Zanthoxylum rhetsa Stem Bark Extract Inhibits LPS-induced COX-2 and iNOS expression in RAW 264.7 Cells via the NF-κB Inactivation

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Abstract — The methanol extract of Zanthoxylum rhetsa (MZRR) were evaluated for its ability to suppress the formation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. MZRR presented an inhibition of LPS-induced production of nitric oxide (NO) and prostaglandin E₂ (PGE₂) in RAW 264.7 macrophages. Western blotting and RT-PCR analyses demonstrated that MZRR significantly inhibited the protein and mRNA expressions of iNOS and COX-2 in LPS-activated macrophages in a dose-dependent manner. LPS-induced COX-2, iNOS, and nuclear factor kappa beta (NF-κB) activity were also decreased in the presence of MZRR. The production of tumor necrosis factor-α (TNF-α), the mRNA expression levels of pro-inflammatory cytokines, including TNF-α and IL-1β, were reduced after MZRR administration in a dose dependent-manner. These results suggest that the MZRR extract involved in the inhibition of iNOS and COX-2 via the NF-κB pathway, revealing a partial molecular basis for anti-inflammatory properties of the MZRR extract.

Keywords — Zanthoxylum rhetsa, COX-2, iNOS, NF-κB, RAW 264.7

Introduction

Zanthoxylum rhetsa (Roxb.) DC. (Rutaceae) is a tropical shrub native to Vietnam and China. The Z. rhetsa plant has been used traditionally as an anti-diabetic, anti-spasmodic, diuretic, anesthetic agent (Hsieh, 1993; Chi, 1997; Loi, 2001). In 2002, Rahman, et al. reported that the Z. rhetsa extract also has considerable anti-nociceptive and anti-diarrheal activities (Rahman, et al., 2002). In 2009, Yadav et al. reported the therapeutic effect of the Z. rhetsa extract against experimental Hymenolepis diminuta (Cestoda) infections in rats (Yadav and Tangpu, 2009). The Z. rhetsa extract contains terpenoids, xanthyletin and sesamin, alkaloids, flavonoids, and essential oils (Mathur et al., 1967; Dharmaratne et al., 1998; Ahsan et al., 2000; Joy et al., 2006; Pai et al., 2009; Thipthaviphone et al., 2009). Besides, several reports have been published to assess the pharmacological activities of some species from genus Zanthoxylum such as: Z. ailanthoides, Z. riedelianum, Z. xanthoxyloides, and Z. schinifolium (Lee et al., 2006; Lima et al., 2007; Prumpeh and Mensah-Antipoe, 2008; Cao et al., 2009). However, anti-inflammatory activity of Z. rhetsa has not yet been studied. Thus, this study was investigated the effect of Z. rhetsa extract on the expression of COX-2, iNOS, TNF-α, and IL-1β using LPS-stimulated RAW 264.7 macrophages.

Cyclooxygenase (PGH₂ synthase, COX) donates 2 oxygen molecules to arachidonic acid to form PGG₂ by peroxidation, which in turn is reduced to PGH₂. This leads to the formation of PGE₂, a bioactive prostanoid, via concerted activation of PGE synthase (PGES). COX is the molecular target for regulating pathological conditions such as allergic diseases and rheumatoid arthritis (Goetzl et al., 1995). There are two isoforms of cyclooxygenase: i.e. COX-1 and COX-2 (Hla and Neilson, 1992). COX-1 functions as a housekeeping gene and is constitutively expressed in most human tissues. Whereas, COX-2 protein is only slightly expressed in most normal mammalian tissues in response to physical, chemical, and biological
stimuli, including UV light exposure, dioxin, and LPS insult (Arias-Negrete et al., 1995). Over expression of COX-2 has been related to chronic inflammation, angiogenesis, and carcinogenesis (Tsujii et al., 2001). Nuclear factor-κB (NF-κB) is one of transcription factor binding sites in promoter region of the COX-2 gene (Kosaka et al., 1994). In addition, the transcriptional activation of NF-κB is an important process for the expression of the pro-inflammatory cell adhesion molecules (Ledebur and Parks, 1995; Ross, 1999).

IL-1 is one of primarily pro-inflammatory cytokines by their ability to stimulate the expression of genes associated with inflammation and autoimmune diseases. For IL-1 (IL-1α and IL-1β), the most salient and relevant properties are the initiation of COX-2, type 2 phospholipase A, and iNOS (Dinarello, 2002). This accounts for the large amount of PGE2 and NO produced by cells exposed to IL-1 or in animals or humans injected with IL-1. Another important member of the pro-inflammatory IL-1 family is IL-18. IL-1α and TNFα share several biological properties but the salient difference is that the TNF receptor signaling induces programmed cell death whereas IL-1 receptor signaling does not (Dinarello, 2002).

In the present study, we found that COX-2 expression was selectively down-regulated by the methanol extract of stem bark of *Zanthoxylum rhetsa* (MZRR) in RAW 264.7 macrophages. Furthermore, we demonstrated that MZRR-inhibited COX-2 expression was transcriptionally regulated by four distinct transcription factors, including NF-κB, AP-1, IL-1β, and TNF-α.

**Materials and Methods**

**Plant materials** – The dried stem bark of *Zanthoxylum rhetsa* (ZRR) was purchased from a local medicinal herb market (Lan Ong, Hanoi, Vietnam). Voucher specimens (VIET-0920) were verified by Dr. Tran Van On and deposited in the Herbarium of Department of Botany (Hanoi University of Pharmacy).

**Preparation of the MZRR extract** – The dried powder of ZRR (100 g) was extracted three times with hot MeOH (1 L each time) using an ultrasonic apparatus for 3 h each time. The combined extract was then passed through a No. 1 Whatman filter (Whatman Inc., Hilllsboro, OR, USA). The filtrate was evaporated to dryness in vacuo at 40 °C to obtain a residue (15 g, 15%; wt:wt) named as MZRR and was stored at −20 °C.

**Chemicals** – The chemicals and cell culture materials were obtained from the following sources: *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) from Sigma Co.; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assay kit from Roche Co.; Lipofect AMINE Plus, RPMI 1640, fetal bovine serum, and penicillin-streptomycin solution from Life Technologies, Inc.; pGL3-NF-κB and the luciferase assay system from Promega; pCMV-β-gal from Clontech; enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, and TNF-α from R&D Systems; Primary antibodies (anti-COX-2, COX-2 and anti-β-actin) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-murine iNOS antiserum from Transduction Laboratories (Lexington, KY) and secondary antibody (HRP-linked anti-rabbit IgG) from Cell Signaling Technology (Beverly, MA). All other chemicals were of the highest commercial grade available.

**Cell culture** – RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured at 37 °C in 5% CO2/95% air in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. For all experiments, cells were grown to 80% - 90% confluency and subjected to no more than 20 cell-passages.

**MTT cell viability assay** – Viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/mL) for 4 h. Media were then removed and the formazan crystals produced were dissolved by adding 200 μl of dimethylsulfoxide. Absorbance was assayed at 540 nm and cell viabilities were expressed as ratios versus untreated control cells.

**Measurement of nitrite** – RAW 264.7 cells (5 × 10⁵ cells/mL) were cultured in 48-well plates. After incubating the cells for 24 h, the level of NO production was determined by assaying the culture supernatants for nitrite, which is the stable reaction product of a reaction between NO and molecular oxygen using a Griess reagent as described previously (Lee et al., 2007). Absorbance was measured at 540 nm after incubating for 10 min.

**RNA preparation and mRNA analysis by reverse transcription-polymerase chain reaction** – The RAW 264.7 cells were cultured with either endosulfan or LPS at for 6 h. The total cellular RNA was isolated using the acidic phenol extraction procedure cDNA synthesis, semi-quantitative RT-PCR for IL-1β, TNFα, and β-actin mRNA, and the analysis of the results were carried out as described previously (Han et al., 2007). The PCRs were electrophoresed through a 2.5% agarose gel and visualized with ethidium bromide staining and UV irradiation.

**Transfection assays** – The RAW 264.7 cells (5 × 10⁵ cells/mL) were plated in each well of a 24-well plate.