

Original Article

Veterinary Acupuncture and  
Complementary Medicine



# Anti-microbial and anti-inflammatory effects of Cheonwangbosim-dan against *Helicobacter pylori*-induced gastritis

Hee-Seon Park <sup>1</sup>, Hye-Yun Jeong <sup>1</sup>, Young-Suk Kim <sup>1</sup>, Chang-Seob Seo <sup>2</sup>,  
Hyekyung Ha <sup>2</sup>, Hyo-Jung Kwon <sup>1,\*</sup>

<sup>1</sup>Department of Veterinary Pathology, College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Korea

<sup>2</sup>Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon 34054, Korea



Received: Oct 10, 2019

Revised: Jan 26, 2020

Accepted: Jan 26, 2020

\*Corresponding author:

Hyo-Jung Kwon

Department of Veterinary Pathology, College of Veterinary Medicine, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Korea.

E-mail: hyojung@cnu.ac.kr


© 2020 The Korean Society of Veterinary Science

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.


ORCID iDs

Hee-Seon Park 


<https://orcid.org/0000-0002-8614-1677>

Hye-Yun Jeong 


<https://orcid.org/0000-0003-4757-2287>

Young-Suk Kim 

<https://orcid.org/0000-0003-4559-2719>

Chang-Seob Seo 

<https://orcid.org/0000-0002-8156-446X>

Hyekyung Ha 

<https://orcid.org/0000-0002-7326-6366>

Hyo-Jung Kwon 

<https://orcid.org/0000-0001-5927-1970>

## ABSTRACT

**Background:** There are various *Helicobacter* species colonizing the stomachs of animals. Although *Helicobacter* species usually cause asymptomatic infection in the hosts, clinical signs can occur due to gastritis associated with *Helicobacter* in animals. Among them, *Helicobacter pylori* is strongly associated with chronic gastritis, gastric ulcers, and gastric cancers. As the standard therapies used to treat *H. pylori* have proven insufficient, alternative options are needed to prevent and eradicate the diseases associated with this bacterium. Cheonwangbosim-dan (CBD), a traditional herbal formula that is popular in East Asia, has been commonly used for arterial or auricular flutter, neurosis, insomnia, and cardiac malfunction-induced disease.

**Objectives:** The present study investigated the antimicrobial effect of CBD on *H. pylori*-infected human gastric carcinoma AGS cells and model mice.

**Methods:** AGS cells were infected with *H. pylori* and treated with a variety of concentrations of CBD or antibiotics. Mice were given 3 oral inoculations with *H. pylori* and then dosed with CBD (100 or 500 mg/kg) for 4 weeks or with standard antibiotics for 1 week. One week after the last treatment, gastric samples were collected and examined by histopathological analysis, real-time quantitative polymerase chain reaction, and immunoblotting.

**Results:** Our results showed that CBD treatment of AGS cells significantly reduced the *H. pylori*-induced elevations of interleukin-8, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). In the animal model, CBD treatment inhibited the colonization of *H. pylori* and the levels of malondialdehyde, inflammation, proinflammatory cytokines, iNOS, and COX-2 in gastric tissues. CBD also decreased the phosphorylation levels of p38 mitogen-activated protein kinase family.

**Conclusions:** This study suggests that CBD might be a prospective candidate for treating *H. pylori*-induced gastric injury.

**Keywords:** Cheonwangbosim-dan; *Helicobacter pylori*; gastritis

### Funding

The present research was supported by a grant from the Korea Institute of Oriental Medicine (KIOM; Evaluation of Herb-Drug Interactions, K16252).

### Conflict of Interest

The authors have no conflicts of interest to declare.

### Authors Contributions

Conceptualization: Kwon HJ; Data curation: Park HS, Jeong HY, Kim YS; Formal analysis: Park HS; Methodology: Jeong HY, Kim YS; Project administration: Kwon HJ; Resources: Seo CS, Ha H; Writing - original draft: Seo CS, Ha H, Park HS; Writing - review & editing: Kwon HJ.

## INTRODUCTION

The microaerophilic gram-negative bacterium, *Helicobacter pylori*, is a spiral organism that is strongly associated with chronic gastritis, gastric ulcers, and gastric cancers [1]. *H. pylori* prevalence is often higher than 50% in southern and eastern Europe, South America and Asia, while about a third of adults are still infected in northern Europe and North America [2]. Proton pump inhibitor-clarithromycin-based triple therapy is the first-line choice for eradicating *H. pylori*, but the eradication rate of this treatment against *H. pylori* has decreased to around 80%, mainly due to antibiotic resistance [3]. Given that the current standard therapies for treating *H. pylori* are insufficient, we need to identify alternative options to prevent and eradicate the diseases associated with this pathogen [4].

There are various *Helicobacter* species including *H. pylori*, *H. felis*, *H. bizzozeronii*, and *H. salomonis* colonizing the stomachs of animals [5]. Although gastritis in human and animal is caused by *Helicobacter*, they usually induce asymptomatic infection in the hosts. But, clinical signs such as chronic vomiting and weight loss in pet animals can occur due to gastritis associated with *Helicobacter* [6]. While treating *Helicobacter* infection in dogs and cats is still controversial, a study suggested cats may be a potential natural reservoir of *H. pylori* and cats with *H. pylori* infection induce more severe gastritis than those with *H. felis* or *H. heilmannii* [7].

*H. pylori* colonization induces an inflammatory reaction in the gastric mucosa; this is mediated by neutrophils and mononuclear cells and is characterized by the up-regulation of various cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and tumor necrosis factor alpha (TNF- $\alpha$ ) [8]. In gastric mucosa, inflammatory cytokines have been shown to generate reactive oxygen species (ROS) [9]. During the inflammatory response, ROS impair the important biomolecules, such as DNA, lipids, and proteins leading to gastric mucosal injury, while conversely triggering the production of malondialdehyde (MDA), which is a cytotoxic molecule [10]. This inflammatory reaction is accompanied by the up-regulation of nitric oxide (NO) and prostaglandin E<sub>2</sub>, which are induced by the overexpression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) [11].

The herbal remedy called Cheonwangbosim-dan (CBD) in Korean and Tian Wang Bu Xin Dan in Chinese is one of the most popular traditional herbal formulas in East Asian countries. CBD consists of 15 medicinal herbs: *Rehmannia glutinosa*, *Coptis japonica*, *Acorus gramineus*, *Panax ginseng*, *Angelica gigas*, *Schisandra chinensis*, *Asparagus cochinchinensis*, *Liriope platyphylla*, *Thuja orientalis*, *Ziziphus jujuba*, *Scrophularia buergeriana*, *Poria cocos*, *Salvia miltiorrhiza*, *Platycodon grandiflorum*, and *Polygala tenuifolia* [12]. It has been commonly used to treat arterial or auricular flutter, neurosis, insomnia, and cardiac malfunction-induced disease [13]. CBD has been shown to have a variety of biological activities, including neurological, vasorelaxant, and hypotensive effects [14]. However, the effect of CBD against *H. pylori* has not been examined. In the present work, we therefore examined whether CBD has antimicrobial activity against *H. pylori* in AGS cells and model mice that has been used extensively in *Helicobacter* research [15].

## MATERIALS AND METHODS

### Chemicals, materials and CBD preparation

Berberine chloride (PubChem CID: 12456, purity 98.0%), cinnamic acid (PubChem CID: 444539, purity 99.5%), and coptisine chloride (PubChem CID: 72321, purity 98.0%) were

purchased from Wako Pure Chemical Co. (Japan). (*E*)-harpagoside (PubChem CID: 5281542, purity 98.0%),  $\beta$ -asarone (PubChem CID: 5281758, purity 98.1%), nodakenin (PubChem CID: 73191, purity 98.0%), and 5-(hydroxymethyl)-2-furaldehyde (5-HMF; PubChem CID: 237332, purity 99.0%) were purchased from ChemFaces (China), Carl Roth (Germany), NPC Bio Technology (Korea), and Merck KGaA (Germany), respectively. High-performance liquid chromatography (HPLC)-grade acetonitrile and water were purchased from J.T. Baker (USA) and anhydrous acetic acid was purchased from Merck KGaA.

Powdered CBD water extract was prepared by the Korea Institute of Oriental Medicine (Korea). Crude preparations of 15 herbs (**Table 1**) were extracted with a 10-fold mass of distilled water at 100°C for 2 h under pressure (98 kPa) using an electric extractor (COSMOS-660; Kyungseo Machine Co., Korea). The extracted water solution was filtered through a standard sieve (No. 270, 53  $\mu$ m; Chung Gye Sang Gong Sa, Korea) and freeze-dried using a vacuum control freeze dryer (PVT100; IlShinBioBase, Korea) to yield a powder of water decoction. Preparation of CBD water extract is described in The Korean Herbal Pharmacopoeia with regard to standard manufacturing processes [16]. In addition, we previously reported a study on the simultaneous analysis using HPLC and LC-MS/MS for quality assessment of CBD [17,18].

### HPLC analysis of CBD

Chromatographic analysis of 5-HMF, coptisine chloride, berberine chloride, nodakenin, (*E*)-harpagoside, cinnamic acid, and  $\beta$ -asarone was performed with a Prominence LC-20A series HPLC system (Shimadzu Co., Japan), which consisted of a solvent delivery unit (LC-20AT), online degasser (DGU-20A<sub>3</sub>), column oven (CTO-20A), auto sample injector (SIL-20AC), and photo-diode array (PDA) detector (SPD-M20A). The measured data were processed using LCsolution software (Version 1.24; Shimadzu). The major components of the CBD sample were separated using a Gemini C<sub>18</sub> column (250 mm  $\times$  4.6 mm; 5  $\mu$ m particle size; Phenomenex, USA) at 40°C. The mobile phases consisted of (A) distilled water and (B) acetonitrile, both containing 1.0% (v/v) acetic acid. The gradient condition was as follows: 5–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min, and 100–5% B for 45–50 min. The flow rate and injection volume were 1.0 mL/min and 10  $\mu$ L, respectively.

**Table 1.** Composition of Cheonwangbosim-dan

Herbal name	Scientific name	Origin	Amount (g)
Rehmanniae Radix	<i>Rehmannia glutinosa</i>	Andong, Korea	15.0
Coptidis Rhizoma	<i>Coptis japonica</i>	China	7.5
Acori Graminei Rhizoma	<i>Acorus gramineus</i>	Jeju, Korea	3.75
Ginseng Radix Alba	<i>Panax ginseng</i>	Yeongju, Korea	1.875
Angelicae Gigantis Radix	<i>Angelica gigas</i>	Bonghwa, Korea	1.875
Schizandrae Fructus	<i>Schisandra chinensis</i>	Samcheok, Korea	1.875
Asparagi Tuber	<i>Asparagus cochinchinensis</i>	China	1.875
Liriope Tuber	<i>Liriope platyphylla</i>	Miryang, Korea	1.875
Thujae Semen	<i>Thuja orientalis</i>	China	1.875
Zizyphi Semen	<i>Zizyphus jujuba</i>	China	1.875
Scrophulariae Radix	<i>Scrophularia buergeriana</i>	Uiseong, Korea	1.875
Poria Sclerotium	<i>Poria cocos</i>	Pyeongchang, Korea	1.875
Salviae Miltiorrhizae Radix	<i>Salvia miltiorrhiza</i>	China	1.875
Platycodi Radix	<i>Platycodon grandiflorum</i>	Muju, Korea	1.875
Polygalae Radix	<i>Polygala tenuifolia</i>	China	1.875
Total amount	-	-	48.75

### Bacterial strains and cell culture

*H. pylori* strains SS1, 26695, PED503G, and PED3582GA were supplied by the *H. pylori* Korean Type Culture Collection (Gyeongsang National University School of Medicine, Korea). Each strain was grown on brucella agar plates (Difco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), in 10% CO<sub>2</sub> and 100% humidity at 37°C. Human gastric carcinoma AGS (ATCC CRL 1739; ATCC, USA) cells grown in antibiotic-free RPMI-1640 or DMEM (Gibco) supplemented with 10% FBS. CBD, amoxicillin, or clarithromycin at a variety of concentrations were incubated with *H. pylori* suspensions at OD<sub>600</sub> 1.0 (1 × 10<sup>8</sup> colony-forming unit [CFU]/mL) for 24 h, 100 μL of each bacterial suspension was added to AGS cells, and incubation was continued for 6 h. Supernatants were assessed for the IL-8 level, and proteins were collected from AGS cells.

### Disk agar diffusion method

The antimicrobial ability of CBD was tested by the disk agar diffusion method. The CBD extract was dissolved in phosphate buffered saline (PBS) and impregnate 6-mm blank paper disks. As positive controls, we used 2 μg of amoxicillin or clarithromycin (Oxoid, UK). The disks were placed on plates that had been spread with brucella agar (Difco) containing 10% FBS and top coated with 0.1 mL of *H. pylori* suspension at OD<sub>600</sub> 6.0. The plates were incubated in 10% CO<sub>2</sub> at 37°C and 100% humidity for 48 h. The diameter of the inhibition zone was recorded for each *H. pylori* strain.

### Determination of the minimum inhibitory concentration (MIC)

The broth-dilution method was used to measure the MIC. *H. pylori* were suspended at OD<sub>600</sub> 1.0 in 10% FBS supplemented-brucella broth and incubated with CBD, amoxicillin, or clarithromycin at a variety of concentrations at 37°C. After 48 h, 100 μL of bacterial suspension was mixed with 100 μL urease reagent (3 mM PBS, pH 5.8, 2% urea, and 7 μg/mL phenol red, pH 5.0). The resulting mixture was incubated for 4 h at 37°C, and a plate reader (Infinite M200 Pro; TECAN, Switzerland) was used to measure the MIC, with absorption assessed at 560 nm.

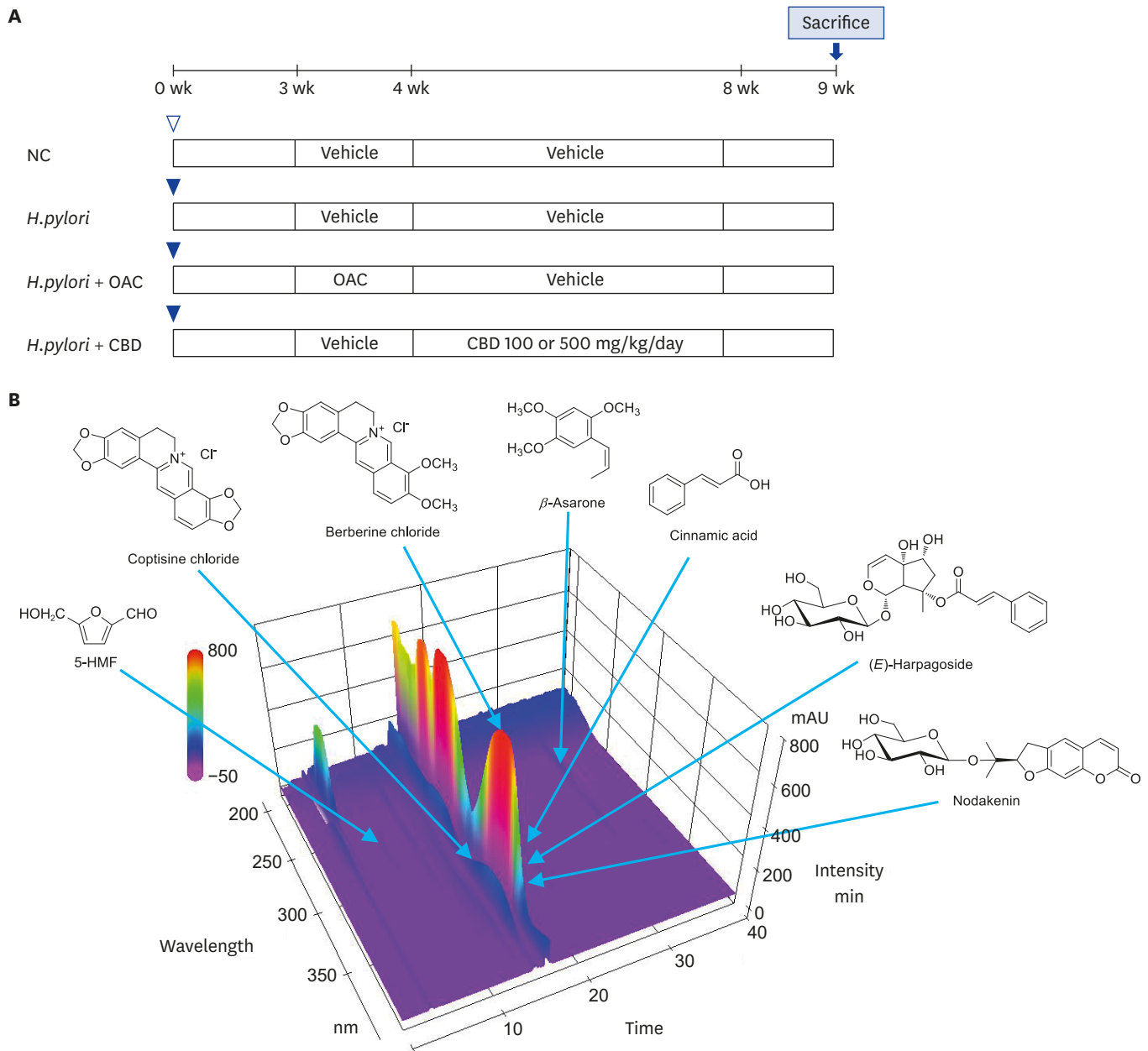
### Enzyme-linked immunosorbent assay (ELISA) detection of IL-8

Secreted IL-8 was measured using an ELISA kit (R&D Systems, USA). Briefly, AGS cells (2 × 10<sup>5</sup>/well) were seeded in 6-well plates. Suspensions of *H. pylori* at OD<sub>600</sub> 1.0 were treated with various concentrations of CBD, amoxicillin, or clarithromycin for 24 h, and then applied to the AGS cells for 6 h. Supernatants were collected, and the levels of secreted IL-8 were measured as described by the manufacturer.

### Animal experiment

Male 4-week-old C57BL/6 mice (Orient Bio Co., Korea) were maintained on standard rodent chow with a 12 h light/dark cycle. All protocols involving animals were approved by the Animal Experimental Ethics Committee of Chungnam National University (Korea; approval No: CNU-00444). We conducted a preliminary trail with 3 doses of CBD (100, 500, and 1,000 mg/kg) to identify the optimum dose. Based on these experiments, we chose a dose of 100 mg/kg/day as a low concentration or 500 mg/kg/day as a high concentration, because these doses reduced *H.pylori*-induced gastritis-related parameters (data not shown).

The mice were allocated to 5 groups (n = 10/group) randomly as per the scheme shown in **Fig. 1A**. Mice in the uninfected control group received PBS, while those in the infected groups were infected orally with *H. pylori* SS1 (1 × 10<sup>9</sup> CFU/mL/mouse) 3 times at 2-day intervals. Two weeks



**Fig. 1.** Animal experiment and 3D chromatogram. (A) Experimental protocol for development of *H. pylori*-infected murine model. Mice received intragastric inoculations of *H. pylori* SS1 ( $1 \times 10^9$  CFU/mouse; arrow heads) or vehicle (empty arrow head) 3 times at 2 day intervals and treatment with CBD (100 or 500 mg/kg) or OAC. (B) 3D chromatogram of CBD obtained using HPLC-PDA. 3D, three-dimensional; CBD, Cheonwangbosim-dan; CFU, colony-forming unit; NC, normal control; OAC, omeprazole (400  $\mu$ mol/kg), amoxicillin (28.6 mg/kg), and clarithromycin (14.3 mg/kg); HPLC-PDA, high-performance liquid chromatography photo-diode array; 5-HMF, 5-(hydroxymethyl)-2-furaldehyde.

after inoculation, mice in the infected control group received PBS for 7 days, mice in the triple therapy group were treated daily by gavage for 1 week with omeprazole (400  $\mu$ mol/kg), amoxicillin (28.6 mg/kg), and clarithromycin (14.3 mg/kg) (OAC), and mice in the CBD-treated groups were orally gavaged each day with the indicated concentrations of CBD for 4 weeks. The animals had no access to food or water for 6 h before and after each inoculation. At 1 week after the last treatment, the mice were sacrificed.

### Histopathological examination

Stomach tissues fixed with 10% neutral buffered formalin were washed, processed, and embedded in paraffin. Blocks were sectioned (4  $\mu\text{m}$ ) and stained with hematoxylin and eosin for examination by microscopy. The inflammation scores were graded as previously described [19].

### Quantification of DNA from *H. pylori*

Gastric samples were subjected to extraction of genomic DNA using a QIAamp DNA Mini Kit (Qiagen, USA) as per the manufacturer's protocol. Quantification of *H. pylori* 16S ribosomal DNA (rDNA) was performed using TaqMan Master Mix (Life Technologies, USA) and an Applied Biosystems 7500 Real-Time PCR System (Life Technologies). The fold changes of target gene expression were calculated relative to that of the endogenous control (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]).

### Measurement of MDA

Frozen stomach tissues were homogenized with lysis buffer and centrifuged at  $12,000 \times g$  and  $4^\circ\text{C}$  for 10 min. Supernatants were collected and the level of MDA was quantified using an appropriate assay kit (Cell Biolabs, USA) according to the manufacturer's protocol.

### Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated with the TRIZOL reagent (Thermo Scientific, USA) and complementary DNA was generated from 1  $\mu\text{g}$  total RNA using a ReverTraAce qPCR RT kit (TOYOBO, Japan) according to the manufacturer's protocol. PCR was performed using SYBR Green PCR Master Mix and an Applied Biosystems 7500 Real-Time PCR System (Life Technologies). The fold change in the expression of the target gene relative to that of GAPDH was assessed using the  $2^{-\Delta\Delta\text{Ct}}$  method.

### Western blotting analysis

Proteins were resolved by 8–12% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P PVDF Membranes (Millipore, USA) by electroblotting. The membranes were blocked with 5% bovine serum albumin for 1 h and then incubated with anti-iNOS, anti-COX-2, anti-phospho p38 mitogen-activated protein kinase (MAPK), anti-p38 MAPK, anti-phospho-c-Jun N-terminal kinase (JNK), anti-JNK, anti-phospho-extracellular signal-regulated kinases 1/2 (ERK1/2), anti-ERK1/2 (all from Cell Signaling Technology and diluted 1:1,000), or anti- $\beta$ -actin (Sigma, 1:1,500) antibodies overnight at  $4^\circ\text{C}$ . The membranes were washed, incubated with anti-mouse or -rabbit secondary antibodies for 2 h at room temperature, washed again, and visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

### Statistical analysis

The results are expressed as the means  $\pm$  standard deviation. One-way analysis of variance followed by Tukey's multiple comparison test were used to compare groups. The  $p < 0.05$  was considered as representing a significant difference.

## RESULTS

### HPLC analysis of the CBD extract

A 3-dimensional (3D) chromatogram of the CBD sample was obtained using an established HPLC–PDA method [18]. The 7 marker compounds, 5-HMF, coptisine chloride, berberine

chloride, nodakenin, (*E*)-harpagoside, cinnamic acid, and  $\beta$ -asarone, were eluted within 40 min; their retention times were 7.81, 14.78, 16.36, 17.29, 22.30, 23.76, and 32.84 min, respectively. The 3D HPLC chromatogram of a representative CBD sample is shown in **Fig. 1B**. The concentrations of 5-HMF, coptisine chloride, berberine chloride, nodakenin, (*E*)-harpagoside, cinnamic acid, and  $\beta$ -asarone in the CBD sample, as assessed using HPLC–PDA, were 0.25, 2.54, 12.74, 1.27, 0.26, 0.09, and 1.49 mg/freeze-dried g, respectively.

### CBD has antimicrobial susceptibility against *H. pylori*

The antimicrobial susceptibility of CBD against 4 strains of *H. pylori* was investigated using the disk agar diffusion method. The growth inhibition zones of amoxicillin and clarithromycin against the 4 tested strains were showed in **Table 2**. Interestingly, strain PED3582GA showed resistance against clarithromycin (inhibition zone, 0 mm at 2  $\mu$ g/disk). CBD showed anti-*H. pylori* activity against all 4 strains, with growth inhibition zones in the range of 11–29 mm at 2,000  $\mu$ g/disk. The MICs of CBD and the tested antibiotics against 4 *H. pylori* strains are presented in **Table 3**.

### Secretion of IL-8 by AGS cells is suppressed by CBD

As IL-8 is known to play a critical role in the immunopathology of *H. pylori*-associated gastroduodenal disease [20], we evaluated the effect of CBD on IL-8 secretion. *H. pylori* infection was associated with a significant increase in IL-8 concentration in the conditioned medium of AGS cells; however, this increase was suppressed in a dose-dependent manner by CBD, amoxicillin, or clarithromycin (**Fig. 2A**).

### CBD decreases the expression of COX-2 and iNOS in AGS cells

As a previous study indicated that *H. pylori* infection up-regulates the levels of COX-2 and iNOS in host cells or tissues [21], we evaluated the levels of these proteins. As expected, infection of AGS cells with *H. pylori* increased the expression levels of COX-2 and iNOS. However, CBD, amoxicillin, or clarithromycin decreased these changes (**Fig. 2B**).

### CBD attenuates the histological changes induced by *H. pylori*

Next, we tested the therapeutic effect of CBD against *H. pylori* infection in an animal model. As shown in **Fig. 3A**, inflammatory cells (e.g., macrophages, lymphocytes, and neutrophils) were infiltrated in gastric mucosa and submucosa of *H. pylori*-infected mice compared to uninfected controls, and treatment with OAC or CBD suppressed these changes. In addition, the inflammation scores were reduced in infected mice treated with OAC or CBD compared to infected mice that did not receive these treatments (**Fig. 3B**).

**Table 2.** Inhibition zone diameters determined by the disk agar diffusion method

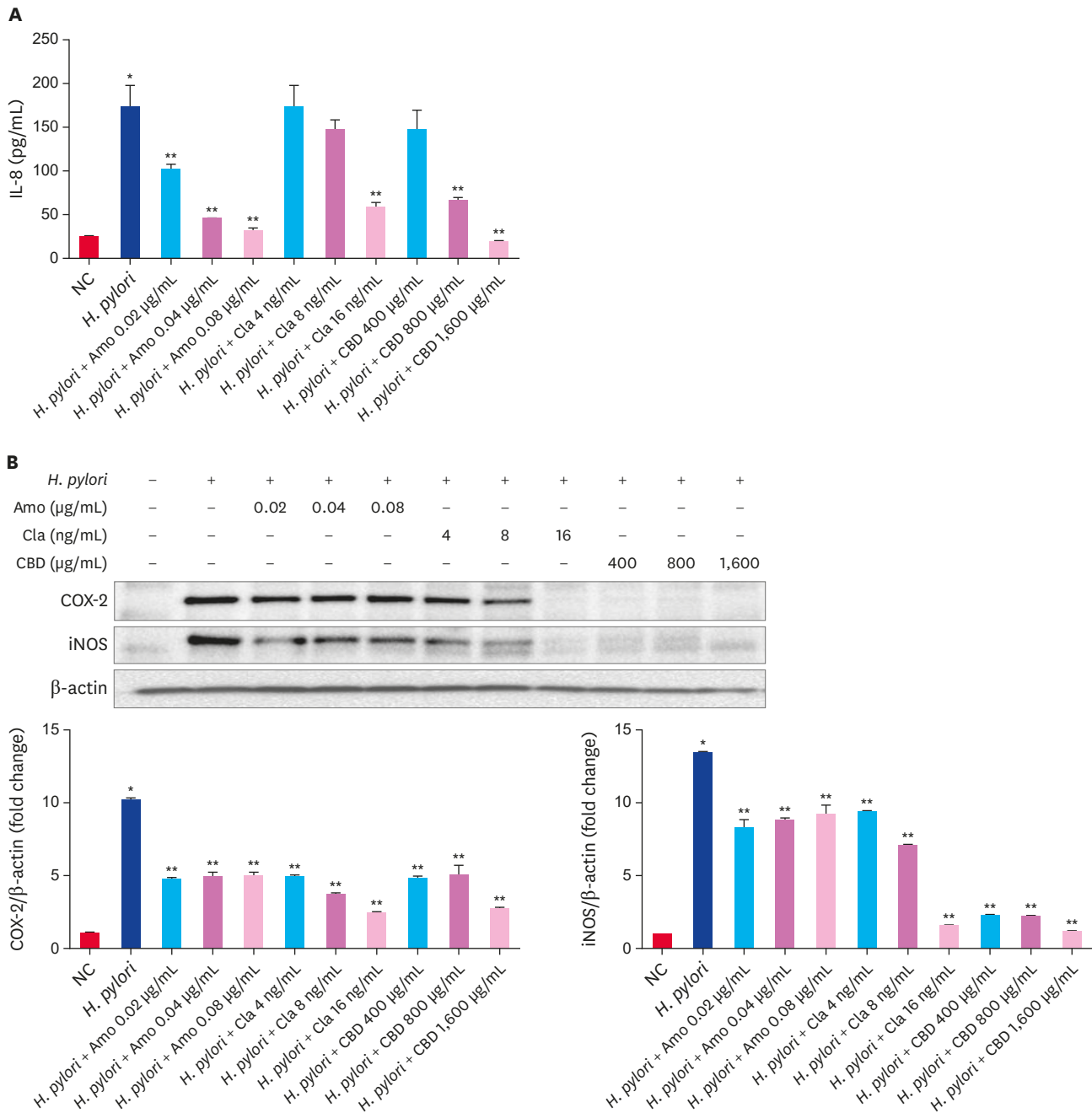
Extract/antibiotics	Diameter of growth inhibition zone (mm)			
	26695	SS1	PED3582GA	PED503G
CBD (2,000 $\mu$ g)	11	22	29	15
Amoxicillin (2 $\mu$ g)	30	25	38	36
Clarithromycin (2 $\mu$ g)	46	56	0	4

CBD, Cheonwangbosim-dan.

**Table 3.** MIC

Extract/antibiotics	MIC			
	26695	SS1	PED3582GA	PED503G
CBD ( $\mu$ g/mL)	800	400	1,600	400
Amoxicillin ( $\mu$ g/mL)	0.0313	0.1250	0.0625	0.0039
Clarithromycin ( $\mu$ g/mL)	0.0039	0.0020	32	0.0020

MIC, minimum inhibitory concentration; CBD, Cheonwangbosim-dan.



**Fig. 2.** The effect of CBD on the secretion of IL-8 and the expression of COX-2 and iNOS in AGS cells infected with *H. pylori*. Suspensions of *H. pylori* at OD<sub>600</sub> 1.0 were treated with CBD (400–1,600 µg/mL), Amo (0.02–0.08 µg/mL), or Cla (4–8 ng/mL) for 24 h and applied to AGS cells for 6 h. (A) IL-8 levels in AGS cells. (B) The protein levels of COX-2 and iNOS in AGS cells. β-actin was used as an endogenous control.

All data are presented as the mean ± standard deviation.

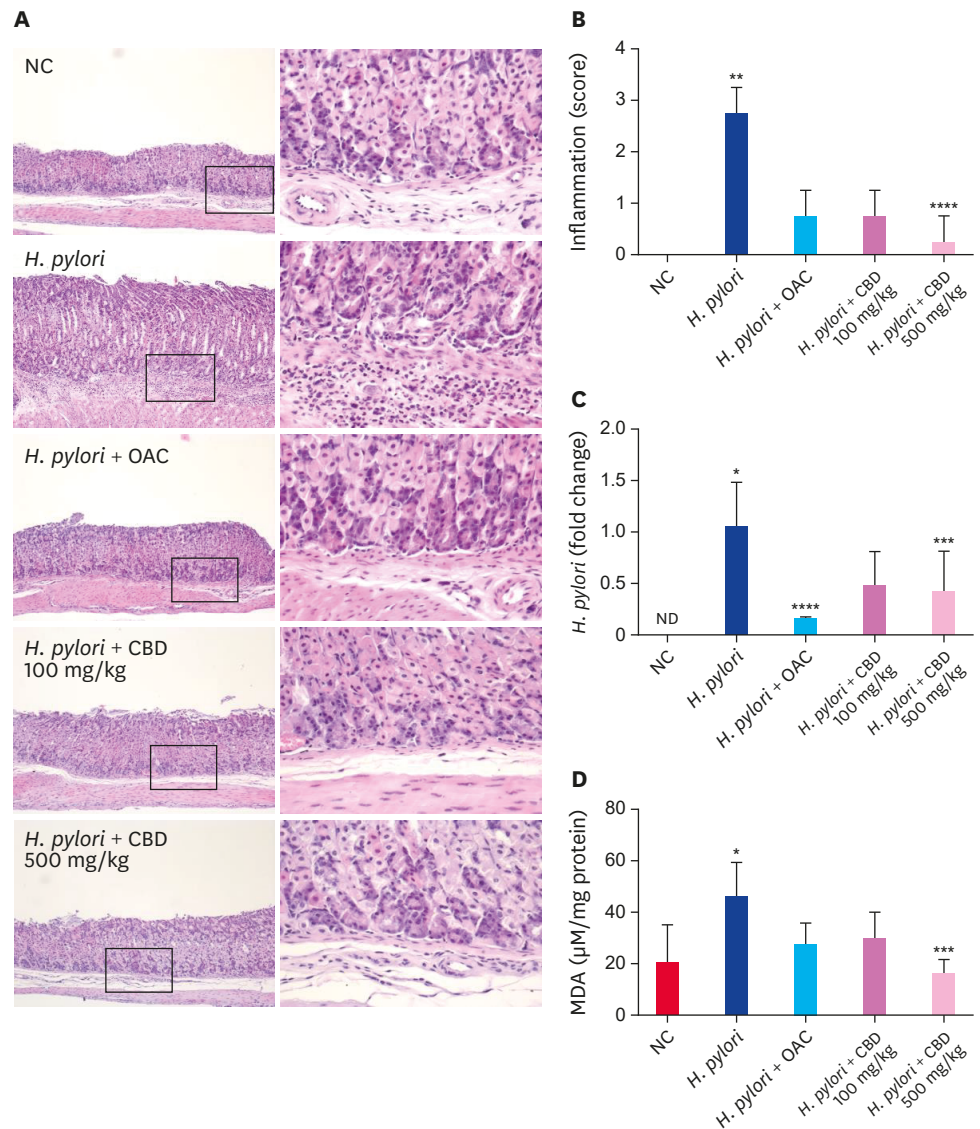
CBD, Cheonwangbosim-dan; IL, interleukin; COX-2, cyclooxygenase-2; iNOS, nitric oxide synthase; Amo, amoxicillin; Cla, clarithromycin; NC, normal control.

\**p* < 0.01 vs. the control group; \*\**p* < 0.01 vs. the *H. pylori* group.

### CBD reduces colonization of *H. pylori* in mice

To investigate the colonization of *H. pylori* to the stomachs of mice, we quantified *H. pylori* 16s rDNA in stomach samples obtained from the various groups. Our results revealed that the normal control group had no detectable level of *H. pylori*, the infected group had a detectable





**Fig. 3.** CBD attenuates the histological changes, bacterial colonization, and oxidative stress induced by *H. pylori*. (A) Formalin-fixed stomach sections were stained with hematoxylin and eosin and examined under a microscope ( $\times 40$  and  $\times 100$ ). (B) Inflammation scores obtained from histological analysis. (C) The relative levels of *H. pylori* 16S ribosomal DNA were quantified. (D) MDA level of in gastric tissues of mice infected with *H. pylori*. All data are presented as the mean  $\pm$  standard deviation. CBD, Cheonwangbosim-dan; IL, interleukin; MDA, malondialdehyde; ND; not detected; OAC, omeprazole, amoxicillin, and clarithromycin; NC, normal control. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group; \*\*\* $P < 0.05$ , \*\*\*\* $P < 0.01$  vs. the *H. pylori* group.

level of *H. pylori* colonization, and this level was reduced in groups treated with OAC or CBD compared to the infected group (Fig. 3C).

### CBD reduces MDA level in mice

The early stages of *H. pylori* infection are characterized by an increase in the MDA level, which is due to oxidative changes and contributes considerably to mucosal damage [22]. Here, we observed that infection of *H. pylori* increased the MDA level in mice, whereas treatment with OAC or CBD reduced this level (Fig. 3D).

### CBD suppresses cytokine expression in mice

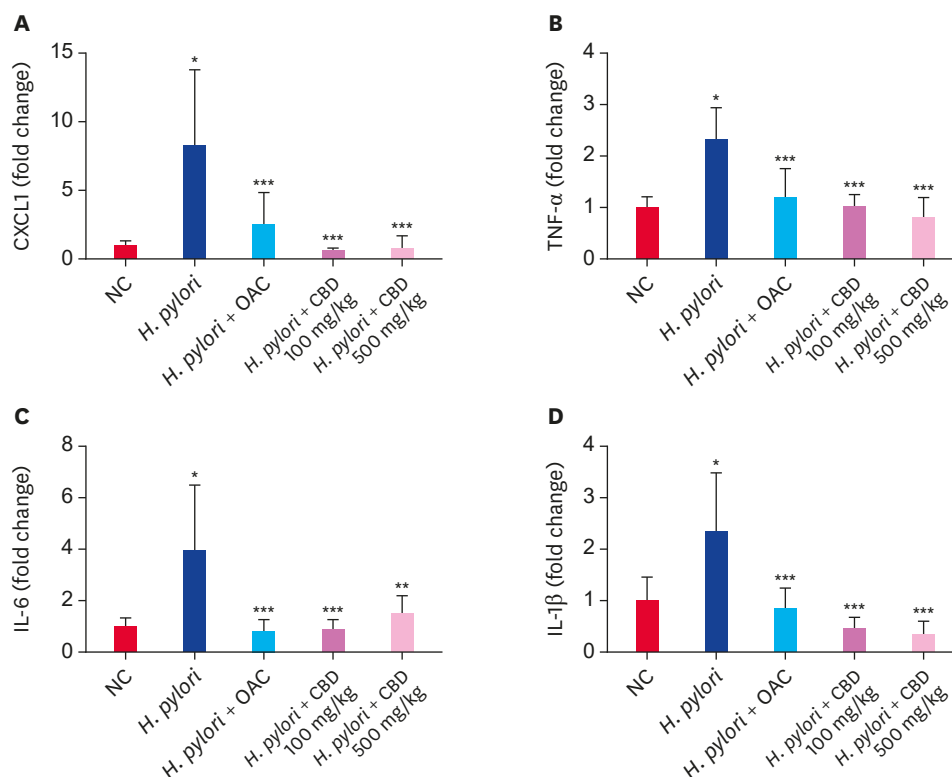
To evaluate the effect of CBD on pro-inflammatory cytokine production, we measured the gastric levels of C-X-C motif chemokine ligand 1 (CXCL1), TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. We found that *H. pylori* infection significantly increased the expression levels of CXCL1, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, but that these increases were significantly inhibited by treatment with OAC or CBD (Fig. 4).

### CBD decreases the expression levels of COX-2 and iNOS in mice

The trend in COX-2 and iNOS expression levels that we observed in our animal model resembled those that we recorded in AGS cells. Specifically, expression levels of COX-2 and iNOS in mice increased following *H. pylori* infection, but treatment with OAC or CBD reduced this effect (Fig. 5A and B).

### CBD reduces the activation of MAPK family members in mice

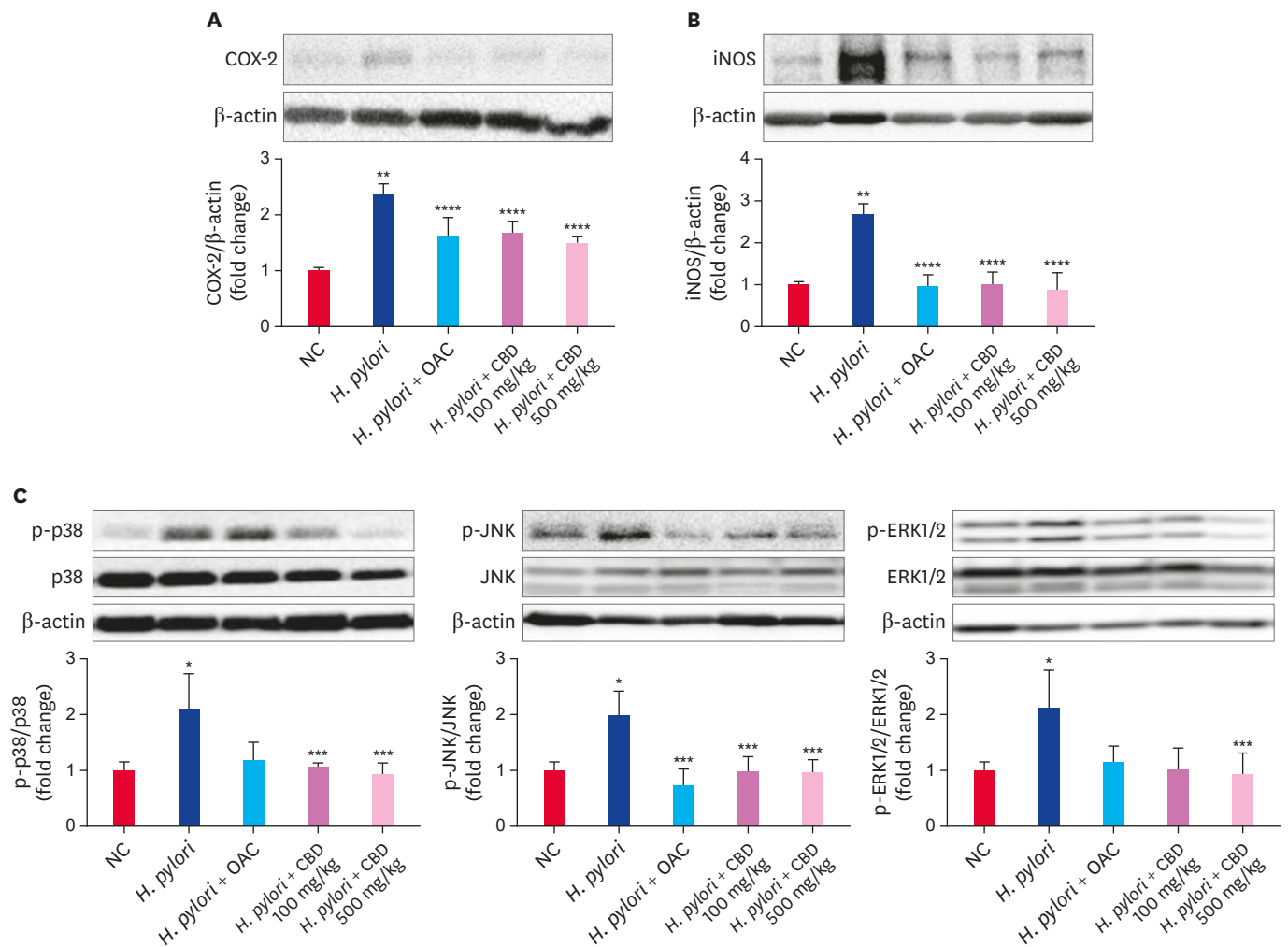
In an effort to determine the mechanism underlying the antimicrobial and anti-inflammatory effects of CBD, we analyzed the protein levels of various MAPK family members. We found that the phosphorylation levels of p38 MAPK, ERK1/2, and JNK were elevated in *H. pylori*-infected mice compared to normal control mice, but treatment OAC or CBD significantly suppressed these activating phosphorylations (Fig. 5C).



**Fig. 4.** CBD suppresses the mRNA expression levels of cytokines in gastric tissues of mice infected with *H. pylori*, as assessed by RT-qPCR. (A) The mRNA levels of CXCL1. (B) The mRNA levels of TNF- $\alpha$ . (C) The mRNA levels of IL-6. (D) The mRNA levels of IL-1 $\beta$ . All data are presented as the mean  $\pm$  standard deviation.

CBD, Cheonwangbosim-dan; OAC, omeprazole, amoxicillin, and clarithromycin; mRNA, messenger RNA; RT-qPCR, real-time quantitative polymerase chain reaction; IL, interleukin; CXCL1, C-X-C motif chemokine ligand 1; TNF- $\alpha$ , tumor necrosis factor alpha; NC, normal control.

\* $P < 0.01$  vs. the NC group; \*\* $P < 0.05$ , \*\*\* $P < 0.01$  vs. the *H. pylori* group.



**Fig. 5.** CBD suppresses the protein expression levels of COX-2, iNOS and the phosphorylation levels of MAPK family members in gastric tissues of mice infected with *H. pylori*. (A) Expression of COX-2 in stomach tissues. (B) Expression of iNOS in gastric tissues. (C) Expression levels of phosphorylated and total p38 MAPK, JNK, and ERK1/2 in stomach tissues. The protein levels were analyzed by immunoblotting.  $\beta$ -actin was used as an endogenous control. All data are presented as the mean  $\pm$  standard deviation.

CBD, Cheonwangbosim-dan; COX-2, cyclooxygenase-2; iNOS, nitric oxide synthase; NC, normal control; MARK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinases 1/2; OAC, omeprazole, amoxicillin, and clarithromycin.

\* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group; \*\*\* $P < 0.05$ , \*\*\*\* $P < 0.01$  vs. the *H. pylori* group.

## DISCUSSION

The possibility of zoonotic transmission of *H. pylori* has been suggested. A recent studies reported high presence of *H. pylori* strains in stomach of domestic animals, milk and meat similar to vac A genotypes in human being, suggesting food with animal origin may be its reservoirs [23,24]. There is currently an unmet need for potent therapeutic agents against *H. pylori* that can avoid the increasing issues of antimicrobial resistance while ideally offering lower cost and fewer side effects and contraindications [25]. Many natural products such as *Qualea parviflora* and *Byrsonima intermedia* have been reported their antimicrobial activity against *H. pylori*, and various plants and substances derived from alternative sources have been treated for gastrointestinal diseases [26].

In our HPLC–PDA data 7 major components of CBD are 5-HMF, coptisine chloride, berberine chloride, b-asarpme, cinnamic acid, (*E*)-harpagoside and nodakenin. In the previous studies, 5-HMF showed protective effect against oxidative injury and mitigated inflammation via MAPK pathway [27,28]. Especially, coptisine inhibited *H. pylori* by inhibiting urease activity [29]. Berberine also inhibited enterobacterial growth in colitis mouse model [30]. Each component contributed to anti-*H. pylori* effect, but further study will be needed to clarify which components is responsible for the anti-inflammatory activity and anti-microbial activity of CBD against *H. pylori*-induced gastritis.

In the present study, we evaluated the efficacy of CBD against 4 different *H. pylori* strains (26695, SS1, PED3582GA, and PED503G); its MIC ranged from 400 to 1,600 µg/mL and the growth inhibition zones obtained in our disk assay ranged from 11 to 29 mm. Amoxicillin standard by European Committee on Antimicrobial Susceptibility Testing (EUCAST) to MIC breakpoints are susceptible ≤ 0.125 mg/L and resistant > 0.125 mg/L. Clarithromycin standard by EUCAST to MIC breakpoints are susceptible ≤ 0.25 mg/L and resistant > 0.5 mg/L [31]. CLSI provides a guideline only for an agar dilution method. Disk diffusion criteria for antimicrobial susceptibility testing of *H. pylori* have not yet been defined. It is not standardized and can be only susceptible or resistant [32]. PED3582GA exhibited no growth inhibition zone for clarithromycin, but was greatly inhibited by CBD comparing to other strains. This suggests that CBD might has better antimicrobial susceptibility for this strain.

IL-8 plays a critical role in the response of gastric epithelial cells to *H. pylori* infection, and its level is remarkably elevated in the gastric mucosal tissues of *H. pylori*-positive patients [33]. ROS mediate the expression of IL-8 that can induce proinflammatory cytokines, such as IL-1β, IL-6, IL-12, interferon-γ, and TNF-α, which regulate the inflammatory response [34]. In this study, CBD significantly reduced the *in vitro* secretion of IL-8 by AGS cells. *In vivo*, we found that OAC or CBD treatment decreased the *H. pylori*-induced up-regulations of pro-inflammatory cytokines (CXCL1, IL-1β, IL-6, and TNF-α) and inflammatory cell infiltration into stomach tissues.

The chemotactic ability of IL-8 can attract cells to sites of infection and inflammation [35]. The inflammatory cells recruited to the stomach during *H. pylori* infection can induce ROS; this triggers lipid peroxidation, which is associated with different gastroduodenal diseases [36]. MDA, which is a lipid peroxidation product that can serve as a biomarker for oxidative stress, is reportedly elevated in the gastric mucosa of patients infected with *H. pylori* [22]. Furthermore, the MDA level of gastric juice was proposed to be related with the severity of gastric disease [37]. Here, we show that CBD treatment dose-dependently reduced the inflammation score and *H. pylori* colonization in parallel with its ability to reduce the MDA level in mice. These results indicate that CBD effectively ameliorates oxidative stress, thereby decreasing the risk for gastric injury.

To further validate the anti-inflammatory effect of CBD, we evaluated COX-2 and iNOS, which are important regulators of mucosal inflammation in *H. pylori*-induced gastric injury [38]. In *H. pylori*-infected AGS cells and the stomach tissues of *H. pylori*-infected mice, the protein expression levels of COX-2 and iNOS were up-regulated compared to the control levels, and these changes were suppressed by CBD or antibiotics. As the severity of gastric injury is associated with the protein expression levels of COX-2 and iNOS in stomach tissues, the inhibition of these inflammatory enzymes may be a valuable strategy for treating the gastric diseases induced by *H. pylori* [39].

The MAPK family includes 3 major subclasses: p38, ERK1/2, and JNK [12]. The MAPKs are believed to mediate *H. pylori*-induced IL-8 expression and regulate transcription factors (e.g., NF- $\kappa$ B and AP-1) that modulate cytokine gene expression [40]. Here, we found that *H. pylori* infection activated MAPK signaling, as represented by increased phosphorylation of p38, ERK1/2, and JNK, and that these *H. pylori*-induced activations were inhibited by CBD or OAC. Since the MAPK family members regulate inflammatory and immune responses [28], their inhibition may contribute to reducing the proinflammatory milieu to suppress inflammatory events. We herein demonstrate that CBD suppresses the *H. pylori*-induced production of pro-inflammatory mediators, as well as its ability to trigger oxidative stress and MAPK activation. Taken together, our results reveal that CBD could prove useful for treating *H. pylori*-induced gastric injury.

## REFERENCES

1. Hooi JKY, Lai WY, Ng WK, Suen MMY, Underwood FE, Tanyingoh D, Malfertheiner P, Graham DY, Wong VWS, Wu JCY, Chan FKL, Sung JY, Kaplan GG, Ng SC. Global prevalence of *Helicobacter pylori* infection: systematic review and meta-analysis. *Gastroenterology*. 2017;153(2):420-429.  
[PUBMED](#) | [CROSSREF](#)
2. Eusebi LH, Zagari RM, Bazzoli F. Epidemiology of *Helicobacter pylori* infection. *Helicobacter*. 2014;19 Suppl 1:1-5.  
[PUBMED](#) | [CROSSREF](#)
3. O'Connor A, Lamarque D, Gisbert JP, O'Morain C. Treatment of *Helicobacter pylori* infection 2017. *Helicobacter*. 2017;22 Suppl 1.  
[PUBMED](#) | [CROSSREF](#)
4. Ayala G, Escobedo-Hinojosa WI, de la Cruz-Herrera CF, Romero I. Exploring alternative treatments for *Helicobacter pylori* infection. *World J Gastroenterol*. 2014;20(6):1450-1469.  
[PUBMED](#) | [CROSSREF](#)
5. Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, Decostere A, Ducatelle R. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. *Clin Microbiol Rev*. 2009;22(2):202-223.  
[PUBMED](#) | [CROSSREF](#)
6. Sykes J, Greene C. *Infectious Diseases of the Dog and Cat*. 4th ed. St. Louis: Saunders; 2012, p 374-380.
7. Neiger R, Simpson KW. *Helicobacter* infection in dogs and cats: facts and fiction. *J Vet Intern Med*. 2000;14(2):125-133.  
[PUBMED](#) | [CROSSREF](#)
8. Sharma SA, Tummuru MK, Miller GG, Blaser MJ. Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation *in vitro*. *Infect Immun*. 1995;63(5):1681-1687.  
[PUBMED](#) | [CROSSREF](#)
9. Handa O, Naito Y, Yoshikawa T. *Helicobacter pylori*: a ROS-inducing bacterial species in the stomach. *Inflamm Res*. 2010;59(12):997-1003.  
[PUBMED](#) | [CROSSREF](#)
10. Tiwari SK, Manoj G, Sharma V, Sivaram G, Saikant R, Bardia A, Sharma VK, Abid Z, Khan AA, Habeeb MA, Habibullah CM, Kumar BS, Nandan A. Relevance of *Helicobacter pylori* genotypes in gastric pathology and its association with plasma malondialdehyde and nitric oxide levels. *Inflammopharmacology*. 2010;18(2):59-64.  
[PUBMED](#) | [CROSSREF](#)
11. Slomiany BL, Slomiany A. Induction in gastric mucosal prostaglandin and nitric oxide by *Helicobacter pylori* is dependent on MAPK/ERK-mediated activation of IKK- $\beta$  and cPLA2: modulatory effect of ghrelin. *Inflammopharmacology*. 2013;21(3):241-251.  
[PUBMED](#) | [CROSSREF](#)
12. Kim OS, Kim Y, Yoo SR, Lee MY, Shin HK, Jeong SJ. Cheonwangbosimdan, a traditional herbal formula, inhibits inflammatory responses through inactivation of NF- $\kappa$ B and induction of heme oxygenase-1 in raw264.7 murine macrophages. *Int J Clin Exp Med*. 2016;9(2):1692-1699.
13. Kim NJ, Kong YY, Chang SW. Studies on the efficacy of combined preparation of crude drugs (XXXVII). The effects of chunwangboshimdan on the central nervous system and cardio-vascular system. *Korean J Pharmacogn*. 1988;19(3):208-215.

14. Kim B, Jo C, Choi HY, Lee K. Vasorelaxant and hypotensive effects of Cheonwangbosimdan in SD and SHR rats. *Evid Based Complement Alternat Med*. 2018;2018:6128604.  
[PUBMED](#) | [CROSSREF](#)
15. Thompson LJ, Danon SJ, Wilson JE, O'Rourke JL, Salama NR, Falkow S, Mitchell H, Lee A. Chronic *Helicobacter pylori* infection with Sydney strain 1 and a newly identified mouse-adapted strain (Sydney strain 2000) in C57BL/6 and BALB/c mice. *Infect Immun*. 2004;72(8):4668-4679.  
[PUBMED](#) | [CROSSREF](#)
16. Shinilbooks Committee. *The Korean Herbal Pharmacopoeia*. 5th ed. Seoul: Shinilbooks; 2016, p 458.
17. Seo CS, Kim OS, Shin HK. Quantitative determination and antioxidant effects of Cheonwangbosimdan. *Korean J Pharmacogn*. 2014;45(4):300-314.
18. Seo CS, Shin HK. Development and validation of a high-performance liquid chromatographic method for the simultaneous quantification of marker constituents in Cheonwangbosimdan. *Nat Prod Commun*. 2014;9(12):1751-1754.  
[PUBMED](#) | [CROSSREF](#)
19. Park HS, Wijerathne CU, Jeong HY, Seo CS, Ha H, Kwun HJ. Gastroprotective effects of Hwanglyeonhaedok-tang against *Helicobacter pylori*-induced gastric cell injury. *J Ethnopharmacol*. 2018;216:239-250.  
[PUBMED](#) | [CROSSREF](#)
20. Crabtree JE, Lindley IJ. Mucosal interleukin-8 and *Helicobacter pylori*-associated gastroduodenal disease. *Eur J Gastroenterol Hepatol*. 1994;6 Suppl 1:S33-S38.  
[PUBMED](#)
21. Huang HL, Ko CH, Yan YY, Wang CK. Antiadhesion and anti-inflammation effects of noni (*Morinda citrifolia*) fruit extracts on AGS cells during *Helicobacter pylori* infection. *J Agric Food Chem*. 2014;62(11):2374-2383.  
[PUBMED](#) | [CROSSREF](#)
22. Drake IM, Mapstone NP, Schorah CJ, White KL, Chalmers DM, Dixon MF, Axon AT. Reactive oxygen species activity and lipid peroxidation in *Helicobacter pylori* associated gastritis: relation to gastric mucosal ascorbic acid concentrations and effect of *H pylori* eradication. *Gut*. 1998;42(6):768-771.  
[PUBMED](#) | [CROSSREF](#)
23. Saeidi E, Sheikhshahrokh A. *vacA* genotype status of *Helicobacter pylori* isolated from foods with animal origin. *Biomed Res Int*. 2016;2016:8701067.  
[PUBMED](#) | [CROSSREF](#)
24. Momtaz H, Dabiri H, Souod N, Gholami M. Study of *Helicobacter pylori* genotype status in cows, sheep, goats and human beings. *BMC Gastroenterol*. 2014;14(1):61.  
[PUBMED](#) | [CROSSREF](#)
25. Vega AE, Wendel GH, Maria AO, Pelzer L. Antimicrobial activity of *Artemisia douglasiana* and dehydroleucodine against *Helicobacter pylori*. *J Ethnopharmacol*. 2009;124(3):653-655.  
[PUBMED](#) | [CROSSREF](#)
26. Bonifácio BV, dos Santos Ramos MA, da Silva PB, Bauab TM. Antimicrobial activity of natural products against *Helicobacter pylori*: a review. *Ann Clin Microbiol Antimicrob*. 2014;13(1):54.  
[PUBMED](#)
27. Li MM, Wu LY, Zhao T, Xiong L, Huang X, Liu ZH, Fan XL, Xiao CR, Gao Y, Ma YB, Chen JJ, Zhu LL, Fan M. The protective role of 5-HMF against hypoxic injury. *Cell Stress Chaperones*. 2011;16(3):267-273.  
[PUBMED](#) | [CROSSREF](#)
28. Kong F, Lee BH, Wei K. 5-hydroxymethylfurfural mitigates lipopolysaccharide-stimulated inflammation via suppression of MAPK, NF- $\kappa$ B and mTOR activation in RAW 264.7 cells. *Molecules*. 2019;24(2):275.  
[CROSSREF](#)
29. Li C, Huang P, Wong K, Xu Y, Tan L, Chen H, Lu Q, Luo C, Tam C, Zhu L, Su Z, Xie J. Coptisine-induced inhibition of *Helicobacter pylori*: elucidation of specific mechanisms by probing urease active site and its maturation process. *J Enzyme Inhib Med Chem*. 2018;33(1):1362-1375.  
[PUBMED](#) | [CROSSREF](#)
30. Lee IA, Hyun YJ, Kim DH. Berberine ameliorates TNBS-induced colitis by inhibiting lipid peroxidation, enterobacterial growth and NF- $\kappa$ B activation. *Eur J Pharmacol*. 2010;648(1-3):162-170.  
[PUBMED](#) | [CROSSREF](#)
31. The European Committee on Antimicrobial Susceptibility Testing. *Breakpoint Tables for Interpretation of MICs and Zone Diameters*. version 9.0 [Internet]. Växjö: The European Committee on Antimicrobial Susceptibility Testing; [http://www.eucast.org/clinical\\_breakpoints](http://www.eucast.org/clinical_breakpoints). Updated 2019.
32. Clinical and Laboratory Standards Institute (CLSI). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*. 3rd ed. Wayne: CLSI; 2016.

33. Backert S, Clyne M. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter*. 2011;16 Suppl 1:19-25.  
[PUBMED](#) | [CROSSREF](#)
34. Lee KE, Khoi PN, Xia Y, Park JS, Joo YE, Kim KK, Choi SY, Jung YD. *Helicobacter pylori* and interleukin-8 in gastric cancer. *World J Gastroenterol*. 2013;19(45):8192-8202.  
[PUBMED](#) | [CROSSREF](#)
35. Siddiqui RA, Akard LP, Garcia JG, Cui Y, English D. Chemotactic migration triggers IL-8 generation in neutrophilic leukocytes. *J Immunol*. 1999;162(2):1077-1083.  
[PUBMED](#)
36. Berg K, Chatterjee A, Yasmin T, Shara M, Bagchi D. Cytokine expression due to *Helicobacter pylori* in a tissue culture model. *Mol Cell Biochem*. 2007;300(1-2):171-175.  
[PUBMED](#) | [CROSSREF](#)
37. Wang YK, Chiang WC, Kuo FC, Wu MC, Shih HY, Wang SSW, Liu CJ, Chen YH, Wu DC, Su WW, Huang YL. Levels of malondialdehyde in the gastric juice: its association with *Helicobacter pylori* infection and stomach diseases. *Helicobacter*. 2018;23(2):e12460.  
[PUBMED](#) | [CROSSREF](#)
38. Fu S, Ramanujam KS, Wong A, Fantry GT, Drachenberg CB, James SP, Meltzer SJ, Wilson KT. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology*. 1999;116(6):1319-1329.  
[PUBMED](#) | [CROSSREF](#)
39. Cho SO, Lim JW, Kim KH, Kim H. Involvement of Ras and AP-1 in *Helicobacter pylori*-induced expression of COX-2 and iNOS in gastric epithelial AGS cells. *Dig Dis Sci*. 2010;55(4):988-996.  
[PUBMED](#) | [CROSSREF](#)
40. Israel DA, Peek RM. Pathogenesis of *Helicobacter pylori*-induced gastric inflammation. *Aliment Pharmacol Ther*. 2001;15(9):1271-1290.  
[PUBMED](#) | [CROSSREF](#)