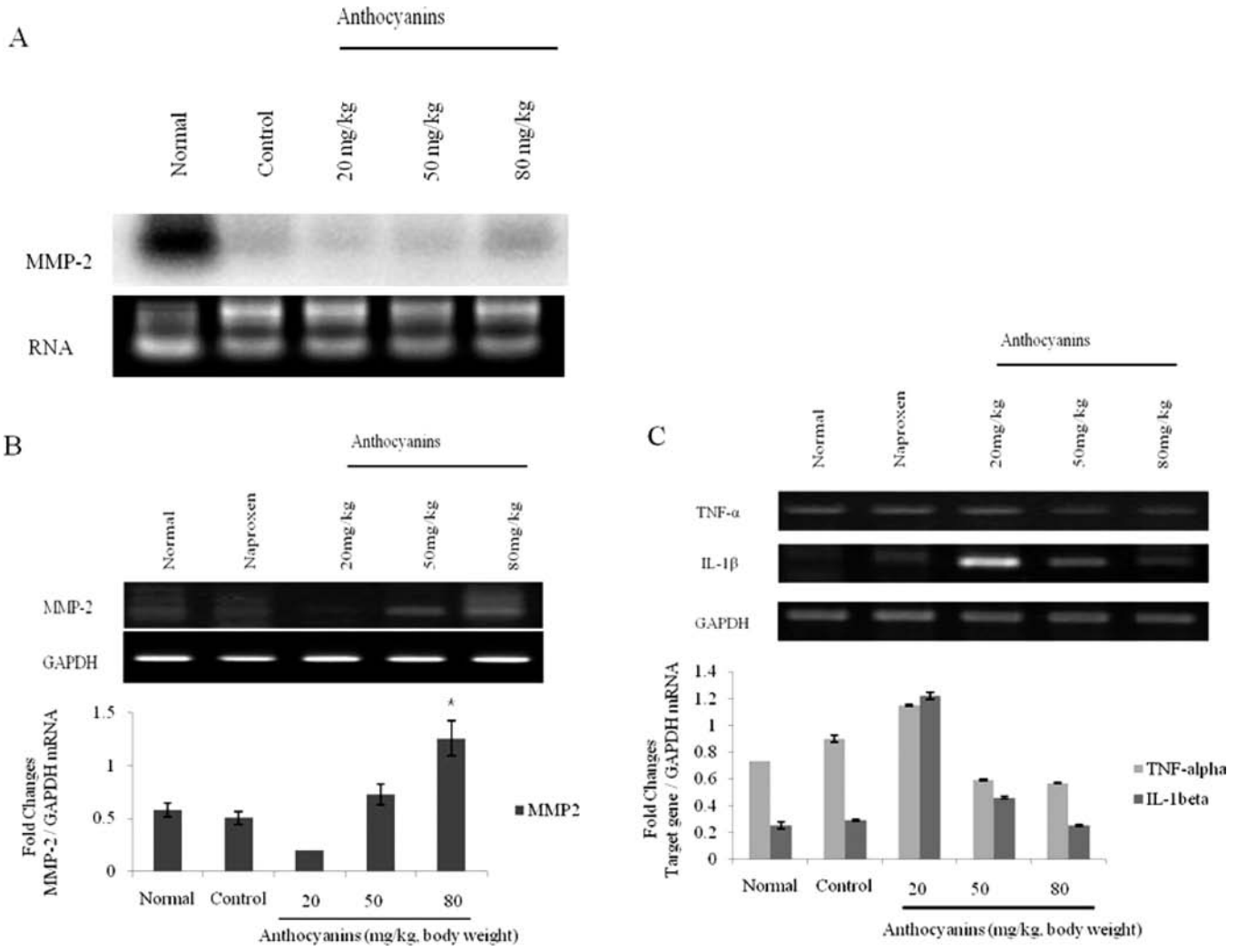

Figure 6. Effect of anthocyanins from *R. coreanus* on expression of MMP-2 and proinflammatory molecules in naproxen-treated rats. (A) Gastroprotective effect of anthocyanins on related MMP-2 activity was assessed by Northern blotting. (B) RT-PCR analysis of MMP-2 mRNA expression in rat gastric tissues. (C) RT-PCR analysis of TNF-α, and IL-1β mRNA expression in rat gastric tissues. PCR using GAPDH primers was done as a positive control. Results are reported as the mean $\pm$ SDs ($n = 5$). The statistical significance of the assay was evaluated using the t test (*, $P < 0.05$; **, $P < 0.01$ as compared with control rats).

damage due to oxidative stress. Also, the anti-inflammatory action of anthocyanins due to the regulation of MMP-2 at the level of secretion is important in ECM remodeling.

In summary, the results of this study demonstrate that the antiulcer effect of anthocyanins from *R. coreanus* causes the reversal of naproxen-induced gastric epithelial cell damage through the prevention of radical scavenging and inhibition of lipid peroxidation. Furthermore, anthocyanins have an antiulcer effect due to their regulation of MMP-2 activity. Also, anthocyanins can regulate the expression of proinflammatory molecules in the ROS- and MMP-2-mediated pathways for antiulcer action. Taken together, these findings suggest a beneficial mechanism of anthocyanins from *R. coreanus* in the prevention of gastric ulceration.

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Notes

We declare that we have no conflicts of interest.

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