Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(5): 316-321

ISSN- 0975 1556

Research Article

In Vitro Study of NF-κB and Pro-Inflammatory Cytokines Declining Levels by Polyherbal EMSA Eritin

Muhaimin Rifa I[1*], Yuyun Ika Christina[1], Qonitatul Khasanah[1], Agung Pramana Warih Marhendra[2], Hideo Tsuboi[3]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia.*
[2]*Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia*
[3]*Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

Available Online: 1st May, 2016

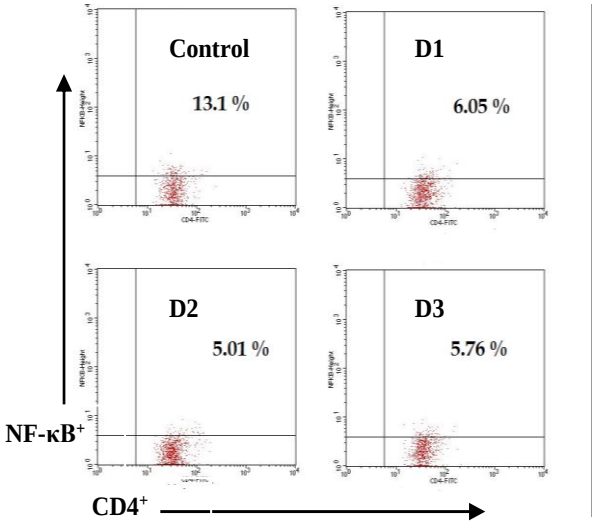
ABSTRACT
Inflammation is an immunological response which plays pivotal role in antigen elimination, but it also causes detrimental effect to the surrounding tissue. Inflammation pathway is activated by the molecular signaling which regulate both pro- and anti-inflammatory cytokines. NF-κB is a transcription factor which regulates the transcription of several genes that regulate growth, angiogenesis, and survival, including the transcription of inflammatory cytokines. In the present study, we examined the effect of polyherbal EMSA Eritin focusing on immunological function with aim of highlighting the production of pro-inflammatory cytokines and the expression of transcriptional factor NF-κB in vitro. EMSA Eritin is a polyherbal consisted of soybean, red rice, and coconut water extract. Splenocytes from healthy mice were cultured for 5 days in RPMI-1640 medium. On day 5, the cultured cells were harvested and analyzed by flow cytometry. EMSA Eritin was able to inhibit the expression of NF-κB in T cells and also the production of proinflammatory cytokines marked by the decreasing levels of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ T cells. Previous study indicated that EMSA Eritin is able to decrease NF-κB and pro-inflammatory cytokines in irradiated mice (in vivo study) with sublethal dose. Hereby, our study suggests that EMSA Eritin is a potential herbal medicine which act as an anti-inflammatory agent.

Keywords: EMSA Eritin; in vitro; IFN-γ; NF-κB; TNF-α

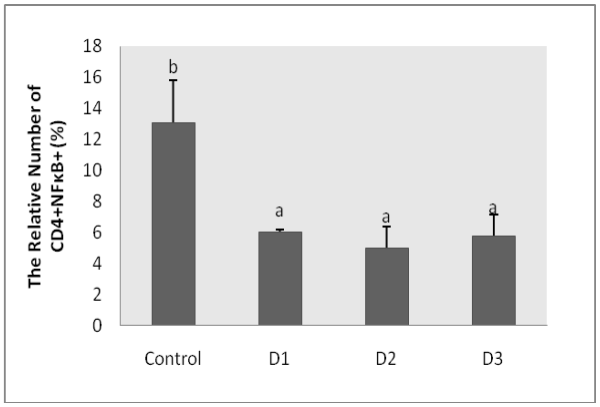
INTRODUCTION

Inflammation is commonly known as the swelling, redness, heat and pain after various form of injuries. Inflammation response is the most important natural defense mechanisms against internal and external threats which is triggered when innate immune cells detect infection or tissue damage. Tissue damage leading to inflammation may occur through physical factors, such as physical injury, trauma, ionizing radiation, burns or excessive cooling. Inflammation also caused by biological factors such as pathogens infection, hypersensitive, and stress[1]. Innate immune cells residing in tissues, such as macrophages, fibroblasts, mast cells, and dendritic cells, including circulating leukocytes, monocytes and neutrophils recognize the pathogen invasion or cell damage with intracellular or surface-expressed pattern recognition receptors (PRRs)[2]. Lipopolysaccharide (LPS) is a bacterial endotoxin which has a potent initiator of inflammatory responses and serves as an indicator of bacterial infection[3]. The bacterial endotoxin could induce the inflammatory response. The inflammatory response is characterized by activation of various signaling pathways that regulate expression of both pro- and anti-inflammatory mediators in leukocytes recruited. Currently, most of our knowledge of signaling in inflammation is gained from studying members of the IL-1 and TNF receptor families and the Toll-like microbial pattern recognition receptors (TLRs), which belong to the IL-1R family. IL-1 and TNFα represent the pro-inflammatory cytokines that are rapidly released after tissue injury or infection[4]. NFkB is a ubiquitous transcription factor and pleiotropic regulator of numerous genes involved in the immune and inflammatory responses. The nuclear factor NF-κB pathway has long been considered a prototypical pro-inflammatory signaling pathway, largely based on the role of NF-κB in the expression of pro-inflammatory genes including cytokines, chemokines, and adhesion molecules[5]. The present study was designed to investigate the effect of polyherbal EMSA Eritin extract on NF-κB activation on T cell and proinflammatory cytokines production. EMSA Eritin consisted of soybean, red rice and coconut water extract. Previous study indicated that EMSA Eritin had an ability to decrease the NF-κB and pro-inflammatory cytokines levels in irradiated mice[6]. In this study, we focused on the effect of EMSA Eritin in decreasing the inflammation marker in controlled and limited condition or culture environment.

**Author for Correspondence*

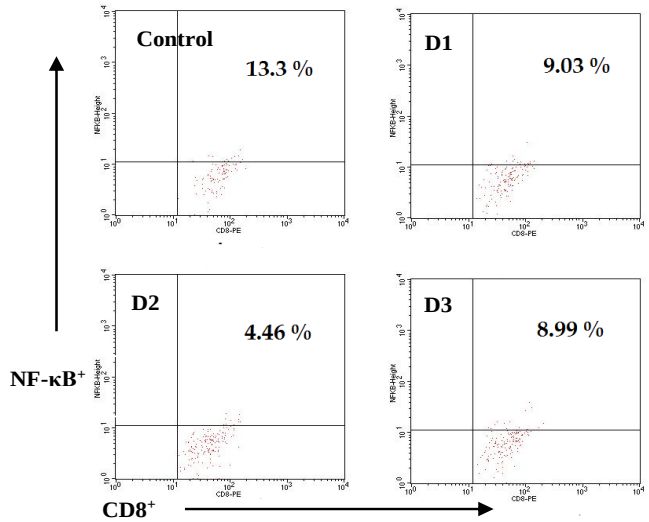


(A)

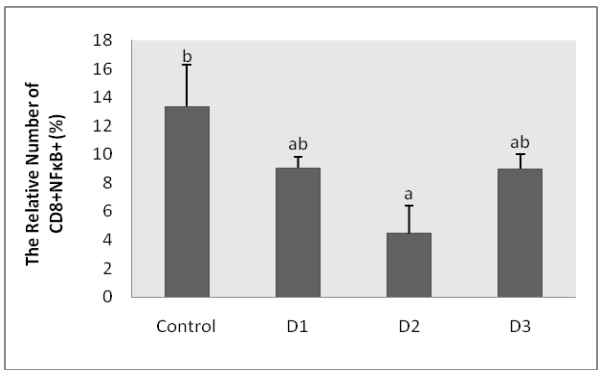


(B)

Figure 1: EMSA Eritin blocks the expression of NF-κB in CD4 T cells in vitro. (A) Spleen cells ($2x10^6$) were obtained from 4-week-old BALB/c mice and cultured in 48-well plates for 5 days in a RPMI-1640 medium containing anti-CD3 stimulant and LPS. Flow cytometry analysis showed the expression of NF-κB was decreased in all EMSA Eritin doses. EMSA Eritin was able to normalize NFκB production in CD4 T cells (B). The bars are calculation of the number CD4 T cells expressing positive NF-κB on the spleen cells of mice in vitro. It was shown that treatment of EMSA Eritin has smaller number of transcription factor (NF-κB) in all dose EMSA Eritin treatments than the control group. (Control: without EMSA Eritin treatment, EMSA Eritin D1 : 0.025 mg/mL, D2 : 0.25 g/mL and D3 : 2.5 mg/mL).



(A)



(B)

Figure 2: EMSA Eritin also blocks the expression of NF-κB in CD8 T cells in vitro. (A) Spleen cells ($2x10^6$) were obtained from 4-week-old BALB/c mice and cultured in 48-well plates for 5 days in a RPMI-1640 medium containing anti-CD3 stimulant and LPS. Flow cytometry analysis showed the expression of NF-κB was decreased in D2 significantly compared with control. (B). The bars are calculation of the number of CD8 T cells expressing positive NF-κB in the spleen cells of mice in vitro after administration of EMSA Eritin. (Control: without EMSA Eritin treatment, EMSA Eritin D1: 0.025 mg/mL, D2: 0.25 g/mL and D3: 2.5 mg/mL).

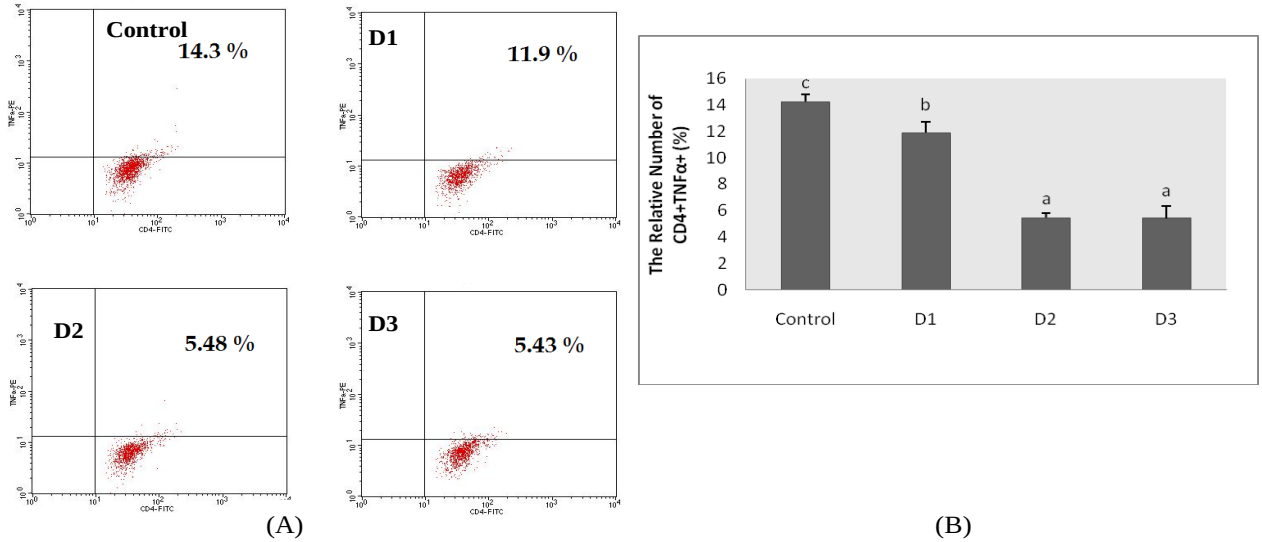
MATERIALS AND METHODS

EMSA Eritin extraction

Red rice and soybean were washed and dried in a vacuum oven at 40^0C. Then, mashed to obtain the particles of 60-mesh size (250 mm). The powder was extracted using water with ratio 1:10 (materials: water) at 50^0C for 2 hours. The extract and coconut water were evaporated by freeze drying for 24 hours at -60^0C. And the last step was mixing the red rice rice and soybean extract with coconut water powder.

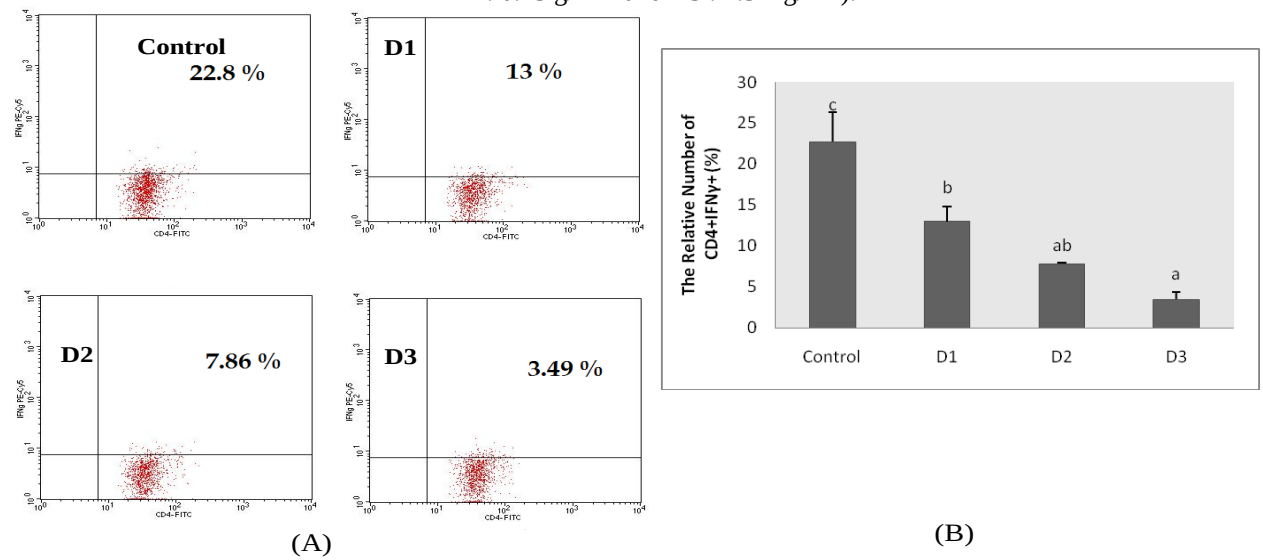
Medium Preparation

T cells were cultured in Roswell Park Memorial Institute-1640 (RPMI) supplemented with 10% Fetal Bovine Serum (FBS), glutamin (30 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), anti-CD3 (2% culture supernatant, lipopolysaccharide, LPS (10 ng/mL), and 2-Mercaptoethanol (2-ME, 5 x 10^{-5} M) with EMSA Eritin concentration were 0, 0.025, 0.25 and 2.5 mg mL^{-1}.



(A) (B)

Figure 3: EMSA Eritin decreased the level of CD4 T cells producing TNF-α in vitro (A) Spleen cells ($2x10^6$) were obtained from 4-week-old BALB/c mice and cultured in 48-well plates for 5 days in a RPMI-1640 medium containing anti-CD3 stimulant and LPS. Flow cytometry analysis showed the expression of TNF-α was decreased in all EMSA Eritin treatments. (B). The bars are calculation of the number of CD4 T cells expressing positive TNF-α on the mice spleen cells received EMSA Eritin. D2 and D3 suppress the level of TNF-α significantly compared with D1. (Control: without EMSA Eritin treatment, EMSA Eritin D1: 0.025 mg/mL, D2 : 0.25 g/mL and D3 : 2.5 mg/mL).



(A) (B)

Figure 4: IFN-γ produced by CD4 T cells was also decreased after administration of EMSA Eritin. (A). Spleen cells ($2x10^6$) were obtained from 4-week-old BALB/c mice and cultured in 48-well plates for 5 days in a RPMI-1640 medium containing anti-CD3 stimulant. Flow cytometry analysis showed the expression of IFN-γ was decreased in all EMSA Eritin treatments. (B). The bars are calculation of the number of CD4 T cells expressing positive IFN-γ on the mice spleen cells received EMSA Eritin. All dose of EMSA Eritin showed the same result to suppress the level of IFN-γ. Data are mean of ± SD values of 5 mice in each group. (Control: without EMSA Eritin treatment, EMSA Eritin D1: 0.025 mg/mL, D2 : 0.25 g/mL and D3 : 2.5 mg/mL).

Medium filtered by millipore membrane 0.20 µm. All of that procedure had done with aseptic method in Laminar Air Flow (LAF).

Isolation of lymphocyte

In this study we used 4 week-old BALB/c mice, which were maintained in pathogen free facility, Biology Department, Faculty of Sciences, Brawijaya University, Malang, Indonesia. The mice were dislocated and the spleen was isolated. The spleen were washed two times with phosphate-buffered saline (PBS) in Petri dish. The spleen were pressed clockwise using steril syringe base. Homogenates were then mixed with 10 mL PBS to obtain the lymphocytes. The suspense was centrifuged at 2500 rpm, at 10°C, for 5 min. Supernatant was discarded, while the pellet resuspended in 1 mL of RPMI-1640 medium. The cell suspension was taken 5 µL, added with 95 µL (20×dilution) of evans blue 10x and homogenized with pipette. Cells were counted using haemocytometer with

microscope. The formula for counting the number of lymphocyte is: Σ Cells = Σ the cell count×5×dilution×10^4 cells mL^{-1}.

Cell Culture

The cells (1.5 – 2 x 10^6 cell / ml) were cultured in 48-well plates and divided into four groups based on the different dose of EMSA Eritin: 0 mg mL^{-1} (K); 0.025 mg mL^{-1} (D1); 0.25 mg mL^{-1} (D2) and 2.5 mg mL^{-1} (D3). Medium for control, D1, D2 and D3 were added with 3 million mL^{-1} of cell and then mixed gently. The cells were cultured in an incubator (5% CO_2 at 37°C) for 5 days. The cells were harvested by pipetting medium of each treatment and moved it into 15 mL polypropylene tube. The homogenate were centrifuged at 2500 rpm, 10°C for 5 min. Pellet were resuspended with 1 mL of PBS and continued to intracellular staining procedure.

Intracellular staining and Flow cytometry analysis

The following combinations of antibody for intracellular staining were A: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8 and PE/Cy5-conjugated rat anti-mouse NF-κB, and B: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse TNF-α and PE/Cy5-conjugated rat anti-mouse IFNg. Cells were incubated with extracellular antibodies for 20 min in the ice box at 4°C. After incubation, the suspension was washed and the pellet was resuspended in cytofix buffer (50 µL) for 20 min in dark conditions at 4^0C. Then the suspension was resuspended in 500 µL wash-perm and centrifuged at 2500 rpm, 10°C for 5 min. Supernatant was discarded and the obtained pellet was subjected to intracellular staining for 20 min, at 4^0C. Each sample was transferred into flow cytometry cuvet and then analyzed with flow cytometer (FACSCalibur; BD Biosciences, New Jersey, USA), and calculated using BD CellQuest PRO software.

Data Analysis

Data were analyzed by BD CellQuest PRO software then tabulated and analyzed statistically. Data were analyzed using SPPS 16.0 for Windows. Oneway ANOVA test was used to assess the statistical difference between the control group and the different dose of EMSA Eritin groups ($p<0.05$ was defined as statistically significant). If the obtained results are significant and then analyzed with TukeyTest

RESULTS

EMSA Eritin blocks NF-κB on T lymphocyte

NF-κB plays a crucialroles in immune response and regulate the expression of pro-inflammatory cytokines. EMSA Eritin had an ability to suppress the expression of NF-κB in $CD4^+$ and $CD8^+$ T cells in vitro. NF-κB in $CD4^+$ T cells were decreased in all dose of EMSA Eritin (6.05%, 5.01%, and 5.76%) compared with control 13.1% (Fig.1). In this experiment we also found that EMSA Eritin is not only decrease the level of NF-κB on $CD4^+$ T cells but also decrease NF-κB in CD8 T cells. D2 showed the significant decrease in the relative number of $CD8^+$NF-$κB^+$ T cells (4.46%) compared to control (13.3%) (Fig. 2). NF-κB that produced by CD4 and CD8 T cells was decreased in all doses of EMSA Eritin treatments.

EMSA Eritin depleted the level of proinflammatory cytokines on T lymphocyte

In this experiment we demonstrated that administration of EMSA Eritin in cell culture was able to decrease the pro-inflammatory cytokines. Interestingly, administration of EMSA Eritin is not only suppressed TNF-α but also IFN-γ produced by CD4 T cells. In control without EMSA Eritin treatment, the level of TNF-α is 14.3% of the population CD4 T cells (Fig 3.A). TNF-α decreased significantly in D2 (5.48%) and D3 (5.43%) compared to control ($p<0.05$) (14.3%). Furthermore, the number of IFN-γ that produced by CD4 T cells in EMSA Eritin treatment was markedly decreased from D1 (0.025 mg/ml) to D3 (2.5 mg/ml) respectively (13%, 7.86%, 3.49%; Figure 4.A), compared with control (22.8%). Based on ANOVA (Figure 4.B), the relative number of IFN-γ cytokines in all dose treatment of EMSA Eritin showed significant difference compared to control ($p<0.05$). It was indicated that all dose treatment of EMSA Eritin suppressed the level of TNF-α on BALB/c mice lymphocyte in culture condition. In this study we reported that the administration of EMSA Eritin in vitro was able to suppressed the expression of TNF-α and IFN-γ from CD4 T cells and also suppressed the NF-κB which are produced by CD4 and CD8 T cells. causes of inflammation reaction is endotoxin which is a complex lipopolysaccharide (LPS). This endotoxin is a major component of the outer membrane of most gram-negative bacteria. In some cases, LPSs are considered to be important in a number of inflammation conditions, such as ARDS and cystic fibrosis[9,10]. LPS potentiates to stimulate the activity of many leukocyte types. The binding of LPS to its receptor (CD14) on the cell membrane induces the downstream pathway and activates the transcription factor to regulate the production of pro-inflammatory cytokines such as TNF-α, IFN-γ IL-1, or IL-8, and ROS and implicated in the pathogenesis of inflammation[11,12]. In response to endotoxin stimulation, some of innate immune cells for instance macrophages and mononuclear phagocytes produce and release various of cytokines, including tumor necrosis factor (TNF) and interleukins both in vivo and in vitro[13]. Morris *et al.*[3]

DISCUSSION

In the present study, we demonstrated that LPS was able to induce the inflammatory response marked by the increasing level of transcription factor, NF-κB and pro-inflammatory cytokines, IFN-γ and TNF-α. NF-κB regulates the gene expression of various cytokines, chemokines, growth factors, and cell-adhesion molecules[7]. The main factor of NF-κB activation is the dissociation of IκB from p50-p65 heterodimer which is the NF-κB complex. IκB is an inhibitor protein and is retained in the cytoplasm. Ligand binding to the receptor initiates the phosphorylation and regulates the IκB subunit to activate the heterodimer, followed by translocation of the active dimmer to the nucleus, where it binds to the NF-κB motif of a gene promoter and functions as a transcriptional regulator[8]. One of the major

reported that endotoxin levels as low as 100 ng/ml could stimulate the proliferation of human T cells and their production of lymphokines. In the current study, we showed the importance of EMSA Eritin as anti-inflammatory agent in LPS-induced cultured cell. EMSA Eritin is a polyherbal consisted of soybean, red rice and coconut water extract. This herbal combination has a wide range of important components such as genistein, cytokinin, nicotinic acid, pantothenic acid, biotin, riboflavin, folic acid, thiamine B1, vitamin C, pyridoxine, daidzein, glycitein, phenolic acids, and anthocyanins. The content of EMSA Eritin might contribute to the depleting levels of some pro-inflammatory cytokines although the mechanism are not known in details. The largest composition in EMSA Eritin is soybean which contains some isoflavones including genistein, daidzein and glycitein. Genistein is the most abundant isoflavone in soybean that exhibit an anti-inflammatory effect both in vivo and in vitro[14-16]. Hooshmand[17] reported that genistein was reported to inhibit the production of pro-inflammatory molecules in human chondrocytes. Some flavonoids were reported to have potential to inhibit NO production in response to inflammatory reaction[18-22]. The anti-inflammatory potential of genistein have also been studied in vivo in rat with LPS-induced by inhibiting the septic response[21]. Here we found that the effective compounds in EMSA Eritin inhibits the activation of NF-κB and regulates the production of pro-inflammatory cytokines, including IFN-γ and TNF-α. To our knowledge, the mechanism of genistein and daidzein in inhibiting the activation of NF-κB are not known but may be associated with inhibition of phosphorylation of STAT-1 or the kinase receptor JAK2 and most probably regulatory T cell involved in the mechanism of action[23-25]. In summary, we reported that EMSA Eritin plays a pivotal role in exhibiting the anti-inflammatory response in LPS-induced cultured cell. Although our findings provide a result for the possible use of EMSA Eritin, further studies might be important regarding to determine the mechanism of effective compound in EMSA Eritin in inhibiting the inflammation response.

ACKNOWLEDGEMENTS

The author would like to thank to Mr. Nashi Widodo Ph.D. Med. Sc for careful reading and critical comments on this manuscript and also to all fellow researchers in Animal Physiology Laboratory, University of Brawijaya, which provide much help in the process of this research.

Ethics

This study has received ethical eligibility certificate (Ethical Clearance) from The Research Ethics Committee (Animal Care and Use Committee) Brawijaya University No. KEP-255-UB.

REFERENCES

1. Heimdall JH, Aarstad HJ, Olofsson J (2000) Peripheral blood-lymphocyte and monocyte function and survival in patients with head and neck carcinoma. Laryngoscope. 110:1-6
2. Abbas AK, Litchman AH (2005) Cellular and molecular immunology 5th edition. Elsevier Saunders Company. Philadelphia.
3. Morris DD, Crowe N, Moore JN, Moldawer LL (1992) Endotoxin-Induced Production of Interleukin 6 by Equine Peritoneal Macrophages In Vitro. Am. J. Vet. Res. 53:1298-1301.
4. Coppack SW (2001) Pro-inflammatory cytokines and adipose tissue. Proc. Nutr. Soc. 60(3): 349-56.
5. Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 104 (4): 487-501.
6. Christina YI, Ibrahim M, Rifa'i M (2015) Polyherbal EMSA Eritin blocks nuclear factor-kappa B (NF-κB) and proinflammatory cytokines in irradiated mice. American Journal of Immunology. 11(1):17-25
7. Chen F, Demersl M, Valltahanf, Luy (1999) Vanadate induction of NF-kappa B involves Ikappa B kinase beta and SAPK/ERK kinase 1 in macrophages. J. Biol. Chem. 274: 20307-20312.
8. Schmid JA, Birbach A. (2008) IkappaB kinase beta (IKKbeta/IKK2/IKBKB)-A key molecule in signaling to the transcription factor NF-kappa B. Cytokine Growth Factor Rev. 19: 157-165.
9. Hutchison ML, Bonell EC, Poxton IR, and Govan JR. (2000) Endotoxic activity of lipopolysaccharides isolated from emergent potential cystic fibrosis pathogens. FEMS Immunol Med Microbiol. 27: 73–77.
10. Thorn J. (2001) The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflamm Res. 50: 254–261.
11. DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, Remick DG (1993) Regulation of interleukin 8 gene expression by oxidant stress. J Biol Chem. 268: 25568–25576.
12. Rahman I (2003) Oxidative stress, chromatin remodeling and gene transcription in inflammation and chronic lung diseases. J Biochem Mol Biol. 36: 95–109.
13. Morrison DC, Dinarello CA, Munford RS, Natanson C, Danner R, Pollack M, Spizer JJ, Ulevitch RJ, Vogel SN, McSweegan E (1994) Current Status of Bacterial Endotoxins. ASM News, 60:479-484.
14. Ruetten H, Thiemerman NC (1997) Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase. Br. J. Pharmacol. 122: 59-70.
15. Middletone Jr, Kandaswamic, Theoharidest C (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52: 673-751.
16. Havsteen BH (2002) The biochemistry and medical signifi-cance of the flavonoids. Pharmacol. Ther. 96: 67-202.
17. Hooshmand S, Soung Do Y, Lucas EA, Madihallys V, Levensonc W (2007) Genistein reduces the production of proinflammatory molecules in human chondrocytes. J. Nutr. Biochem. 18: 609-614.
18. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen

CF, Lin JK. (1999) Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20(10): 1945–1952.
19. Wang J, Mazza G. (2002) Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-γ-activated RAW 264.7 macrophages, Journal of Agricultural and Food Chemistry. 50(4): 850-857
20. Autore G, Rastrelli L, Lauro MR. (2001) Inhibition of nitric oxide synthase expression by a methanolic extract of Crescentia alata and its derived flavonols. Life Sciences. 70(5): 523–534.
21. Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP. (1999) Effects of naturally occurring flavonoids on nitric ox-ide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochemical Pharmacology. 58(5): 759–765.
22. Raso GM, Meli R, Di Carlo G, Pacilio M, Di Carlo R (2001) Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life Sciences. 68(8): 921–931.
23. Akiyama T, Ishida J, Nakagawa S (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases, Journal of Biological Chemistry. 262(12): 5592–5595.
24. Rifa'i M, Widodo N. 2014. Significance of propolis administration for homeostasis of $CD4^{+}CD25^{+}$ immunoregulatory T cells controlling hyperglycemia. Springer 3(1):1-8.
25. Rifa'i M. 2013. $CD4^{+}CD25^{+}$ Regulatory T Cells Preventing Detrimental Autoimmune Reactions. Open Autoimmunity Journal 5(1-5).