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The dichloromethane fraction from *Mahonia bealei* (Fort.) Carr. leaves exerts an anti-inflammatory effect both *in vitro* and *in vivo*

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ABSTRACT

Ethnopharmacological relevance: *Mahonia bealei* has a long history of medical use in traditional Chinese medicine for the treatment of inflammatory-associated diseases. Despite numerous phytochemical and pharmacological studies, there is a lack of systematic studies to understand the cellular and molecular mechanisms of the anti-inflammatory activity of this plant.
Aim of study: This study aimed to evaluate the anti-inflammatory activity of the dichloromethane fraction from *M. bealei* leaves (MBL-CH).
Materials and methods: RAW 264.7 cells were pretreated with different concentrations of MBL-CH for 30 min prior to treatment with 1 μg/ml of lipopolysaccharide (LPS). The nuclear factor κB (NF-κB) pathway and subsequent production of inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumour necrosis factor (TNF)-α were investigated. Furthermore, the *in vivo* mouse model of LPS-induced acute lung injury (ALI) was employed to study the anti-inflammatory effects of MBL-CH.
Results: Pre-treatment with MBL-CH significantly inhibited the LPS-stimulated secretion of NO, PGE_2, and TNF-α into the culture medium, as well as the mRNA levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α, which were associated with a reduction in the phosphorylation of IκBα, Akt, and PI3K and inhibition of the transcriptional activity of NF-κB. Furthermore, *in vivo* experiments revealed that MBL-CH attenuated LPS-stimulated lung inflammation in mice.
Conclusion: Taken together, our findings indicate that MBL-CH attenuates LPS-stimulated inflammatory responses in macrophages by blocking NF-κB activation through interference with activation of the PI3K/Akt pathway, providing scientific evidence that the plant can be employed in traditional remedies.

1. Introduction

Inflammation is a ubiquitous defensive response to invading pathogens that involves the ingress of leukocytes into sites of inflammation. Macrophages are major immune effector cells that play essential roles in the initiation and propagation of inflammatory responses against bacterial infection (Zimmermann et al., 2012). Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is a potent activator of macrophages, which stimulate signalling through Toll-like receptor 4 (TLR4) (Aderem and Ulevitch, 2000). Upon forming a complex with CD14, LPS triggers subsequent signal transmission events, leading to the activation of mitogen-activated protein kinases (MAPKs), such as p38 MAPK, c-Jun N-terminal kinase (JNK),

Abbreviations: AP-1, activator protein-1; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; IRF-3, phosphoinositide-3-kinase-3; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MBL-CH, dichloromethane fraction from *Mahonia bealei* leaves; NF-κB, nuclear factor –κB; NO, nitric oxide; PGE_2, prostaglandin E_2; PI3K, phosphoinositide-3-kinase; PVDF, polyvinylidene fluoride; TLR4, toll-like receptor 4

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extracellular signal-regulated kinase (ERK), and phosphoinositide-3-kinase (PI3K)/Akt, as well as their associated inflammatory transcription factors, such as interferon regulatory factor (IRF)-3, nuclear factor (NF)-κB, and activator protein (AP)-1 (Hong et al., 2015). LPS-induced activation of macrophages is involved in the secretion of various inflammatory products, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), tumour necrosis factor-alpha (TNF-α), granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin-1 beta (IL-1β), IL-2, IL-6, and other inflammatory mediators (Ribeiro et al., 2015). Chronic inflammation is implicated in the pathogenesis of many diseases, such as cancer, gastritis, atherosclerosis, rheumatoid arthritis, hearing loss, diabetes, and septic shock (Dandona et al., 2004; Diederichs et al., 2011; Shacter and Weitzman, 2002a, 2002b). Acute lung injury (ALI) is an inflammatory disorder and commonly characterized by pulmonary edema, severe hypoxemia, and neutrophil accumulation in the lung (Goodman et al., 2003). Increasing evidence has revealed that LPS plays critical roles in the development of ALI that associated with the activation of TLR4 in macrophages by secreting various pro-inflammatory cytokines (Liu et al., 2015; Zhong et al., 2013). Thus, these cytokines may reflect the degree of inflammation and provide information to aid in investigating the effect of pharmacological agents on the inflammatory process. Hence, efforts are focused on the identification of herbal medicine candidates with potential anti-inflammatory activity and minimal side effects and that can negatively modulate the production of such cytokines (Jeong et al., 2013; Joo et al., 2014; Lim et al., 2015).

Mahonia is a flowering evergreen tree of the family Berberidaceae. There are approximately 60 recorded species of the genera *Mahonia*, mainly distributed in East and Southeast Asia; 30 are distributed in the Southwest region of China (Flora of China Editorial Committee, 2001). *Mahonia* has a long history of medical use in traditional Chinese medicine for the treatment of tuberculosis, dysentery, jaundice, periodontitis, and red urine. Two of the earliest literature sources in China, "Zhi Wu Ming Shi Tu Kao" and "Zhong Hua Ben Cao", mentioned the ethnopharmacological uses of this plant. Two species of *Mahonia*, *M. bealei* (Fort.) Carr. and *M. fortunei* (Lindl.) Fedde, are officially listed in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2010). Furthermore, various formulations of this plant were developed for medicinal products, such as "Gong Lao Qu Huo Pian", "Fu Ke Qian Jin Pian", "Chang Wei Shi Jiao Nang", and "Zhi Chuang Pian" (He and Mu, 2015). Recent investigations have suggested that *M. bealei* (Fort.) Carr. possesses a wide range of pharmacological properties, including cytotoxic, antioxidative, anti-influenza, and anti-gastrin effects. Thus far, the main active constituents, including alkaloids such as berberine, isotetrandrine, oxyacanthine, jatrorrhizine, and epiberberine, have been isolated from *M. bealei* (Hu et al., 2011; Zeng et al., 2006; Zhang et al., 2014).

Despite numerous phytochemical and pharmacological studies, there has been no systematic approach to understand the cellular and molecular mechanisms of the anti-inflammatory activity of this plant. Therefore, in the present study, we investigated the anti-inflammatory activity of *M. bealei* by studying molecular/biochemical pathways in LPS-activated macrophages and in an *in vivo* acute inflammatory model.

2. Materials and methods

2.1. Plant material and chemicals

M. bealei (Fort.) Carr. leaves were collected from Guizhou Province, China in 2013 and were authenticated by Dr. Haifeng Wu (Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Institute of Medicinal Plant Development, Peking Union Medical College and Chinese Academy of Medical Sciences). A voucher specimen (MBL-2013) was deposited in School of Life Sciences, Huaiyin Normal University, China. 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), pyrrolidine dithiocarbamate (PDTC), LY294002, dimethyl sulfoxide (DMSO), Griess reagents, and LPS were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Bioworld (Bioplus Fine Research Chemicals, Dublin, OH, USA). All reagents for RNA extraction and first-strand cDNA synthesis were obtained from Invitrogen (Carlsbad, CA, USA). Protein marker was purchased from New England Biolabs (Beverly, MA, USA). RPMI medium 1640, trypsin-EDTA, and penicillin–streptomycin solution were from Gibco BRL (Life Technologies, China). Polyvinylidene fluoride (PVDF) membrane was obtained from BioRad (Hercules, CA, USA). The indicated primary antibodies and actin were obtained from Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were provided by Abcam (Cambridge, MA, USA). Enhanced chemiluminescence (ECL) detection reagent was from ComWin Biotech Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2 and TNF-α were from R&D Systems (Minneapolis, MN, USA). All other chemicals were of analytical grade.

2.2. Preparation of extracts

The air-dried *Mahonia bealei* (Fort.) Carr. leaves (3.5 kg) were pulverized and extracted with ethanol using ultrasonic-assisted extraction. The residue was re-extracted twice (each for 30 min), then the resulting extracts were combined, filtered through filter paper (100 mm; Whatman, Maidstone, UK), and evaporated using a vacuum rotary evaporator (RE-3000; YaRong Biochemistry Instrument Factory, Shanghai, China) at 40 °C to produce a crude extract of 470.44 g. The crude extract was suspended in 2 l deionized water and then partitioned three times with solvent–solvent extraction using *n*-hexane (3 × 1 l), CH_2Cl_2 (3 × 1 l), EtOAc (3 × 1 l), and water-saturated *n*-BuOH (3 × 1 l). These five phases were evaporated using a vacuum rotary evaporator. This procedure produced *n*-hexane (30.66 g), CH_2Cl_2 (43.40 g), EtOAc (37.44 g), *n*-BuOH (159.35 g), and H_2O (169.59 g) soluble fraction powders.

2.3. Cell line and cell culture

RAW 264.7 cells, a mouse macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained at RPMI 1640 medium supplemented with 10%v/v FBS, and 1% penicillin–streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin). Cultures were maintained in 25 cm^2 culture flasks at 37 °C in a humidified 5% CO_2 incubator (MCO-15AC CO_2 incubator, SANYO, Osaka, Japan) and passaged every 2–3 days to maintain logarithmic growth. The fractions were freshly dissolved in absolute dimethyl sulfoxide (DMSO); the final concentration of DMSO in experimental media was not in excess of 0.1% at any time during the experiment.

2.4. Preparation of peritoneal macrophages

C57BL/6 male mice (6–8 weeks old, 17–21 *g*) were obtained from Laboratory Animal Service Center of Yangzhou University and housed at a room temperature of 20–25 °C under relative humidity (50 ± 10%) with a 12 h light/12 h dark cycle. All animals were allowed free access to a commercial stock diet and water throughout the experiment. All experiments were performed in accordance with guidelines established by the Animal Care and Use Committee of Nanjing Medical University. Peritoneal macrophages were obtained according to the methods reported previously (Shen et al., 2013). Mice were injected (i.p.) with 4% thioglycollate broth (1 ml/each) for 4 days. Then, the mice were euthanized by cervical dislocation, and the peritoneal macrophages were harvested with 10 ml ice cold PBS. After centrifugation the cells were resuspended in RPMI 1640 medium supplemented with 10%v/v FBS, and 1% penicillin–streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin) and incubated in a culture plate. Non-adherent cells were removed by washing three times with sterile PBS.

2.5. Cell viability

Cell viability was determined using the MTT assay (Hu et al., 2014). Briefly, cells were seeded at a density of 1×10^5 cells in a 96-well flat-bottom plate (Corning, Suzhou, China) and incubated overnight. Then, the cells were treated with test samples for 24 h. At the end of incubation, MTT stock solution (5 mg/ml) was added to each well and the cells were cultured for another 3 h. The formazan crystals in each well were dissolved in lysis buffer (containing 10% SDS and 0.01 M hydrochloric acid) for overnight at 37 °C. The absorbance at 550 nm was measured by an Infinite M200 Pro spectrophotometer (Tecan, Switzerland). The data are expressed as the percentage of the control optical density (OD) values for each experiment by the following formula:

$$\text{Cell viability } (\%) = (OD_s/OD_v \times 100)\%$$

OD_s and OD_v indicated the optical density of cell lines incubated with test samples and vehicle control, respectively.