



In vivo and *in vitro* anti-inflammatory effects of Zao-Jiao-Ci (the spine of *Gleditsia sinensis* Lam.) aqueous extract and its mechanisms of action



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ABSTRACT

Ethnopharmacological relevance: Zao-Jiao-Ci (ZJC), as the spine of Chinese Honey locust (*Gleditsia sinensis* Lam.), is traditionally used as Chinese medicine to reduce inflammation.

Aim of the study: The present study aimed to investigate an anti-inflammatory effect of ZJC aqueous extract both *in vitro* and *in vivo*, as well as its underlying mechanisms.

Materials and methods: Anti-inflammatory effect of ZJC aqueous extract was evaluated by using carrageenan-induced paw edema in rats. In addition, the inhibitory effects of ZJC on nitric oxide production, intracellular reactive oxygen species production, pro-inflammatory mediator expression and prostaglandin E₂ (PGE₂) production were determined by using LPS-activated RAW 264.7 cells. The anti-oxidant activity of ZJC was assessed using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid assay.

Results: ZJC aqueous extract showed significant suppressive effect on paw edema in rats at 100 mg/kg. Moreover, ZJC aqueous extract decreased the expression of cyclooxygenase (COX)-2 and significantly decreased the PGE₂, tumor necrosis factor- α , interleukin (IL)-1 β and IL-6 production in LPS-activated macrophages in dose-dependent manners. ZJC aqueous extract inhibited the mRNA expression of these inflammatory cytokines as well. Furthermore, ZJC aqueous extract was found as an anti-oxidant and could inhibit ROS production in the LPS-induced cells.

Conclusions: These findings show the potential of ZJC aqueous extract as a naturally occurring COX-2 inhibitor to reduce inflammation.

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1. Introduction

Inflammation is an adaptive response from the human body to external challenge and harmful stimuli such as tissue damage and infection, which leads to the release of inflammatory mediators and finalizing the restoration of tissue structure and function (Nathan, 2002). Macrophages play a crucial role in both non-specific and acquired immune response. With the induction of

mitogens, inflammatory cytokines, ionizing radiation, and/or bacterial lipopolysaccharides (LPS), macrophages release various cytokines and pro-inflammatory molecules. LPS is a typical structure at the outer membrane of the gram-negative bacteria. As a well-known activator of macrophage, LPS can initiate Toll-like receptor 4 (Anwar et al., 2013) and produce pro-inflammatory mediators [e.g. tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6)], chemokines (e.g. CCL2/MCP-1), nitric oxide (NO), and prostaglandins (PGs), to elicit the inflammatory response (Kim et al., 2008). The toxic effects of reactive oxygen species (ROS) are inducing extensive tissue damage through lipid peroxidation, oxidation of amino acid side chains, protein cross-linking, and DNA damage (Wagner et al., 2004). Meanwhile, endogenous ROS and reactive nitrogen species (RNS) such as hydrogen peroxide and nitric oxide are important mediators of natural physiological processes (Hoffman et al., 2015). In the process of host defense against microbial and tumor cells, the

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inflammatory cells could release ROS and RNS which activate cell signaling pathways as messenger molecules of the autocrine or paracrine system (Saran and Bors, 1989; Suzuki et al., 1997). They have important roles in different inflammatory cells as regulators of signal transduction, activators of key transcription factors, and modulators of gene expression and apoptosis. Their excessive production may result in tissue injury and inflammation (Halliwell et al., 1992). It has been reported that ROS are critical for LPS-induced inflammation through activation of JAK-STATs signaling pathway (Kacimi et al., 2011; Qi et al., 2013). Thus, inhibition of ROS production would be a potential strategy for anti-inflammation.

LPS conjugate to Toll-Like receptor 4 (TLR4) to form LPS-TLR4 signalosome complex on the cellular surface, leading to the rapid and coordinated activation of various intracellular signaling pathways. In this process, two main signaling pathways of I κ B kinase (IKK) and mitogen-activated protein kinases (MAPKs) are activated. Upon LPS stimulation, IKK phosphorylates the inhibitory protein I κ B (Baeuerle, 1998). This phosphorylation triggers degradation of I κ B of the ubiquitin system (Brown et al., 1995), which releases the transcription factor NF- κ B and induces transcription of numerous target genes for inflammation (Li and Verma, 2002). Alternatively, MAPK families including p38, ERK1/2, and c-JNK are a group of serine/threonine kinases which are activated in response to diverse extracellular stimuli such as LPS and ROS for cellular signal transduction from the cell surface to the nucleus (Kaminska, 2005; Kirkwood et al., 2009). Phosphorylation and activation of MAPKs have been implicated in the signaling pathways relevant to LPS-induced inflammation, which suggested that MAPKs are also important targets for anti-inflammatory molecules (Beutler and Cerami, 1989). Direct blockade of IKK, JNK, and p38 MAPK have been demonstrated for anti-inflammatory effects (Novoselova et al., 2014).

Gleditsia sinensis Lam. is well known as Chinese Honey locust, and has been widely used for centuries. Both of its spines ("Zao-Jiao-Ci" in Chinese; ZJC) and fruits were used as traditional medicine with valuable bioactivities. According to the TCM ancient books "Ben Cao Gang Mu" and "Pu Ji Fang", its spines are used for the treatment of carbuncle, scabies, ulcer, sepsis, and skin diseases, while its fruits are mainly used to treat various respiratory symptoms (productive cough, asthma), apoplexy, headache, coma, epilepsy and scabies (Dai et al., 2005; Kuang and Zhang, 2005; Chinese Pharmacopoeia Commission, 2015; Zhang et al., 2016). Most of the compounds isolated and elucidated from the spines and fruits of *Gleditsia sinensis* showed strong bioactivities such as anti-inflammation, anti-allergic, anti-tumor, anti-angiogenesis, antibacterial and antifungal activity, etc. (Shin and Kim, 2000; Long et al., 2006; Zhou et al., 2007a, 2007b; Gao et al., 2008; Ha et al., 2008; Lee et al., 2009; Yi et al., 2012, 2015; Zhang et al., 2016). However, the major components in the spines and fruits are different. In the spines, the most characteristic constituents are flavonoids, phenolics and their derivatives; while in the fruits, the main components are triterpenoid saponins (Zhang et al., 1999; Li et al., 2007; Zhou et al., 2007a).

Previous studies have indicated that ZJC (the spines of *Gleditsia sinensis*) aqueous extract and different organic solvents extract could reduce inflammation through inhibiting NF- κ B activation, and phosphorylation of ERK1/2 and c-JNK in lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages (Ha et al., 2008; Seo et al., 2015). Nevertheless, relatively little attention was paid to the *in vivo* effect of ZJC aqueous extract on COX-2 and its molecular mechanisms in detail. Moreover, the possibility that the antioxidant activity of ZJC aqueous extract could contribute to its anti-inflammatory activity has never been examined. Therefore, in the present study, we aimed to investigate an anti-inflammatory effect of ZJC aqueous extract both *in vitro* and *in vivo*, as well as its underlying mechanisms.

2. Material and method

2.1. Reagents

RAW 264.7 murine macrophage cell line, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and streptomycin/penicillin solution were purchased from American type culture collection (Manassas, VA, USA). 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA), and Griess Reagent System were purchased from Sigma (St. Louis, MO, USA). Mouse β -actin, iNOS, COX-2 and I κ B- α (Ser32) were purchased from BD Pharmingen (San Diego CA, USA). Rabbit Phospho-MAPK Family Antibody Sample (p-P38, p-ERK) and rabbit Phospho-c-Jun (Ser73) antibody were obtained from Cell Signaling Technology (Danvers, MA). Anti-rabbit-HRP antibodies, anti-mouse-HRP antibodies and ECL detection kit were purchased from GE Healthcare (Waukesha, WI, USA).

2.2. Preparation of ZJC aqueous extract

The raw herb of the spine of *Gleditsia sinensis* Lam. was purchased from Jiangsu province, China. It was chemically authenticated using thin layer chromatography in accordance to the Chinese Pharmacopoeia 2015, and its specimen was deposited in the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong (The voucher specimen number: 2015-3489). For extraction, the raw herb was cut into small pieces, soaked with 10-fold of water (v/w) for 1 h, and followed by heat extraction at 100 °C for 1 h. Subsequent extractions were carried out with 10-fold of water (v/w) for another 1 h. The supernatant was combined and concentrated under reduced pressure to obtain the dry powdered extract. The extracts were stored at –20 °C before use. The yield of aqueous extract from ZJC raw herb was about 8.3% (w/w).

2.3. Chemical standardization of aqueous extract by LC-MS

Chemical analysis of the extract was performed by liquid chromatography–mass spectrometry (LC-MS) which was equipped with an Agilent 1290 Infinity HPLC system (Santa Clara, CA, USA) containing an online degasser, a binary-pump, an autosampler and a diode array detector. An Alltima HPLC C18 column (250 mm $\times$ 4.6 mm, 5 μ m) with a pre-column filter was used. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The linear gradient elution system was as follows: 0–2 min, 2% B; 2–30 min, 2–15% B. The flow rate was set at 0.7 ml/min. The column temperature was maintained at 40 °C, and the injection volume was 2 μ l. Mass spectra of dihydrofisetin and quercetin were recorded in the positive ionization mode using an electrospray (ESI) ionizing source of Agilent 6530 Accurate-Mass QTOF mass spectrometer with nitrogen as drying gas (Santa Clara, CA, USA). Spray chamber parameters were as follows: gas temperature, 350 °C; drying gas flow, 11 L/min; nebulizer pressure, 55 psi; capillary, 4200 V; scan range, *m/z* 100–1200. Chemical identification was carried out by comparing the retention times and mass spectra of targeted peaks with those of the authentic standards. Data were analyzed using Agilent Mass Hunter Workstation v.B.05.00 software (Santa Clara, CA, USA).

2.4. Carrageenan-induced paw edema in rats

Male Sprague Dawley rats (around 200 g) were used in this experiment. Animals were allowed free access to commercial pellet chow and water *ad libitum*. They were maintained at a temperature (22 $\pm$ 1 °C) and humidity (55 $\pm$ 10%) controlled room

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