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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 E_2 (PGE_2) 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 *Hymenolepsis 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; Prempeh and Mensah-Attipoe, 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

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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 (Tsuji *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-1 is distinct from TNF. IL-1 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., Hillsboro, OR, USA). The filtrate was evaporated to dryness in vacuo at 40 ºC to obtain a residue (15 g, 15%; wt:wt) named as MZRR and was stored at –20 ºC.

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-2yl)-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 fromR&D Systems; Primary antibodies (anti-COX-2, COX-2 and anti-β-actin) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-murine iNOS antiserum by 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 ºC in 5% CO_2/95% 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 (5×10^5 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-lβ, 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 (5×10^5 cells/mL) were plated in each well of a 24-well plate.

Twelve hours later, the cells were transiently co-transfected with the plasmids, pGL3-NF-κB, and pCMV-β-gal, using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, a transfection mixture containing pGL3-NF-κB and of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent, and added to the cells. After 18 h, the cells were treated with LPS with or without endosulfan for 18 h, and then lysed. The luciferase and β-galactosidase activities were determined as described previously (Kim *et al.*, 2004). The luciferase activity was normalized to the β-galactosidase activity, and the results are expressed relative to the activity of the control.

Western blot analysis – Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described elsewhere (Zang *et al.*, 2006). Briefly, the cell lysates were fractionated by 10% gel electrophoresis, transferred electrophoretically to nitrocellulose paper that had previously been incubated with the primary antibody, and then with the horseradish peroxidase-conjugated secondary antibodies. Finally, the papers were developed using an ECL chemiluminescence detection kit.

Statistical analysis – All the experiments were repeated at least three times. The data is presented as means ± SD for at least three different sets of plates and treatment groups. A Student's t-test was used to examine the statistical significance of the differences. $P < 0.05$ was considered significantly.

Results and Discussion

MZRR inhibits prostaglandin and nitrite production and iNOS protein expression in LPS-activated macrophages – The cytotoxicity of MZRR to RAW 264.7 cells were examined by MTT assay. Cell viability was not significantly altered by MZRR up to 30 μg/mL (Fig. 1). Thus, we treated cells with MZRR in the concentration range 5 - 20 μg/mL during subsequent experiments.

To invest the anti-inflammatory effects of MZRR, the MZRR extract was examined with regard to its effect on prostaglandin and nitrite production in LPS-activated RAW 264.6 macrophages. As shown in Fig. 2A, the LPS-induced PGE_2 production was markedly suppressed by treatment with 20 μM of MZRR, and the result was dose dependent. In addition, to assess the NO-blocking effect of MZRR, we monitored nitrite levels in culture media after stimulating cells with LPS (1 μg/mL) in the presence or absence of MZRR for 48 h. LPS stimulation caused a significant accumulation of nitrite in culture media at

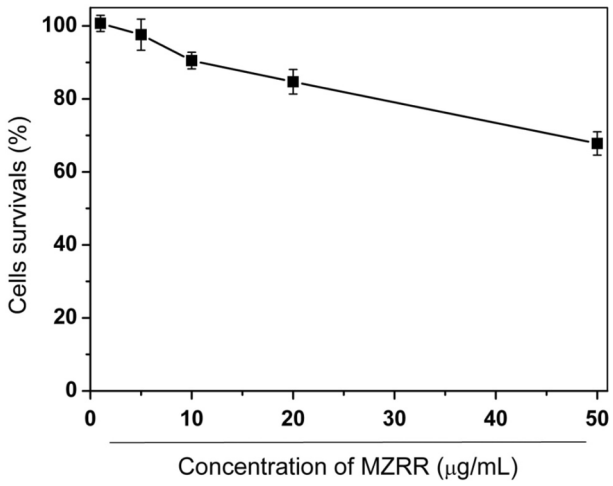

Fig. 1. The effect of MZRR on cell survival in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured in the presence of the indicated concentrations of MZRR for 48 h. Cell survival was determined using an MTT assay.

24 h. However, pretreatment with MZRR (5 - 20 μg/mL) significantly attenuated LPS-induced nitrite production in a concentration-dependent manner (Fig. 2B). We then examined whether the inhibition of NO production by MZRR is due to iNOS transcription. Western blot analysis and RT-PCR showed that exposure of RAW 264.7 cells to LPS (1 μg/mL) for 18 h increased iNOS protein and gene levels versus un-stimulated controls. However, MZRR treatment at 5 - 20 μg/mL significantly reduced the LPS-induced iNOS protein and gene expression (Fig. 2C and 2D). Consistent with the Western blot analysis result, RT-PCR analysis revealed that iNOS luciferase activity was significantly inhibited by MZRR (5 - 20 μg/mL) in RAW 264.7 macrophages (Fig. 2E).

MZRR decreases COX-2 expression in LPS-activated macrophages – The effect of various MZRR on the protein and gene expression of COX-2 induced by LPS was determined in RAW 264.7 macrophages. Western blot analyses and RT-PCR revealed that COX-2 gene and protein expression were up-regulated by LPS treatment in RAW 264.7 macrophages for 24 h. However, MZRR treatment at 5-20 μg/mL significantly inhibited COX-2 protein and gene expression level (Fig. 3A and 3B). Furthermore, COX-2 luciferase activity was also reduced by 5 - 20 μg/mL of MZRR treatment (Fig. 3C).

MZRR inhibits LPS-induced TNF-α, IL-1β, and AP-1 production and suppress activation of NF-κB in LPS-activated macrophages – LPS treatment (1 μg/mL, 18 h) caused almost 4.3-fold increase in NF-κB reporter activity as compared with the un-stimulated control (Fig. 4A) and pretreatment of RAW 264.7 macrophages with

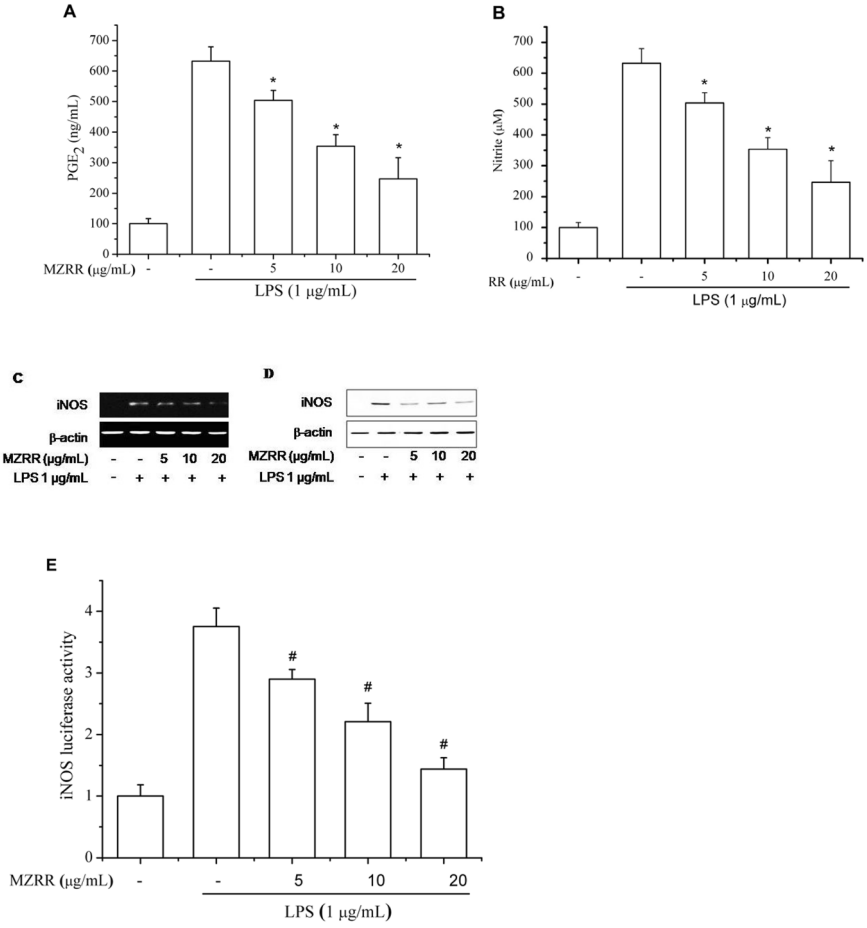

Fig. 2. (A and B) Effect of MZRR on PGE_2 and NO production in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured for 24 h in the presence of the media alone, with the indicated concentrations of MZRR, or with LPS (1 μg/mL). The level of PGE_2 and NO production was determined. *$P < 0.05$, significantly different from LPS-treated cells. (C and D) Effect of MZRR on iNOS gene and protein expression. The RAW 264.7 macrophages were cultured for 6 h in the presence of media alone, with the indicated concentrations of MZRR, or with LPS (1 μg/mL). The cells were lysed and conformed by RT-PCR and Western blot analysis. (E) Effects of MZRR on iNOS luciferase gene expression in RAW 264.7 macrophages. Cells were transiently cotransfected with iNOS and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of MZRR or LPS (1 μg/mL) for 12 h. The cells were harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and is expressed relative to the activity of the control. Each bar shows means ± SD of triplicate. $^{\#}P < 0.05$, significantly different from LPS-treated cells.

Fig. 2. Suppression of COX-2 induction by MZRR. (A and B) Effect of MZRR on COX-2 gene and protein expression. The RAW 264.7 macrophages were cultured for 6 h in the presence of media alone, with the indicated concentrations of MZRR, or with LPS (1 μg/mL). The cells were lysed and COX-2 expression was detected by RT-PCR and Western blot analysis. (C) Effects of MZRR on COX-2 luciferase activity. Cells were transiently cotransfected with COX-2 and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of MZRR or LPS (1 μg/mL) for 12 h. The cells were harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and is expressed relative to the activity of the control. Each bar shows means ± SD of triplicate. $^{\#}P < 0.05$, significantly different from LPS-treated cells.

5 - 20 μg/mL of MZRR significantly inhibited the increase in NF-κB reporter activity induced by 1 μg/mL LPS (Fig. 4A). Moreover, LPS-induced TNFα and IL-1β gene expression were significantly altered by MZRR (Fig. 4B), however unchangeable was observed to IL-6 caused by MZRR (data not shown). AP-1 is another transcription factor involved in regulating the COX-2 expression, iNOS gene, and carcinogenesis (Zeng *et al.*, 2002; Chun *et al.*, 2004). In this study, LPS treatment increased activity of AP-1, but this increasing was inhibited in the presence of 5 - 20 μg/mL of MZRR (Fig. 4C), which suggests that the inhibitory effect of MZRR on iNOS induction is associated with the blocking of the AP-1 activation process. These results demonstrated that MZRR

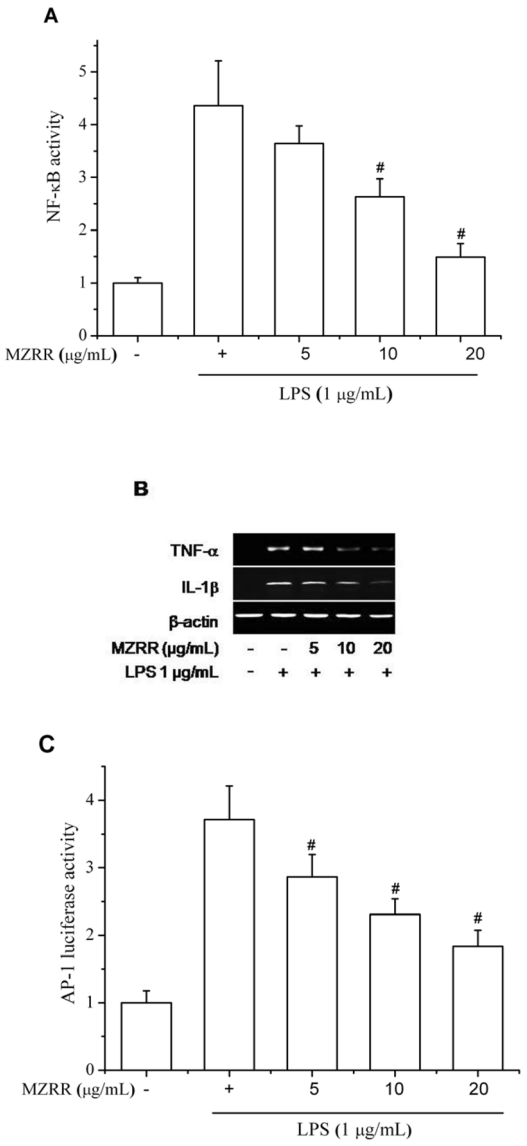

Fig. 3. (A) Effects of MZRR on NF-κB luciferase activity. Cells were transiently cotransfected with NF-κB and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of MZRR or LPS (1 μg/mL) for 12 h. The cells were harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and is expressed relative to the activity of the control. Each bar shows means ± SD of triplicate. $^{\#}P < 0.05$, significantly different from LPS-treated cells. (B) TNF-α and IL-1β mRNA expression were inhibited by MZRR. The RAW 264.7 macrophages were cultured for 6 h in the presence of media alone, with the indicated concentrations of MZRR, or with LPS (1 μg/mL). TNF-α and IL-1β mRNA expression were measured by RT-PCR. (C) Effects of MZRR on AP-1 luciferase activity. Cells were transiently cotransfected with AP-1 and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of MZRR or LPS (1 μg/mL) for 12 h. The cells were harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and is expressed relative to the activity of the control. Each bar shows means ± SD of triplicate. $^{\#}P < 0.05$, significantly different from LPS-treated cells.

selectively inhibits the NF-κB activation process and pro-inflammation cytokines, and suggest that this is associated with an abrogation of COX-2 and iNOS induction by MZRR.

Discussion

Herbal medications have a long tradition in the treatment of inflammatory diseases. Since the mechanism of anti-inflammatory effects of *Z. rhetsa* has not been reported to the present time, we evaluated the effects of *Z. rhetsa* methanol extract on the expression of genes COX-2, iNOS, and TNF-α genes on RAW 264,7 macrophages activated by LPS and inhibition of NF-κB activation in RAW 264,7 macrophages.

Pro-inflammatory cytokine upregulation and excessive NO production play vital roles in a severe inflammatory disease and the inhibition of pro-inflammatory cytokines and iNOS gene expression in inflammatory cells could beneficially suppress excessive inflammatory reactions (Southan and Szabo, 1996). NF-κB is one of the pivotal regulators of pro-inflammatory gene expression and it induces the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, COX-2, and iNOS. NF-κB is highly activated at sites of inflammation in diverse diseases, such as rheumatoid arthritis, inflammatory bowel diseases (Li and Verma, 2002). Specific inhibition of NF-κB activity has been shown consistently to be effective at controlling inflammatory diseases in several animal models (Li and Verma, 2002). In this study, MZRR are highly active in suppressing expression of both iNOS and COX-2 mRNA and protein at concentrations and also markedly attenuate cytokine production, suggesting that there may be common mechanic basis for this action. Suppression of activation of NF-κB by active MZRR may, at least in a part, account for this, because there are known to be NF-κB response elements on the promoters for both the iNOS and the COX-2 genes (Båge *et al*., 2010; Carothers *et al*., 2010; Diaz-Guerra *et al*., 1996; Xie *et al*., 1993). Our results partially explained that MZRR inhibition of LPS-induced expression of COX-2, iNOS, and TNF-α genes occur through blocking of NF-κB activation although inhibition of another factor (AP-1), the interferon response element and γ-activated site may be involved. Consistent with previous study reported that a significant anti-inflammatory agent (lupeol) had been isolated from the stem bark of *Z. rhetsa* and this compound had been demonstrated to be a novel anti-inflammatory and anti-cancer dietary triterpene (Dharmaratne *et al*., 1998; Saleem, 2009). Especially, the molecular mechanism of action of

lupeol indicated that its anti-inflammatory effect is involve in NF-κB pathway (Saleem, 2009).

In summary, the results of the present study indicate that the stem bark of *Z. rhetsa* methanol extract is a high potent inhibitor of iNOS, COX-2, and TNF-α via gene expression by blocking NF-κB activation. Our result therefore provides a mechanistic basis for the anti-inflammatory effect of the stem bark of *Z. rhetsa*. The anti-inflammatory potential may represent a novel mechanism of action of this traditionally employed herbal medicine. Further studies are required to investigate for the precise active constituents responsible for such activity.

Acknowledgments

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