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Anti-inflammatory Potential of Flavonoid Contents from Dried Fruit of *Crataegus pinnatifida* in Vitro and in Vivo

ERL-SHYH KAO,† CHAU-JONG WANG,† WEA-LUNG LIN,‡ YU-FANG YIN,† CHI-PIN WANG,# AND TSUI-HWA TSENG*,§

Institute of Biochemistry and Biotechnology, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Chien Kuao N. Road, Taichung, 402 Taiwan; Departments of Pathology and Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan; and School of Applied Chemistry, College of Health Management, Chung Shan Medical University, No. 110, Sec. 1, Chien Kuao N. Road, Taichung, 402 Taiwan

The dried fruits of *Crataegus pinnatifida*, a local soft drink material and medical herb, demonstrated antioxidant effect in a previous study. The present study investigates the anti-inflammatory potential of flavonoid contents from dried fruit of *C. pinnatifida* (CF-Fs). The preliminary investigation showed that CF-Fs (0.25−0.75 mg/mL) decreased the release of PGE_2 and nitric oxide as induced by lipopolysaccharide (LPS, an endotoxin) in macrophage RAW 264.7 cells. The in vivo assay showed that pretreatment of rats with CF-Fs (50−200 mg/kg dosed by gavage) for 5 days significantly decreased the serum levels of the hepatic enzyme markers alanine aminotransferase and aspartate aminotransferase induced by the 6-h treatment with LPS (ip; 5 mg/kg). Histopathological evaluation of the rat livers revealed that CF-Fs reduced the incidence of liver lesions such as neutrophil infiltration and necrosis induced by LPS. Furthermore, it was found that pretreatment with CF-Fs decreased the hepatic expression of iNOS and COX-2 induced by LPS in rats. These results demonstrate that CF-Fs present anti-inflammatory potential in vitro and in vivo and that they may play a role in hepatoprotection.

KEYWORDS: ***Crataegus pinnatifida*; macrophage; lipopolysaccharide; inducible nitric oxide synthase; cyclooxygenase-2**

INTRODUCTION

The fruits of *Crataegus pinnatifida* (Rosaceae) have been used traditionally as a peptic agent in oriental medicine and recently as a local soft drink material. In addition, it is believed that preparations of leaves or fruits of *C. pinnatifida* improve the heart function when there are indications of declining cardiac performance, deficiency in coronary blood supply, and mild forms of arrhythmia (*1*, *2*). Recently, we demonstrated that a hot-water extract of *C. pinnatifida* exhibited a free radical quenching capacity and an inhibitory effect on low-density lipoprotein (LDL) oxidation in both cell and cell-free systems (*3*). However, other bioactivities remain unclear.

Flavonoids are the most common and widely distributed group of plant phenolics, with >5000 different flavonoids having been described to date. They are found in abundance in fruits, vegetables, and plant-derived beverages such as tea (*4*), and humans have been estimated to consume ∼1 g of flavonoids/day. Consumption of a traditional diet rich in flavonoids is correlated with the decrease of the risk of some diseases in Asia (*5*). The flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers, as well as being suggested as powerful chain-breaking antioxidants (*6*, *7*). In addition, flavonoids display a remarkable array of biochemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems, including anti-inflammatory (*8*), anticarcinogenic (*9*), antiallergic and antiviral (*10*, *11*), cytotoxic (*12*), and cytostatic activities (*13*).

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is the triggering factor for multiple-organ failure during septic shock. Macrophage activation by bacterial LPS promotes the secretion of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and of secondary mediators, such as leukotrienes and prostaglandins (PGs). These substances are important regulators of both innate and adaptive immunity. However, their uncontrolled expression can cause acute or chronic inflammatory syndromes. An acute inflammatory syndrome induced by these mediators is the septic shock syndrome, which is characterized by fever, hypotension, disseminated intravascular coagulation, and multiple-organ failure. Moreover, LPS induced inducible

* Corresponding author (telephone 886-4-24730022; fax 886-4-23248189; e-mail tht@csmu.edu.tw).
† Institute of Biochemistry and Biotechnology.
‡ Department of Pathology.
# Department of Laboratory.
§ School of Applied Chemistry.

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nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in rat liver, and the COX enzyme possesses both cyclooxygenase and peroxidase functions (*14*).

COX-2 is expressed at very low levels and is strongly induced by growth factors and several activated oncogenes (*14*, *15*). The significance of COX-2 in prostaglandin synthesis and inflammation is highlighted by the observation that COX-2 inhibitors block the synthesis of PGE_2, and as a result, they inhibit inflammation and confer analgesia (*16*). Moreover, homozygous deletion of the COX-2 gene in mice leads to alleviation of hepatocellular toxicity caused by LPS administration, suggesting an important physiological role for this enzyme in LPS-induced pathology (*17*).

The pivotal role of nitric oxide (NO) as a messenger and effecter molecule in a variety of tissues has been demonstrated in recent years (*18*). NO has been identified as a neurotransmitter in the central nervous system and a potent vasorelaxant that physiologically regulates blood pressure through modulating muscular tone (*19*). NO also has been defined as an important molecule in inflammation and sepsis (*20*). NO is produced by nitric oxide synthase (NOS), a family of enzymes composed of three isoforms encoded by distinct genes. Neuronal NOS (type I, nNOS) and endothelial NOS (type III, eNOS) are Ca^{2+}- and calmodulin-dependent constitutive isoforms. Of these, nNOS has a function in neurotransmission, eNOS plays an important role in vasorelaxation, and the NO produced by the endothelium has antithrombotic properties. iNOS, also known as type II NOS, is not expressed under normal conditions. After exposure to endogenous and exogenous stimulators, iNOS can be induced quantitatively in various cells such as macrophages, smooth muscle cells, and hepatocytes to trigger several disadvantageous cellular responses, as well as causing some diseases including inflammation, sepsis, and stroke (*21*). Therefore, NO production induced by iNOS may reflect the degree of inflammation and provide a measure to assess the effect of drugs on the inflammatory process. In the liver, LPS activates iNOS of Kupffer cells, endothelial cells, and hepatocytes to promote NO production (*22*). Recently, it was demonstrated that inhibitors of iNOS might offer some protection in LPS-induced hepatic toxicity (*23*) and that natural antioxidants such as curcumin (*24*), resveratrol (*25*), and tea polyphenols (*26*) exhibit inhibition effects on LPS-induced iNOS and hepatic damage.

Therefore, inhibiting COX-2 and iNOS expressions is anticipated to exert chemopreventive effects. In this study, we investigate the anti-inflammatory potential of the flavonoid contents from dried fruits of *C. pinnatifida* (CF-Fs) in vitro and in vivo. The results suggest that *C. pinnatifida* may play a role in preventing inflammation and cancer.

MATERIALS AND METHODS

Chemicals. LPS (endotoxin from *Escherichia coli*, serotype 0127: B8), lipophilic Sephadex LH-20, kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Hyperoside (Hyperin; quercetin 3-galactoside) was purchased from Extrasynthese (Genay Cedex, France). Prostaglandin E2 immunoassay kit (R&D Systems), anti-iNOS, anti-COX-2, and anti-α-tubulin antisera (Transduction Laboratories, Lexington, KY) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated.

HPLC Analysis. Dried fruits of *C. pinnatifida* weighing 720 g were extracted twice with 2000 mL of water at 100 °C for 1 h. The extracted solution was filtered and then dried to obtain ~176 g of product that was termed CF-HW. One milligram per milliliter of CF-HW was prepared for flavonoid analysis by HPLC. HPLC analysis was performed using a Hitachi L7100 system with a 5 μm ODS-Hypersil column (250 × 4.6 mm). The mobile phase was 24% of methanol and 76% of 0.5% (v/v) orthophosphoric acid in water. The detection wavelength was set at 280 nm and the flow rate to 1.0 mL/min. Quantitation was carried out by the absolute calibration method on the basis of the area at 280 nm using hyperoside and rutin as standard.

CF-Fs Preparation. Dried fruits of *C. pinnatifida* were extracted twice with hot water as described in the previous study (*3*). Quantitative dried extract was dissolved with methanol and applied to a lipophilic Sephadex LH-20 column with methanol as elution buffer. Then 3 mL of elute was collected in each test tube and analyzed by wavelength scanning. This showed that tubes 8−36 demonstrated two absorbance peaks rounding at 277 and 382 nm that are characteristic of flavonoids (*27*). These elutes were collected and dried as CF-Fs.

Cell Culture. Rat macrophage RAW 264.7 cells were cultured in a humidified atmosphere of 95% air−5% carbon dioxide at 37 °C and using RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% penicillin−streptomycin.

Cell Viability Assay. RAW 264.7 cells were plated in 24-well plates at 5×10^4 cells per well and allowed to adhere to the plate overnight, after which the medium was refreshed. Cells underwent a 24-h treatment with CF-Fs (0−0.75 mg/mL), and then treatment medium was replaced with fresh medium containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4 h. Medium was then removed, and 1 mL of 2-propanol was added to wells to solubilize crystals. The optical density of each sample was read at 563 nm against the blank prepared from cell-free wells.

Nitrite Oxide Measurement. Total nitrites in the culture medium were measured using the Griess reagent as described (*28*). After incubation of RAW 264.7 cells with or without LPS and/or CF-Fs at 37 °C for 12 h, 100 μL of each culture medium was mixed with an equal volume of Griess reagent (1 g/L sulfanilamide and 0.1 g/L *N*-1-naphthylethylenediamine in 2.5% phosphoric acid solution), and incubated at room temperature for 10 min. Absorbance at 540 nm was then read and compared with known standard solutions of $NaNO_2$.

Production of PGE_2. RAW 264.7 cells were plated in 24-well plates at 1×10^6 cells per well, after cells had been allowed to adhere to the plate and the medium had been refreshed without phenol red. After incubation with or without LPS and/or CF-Fs (0−0.75 mg/mL) at 37 °C for 12 h, the production of prostaglandin E_2 (PGE_2) of each culture medium was determined with a commercial competitive PGE_2 ELISA kit.

Animal Treatment. Male Sprague−Dawley rats (260 ± 10 g) were used for the experiments. The rats were provided with food and water ad libitum and divided into six groups (four rats/group). CF-Fs (50, 100, and 200 mg/kg) was given daily to the animals for 5 consecutive days using a gavage. On day 5, 1 h after the CF-Fs treatment, LPS (5 mg/kg) or distilled water as solvent control was injected (intraperitoneally, ip) into each animal. The rats were sacrificed 6 h later by decapitation, and the blood samples were collected for the assays of ALT and AST. The livers were excised from the animals and assayed for the expression of iNOS and COX2, and the pathological histology was determined according to the procedures described below.

Hepatotoxicity Assessment. Hepatic enzymes ALT and AST were used as biochemical markers for early acute hepatic damage. The serum activities of ALT and AST were determined by enzymatic colorimetric methods with commercial kits.

Pathological Histology of Liver. After removal from the animals, hepatic tissue was immediately fixed in 10% buffered formaldehyde and processed for histological examination by conventional methods with hematoxylin and eosin (H&E) stain. The liver lesions observed were classified according to morphology changes, such as neutrophil infiltration and necrosis. The severity of liver damage was evaluated by examining the section under 10 randomly selected high-power fields (×400). The number of fields that had lesions and the areas of the lesions were recorded.

Immunoblot Analysis. Liver tissue was extracted at 4 °C by homogenization in buffer containing 20 mM HEPES, 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin (pH 7.4). The homogenates were then centrifuged at 25000*g* for 30 min at 4 °C. The protein content of the supernatant was

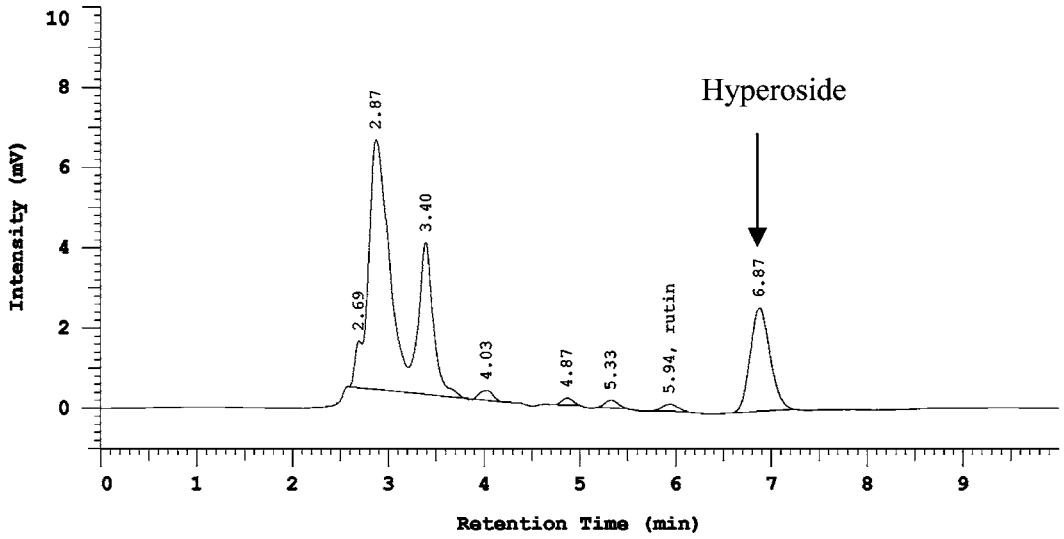

Figure 1. Flavonoid contents analysis of hot-water extracts of *C. fructus* by HPLC.

determinated with the Bio-Rad protein assay reagent using bovine serum albumin as a standard. To perform Western blotting, 80 μg of protein was resolved on 10% SDS-PAGE gels along with the prestained protein molecular weight standards (Bio-Rad). Proteins were then blotted onto an NC membrane (Sartorius) and reacted with primary antibodies (anti-iNOS or COX2 and anti-α-tubulin as internal control). The secondary antibody was a peroxidase-conjugated goat antimouse antibody. The reacted bands were revealed by enhanced chemiluminescence using an ECL commercial kit.

To investigate the effect of hyperoside on iNOS and COX-2 mediated by LPS, RAW 264.7 cells were plated in 10 cm plates at 5×10^6 cells and allowed to adhere to the plate overnight and the medium was refreshed. Then cells were treated with 100 ng/mL of LPS and various concentrations of hyperoside (5−50 μM) for 18 h. Total proteins (50 μg/lane) were prepared and subjected to western blot analysis as described above.

Statistical Analysis. The data were reported as means ± standard deviations (SD) from three repeated determinations and evaluated by analysis of variance (ANOVA) with Dunnett's test. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Analysis of Flavonoid Contents from Dried Fruit of *C. pinnatifida*. Our previous study showed that a hot-water extract of the dried fruit of *C. pinnatifida* (CF-HW) contained numerous polyphenols including flavonoids (6.9%) and procyanidins (2.2%) (*3*). Therefore, we analyzed the flavonoid contents by HPLC and prepared the fraction of flavonoids (CF-Fs) as described in a previous section for the following study. According to calibration standards, the peak at 5.94 min is rutin and the peak at 6.87 min is hyperoside (Hyperin; quercetin 3-galactoside). As analyzed by HPLC, hyperoside is the major flavonoid component in CF-HW and composed of 0.2% of the extract (**Figure 1**). Further study of other flavonoids existing in the *C. pinnatifida* is in progress in our laboratory.

Effect of CF-Fs on LPS-Induced NO and PGE_2 Production in Macrophages. Before testing the effects of CF-Fs on LPS-induced nitrite and PGE_2 production, we tested the cell viability of CF-Fs in RAW 264.7 cells. The results presented in **Figure 2** demonstrate that the concentration of 0.75 mg/mL did not interfere with the survival percentage of macrophages. In the present study, effects of CF-Fs on LPS-induced NO production in RAW 264.7 macrophages were investigated. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO release from the cells.

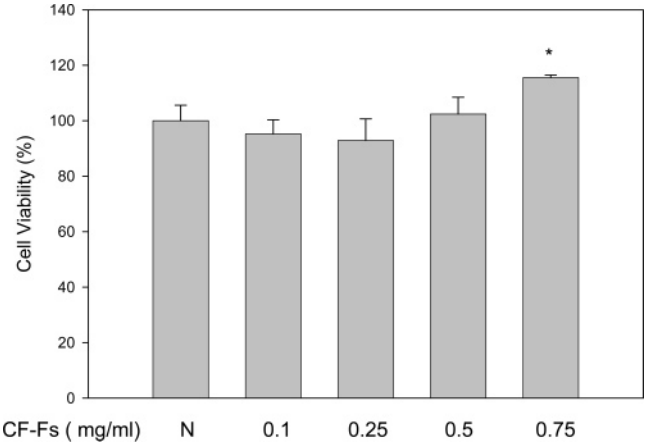

Figure 2. Effect of CF-Fs on cell viability of macrophages. Cells were cultured with various concentrations of CF-Fs for 24 h, and then MTT was added for 4 h and the formazan crystals were determined as described in the text. *, $P < 0.05$, compared with the group normal cells.

After treatment with LPS (100 ng/mL) for 12 h, the nitrite concentration in the medium increased remarkably. When RAW264.7 macrophages were treated with different concentrations of CF-Fs together with LPS (100 ng/mL) for 12 h, the CF-Fs significantly inhibited nitrite production (**Figure 3A**). An increase of PGE_2 production has been demonstrated in the process of LPS treatment. Therefore, we investigated the effects of CF-Fs on LPS-induced PGE_2 production in macrophage. After treatment with LPS (100 ng/mL) for 12 h, the amount of PGE_2 elevated clearly to 30 μg/mL in the medium, and cotreatment of cells with LPS and different concentration of CF-Fs was able to significantly suppress the LPS-induced amount of PGE_2 (**Figure 3B**).

Effect of CF-Fs on LPS-Induced Hepatic Inflammation in Rats. We used male Sprague−Dawley rats for the experiments to determine if CF-Fs regulates LPS-induced hepatic inflammation in vivo. The inflammatory response triggered by LPS is characterized by the release of pro-inflammatory cytokines (e.g., TNF-α, IL-2, and IL-6), which leads to hepatocyte injury and increased plasma levels of AST and ALT (*29*, *30*). The results demonstrated a hepatoprotective effect of CF-Fs on the LPS-induced hepatic damage in rats shown in **Figure 4**. Baseline plasma levels of ALT and AST were normal

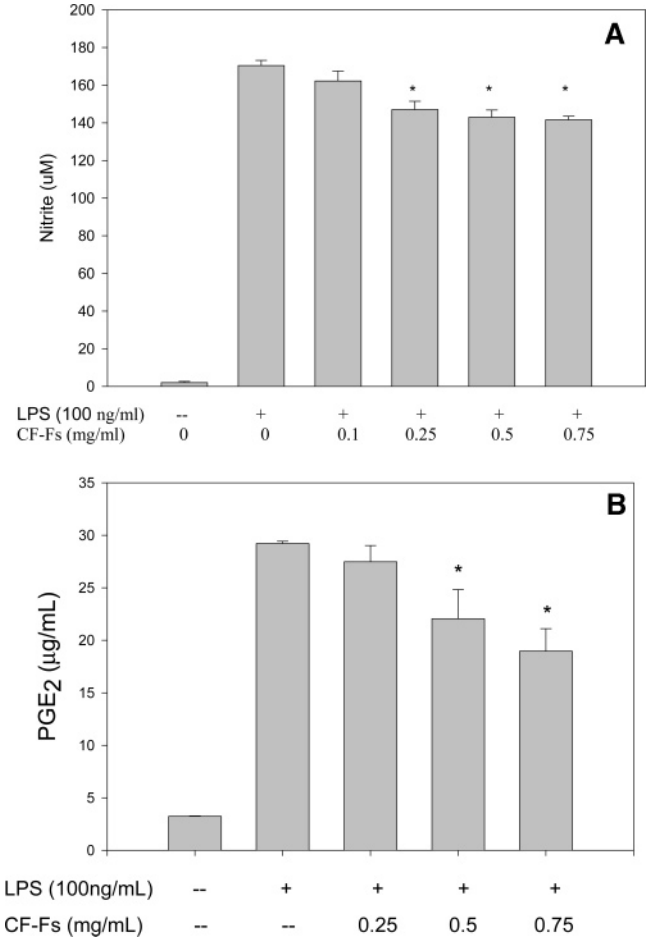

Figure 3. Effect of CF-Fs on LPS-induced nitrite and PGE_2 production in macrophages. Murine macrophages (RAW 264.7), 1×10^6 cells/mL, were stimulated with or without LPS and various concentrations of CF-Fs for 12 h. Supernatants were collected, and then (**A**) total nitrite was determined by Griess reagent or (**B**) PGE_2, by ELISA kit. *, $P < 0.05$, compared with the group stimulated by LPS.

in the groups of control and CF-Fs (200 mg/mL) treated alone. After 6 h of injection of LPS (5 mg/kg), the plasma ALT and AST levels were higher than in rats of the control group. When gavaged with CF-Fs for 5 consecutive days before LPS induction, the AST and ALT of rats were significantly reduced (**Figure 4**).

Effect of CF-Fs on LPS-Induced Hepatic Lesions in Rats. The effect of CF-Fs on the LPS-induced histopathological changes of rat liver was also evaluated after the rats were sacrificed. The liver sections of the control group and CF-Fs alone had no inflammatory cells and no morphological changes of cellular damage (**Figure 5A**,**C**). After LPS administration for 6 h, liver lesions were found in the lobular region, exhibiting necrosis and infiltration by inflammatory neutrophils (**Figure 5B**). In the 10 randomly selected high-power fields (×400), lesions with an area extending over half of the field were observed. The liver pathological changes caused by LPS in the animals pretreated with CF-Fs (100 mg/kg) were reduced (**Figure 5E**). Only approximately 2−3 of 10 fields had lesions observed, and the area of lesion was less than half of the field. The group gavaged with 200 mg/kg CF-Fs exhibited almost no hepatocyte swelling and neutrophil aggregation (**Figure 5F**).

Effect of CF-Fs on Hepatic Expression of iNOS and COX-2 Induced by LPS. In **Figure 3A**, we demonstrated that

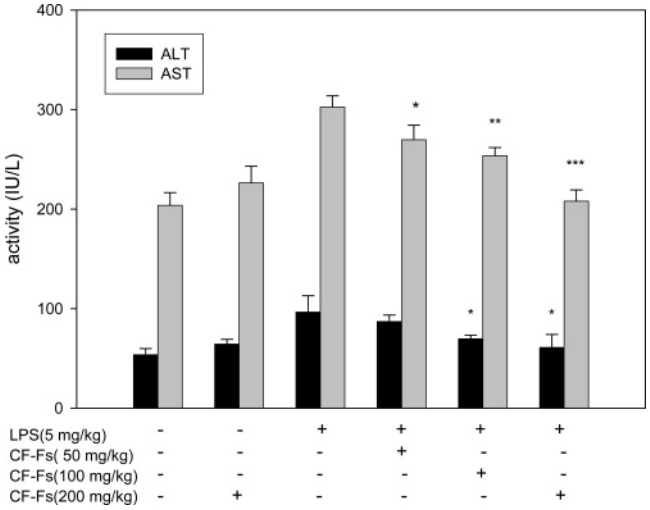

Figure 4. Effects of CF-Fs on the serum enzymes in rats treated with LPS. Rats were treated with distilled water (normal), and 50, 100, and 200 mg/kg CF-Fs by gavage for 5 days. The animals were sacrificed 6 h after the administration of 5 mg/kg LPS (ip), and the sera were prepared for ALT and AST determinations as described in the text. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with the group treated with LPS alone.

CF-Fs can significantly inhibit LPS-induced nitrite production in macrophage. Because NO production induced by NOS and LPS can activate iNOS to promote NO production in the liver, we then observed further if pretreated CF-Fs can regulate iNOS protein expression induced by LPS in rat liver. A Western blot analysis of the iNOS protein level of the liver showed that treatment with LPS induced the expression of the enzyme that was almost undetectable in the control and the group dosed with CF-Fs (200 mg/kg) alone (**Figure 6**). The expression of the iNOS protein (130 kDa) in the liver was weakened by the gavage with a low dose of CF-Fs (50 mg/kg) and was eliminated by a high dose of CF-Fs (200 mg/kg).

Activation of COX-2 gene expression has been demonstrated in the process of LPS treatment. Therefore, we investigated the effects CF-Fs on LPS-induced COX-2 protein expression. Upon LPS (5 mg/mL) treatment for 6 h, hepatic expression of COX-2 protein increased, and gavage with CF-Fs 5 days before LPS injection dose-dependently inhibited COX-2 protein induction in rats (**Figure 6**). The amount of α-tubulin protein as an internal control remained unchanged.

Effect of Hyperoside on LPS-Induced COX-2 and iNOS Protein Expression in Macrophages. To further investigate the important role of hyperoside on anti-inflammation, RAW 264.7 cells were treated with 100 ng/mL of LPS and various concentrations of hyperoside (5−50 μM) for 18 h. Western blotting analysis indicated that COX-2 and iNOS protein expression increased after stimulation with LPS, and both COX-2 and iNOS protein were gradually decreased with increasing concentration of hyperoside (**Figure 7**).

DISCUSSION

Flavonoids are naturally occurring plant polyphenols found in abundance in diets rich in fruits, vegetables, and plant-derived beverages such as tea. Several flavonoids are biochemically active compounds with anti-inflammatory, anticarcinogenic, and free radical scavenging properties. However, the anti-inflammatory effects of some flavonoids are not well established. Recently, some studies have indicated that the flavonoids rutin, quercetin, and its derivate quercetin penta-acetate effectively

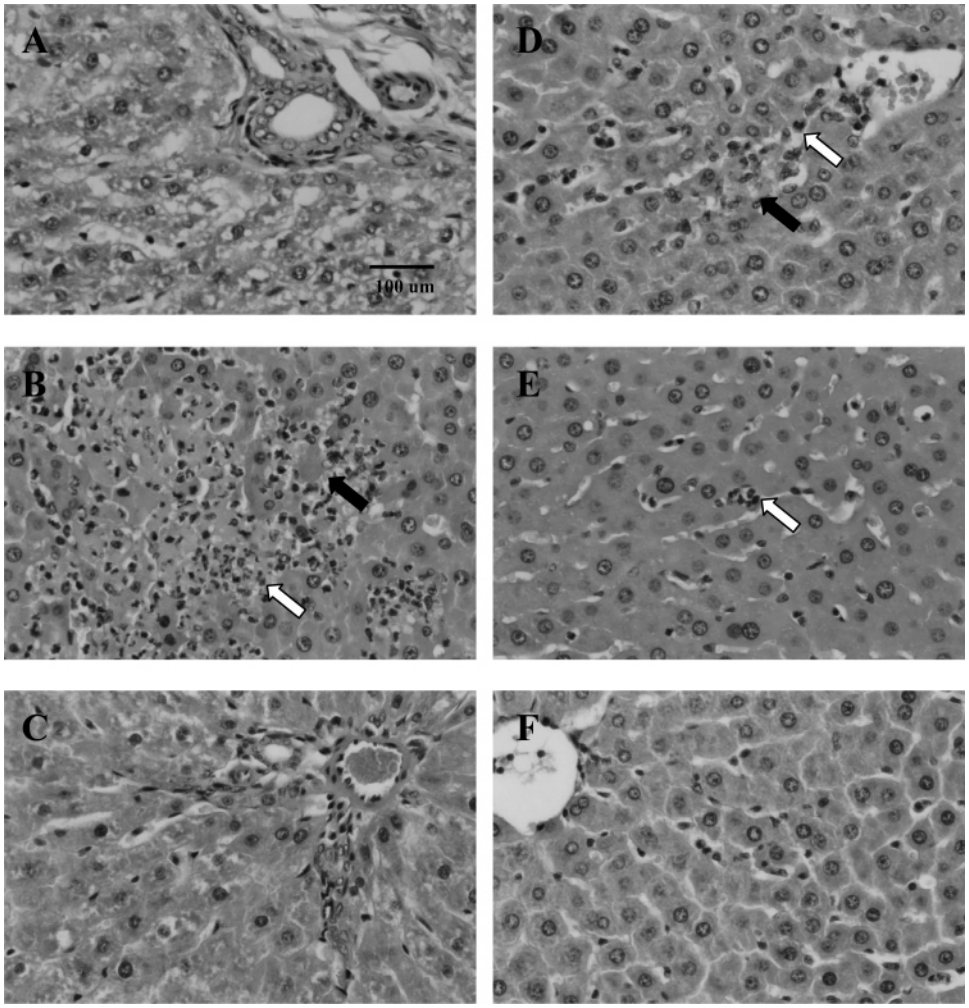

Figure 5. Effect of CF-Fs on LPS-induced hepatic injury in rat: (**A**) section of liver from a control animal treated with solvent; (**B**) section of liver from an animal treated with 5 mg/kg (ip) LPS, showing severe neutrophil leukocyte infiltration (white arrow) and extensive hepatocyte necrosis (black arrow); (**C**) section of liver from an animal treated with 200 mg/kg CF-Fs; (**D**) section of liver from an animal pretreated with 50 mg/kg CF-Fs by gavage and then with LPS, showing focal areas of liver parenchyma with neutrophil infiltration (white arrow) and necrosis (black arrow); (**E**) section of liver from an animal pretreated with 100 mg/kg CF-Fs by gavage and then with LPS, showing focal areas of liver parenchyma with neutrophil infiltration (white arrow); (**F**) section of liver from an animal pretreated with 200 mg/kg CF-Fs by gavage and then with LPS, showing no pathological change except for a few neutrophils in sinusoid.

inhibited LPS-induced NO production and iNOS gene expression in RAW 264.7 macrophages (*31*). According to our previous study (*3*), flavonoids are the major polyphenols in *C. pinnatifida*. Therefore, we suspect that *C. pinnatifida* may play a role in anti-inflammation. The results of this study showed that CF-Fs inhibited LPS-induced inflammation responses in vitro and in vivo. In addition, we analyzed the flavonoid contents of the dried fruit of *C. pinnatifida*, showing the major flavonoid is hyperoside. Although previous studies have indicated that hyperoside (Hyperin; quercetin 3-galactoside) also exists in *Hypericum* species that show hydroxyl radical-scavenging effects (*32*), other biological and pharmacological effects are not well-known. To our knowledge, this is the first demonstration of the anti-inflammatory effect of hyperoside (**Figure 7**).

It is reported that several flavonoids modulate arachidonic acid metabolism in platelet and peritoneal leukocytes (*33*). Cellular mechanisms underlying these effects are still unclear, but are thought to be linked to their antioxidant properties. In our previous study, we demonstrated that a hot-water extract of *C. pinnatifida* containing polyphenols exhibited a free radical quenching capacity (*3*). In the present study, we investigated the effects of CF-Fs on LPS-induced NO and PGE_2 production in macrophages, and evidence indicated that CF-Fs was able to significantly suppress LPS-induced amounts of NO and PGE_2. These data suggest that CF-Fs possesses anti-inflammatory activity in vitro. To determine if CF-Fs regulates LPS-induced hepatic damage in vivo, we used Sprague−Dawley rats as a model and found that CF-Fs inhibited LPS-induced AST and ALT in the plasma. Furthermore, we demonstrated that flavonoids contained in *C. pinnatifida* inhibit both COX-2 and iNOS in a dose-dependent manner. Consistent with curcumin, resveratrol, and tea polyphenols, CF-Fs exhibits an inhibition effect on LPS-induced iNOS and offers some protection in LPS-induced hepatic toxicity (*25*, *26*).

Much evidence suggests that overexpression of COX-2 is strongly related to pathophysiological diseases including inflammation, cancer, multiple sclerosis, and Alzheimer's disease (*34*). On the basis of this information, efforts have been made to develop inhibitors or suppressors for the activity or expression of COX-2. Natural product-derived compounds including resveratrol, curcumin, and other polyphenols were shown to potentially inhibit COX-2 activity (*35*). In this study, we found that hyperoside, the major flavonoid contained in *C. pinnatifida*,

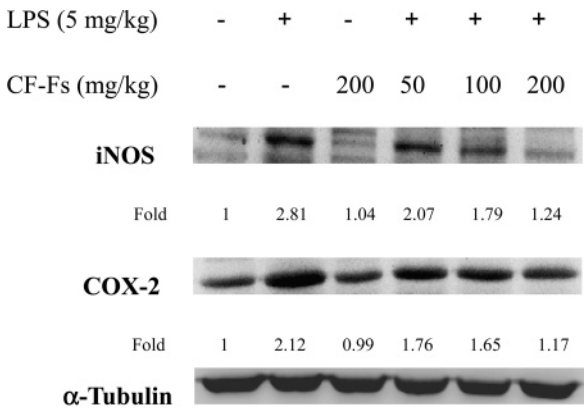

Figure 6. Effect of CF-Fs on the expression of hepatic iNOS and COX-2 after LPS administration in rat. Immunoblotting analysis of iNOS and COX-2 of rat liver was done as described in the text. The data are presented from one rat per group, and each lane contained 80 μg of protein. Other rats in each group showed patterns of protein expression of the iNOS and COX-2 similar to the representation.

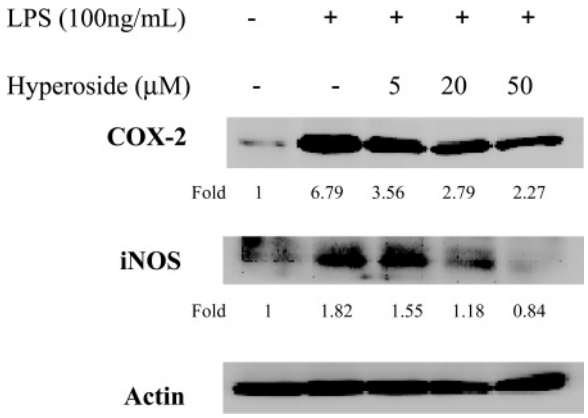

Figure 7. Effect of hyperoside on LPS-induced COX-2 and iNOS protein expression in macrophages. RAW 264.7 cells were stimulated with 100 ng/mL of LPS and various concentrations of hyperoside (5–50 μM) for 18 h. Total cellular protein (50 μg/lane) was separated on 10% SDS–polyacrylamide gels and blotted with antibodies specific for COX-2 and iNOS as described under Materials and Methods.

was a potent COX-2 inhibitor. However, the chemoprevention role of hyperoside needs further investigation.

ABBREVIATIONS USED

CF-Fs, flavonoids of dried fruit of *Crataegus pinnatifida*; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PGE_2, prostaglandin E_2; NO, nitric oxide.

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