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Original Paper

Protective Effects of Morus Root Extract (MRE) Against Lipopolysaccharide-Activated RAW264.7 Cells and CCl_4-Induced Mouse Hepatic Damage

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Key Words
Lipopolysaccharide • Carbon tetrachloride • Morus australis • Morusin

Abstract
Background/Aims: Inflammation is one of the main contributors to chronic diseases such as cancer. It is of great value to identify the potential activity of various medicinal plants for regulating or blocking uncontrolled chronic inflammation. We investigated whether the root extract of *Morus australis* possesses antiinflammatory and antioxidative stress potential and hepatic protective activity. ***Methods:*** The microwave-assisted extractionwere was used to prepare the ethanol extract from the dried root of Morus australis (MRE), including polyphenolic and flavonoid contents. Lipopolysaccharide (LPS)-stimulated RAW264.7 cells was examined the anti-inflammatory and anti-oxidative potential of MRE. CCl_4-induced mouse hepatic damage were performed to detect the hepatic protective potential *in vivo*. Immunohistochemistry (IHC) and western blot assays were used to detect target proteins. ***Results:*** MRE contained approximately 23% phenolic compounds and 3% flavonoids. The major flavonoid component of MRE was morusin. MRE and morusin inhibited lipopolysaccharide-induced production of nitrite and prostaglandin E2 in RAW264.7 cells. MRE and morusin also suppressed the formation of intracellular reactive oxygen species and the expression of iNOS and COX-2. In an *in vivo* study, a thiobarbituric acid reactive substances assay showed that MRE inhibited CCl_4-induced

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oxidative stress and expression of nitrotyrosine. MRE also decreased CCl_4-induced hepatic iNOS and COX-2 expression, as well as CCl_4-induced hepatic inflammation and necrosis in mice. ***Conclusion:*** MRE exhibited antiinflammatory and hepatic protective activity.

Introduction

Inflammation is a complex biological immune response of the body to harmful stimuli, such as pathogens, damaged cells, and other irritants. Acute inflammation is a short-term process that usually lasts for a few hours up to several days and ceases upon the removal of the injurious stimuli. If the progressive destruction persists for months and years, chronic inflammation can be triggered. This degenerative process creates cellular stress and metabolic disorder, thus causing chronic diseases, such as cancer, ageing, and atherosclerosis. Because many inflammatory mediators lead to a wide range of pathological changes, it is difficult to treat inflammation [1]. Therefore, medicinal plants have received great attention and are expected to provide efficacious disease-prevention agents. In pathogenic conditions, abnormally activated macrophages produce excessive amounts of various pro-inflammatory mediators and cytokines that eventually aggravate inflammatory conditions. Lipopolysaccharides (LPSs), a component of the outer membrane of Gram-negative bacteria, are the most common cause of macrophage activation [2-3]. LPS-induced activation of macrophages causes a wide range of pro-inflammatory responses, such as production of pro-inflammatory mediators. Therefore, suppressing aberrant macrophage activation might be a valuable therapeutic target for treating inflammatory disorders.

Liver inflammation is usually attributed to the intensive exposure to endogenous and exogenous reactive oxygen species (ROS) [4]. Trichloromethyl radicals, derived from CCl_4, can initiate oxidative stress, resulting in hepatoxicity [5, 6]. In addition, trichloromethyl radicals can be biotransformed into peroxynitrite, a strong oxidant capable of disrupting the physiological cellular structure and function through peroxidation of cellular lipids, proteins, and DNA. Therefore, CCl_4–induced liver injury has been widely used as a rodent model for the development of hepatic protective agents.

The root bark of the mulberry tree (*Morus* spp., Moraceae) is used in traditional Chinese medicine as an antiinflammatory, antipyretic, antiheadache, and diuretic. From these species, a series of prenylated flavonoids, stilbenes, 2-arylbenzofurans, and other phenolic compounds have been extracted with various biological activities, including antioxidant, anticancer, and antiinflammatory properties [7–12]. Among them, *Morus australis,* one of the major species, is commonly distributed in Taiwan. Its leaves are used to feed silkworms, and its fruit is used as a raw food or to make juice, wine, vinegar, and jam. Moreover, it has been suggested that the extracts from the leaves and fruits have great biological potential for preventing hepatic lipogenesis and obesity [13–14]. The *M. australis* root extract, which contains some flavonoids and stilbenes, has been proposed for use in foods as an antibrowning agent and in cosmetics as a skin-whitening agent [15]. However, the other biological activities of the *M. australis* root have not yet been clarified. In the present study, the antiinflammatory effect of the ethanol extract of the dried root of *M. australis* (MRE) was evaluated *in vitro* and *in vivo*. It was demonstrated that MRE exhibits a hepatic protective effect through antiinflammatory and antioxidative stress potential.

Materials and Methods

Chemicals

RPMI medium, phosphate-buffered saline (PBS), fetal bovine serum, penicillin/streptomycin/neomycin (PSN), and trypsin-EDTA were purchased from Gibco Ltd. (Grand Island, NY, USA). Antibodies against COX-2, iNOS, and actin were obtained from Santa Cruz Biotechnology, Inc., CA, USA. Morusin was synthesized in our laboratory [16]. The dried root of *M. australis* was purchased from a reputable folk medicine store in Taiwan. A prostaglandin E2 and IL-6 immunoassay kit was obtained from R&D Systems (Minneapolis, MN, USA) and a protein assay kit was obtained from Bio-Rad Laboratories (Watford, Herts, UK). LPS (endotoxin from *Escherichia coli*, serotype 0127: B8) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of MRE

MRE was prepared from the dried root of *M. australis,* which was shredded and ground. Then it was extracted with 10 volumes of 95% ethanol using a microwave extractor (Milestone) equipped with a reflux unit and with a nominal maximum power of 300W for 20 min. The mixture was filtered through filter paper (5 μm pore size), and the filtrate was dried using rotary evaporation under vacuum at 40ºC. The percentage yield was 9.5% (w/w).

Assay of total phenolic content

The total phenolic compound content of MRE was spectrophotometrically determined in accordance with the Folin-Ciocalteu procedure by reading the absorbance at 725 nm against a methanol blank. Briefly, samples (20 μL, water added to 1.6 mL) were placed in the test tubes, to which 100 μL Folin-Ciocalteu reagent and 300 μL of sodium carbonate (20%) were then added. The contents were mixed and incubated at 40°C for 40 min. The absorbance at 725 nm was measured. The total phenolic content was expressed as milligrams per gram of MRE for gallic acid.

Assay of total flavonoid content

The total flavonoid content was analyzed by using $AlCl_3$. Briefly, 0.5 mL of MRE was mixed with 0.1 mL of $AlCl_3$ (10%, w/v), 1.5 mL of ethanol, and 0.1 mL of 1M CH_3COOK. The total volume of the mixture was adjusted to 5 mL with distilled water and then mixed well. After 40 min, absorbance at 415 nm was measured and the flavonoid content was estimated by calibration curves using quercetin. The percentage of flavonoid was expressed as quercetin equivalents in milligrams per 100g of fresh weight.

HPLC Analysis

MRE was analyzed using a Hitachi L7100 HPLC system with a 5-μm ODS-Hypersil column (250 x 4.6 mm). The mobile phase was generated from solvent A (acetonitrile) and solvent B (water) using the following gradient program: 5% solvent A and 95% solvent B at 0 min, 90% solvent A and 10% solvent B at 20 min, 95% solvent A and 5% solvent B at 45 min. The detection wavelength was 270 nm.

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. To investigate investigated whether the root extract of Morus australis possessed anti-inflammatory and anti-oxidative stress potential and hepatic protective activity, we pretreated RAW264.7 cells to MRE and morusin for 30 min and then co-treated them with the LPS for 24 h.

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 MRE (0–80 μg/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 the wells to solubilize the crystals. The optical density of each sample was read at 563 nm against the blank prepared from cell-free wells.

Intracellular reactive oxygen species measurement

Relative changes in intracellular ROS were monitored using the fluorescent probe H_2DCF-DA, which diffused through the cell membrane readily and was hydrolyzed by intracellular esterase to nonfluorescent $DCFH_2$, which was then rapidly oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity was proportional to the amount of intracellular ROS. After treatment with MRE and LPS, the RAW 264.7cells was achieved by incubation with H_2DCF-DA for 30 min at a final concentration of 10 μM at 37°C in the dark. Fluorescence was monitored by fluorophotometer (excitation, 485 nm; emission, 528 nm).

Nitrite measurement

Total nitrite in the culture medium was measured using the Griess reagent as described [17]. After incubation of RAW 264.7 cells with or without LPS and/or MRE at 37°C for 24 h, 100 μL of each culture medium was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Absorbance at 550 nm was then read and compared with known standard solutions of $NaNO_2$.

Determination of PGE2

RAW 264.7 cells were plated in 24-well plates at 1×10^6 cells per well, after the 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 MRE (0-40 μg/mL) at 37°C for 24 h, the production of prostaglandin E2 (PGE2) of each culture medium was determined with a commercial ELISA kit.

Preparation of total cell extracts and immunoblots analysis

The cells were collected using trypsin-EDTA and lysed in RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP-40) containing protease inhibitors. After mixing for 30 min at 4°C, the mixtures were centrifuged (10, 000×g) for 10 min at 4°C and the supernatants were collected as whole-cell extracts. The protein content was determined using the Bio-Rad protein assay reagent and bovine serum albumin as a standard. An equal amount of protein from the total cell extracts was boiled for 8 min. The extracts were separated by SDS-polyacrylamide gels and transferred to an NC membrane (Whatman). The blots were blocked in 5% nonfat dry milk/PBS for 1 h at room temperature. The blots then were incubated overnight with primary antibodies, followed by horseradish peroxidase-conjugated goat anti-mouse (or anti-rabbit) IgG for 1h. The immunoreactive bands were revealed by enhanced chemiluminescence with a commercially available ECL kit.

Animal treatment

Male ICR mice (body weight 19–21g) were purchased from GlycoNex Inc. (Taiwan) and maintained in cage housing in a specifically designed pathogen-free isolation facility with a 12/12 h light/dark cycle. Animal care and the general protocols for animal use were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University Animal Ethics Research Board. The mice were intragastric with 150 and 300 mg/kg of MRE thrice weekly for 7 weeks. After MRE treatment for 1 week, the mice of the CCl_4-treated-alone group or the co-treatment with MRE group were given 0.1 mL/mice of CCl_4 (20% CCl_4 in olive oil) via intragastric procedure twice a week for 6 weeks. Those in the control and the MRE-treated-alone groups were given an equal volume of olive oil. The mice were divided randomly into five groups of six mice each. Then, the mice were sacrificed 24 h after the last treatment of CCl_4. The livers were divided into two portions: (1) preserved in 10% formalin for histological and immunohistochemistry examination; (2) frozen for immunoblotting analysis at -80°C.

Liver lipid peroxidation and inflammatory mediator analysis

For the preparation of liver cytosol, 50mg of liver tissues were homogenized at 4°C in 1 mL RIPA lysis buffer containing 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 μg/ml of each leupeptin and pepstatin. A modified thiobarbituric acid reactive species (TBARS) assay was performed to determine the lipid peroxides. Briefly, 100 μL of liver cytosol was added to 200 μL of 10% TCA and then centrifuged at 14, 000 rpm for 10 min. 100 μL of supernatant was reacted with 200 μL 0.67% TBA

at 100°C for 30min and detected at 532 nm, then compared with known standard solutions of 1, 1, 3, 3-tetramethoxypropane. In addition, a volume equivalent to 80 μg proteins of liver cytosol was analyzed by Western immunoblotting assay against anti- COX-2 and anti-iNOS as previously described.

Histopathological and immunohistochemistry analysis

Liver tissues from each mouse were rapidly removed, fixed in 10% neutral-buffered formalin, and processed routinely. Paraffin-embedded sections were cut into sections 4 μm thick. The sections were stained with hematoxylin and eosin (H&E), then examined under a light microscope. Immunohistochemistry (IHC) staining was performed using the primary antibody F4/80 (a global marker for murine macrophages) and nitrotyrosine (oxidative stress marker) and a biotinylated secondary antibody (Vectastain Universal Elite ABC Kit, Burlingame, CA, USA) according to the manufacturer's instruction. Digital images were captured using a digital camera (100× magnification) for each slide.

Statistical Analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc test. *P* values less than 0.05 were considered statistically significant.

Results

Analysis of total phenolic and flavonoid content in MRE and HPLC

Microwave-assisted extraction has received increasing attention as an alternative to traditional solid-liquid extraction methods, mainly due to considerable savings in processing time and solvent consumption. Our preliminary extraction study showed that the yield of microwave-assisted extraction was higher than that of Soxhlet extraction. Thereafter, we used the microwave-assisted extraction to prepare MRE. The polyphenolic compounds, including flavonoids, in the roots of *Morus* spp. exhibit biological activity [7–14]. Thus, we assessed the total polyphenolic and flavonoid content. MRE contained approximately 23% phenolic components (gallic acid as the standard) and 3% flavonoids (quercetin as the standard). Morusin had a retention time of 27.16 min (Fig. 1).

MRE reduces LPS–induced intracellular ROS

To evaluate the antioxidative and antiinflammatory effect of MRE, we used an *in vitro* model with the murine RAW264.7 macrophage cell line. Because MRE showed no cytotoxicity toward RAW264.7 macrophages at concentrations up to 40 μg/mL (Fig. 2A), we used 10, 20, and 40 μg/mL MRE for the rest of the *in vitro* experiments. First, we determined the effect MRE on the production of intracellular ROS induced by LPS. LPS-induced intracellular ROS were reduced significantly by 20

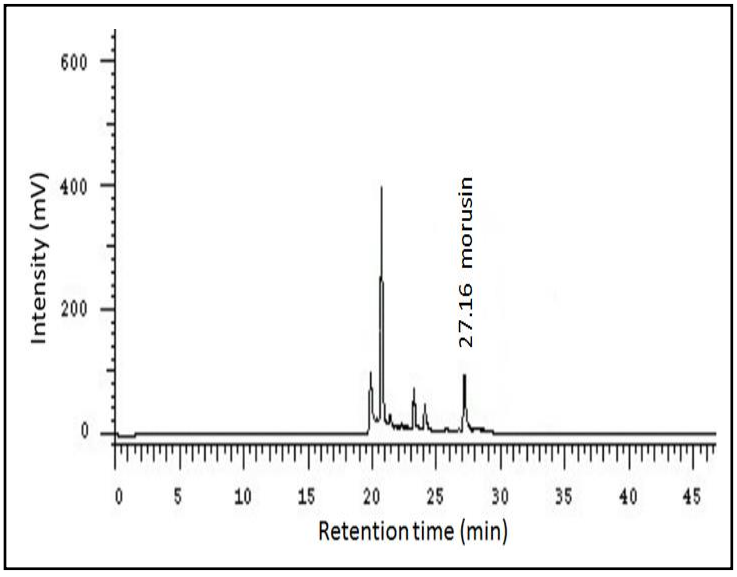

Fig. 1. HPLC analysis of Morus australis extract. For the conditions, see the section of Methods. Retention time peak at 20.82 min is oxyresveratrol and at 27.16 min is morusin.

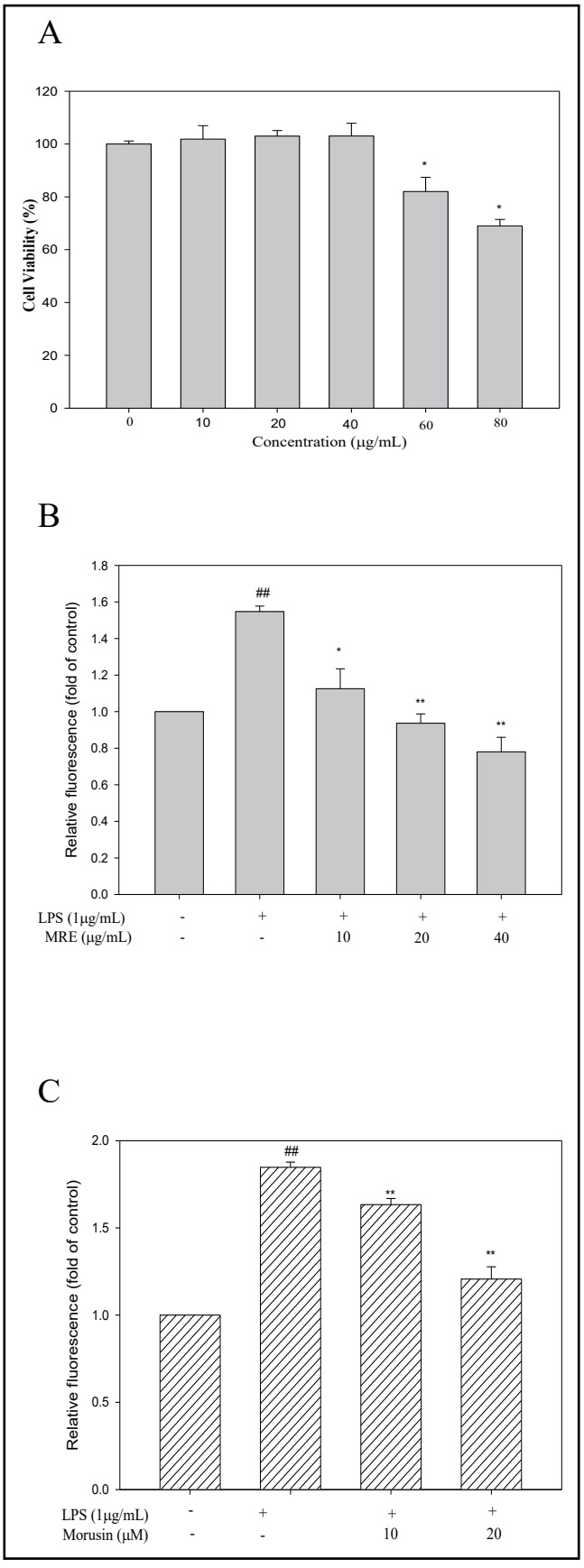

Fig. 2. Effects of MRE on cell viability and LPS-promoted intracellular ROS in RAW-264.7 cells. (A) RAW264.7 cells were treated with 0- 80 μg/mL of MRE for 24 h, after which the cell viability was determined by MTT assay as described in Methods section. The results are reported as the mean±SD of three independent experiments. Data presented as mean ± S.D of three independent experiments. *p<0.05, compared with control (lane 1). RAW-264.7 cells were treated with indicated concentrations of (B) MRE or (C) morusin for 30 min and stimulated with or without LPS for 24 h. Intracellular ROS were determined by the DCF fluorescence intensity as described in the text. Data presented as mean ± S.D of three independent experiments. ##p<0.01, compared with control (lane 1). *p<0.05, **p<0.01, compared with LPS treated alone.

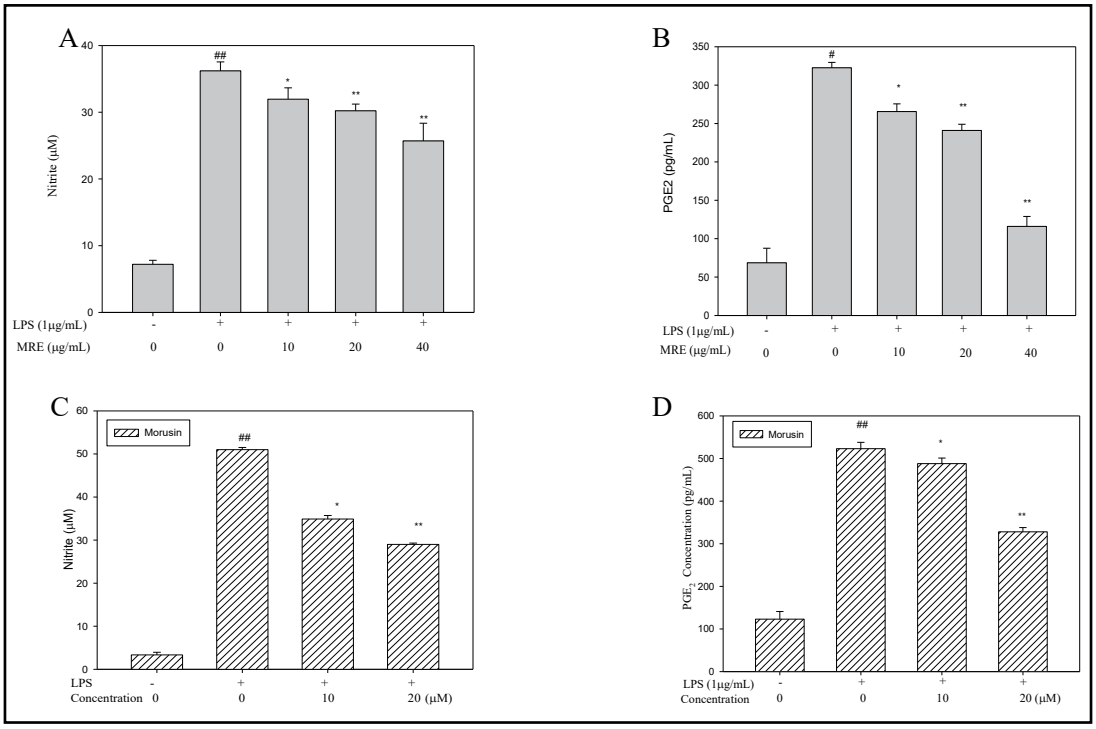

Fig. 3. Effects of MRE and morusin on LPS-stimulated production of nitrite and PGE2 in RAW-264.7 cells. (A) RAW-264.7 cells (5 x 10^5 cells/mL) were treated with indicated concentrations of MRE for 30 min and stimulated with or without LPS for 24 h. Supernatants were collected for nitrite analysis by Griess reagent and determined at 550 nm. (B) PGE_2 analysis was performed by ELISA assay determined at 450 nm respectively. (C) RAW-264.7 cells were treated with indicated concentrations of morusin for 30 min and stimulated with or without LPS for 24 h. Supernatants were collected for nitrite analysis and (D) PGE_2 analysis. Data presented as mean ± S.D of three independent experiments. ##p<0.01, compared with control (lane 1). *p<0.05, **p<0.01, compared with LPS stimulation alone.

and 40 μg/mL MRE (Fig. 2B). In addition, because the antioxidant and antiinflammatory properties of oxyresveratrol, one of the major components of MRE, have been assessed [10], we determined the antioxidant and antiinflammatory activity of morusin. We found that 10 and 20 μM morusin was noncytotoxic (data not shown) and exhibited a significant inhibitory effect on the LPS-induced intracellular ROS (Fig. 2C).

MRE inhibits LPS–stimulated production of inflammatory mediators

Chronic inflammation is associated with many diseases, such as cardiovascular and hepatic disease. We determined the effect of MRE on the LPS-stimulated production of nitrite and prostaglandin E2 (PGE2) by RAW264.7 cells. MRE inhibited LPS-induced nitrite and PGE2 production in a dose-dependent manner (Fig. 3A and 3B). In addition, morusin (10 and 20 μM) significantly inhibited LPS-induced nitrite and PGE2 production (Fig. 3C and 3D). Consistent with the findings for PGE2 and nitrite production, immunoblotting analysis showed that LPS-stimulated COX-2 and iNOS expression in RAW264.7 cells was also reduced by MRE treatment (Fig. 4A). In addition, morusin significantly inhibited LPS-stimulated COX-2 and iNOS expression (Fig. 4B).

Body weight examination

Fig. 5 shows the variation of relative changes in body weight during the 7-week experiment. The body weights of the group that received MRE treatment alone were similar to those of the control group (Fig. 5). In addition, the weights of the livers and kidneys from the group treated with MRE alone were similar to those in the control group (data

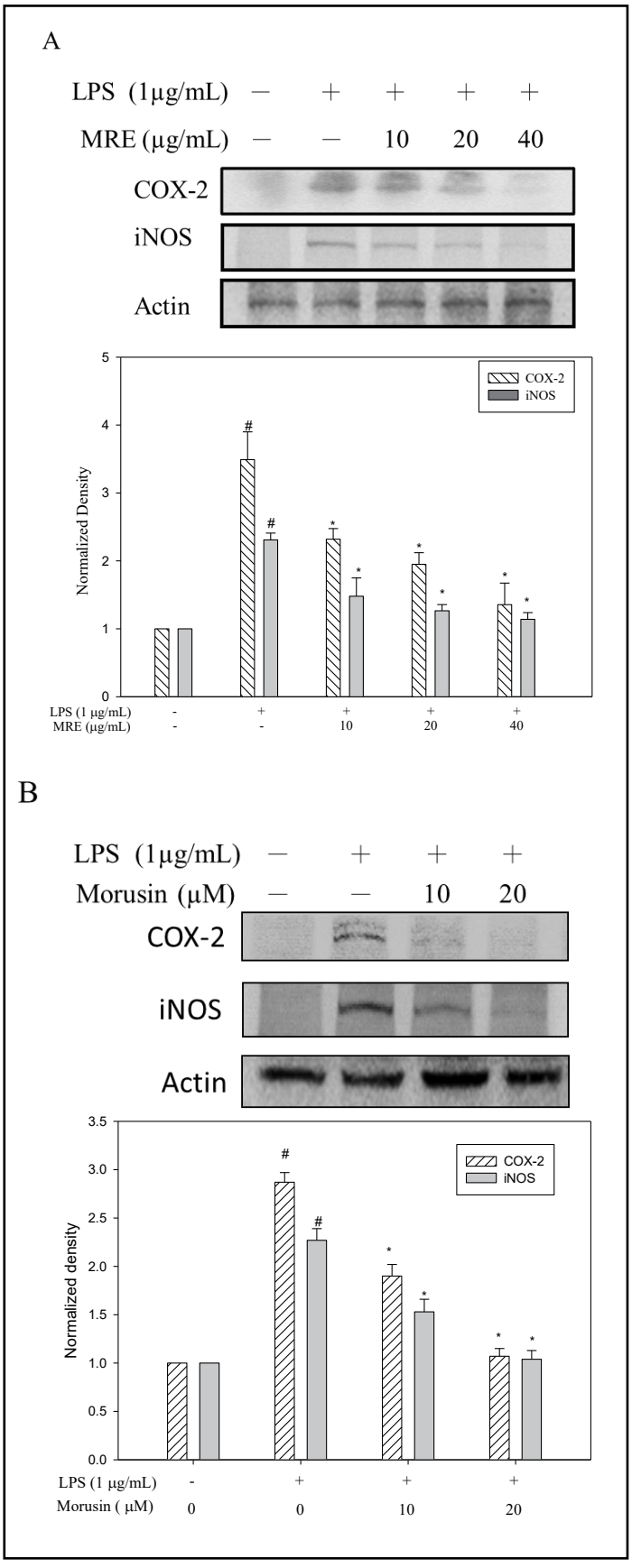

Fig. 4. Effect of MRE and morusin on LPS-stimulated COX-2 and iNOS proteins expression in RAW-264.7 cells by western blotting analysis. Cells were treated with various concentration of MRE or morsuin for 30 min and then incubated with LPS (1μg/mL) for 24 h, and the cells were collected for protein extraction. Protein extract from (A) MRE or (B) morusin pretreatment was subjected to SDS-PAGE, and immunoblotting was performed against anti-COX-2 and anti-iNOS antibodies, and actin as internal control. The average densitometric values of three independent experiments is shown below as means ± SD. #p<0.05, compared with control (lane 1). *p<0.05, compared with LPS stimulation.

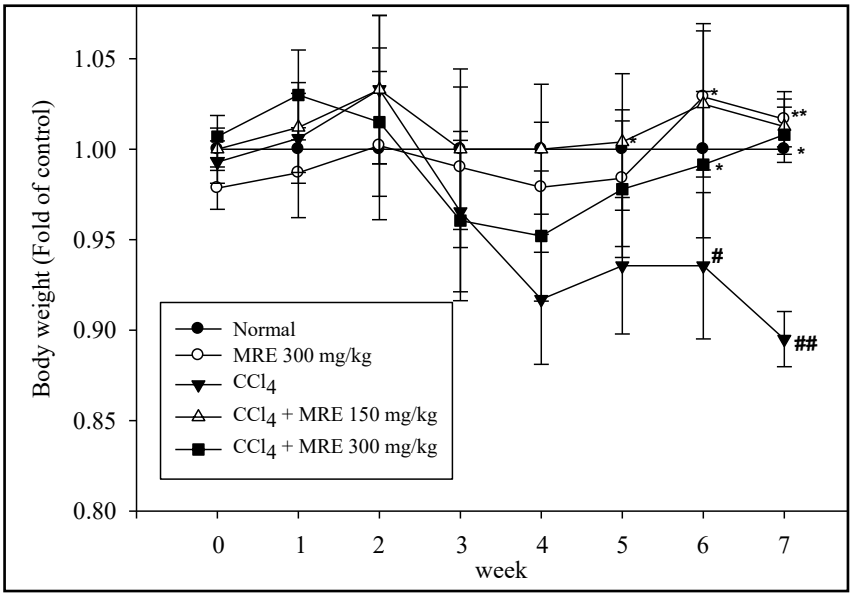

Fig. 5. Effect of MRE on the relative changes in body weight of mice with liver injury induced by CCl_4. #p<0.05, ##p<0.01, compared with control. *p<0.05, **p<0.01, compared with CCl_4 treated alone.

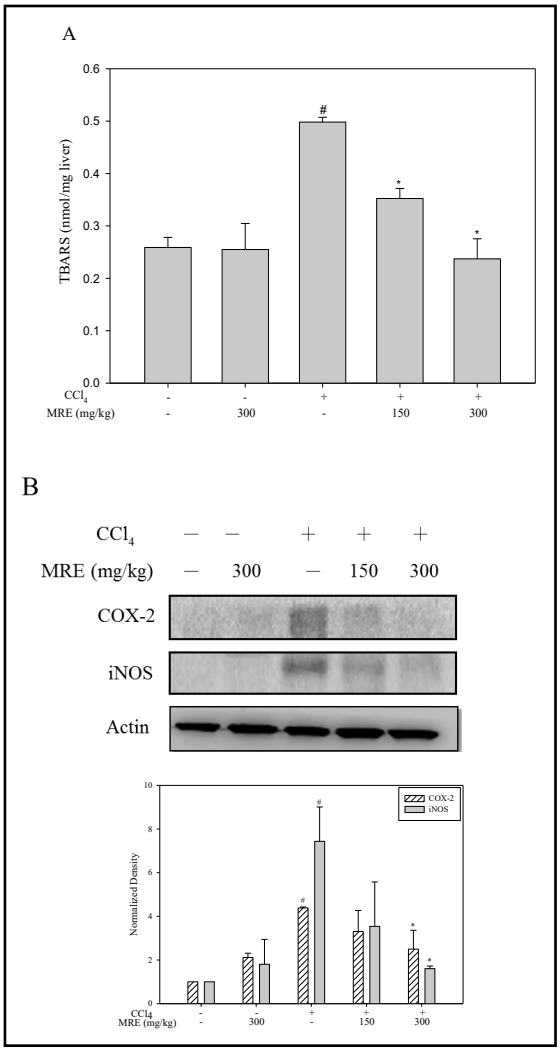

Fig. 6. Effect of MRE on CCl_4 induced hepatic lipid peroxidation and expression of COX-2 and iNOS in the mice. The hepatic homogenates of mice were analyzed as described in the Methods section. (A) The absorbance of TBARS was determined at 532 nm. Data presented as mean ± S.D (n=6). #p<0.05, compared with the control group (lane 1). *p<0.05, compared with the CCl_4 stimulation alone. (B) The representative immunoblotting was performed against anti-COX-2 and anti-iNOS antibodies, and actin as internal control. The average densitometric values was shown below as means ± SD. #p<0.05, compared with control (lane 1). *p<0.05, compared with CCl_4 treated alone.

not shown). This suggests that the treatment with MRE resulted in no toxicity. During the 6- and 7-week experiments, CCl_4 treatment significanly suppressed the increase in body weight compared with the control group, indicating that the growth of CCl_4-treated mice was strongly suppressed. In addition, the CCl_4-induced body weight loss was alleviated in the MRE-treated groups.

MRE ameliorates CCl_4-induced hepatic lipid peroxidation and inflammatory mediator expression

MRE prevented the increase of thiobarbituric acid reactive substances in the liver homogenates of the mice treated with CCl_4 in a dose-dependent manner (Fig. 6A). In addition, Western blot analyses of the iNOS and COX-2 protein levels in the liver showed that treatment with CCl_4 induced expression of both proteins that were undetectable in the control group and the group dosed with MRE alone. The expression of the iNOS and COX-2 protein in the liver was decreased by co-treatment with a low dose of MRE (150 mg/kg), and it was almost abolished by a high dose of MRE (300 mg/kg) (Fig. 6B).

Effect of MRE on CCl_4-induced histopathology and oxidative stress

The morphological changes in liver injury caused by CCl_4 were visualized in H&E stained liver sections. The changes included necrosis and inflammation with neutrophil infiltration (Fig. 7). The degree of necrosis and inflammation were decreased in animals treated with both MRE and CCl_4 compared with those treated with CCl_4 alone. MRE treatment alone did not cause visible changes in the liver. To evaluate the impact of MRE on inflammation and oxidative stress, hepatic macrophages and nitrotyrosine were assessed in the mouse liver using an immunohistochemistry assay. The intensities of F4/80 and nitrotyrosine were higher in the CCl_4-treated group than in the control group. However, co-treatment with MRE decreased the staining of F4/80 and nitrotyrosine, and the high-dose co-treatment of MRE (300 mg/kg) almost abolished both stainings (Fig. 7). These data suggest that MRE protects the liver against CCl_4-induced injury by attenuating inflammation and oxidative stress.

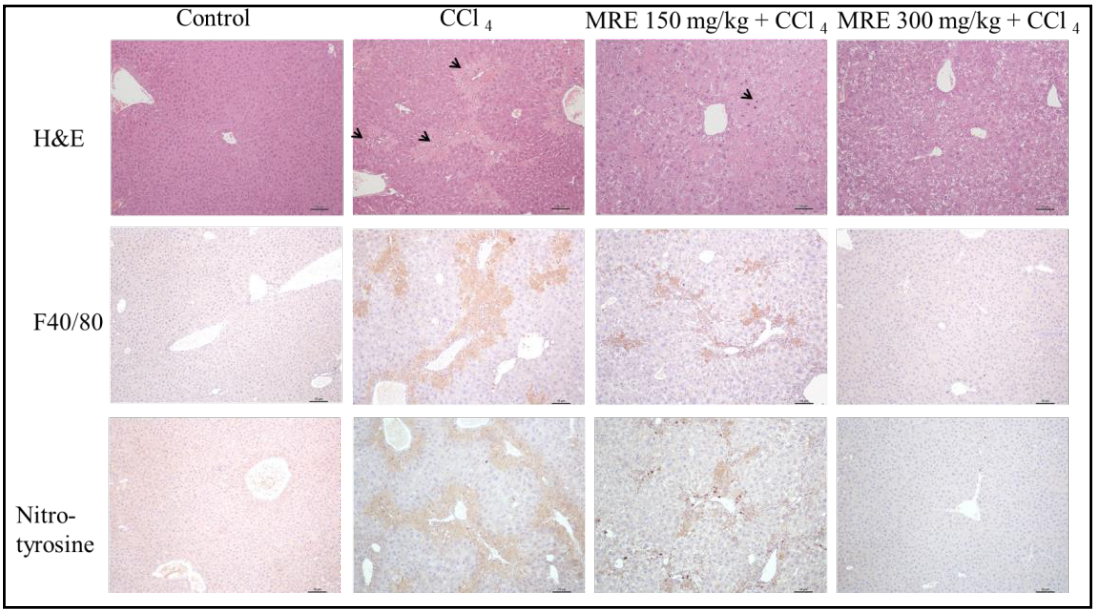

Fig. 7. Effect of MRE on histologic changes and liver Inflammation and oxidative stress induced by CCl_4 in mice. The sections of mouse liver were stained with hematoxylin-eosin (original magnification 100x). Marcrophage and oxidative stress were revealed as indicated by imunohistochemical staining of anti-F40/80 and anti-nitrotyrosine.

Discussion

Much attention has been devoted to identifying phytochemicals in medicinal plants and the diet that have preventive or therapeutic effects against various chronic diseases. *M. australis* exhibits beneficial effects, such as antileukemia, antiinflammatory, and antiobesity activities, as well as tyrosinase inhibition. Twenty-two compounds were isolated from the root extract of *M. australis* by Zheng et al. [15]. Nineteen of the compounds were flavonoids, one was a stilbene (oxyresveratrol), and two were arylbenzofuran derivatives. These compounds have a polyphenolic structure that contributes to many of their bioactivities [18]. Our results showed that the total phenolic content was 23% and the total flavonoid content was 3%. The major components were oxyresveratrol and morusin. Oxyresveratrol has possesses a wide variety of biological activities, such as skin-whitening, antiviral, and neuroprotective effects [15, 19–20]. Soonthornsit et al. reported that oxyresveratrol at concentrations of 5 and 10 μg/mL inhibited LPS-induced nitrite production and the expression of iNOS and COX-2 [12]. Morusin, a prenylated flavonoid, potentially has neuroprotective, anticancer, antioxidative, and ant-colitis activities [21–24]. In this study, the anti-inflammatory effect of ethanol extract from the dried root of *M. australis* (MRE) was evaluated *in vitro* and *in vivo*. In the present study, it showed about one fourth active phenolic components in MRE was morusin, 2% by HPLC analysis while in the following studies we investigate that 40 μg/mL of MRE exhibits a hepatic protection effect through anti-inflammatory and anti-oxidative stress potential as noncytotoxic concentrations. Morusin is one of the major components of MRE, we determined the used concentrations of morusin correlate with its content one tenth. The 4.2 μg/mL (10 μM) of morusin was noncytotoxic concentrations and exhibited a significantly inhibitory effect on the LPS-induced inflammatory effect in RAW264.7 cells. Our data show that morusin also inhibited LPS-induced nitrite production and the expression of iNOS and COX-2. Because pure phytochemicals are expensive, bioactive mixtures may be a realistic alternative for consumers. Our data demonstrated that MRE potently inhibited an LPS–activated inflammatory effect in RAW264.7 cells and CCl_4-induced liver injury in mice. The phenolic constituents, such as oxyresveratrol and morusin, exerted these effects. Therefore, MRE may be valuable as a preventive agent for inflammation-associated chronic disease.

Increased gene expression of iNOS and COX-2 is associated with inflammatory responses [25–26]. NO is synthesized from the amino acid arginine by nitric oxide synthase and induces tissue injury at the inflammatory site. COX-2 catalyses the conversion of arachidonic acid to prostaglandins, which contribute to chronic inflammatory conditions and oxidative stress [27]. Therefore, reduction of iNOS and COX-2 protein expression may be associated with the prevention and treatment of chronic inflammatory disease. In the present study, MRE inhibited iNOS and COX-2 expression induced by LPS stimulation in RAW264.7 cells or by CCl_4 treatment in mouse liver. In addition, a preliminary experiment showed that MRE scavenged free radicals (data not shown). Thus, MRE exerted its antioxidative stress potential by scavenging free radicals and decreasing the expression of iNOS and COX-2. Furthermore, increased nitrotyrosine and lipid peroxidation are indicators of oxidative stress-induced tissue injury. Our animal study demonstrated that MRE decreased CCl_4-induced oxidative stress, resulting in the mitigation of CCl_4-induced liver injury.

Many studies suggest a regular diet of fruit and vegetables as an antioxidative defence as part of a preventive strategy to inhibit, delay, or reverse human carcinogenesis using naturally extracted or synthetic chemical agents, including various phytochemicals [28]. Chemoprevention is an active hepatocellular carcinoma preventive strategy that aims to inhibit, delay, or reverse metabolic function disorders and hepatic injury using naturally extracted or synthetic chemical agents. Our study indicates that *M. australis* root extract may prevent liver damage. We have shown that t-BHP-induced apoptosis, the unfolded protein response, and oxidative stress correspond to the effects of mitochondrial respiratory-chain electron transport [29]. However, further studies of daily consumption of natural MRE products and studies of Chinese herbs, considered as blocking or suppressing agents, are still required to validate these mechanisms of unfolded protein response and oxidative stress corresponding to the effects of mitochondrial respiratory-chain electron transport [30].

Conclusion

We showed that iNOS/COX-2/PGE2 inflammation level and oxidative stress were moderated by morusin. An *in vivo* study demonstrated that MRE decreased the mouse hepatocyte cell loss and macrophages. MRE inhibited liver cell damage based on the oxidative stress levels and the expression of iNOS/COX-2/PGE2 (Fig. 8). These results imply that MRE possesses antiinflammatory and hepatic protective activity. However, the use of MRE in humans needs further clinical investigation.

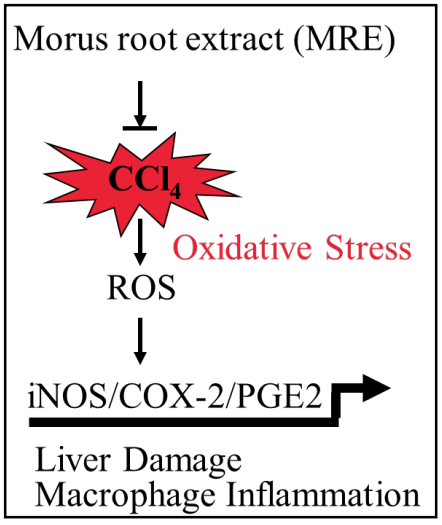

Fig. 8. Schematic presentation of the pathway depicting CCl_4 uptake and intracellular oxidative stress signaling inhibited by MRE leading to decrease of iNOS, COX-2, and their respective secreted metabolites in liver and RAW264.7 cells. The inhibition of effects of CCl_4 on the oxidative stress triggering production of ROS activates iNOS/COX-2/PGE2 complex formation, which was prevented the cells inflammation and increases liver injury in hepatocyte cells.

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Disclosure Statement

There is no financial/commercial conflict of interest.

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