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Anti-inflammatory and Anticancer Properties of Dichloromethane and Butanol Fractions from the Stem Bark of *Broussonetia papyrifera*

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***Broussonetia papyrifera* radix, fruits, leaves, and stems exhibit antioxidant, antinociceptive, antityrosinase, anti-inflammatory, and antiplatelet activities. However, studies of the anticancer and anti-inflammatory activities of *B. papyrifera* stem bark are limited. Yet it is important to evaluate these activities to isolate active compounds for potential medicines and food additive. The dichloromethane fraction of *B. papyrifera* stem bark was found to induce apoptosis-related DNA fragmentation, increase sub-G1 accumulation, increase morphological changes, and inhibit the proliferation of human colon cancer HT-29 cells. Moreover, it significantly increased p53, caspase-3, and Bax expression in HT-29 cells. In addition, the *n*-butanol fraction inhibited nitric oxide production in RAW 264.7 macrophages by suppressing iNOS expression. These results suggest that *B. papyrifera* is a potential natural resource for treating cancer and inflammatory diseases.**

Key words: anti-inflammatory, apoptosis, *Broussonetia papyrifera*, nitrite oxidation, RT-PCR

Broussonetia papyrifera (Moraceae), also known as paper mulberry, is a deciduous tree that grows naturally in the United States and in Asian and Pacific Rim countries such as China and Thailand. The roots, barks, and fruits are all used in traditional Chinese medicines that have antityrosinase activity [Ko *et al*., 2008] and have antioxidant [Mei *et al*., 2009], antinociceptive, and anti-inflammatory effects [Lin *et al*., 2008]. Constituents of this plant exhibit antiplatelet effects [Lin *et al*., 1996], inhibit the PTP1B enzyme [Chen *et al*., 2002], and inhibit aromatase [Lee *et al*., 2001]. There are no reports of the effects of *B. papyrifera* stem bark extract or solvent fractions on cell proliferation.

Apoptosis is an active physiological process resulting in morphological and biochemical changes in the nucleus and cytoplasm [Khan and Mlungwana, 1999]. Thus, apoptosis is the goal in the treatment of malignancies. Novel and effective agents that inhibit the proliferation of tumor cells by inducing apoptosis may be ideal for both cancer chemoprevention and chemotherapy. Apoptosis is characterized by distinct morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies [Wyllie, 1997]. Caspases, a class of cysteine proteases, are central players in the apoptotic process that trigger a cascade of proteolytic cleavage events [Shah *et al*., 2003]. The activation of caspase-3 and p53 are important downstream events in apoptosis [Earnshaw *et al*., 1999].

In this study we investigated the influence of the dichloromethane fraction (DCMF) from *B. papyrifera* stem bark on cellular proliferation and apoptosis using HT-29 cells as a model because there is no significant mechanistic work on the effects of *B. papyrifera* extract on human colon cancer. FACS analysis with annexin V-FITC/IP staining revealed that DCMF induced DNA fragmentation and morphological changes characteristic of apoptosis (e.g., sub-G1 accumulation, the translocation of phosphatidyl serine, and the loss of cell membrane asymmetry) and was thus responsible for a significant cytotoxic effect by inducing apoptosis in the cells. We

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also examined some apoptosis-related gene expression by semi-quantitative RT-PCR analysis.

The inflammatory process, classified as either acute or chronic, involves the activation of monocytes and/or macrophages. The activation of macrophages releases many inflammatory mediators, such as cytokines, nitric oxide (NO), and prostaglandins. Over-induction of NO leads to various harmful responses, including tissue injury, septic shock [Petros *et al*., 1991; Xie *et al*., 1992], apoptosis, and necrosis [Nagai *et al*., 2003]. Thus, inhibiting NO production by blocking iNOS expression may be a useful strategy for treating various inflammatory diseases.

In addition to NO, macrophages also release prostaglandins, which are COX products. COX has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed, whereas COX-2 is barely detectable under normal physiological conditions and can be induced by the same stimulus as iNOS [Aktan, 2004]. Lipopolysaccaride (LPS) is a major inflammatory molecule that triggers the production of proinflammatory toxins, cytokines such as iNOS and COX-2 in various cell types [MacMicking *et al*., 1997]. In the present study, we determined the anti-inflammatory effects of the *n*-butanol fraction (BF) from *B. papyrifera* stem bark methanol extract on LPS-activated RAW 264.7 macrophages by nitrite quantification and by gene expression analysis of iNOS and COX-2.

Materials and Methods

Preparation of solvent fractions of the stem bark. Air-dried *B. papyrifera* stem bark obtained in March 2008 from Henan Xinxiang, China. These materials were confirmed taxonomically by Professor Gui-Fang Xu of Henan Institude of Science and Technology, Henan Xinxiang, China. A voucher specimen has been deposited at the school of life science£¨Henan Institute of Science and Technology. These materials were pulverized using a milling machine and extracted with 80% methanol in water three times at room temperature. The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The methanol extracts was then suspended in water and further fractionated by *n*-hexane (HF), DCMF, ethyl acetate (EAF) and BF in a stepwise manner. Bioactivity was assayed for each of these fractions.

Cancer cell culture and cytotoxicity MTT assay. The human HT-29 cell line was obtained from the American Type Cell Culture (Rockville, MD) and cultured in RPMI-1640 medium (Gibco, MD) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The growth-inhibitory effect of DCMF was measured using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HT-29 cells (2×10^5 cells/mL) were incubated with DCMF (50, 100, 150, or 200 μg/mL) in 96-well plates for 48 h. After incubation, the culture medium was removed and 50 μL MTT reagent (2 mg/mL) was added to each well. The plates were incubated at 37°C for 4 h. DMSO (100%, 150 μL) was added to each well. The metabolized MTT product was dissolved in DMSO and quantified by measuring the optical density at 550 nm on a microplate reader (Dynex Technologies, USA). IC_{50} is the concentration of DCM required to inhibit growth by 50% and was calculated from the equation of the logarithmic line of best fit Microsoft Excel.

Cell cycle analysis. 24 h incubation after seeding, cells were exposed to 0, 50, 100, 150, or 200 μg/mL DCMF for 48 h. Afterward, cells were harvested, washed with PBS, fixed in 70% ethanol for 1 h at −20°C, and then stained with propidium iodide (PI) solution (20 μg/mL PI, 0.1 mM EDTA, 10 μg/mL RNase, and 1% Triton X-100 in PBS) for 30 min in the dark. DNA content was measured using a flow cytometer analysis system (FCAS), and at least 10,000 cells were analyzed for each experimental treatment. PI fluorescence at 585 nm was detected in the FL-2 channel. Samples were initially examined by gating on a FL-2 area versus FL-2 width dot plot to exclude debris and subsequently examined on a DNA content histogram. Data analysis was performed using WinMDI version 2.9 cell cycle analysis software (Scripps Research Institute).

Annexin V-FITC/PI flow cytometric analysis. Phosphatidylserine on the outside of apoptotic cells was determined using an annexin V-FITC apoptosis kit. Briefly, HT-29 cells were plated in 6-well plates with 2 mL medium following treatment with DCMF for 48 h at concentrations of 0, 50, 100, 150, or 200 μg/mL. The cells were collected and resuspended in 100 μL 1Õ annexin binding buffer, 5 μL conjugated annexin V-FITC, and 10 μL PI buffer for 15 min in the dark. Approximately 10^4 cells were analyzed using a FACScan flow cytometer.

RT-PCR analysis of DCMF-treated cells. Total RNA was extracted from cells that had or had not been exposed to DCMF using Trizol reagent. For cDNA synthesis, 2 ìg RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase. Semi-quantitative PCR was performed with oligonucleotides as previously described [Yin *et al*., 2009]. The products of the RT-PCR were separated by electrophoresis using 1.2% agarose gel and stained with ethidium bromide.

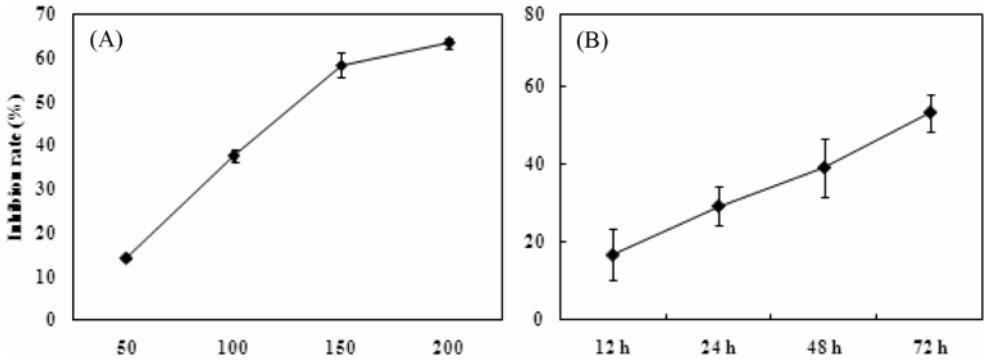

Fig. 1. The growth inhibition ratio of HT-29 cells by 50, 100, 150, or 200 μg/mL DCMF (A) and different time (B). Date were presented as mean±SEM (n=6).

Macrophage RAW 264.7 culture and viability assay. The macrophage RAW 264.7 cell line was incubated in Dulbecco's modified Eagle's medium at 37°C under 5% CO_2 humidified air. Then 1×10^4 cells/mL were seeded into 96-well plates and allowed to adhere for 24 h. After a 24 h treatment with BF (0, 50, 100, or 150 μg/mL), 50 μL MTT was added to each well, followed by incubation for 4 h at 37°C. The resulting color was assayed at 550 nm using a microplate reader.

Measurement of anti-inflammatory activity of BF extracts on LPS-activated RAW 264.7 macrophages by nitrite quantification. Nitrite quantification was performed using 3×10^5 RAW 264.7 cells/mL seeded into 96-well plates and allowed to adhere to the surface overnight. Neither LPS nor samples were added to the control group. After 24 h, cell-free supernatants were collected and NO was determined using the Griess reaction (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); the presence of nitrite was quantified by comparison with a $NaNO_2$ standard curve.

RT-PCR for iNOS and COX-2 mRNA. RAW 264.7 cells were pretreated with BF (0, 50, 100, or 150 μg/mL) for 2 h and then incubated with LPS (1 μg/mL) for 8 h. The RT-PCR primers used (iNOS, COX-2, and GAPDH) were those described [Xu *et al*., 2008].

Statistical analysis. Results are expressed as the mean ±SD of at least three experiments performed using different in vitro cell preparations. Statistically significant differences were determined using a Student's t-test followed by Dunn's test. Statistical significance was set at $p<0.05$.

Results and Discussion

Although many antitumor drugs have been developed, side effects and resistance are still serious problems [Panchal, 1998]. Thus, there is growing interest in the use of naturally occurring compounds for the treatment of various cancers and the development of safer and more effective therapeutic agents [Ramos, 2007]. Use of chemopreventive agents of natural origin, often a part of people's daily diets, may be an inexpensive, effective way of controlling such diseases as colon cancer and inflammation.

Effects of DCMF on cell cytotoxicity. Five fractions were used to examine the inhibition of growth in HT-29 and HEK293 cells using MTT-based assay. Among the five fractions, the DCMF appeared to be the most potent inhibitor of HT-29 cell growth; on HEK293 cells, the DCMF showed non-cytotoxic activity. The DCMF at 50, 100, 150, and 200 μg/mL inhibited HT-29 cell growth rates by 14.21%±0.3, 37.58%±1.4, 58.46%±2.7, and 63.45%±1.1, respectively (Fig. 1), compared with the control. The DCMF inhibited cell growth in a dose-dependent manner. Moreover, the inhibition of cell growth was time dependent. Percent inhibition of the HT-29 cell growth rate following DCMF exposure at 100 μg/mL was 16.83%±0.87, 29.39%±1.03, 39.28%±2.64%, and 53.42%±1.93% after 12, 24, 48, and 72 h, respectively. The effect of the other four extracts on HT-29 cell growth was very weak and not statistically significant.

DCMF induces sub-G1 phase accumulation but not cell cycle arrest. To further evaluate the effects of the DCMF on HT-29 cell proliferation, we determined the cell cycle distribution by flow cytometry. After DCMF treatment, cellular DNA was stained with PI and flow cytometry analysis was performed. The DCMF increased the occurrence of sub-G1 DNA content in a dose-related manner (Fig. 2). However, no cell cycle arrest was observed following DCMF treatments at different doses.

Induction of apoptosis by the DCMF. Because a decrease in cell proliferation may result from the induction of apoptosis, we investigated whether the DCMF induced apoptosis in HT-29 cells. HT-29 cells were treated with DCMF at various concentrations, then annexin V-FITC

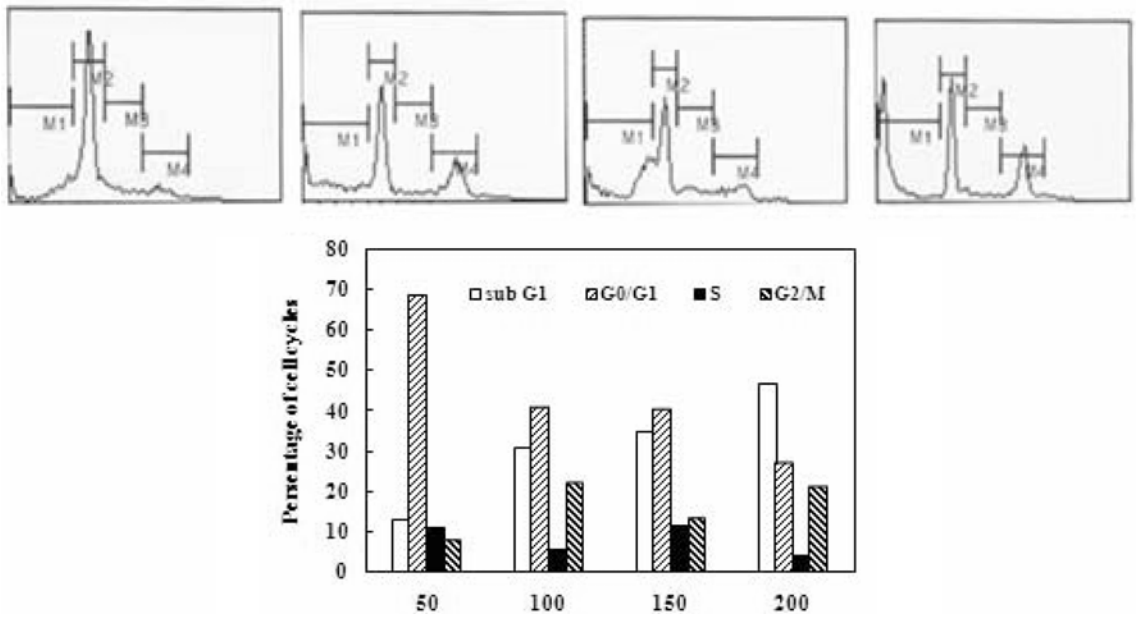

Fig. 2. Flow cytometric analysis of cell cycle progression after DCMF treatment (50, 100, 150, or 200 μg/mL) for 48 h. M1, M2, M3, and M4 in the figures directly correspond to the sub-G1, G0/G1, S, and G2/M phases, respectively. Data are the mean±SD of three independent experiments.

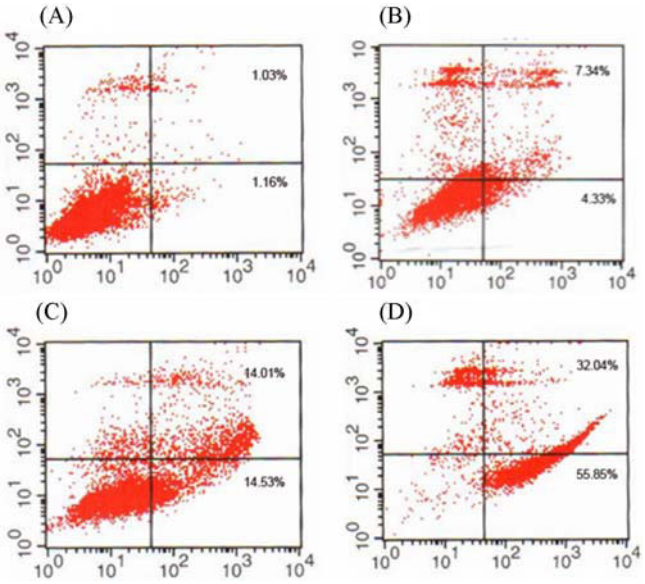

Fig. 3. Flow cytometry analysis of apoptosis after exposure to A: 50 μg/mL; B: 100 μg/mL; C: 150 μg/mL; or D: 200 μg/mL DCMF for 48 h, using annexin V-FITC/PI. The lower right indicates the percentage of early apoptotic cells; the upper right indicates the percentage of necrotic and late apoptotic cells.

and PI fluorescence was determined by flow cytometry (Fig. 3). After treatment with 50, 100, 150, and 200 μg/mL DCMF for 48 h, the percentages of early apoptotic cells (i.e., annexin-V-positive and PI-negative cells) were 1.16, 4.33, 14.53, and 55.85%, respectively. These results suggest that the DCMF inhibited proliferation of HT-29 cells by inducing apoptosis in a concentration-dependent manner.

Effects of the DCMF on caspase cascade mRNA expression in HT-29 cells. The DCMF induced HT-29 cell apoptosis in a dose-dependent manner, but we did not know which apoptotic signal pathway was involved. p53 is a multifunctional transcription factor that regulates critical cellular processes, including proliferation, cell cycle arrest, DNA repair, and apoptosis. Caspase 3 is an executioner caspase in the caspase family of cysteine proteases that induce apoptosis. Caspase 3 activation represents a critical and irreversible point in tumor development [Ayed-Boussema *et al.,* 2008]. Active caspase 3 cleaves some important proteins, such as poly (ADP-ribose) polymerase, to complete the apoptotic process [Earnshaw *et al.,* 1999]. c-Myc is involved with the regulatory proteins in the G1-S phase transition of the cell cycle. c-Myc has been implicated in inducing cyclin D and cyclin E [Hanson *et al.,* 1994]. Bax is a member of the Bcl-2 family of proteins that promote apoptosis. In healthy cells, Bax is located in the cytoplasm, but during apoptosis it is translocated to the mitochondria. Bax is capable of triggering the release of cytochrome c from isolated mitochondria [Chiu *et al.,* 2003].

We analyzed the expression of these regulators at the transcriptional level using RT-PCR. The expression of p53 and caspase-3 was up-regulated after treatment with the DCMF extract, as shown in Fig. 4A, the up-regulation was in a dose-dependent manner except the minor change at the 150 μg/mL DCMF compare with the untreatment.

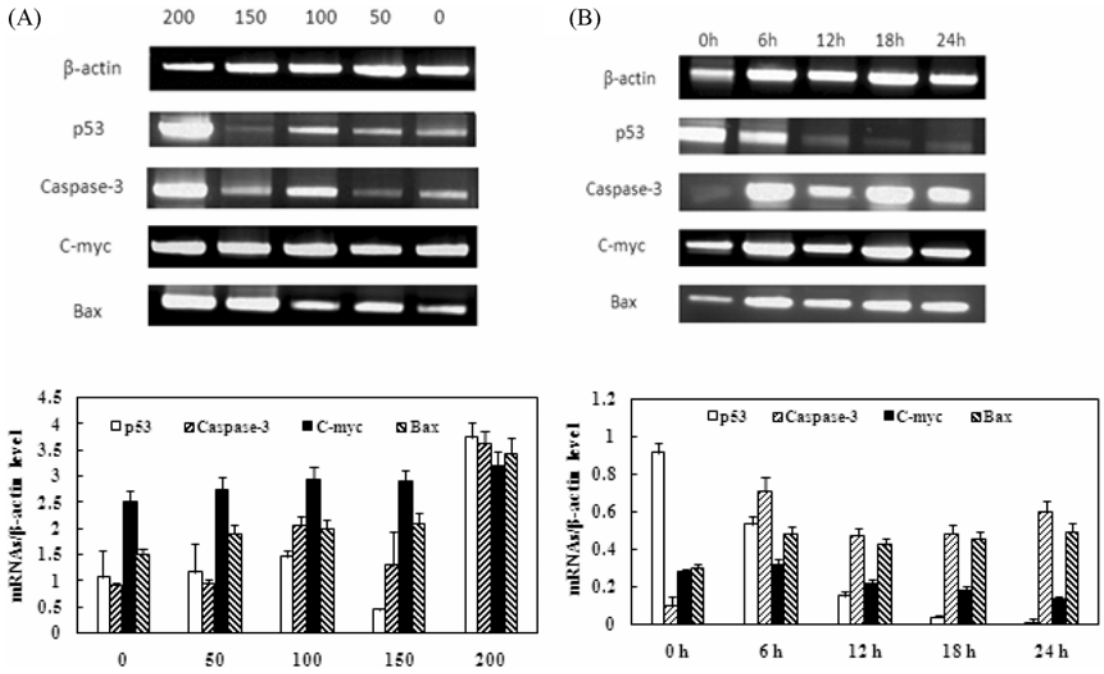

Fig. 4. Effects of the DCMF on apoptosis-related gene expression by semi-quantitative RT-PCR analysis. A. HT-29 cells treated with DCMF (0, 50, 100, 150, or 200 μg/mL) for 48 h. B. HT-29 cells treated with 100 μg/mL DCMF for 0, 6, 12, 18, or 24 h.

The expression of Bax was up-regulated in a dose-dependent manner. However, an effect of the DCMF on the c-Myc level was not obvious. We also examined gene expression after treatment with 100 μg/mL DCMF for 6, 12, 18, and 24 h. p53, caspase-3, and c-Myc were expressed most strongly 6 h after treatment; p53 expression decreased after 6 h, but caspase-3 and c-Myc showed no obvious decrease in expression even after 24 h. Bax expression remained at a constant level (Fig. 4B). This study demonstrates that DCMF only induces sub-G1 accumulation; it does not trigger cell cycle arrest. This conclusion is also supported by the stable expression of c-Myc. The Bax expression results seem to suggest that DCMF-induced apoptosis involves the p53-dependent mitochondrion pathway. To more fully elucidate the mechanisms of DCMF-induced apoptosis, additional studies should focus on the sequential events that induce apoptosis by triggering the signal pathway.

Inhibition of NO production in LPS-stimulated RAW 264.7 macrophages. Lin *et al.* [2008] found that various parts of *B. papyrifera*, including the radix, leaves, fruits, and stem, have anti-inflammatory activity. Our results demonstrate that the stem bark also exhibits anti-inflammatory activity. Among the five fractions tested, only BF showed anti-inflammatory activity. To assess the effects of the BF on LPS-induced NO production in RAW 264.7 macrophages, we treated cells with various concentrations of BF after LPS stimulation of the macrophages. Our results show that BF inhibited LPS-induced NO production in a dose-dependent manner (Fig. 5A). The cytotoxic effects of the BF were also evaluated using MTT assay, but no cell cytotoxic activity was found in the 10-200 μg/mL range.

Effects of BF on iNOS and COX-2 mRNA expression. Because the BF inhibited the production of NO by LPS-treated RAW 264.7 cells, we used RT-PCR to investigate further whether the decrease in NO production was due to down-regulation of iNOS and COX-2 expression at the mRNA level. iNOS and COX-2 expression were induced in the presence of LPS; the BF significantly inhibited iNOS production in a dose-dependent manner (Fig. 5B). No obvious pattern was observed relative to LPS-induced COX-2 expression. However, these treatments did not affect the expression of the housekeeping gene GAPDH.

In summary, the DCMF from *B. papyrifera* can inhibit cell growth and proliferation and induce and enhance apoptosis in HT-29 cells. The RT-PCR results indicate that the DCMF induces apoptosis in HT-29 cells via a p53-dependent mitochondrion signaling pathway. The BF from *B. papyrifera* can inhibit NO production by suppressing iNOS expression in RAW 264.7 macrophages. These results demonstrate the potential use of *B. papyrifera* in the food and pharmacy industries.

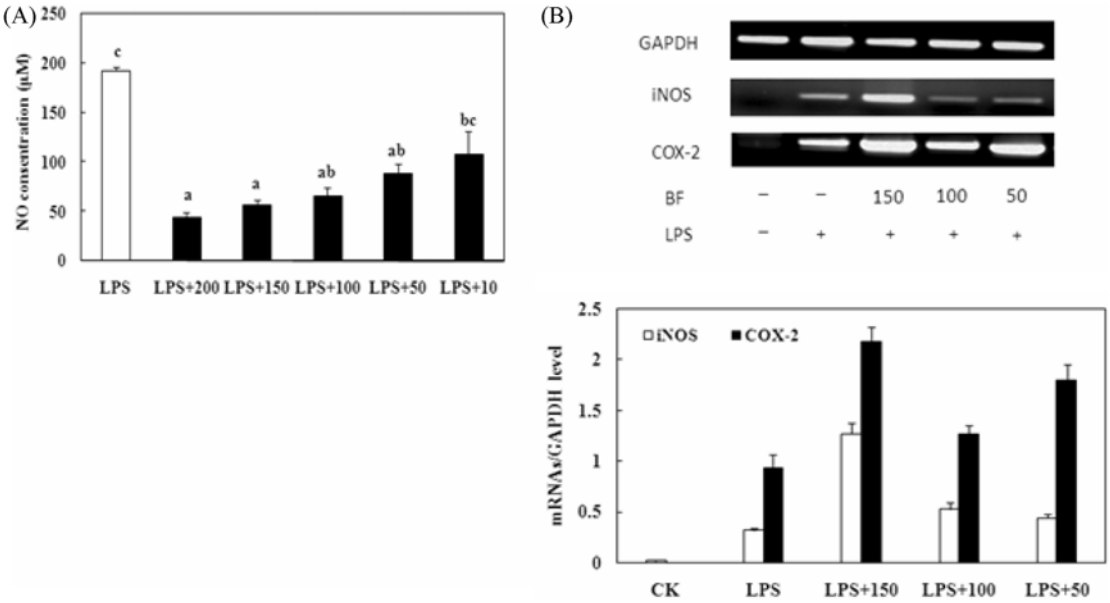

Fig. 5. Effect of BF on NO production (A) and the expression of iNOS and COX-2 by semi-quantitative RT-PCR analysis (B). A. Macrophages were pretreated with BF (0, 10, 50, 100, 150, or 200 μg/mL) for 2 h and then incubated with LPS (1 μg/mL) for 24 h. B. Macrophages were treated with BF (0, 50, 100, or 150 μg/mL).

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