

Original Research Paper

Polyherbal EMSA ERITIN Blocks Nuclear Factor-Kappa B (NF- κ B) and Proinflammatory Cytokines in Irradiated Mice

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Abstract: Radiation therapy is commonly used in cancer treatment that produce deleterious effects, such as normal cell death (apoptotic or necrotic) and suppression of hematopoietic stem cells. This effect was caused by ion rays that can rise to high of free radical. The high of free radical trigger the inflammation of cells. EMSA ERITIN is medicinal herb used as preventative agents or radioprotective to reduce the deleterious effects of radiation. EMSA ERITIN contains soy, red rice and coconut water extract. This study aimed to analyze the effect of polyherbal EMSA ERITIN to the expression of transcription factor of inflammation (NF- κ B). We also observe the proinflammatory cytokines (TNF- α and IFN- γ) in BALB/c mice after radiation. Polyherbal EMSA ERITIN grouped into three doses: Low dose (1.04 mg g⁻¹ BW), optimum dose (3.125 mg g⁻¹ BW) and high dose (9.375 mg g⁻¹ BW). The radiation dose that applied to mice is 5 Gy. The oral administration of EMSA ERITIN started 24 h after radiation and continued until 2 weeks. Data were analyzed using One-way ANOVA (p<0.05) and Tukey test using SPSS 16.0 for Windows. The result showed that the level of NF- κ B, TNF- α and IFN- γ in irradiated mice significantly decreased when the mice received EMSA ERITIN. All dose of polyherbal EMSA ERITIN showed more effective than EPO (Hemapo Epoetin alfa™) treatment to reduced TNF- α , IFN- γ and NF- κ B. So that, EMSA ERITIN is one of potential herbal medicine act as a preventive agent or radioprotective after radiation.

Keywords: EMSA ERITIN, IFN- γ , Ionizing Radiation, NF- κ B, TNF- α

Introduction

Cancer disease in each year increased about 7.6 million people in the world based on International Union Against Cancer (IUCC, 2011). Ionizing radiation commonly used as a main treatment for many types of tumors where radiation therapy is considered the primary non-surgical modality in the curative treatment of cancer (Dewan *et al.*, 2009). Radiation have been described can modulate anti-tumor immune responses (Lara *et al.*, 2013), modifying tumor and it's microinveronment (Frey *et al.*, 2014). Ionizing radiation is commonly used in cancer treatment that produce deleterious effects, such as normal cell death (apoptotic or necrotic) and suppression of hematopoietic stem cells.

Hematopoietic stem cells is known to be sensitive to radiation (Zhou and Man-Tian, 2005). Many natural agents have been investigated on whether they have the efficacy as radioprotective agents in the recent past years. Genistein on soy extract have been extensively

studied among the radioprotective compounds (Zhou and Man-Tian, 2005; Wei *et al.*, 2002). Some studies proved that genistein by *in vivo* and *in vitro* could delay the growth of tumors and induce apoptosis of cancer cells (Wei *et al.*, 2002). Another natural agents, coconut water (*Cocos nuciferus* L.) showed antioxidant activity (Verbeke *et al.*, 2000). The potential anti-cancer properties of specific cytokinins in coconut water could bring encouraging and novel perspectives in finding cures for the different types of cancers (Verbeke *et al.*, 2000; Griffaut *et al.*, 2004; Yong *et al.*, 2009). Kinetin contained coconut water can reduce the formation of reactive oxygen species (Liu *et al.*, 2011).

Gamma radiation can induce the appearance of oxidative stress characterized by the overproduction of Reactive Oxygen Species (ROS) such as Superoxide Radicals (O₂⁻), Hydroxyl Radical (OH) and Hydrogen Peroxide (H₂O₂). ROS will react to the structural and molecular functional organic, such as proteins, lipids and nucleic acids, that causing disruption in cellular metabolism

(Xienia *et al.*, 2000; Al-Rumaih and Al-Rumaih, 2008). Normal tissue damage can be controlled using radioprotective agent. Several studies have been conducted to develop radioprotector that prevent cell and tissue damage. One of radioprotector known is anthocyanins in red rice pigment (Chawla *et al.*, 2006). Anthocyanins in red rice can help mechanisms of protection due to gamma radiation (Al-Rumaih and Al-Rumaih, 2008).

Recently, several studies have demonstrated that the mixtures in various extracts from herbal medicines predicted more effective than a single purified natural product (El-Shemy *et al.*, 2013). So, analysis of the potential polihherbal is very useful and novelty. Here, we report a novel study about the potential of polyherbal extract to reduce inflammation. It's useful for treatment after radiation that we know the level of inflammation is high. The novel polyherbal that we used in this study is EMSA ERITIN. EMSA ERITIN contained of soy, red rice and coconut water extract. This study aimed to investigate the effect of polyherbal EMSA ERITIN to suppress proinflammatory cytokine, TNF- α and IFN- γ in vivo. We also observed the expression of transcription factor of inflammation (NF- κ B).

Materials and Methods

Experimental Design

A total of 18 normal BALB/c male mice, between the ages of 8-9 weeks were used. This study divided into 6 groups of treatment include negative control and positive control (irradiated mice). Determination of EMSA ERITIN doses for in vivo experiments based on the human consumption of 60 kg of body weight that consume about 15 g of EMSA ERITIN. Poly-herbal EMSA ERITIN grouped into three doses: Low dose (1.04 mg g⁻¹ BW), optimum dose (3.125 mg g⁻¹ BW) and high dose (9.375 mg g⁻¹ BW). We also used EPO or Hemapo Epoetin alfa™ 4.16 mg in PBS. EPO or Erythropoietin- α ™ used in this study to compared with EMSA ERITIN treatment.

Total Body Irradiation and Animal Treatment

Radiation exposure conducted at the Laboratory of Radiology, Dr. Saiful Anwar Hospital, Malang. Normal BALB/c mice received 5 Gy of total body irradiation from an open-beam cobalt 60 gamma source. EMSA-Eritrin administrated in mice orally in 24 h after radiation exposure. Administration of EMSA-ERITIN dissolved in distilled water until 1 mL. EMSA-ERITIN administered orally for 2 weeks. EPO was injected intraperitoneally twice a week.

Lymphocyte Isolation

The mice were sectioned after 2 week treatments. Spleen were isolated and crushed clockwise with syringe base. Lymphocyte homogenates were transferred into a new propylene tubes and added 10 mL PBS. Then

centrifuged at 2500 rpm, 4°C for 5 min. Supernatant were removed and pellet resuspended with 1 mL of sterile PBS.

Antibody Staining

Pellet stained with antibodies with extracellular and intracellular staining. The following purified antibodies were used for extracellular staining are Fluorescein Isothiocyanate (FITC)-conjugated anti-mouse CD4 and Phycoerythrin (PE)-conjugated anti-mouse CD8. For extracellular staining, pellet resuspended with 50 μ L of antibodies in sterile PBS. Antibodies for intracellular staining are PE-Cy5 conjugated anti-mouse NF- κ B, PE-Cy5 conjugated anti-mouse IFN- γ and Phycoerythrin (PE) conjugated anti-mouse TNF- α . For intracellular staining, pellet were added with 50 μ L cytofix-cytosperm and incubated for 20 min, 4°C. Then added with 500 μ L washperm and centrifuged 2500 rpm, 4°C, for 5 min. Pellet was resuspended with 50 μ L of antibodies in sterile PBS.

Flow Cytometry Analysis

Flow cytometry analysis was to determine the cell number of CD4⁺NF- κ B⁺, CD8⁺NF- κ B⁺, CD4⁺IFN- γ ⁺ and CD4⁺TNF- α ⁺. Cell moved into the cuvette and mounted on the nozzle flowcytometer (BD FACS Calibur™). Do the settings on the computer with BD Cell Quest Pro software™ and carried connection with flowcytometer (acquiring mode).

Statistical Analysis

Data were analyzed using SPSS 16.0 for Windows. One way ANOVA test was used to assess the statistical difference between the N control group, radiation group and the treatment of EMSA ERITIN groups ($p < 0.05$ was defined as statistically significant). If the obtained results are significant and then analyzed with Tukey Test.

Results

Suppression of NF- κ B

NF- κ B (Nuclear Factor-Kappa B) is a transcription factor that controls a number of genes important in inflammatory processes. EMSA ERITIN which administered in irradiated mice showed immunomodulatory activity as immunosupresant of NF- κ B. NF- κ B showed high expression in irradiated mice compared with normal mice (Fig. 1A) It caused by the induced of gamma rays that were exposed to the mice. Induction of gamma rays can trigger the damaged of cell caused by high of free radical. This activity can be seen through the decrease in the cell number of CD4⁺NF- κ B⁺ T cells in all doses of EMSA ERITIN (Fig. 1A). Based on the ANOVA (Fig. 1B), the relative number of CD4⁺NF- κ B⁺ T cell in all dose of EMSA ERITIN showed lower than EPO treatment ($p < 0.05$). Treatment with EPO has a higher relative number of NF- κ B. The normal dose

(Rad+D2) and high dose (Rad+D3) showed more effective than low dose (Rad+D1) to reduced the level of NF- κ B with the relative number in Rad+D1, Rad+D2 and Rad+D3 are 2.9, 1.7 and 1.55%, respectively.

In this experiment we also found that administered EMSA ERITIN was able to decrease the level of NF- κ B that are produced by CD8 T cells (Fig. 2). All dose of EMSA ERITIN also showed more effective than EPO treatment to suppressed NF- κ B. The relative

number of NF- κ B in negative control is 5.4% and increased after radiation (10.8%). The relative number of CD8⁺NF- κ B⁺ T cell in all doses of EMSA ERITIN are 2.5, 0.4 and 2.2%, respectively. NF- κ B that produced by CD4 and CD8 T cells was decreased in all doses of EMSA ERITIN treatments. It is indicated that EMSA ERITIN can modulate the level transcription factor of inflammation that produced by lymphocytes as the effect after radiation.

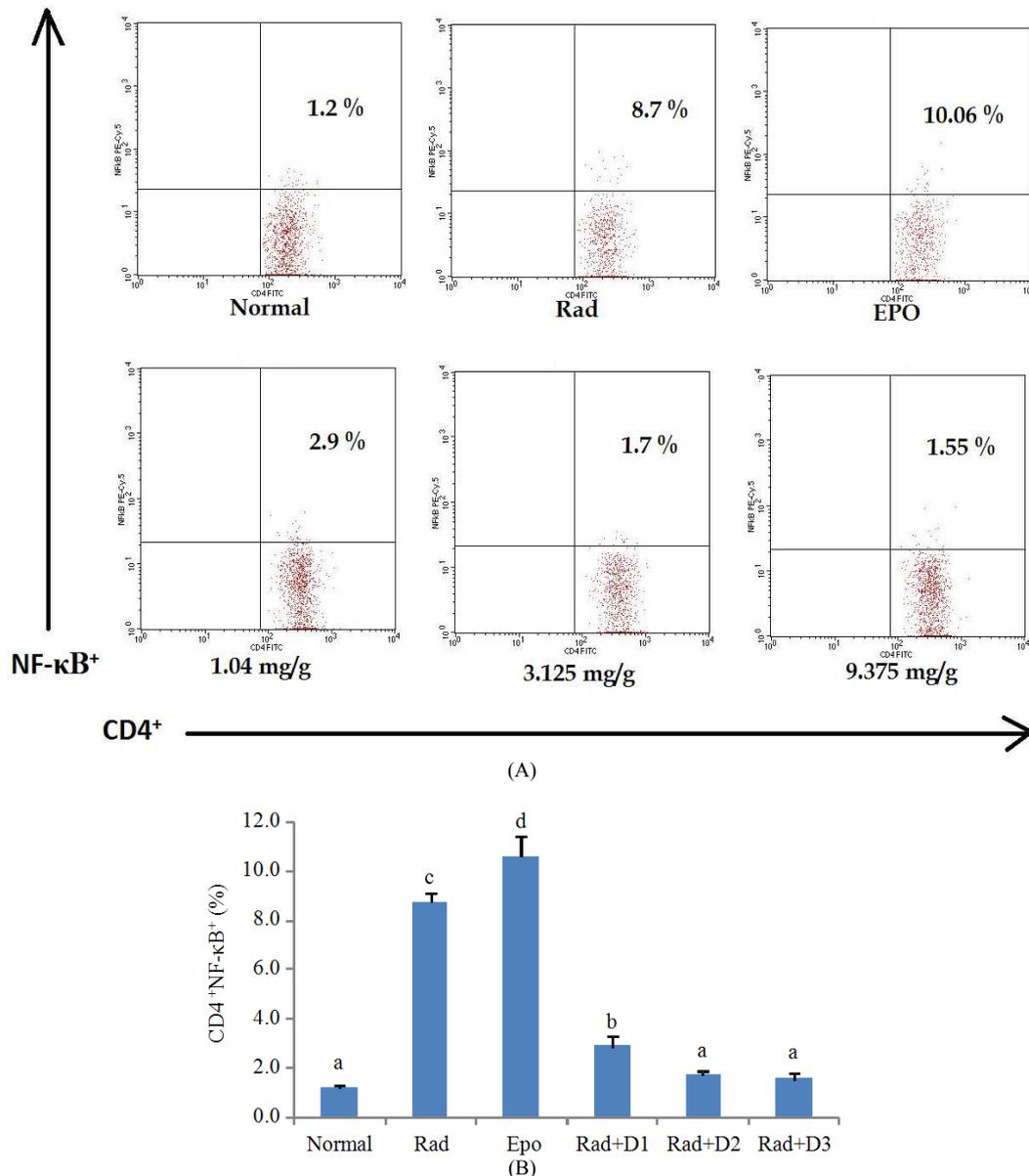


Fig. 1. EMSA ERITIN inhibit NF- κ B production (A) Flow cytometry analysis showed the expression of NF- κ B was decreased in all EMSA ERITIN treatments. EMSA ERITIN normalize NF κ B production in CD4 T cells (B) The bars are calculation of the number of CD4 T cells expressing positive NF- κ B on the spleen cells of mice after EMSA ERITIN treatment. It was shown that treatment of EMSA ERITIN has smaller number of the level transcription factor of inflammation (NF- κ B) in all dose EMSA ERITIN treatments compared with EPO treatment. Data are mean of \pm SD values of 3 mice in each group. (N: normal mice, Rad: Irradiated mice, EPO: Hemapo Epoetin alfaTM treatment, Rad+D1 (low dose): 1.04 mg g⁻¹ BB, Rad+D2 (normal dose): 3.125 mg g⁻¹ BW and Rad+D3 (high dose): 9.175 mg g⁻¹ BW)

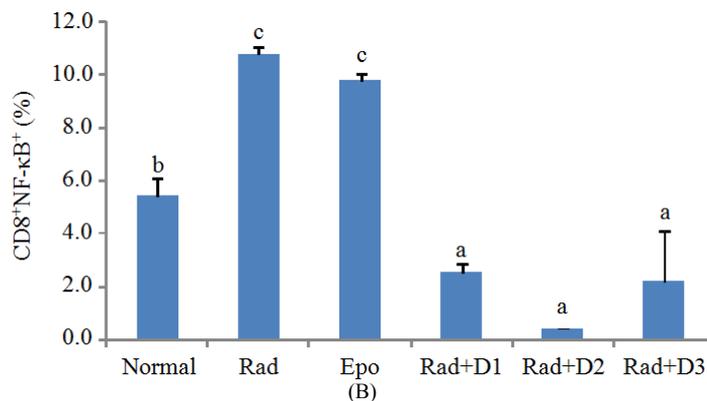
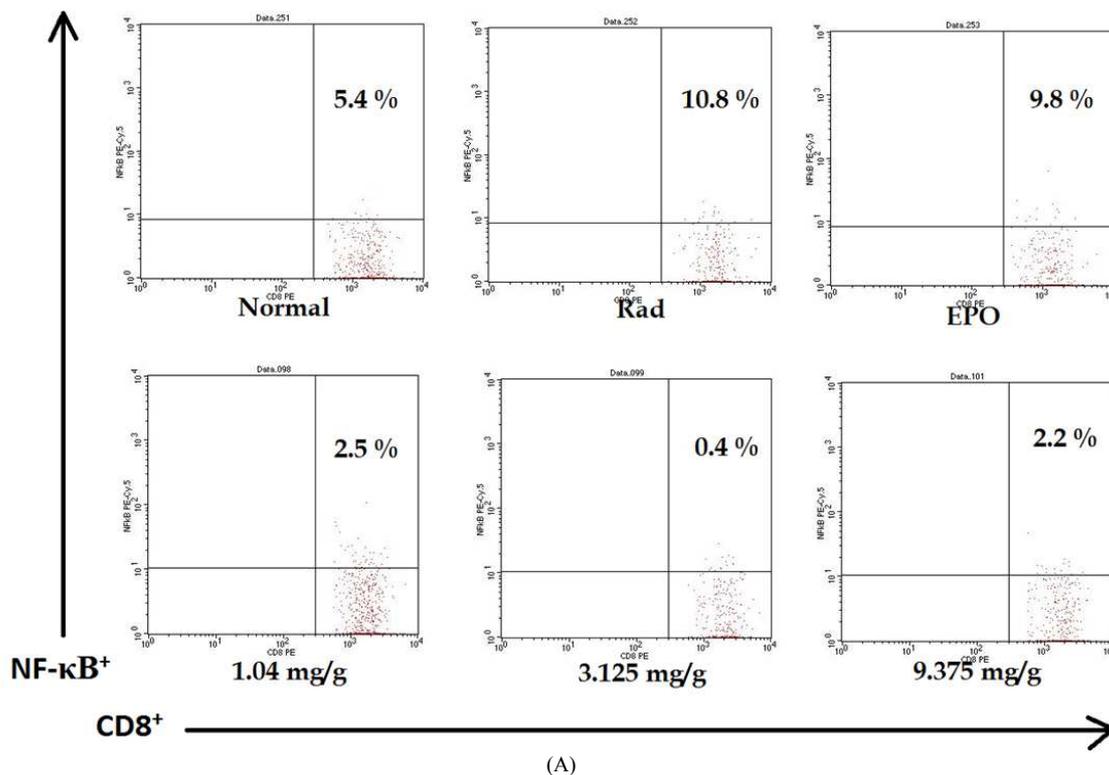
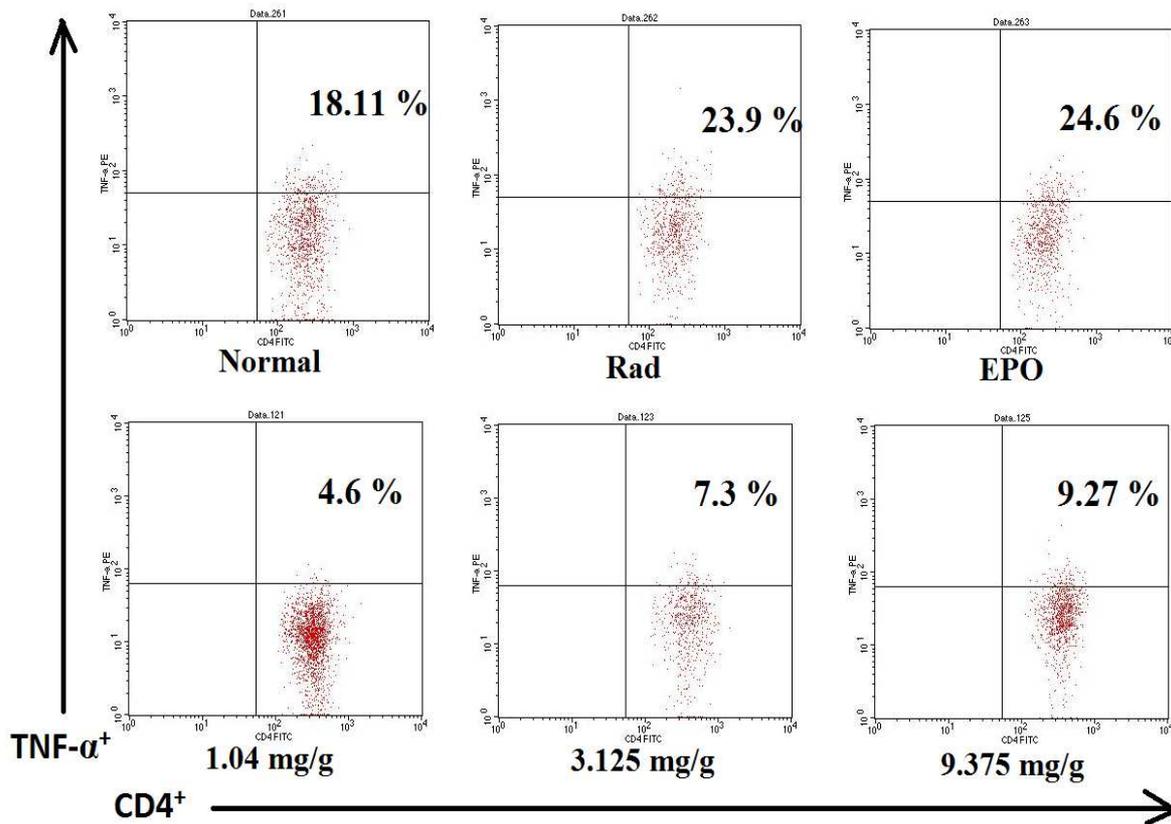


Fig. 2. CD8⁺NF-κB⁺ T cells also decreased in irradiated mice after 2 weeks received EMSA ERITIN. (A). Flow cytometry analysis showed the expression of NF-κB was decreased in all EMSA ERITIN treatments. EMSA ERITIN normalize NF-κB production in CD8 T cells (B) The bars are calculation of the number of CD8 T cells expressing positive NF-κB in the spleen cells of mice after EMSA ERITIN treatment. It was shown that all dose treatment of EMSA ERITIN has the same smaller number of the level transcription factor of inflammation (NF-κB) compared with EPO treatment. Data are mean of ± SD values of 3 mice in each group. (N: Normal mice, Rad: Irradiated mice, EPO: Hemapo Epoetin alfa™ treatment, Rad+D1 (low dose): 1.04 mg g⁻¹ BW, Rad+D2 (normal dose): 3.125 mg g⁻¹ BW and Rad + D3 (high dose): 9.175 mg g⁻¹ BW)

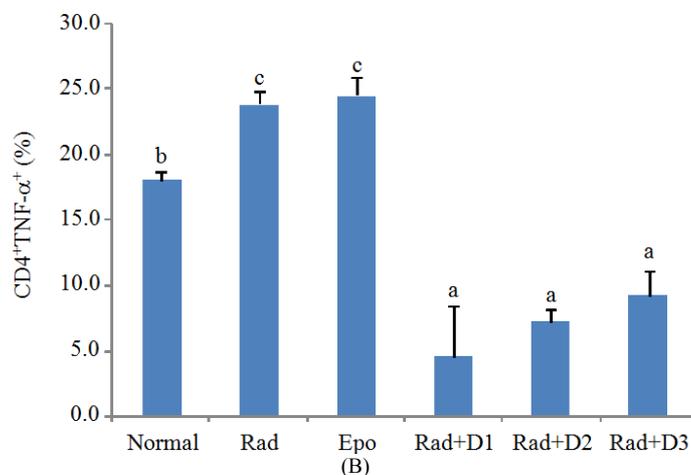
Suppression of Pro-Inflammatory Cytokines

Increasing the activation of NF-κB gene in irradiated mice also increased the production of pro-inflammatory cytokines such as TNF-α. TNF-α expression on CD4 T cells is one marker of pro-inflammatory cytokines. In normal cells, the level of TNF-α generally range between 17.5-18.11% of the population of CD4 T cells (Fig. 3A). TNF-α increased

significantly after mice exposed to radiation compared to normal mice (p<0.05) in Fig. 3 (23.9%). Based on ANOVA (Fig. 3), the relative number of pro-inflammatory cytokines in all dose treatment showed significant difference compared to EPO treatment (p<0.05). All doses of EMSA ERITIN gave the same result with the relative number are 4.6, 7.3 and 9.27%, respectively. EMSA ERITIN is a polyherbal which able to stimulate the expression of TNF-α on CD4 T cells.



(A)



(B)

Fig. 3. The expression of $CD4^+TNF-\alpha^+$ was decreased in irradiated mice after 2 weeks received EMSA ERITIN (A). Flow cytometry analysis showed the expression of $TNF-\alpha$ was decreased in all EMSA ERITIN treatments. (B). The bars are calculation of the number of $CD4$ T cells expressing positive $TNF-\alpha$ on the spleen cells of mice that had been received with EMSA ERITIN. All dose of EMSA ERITIN showed the same result to suppress the level of $TNF-\alpha$. Treatment with EPO cannot suppressed the level of $TNF-\alpha$. Data are mean of \pm SD values of 3 mice in each group. (N: Normal mice, Rad: irradiated mice, EPO: Hemapo Epoetin alfa™ treatment, Rad + D1 (low dose): $1.04 \text{ mg g}^{-1} \text{ BW}$, Rad+D2 (normal dose): $3.125 \text{ g mg}^{-1} \text{ BW}$ and Rad+D3 (high dose): $9.175 \text{ mg g}^{-1} \text{ BW}$)

The level of $IFN-\gamma$ also showed increasing number in mice after radiation (16.4%). But in normal mice the expression of $IFN-\gamma$ range between 1-1.3%

showed in Fig. 4A. Based on ANOVA (Fig. 4B), the relative number of $IFN-\gamma$ cytokines in all dose treatment of EMSA ERITIN showed significant

difference compared to irradiated mice ($p < 0.05$). The low dose ($1.04 \text{ mg g}^{-1} \text{ BW}$), the normal dose ($3.125 \text{ mg g}^{-1} \text{ BW}$) and the highest dose ($2.5 \text{ mg g}^{-1} \text{ BW}$) can suppressed the level of IFN- γ with the relative number are 2.9, 2.7 and 4.82%, respectively. It is indicated

that all dose treatment of EMSA ERITIN can suppressed the level of IFN- γ on lymphocytes mice BALB/c after radiation. We also found that the EPO treatment suppressed the level of IFN γ that are produced by CD4 T cells (2.7%).

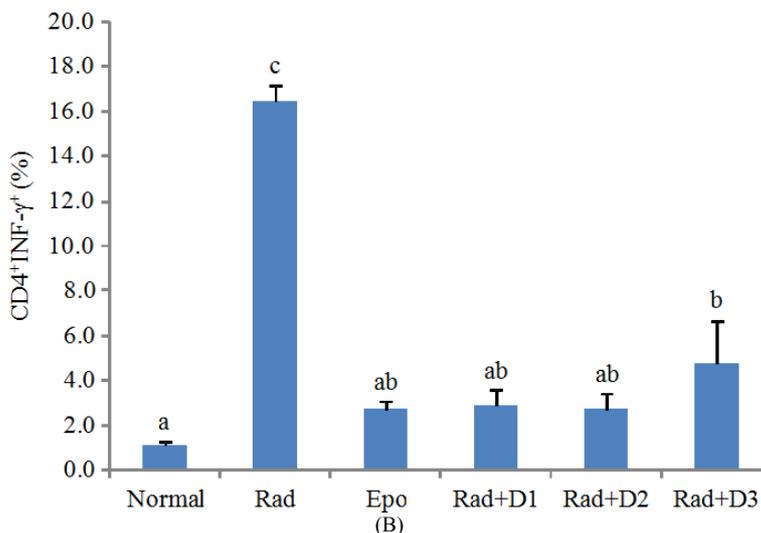
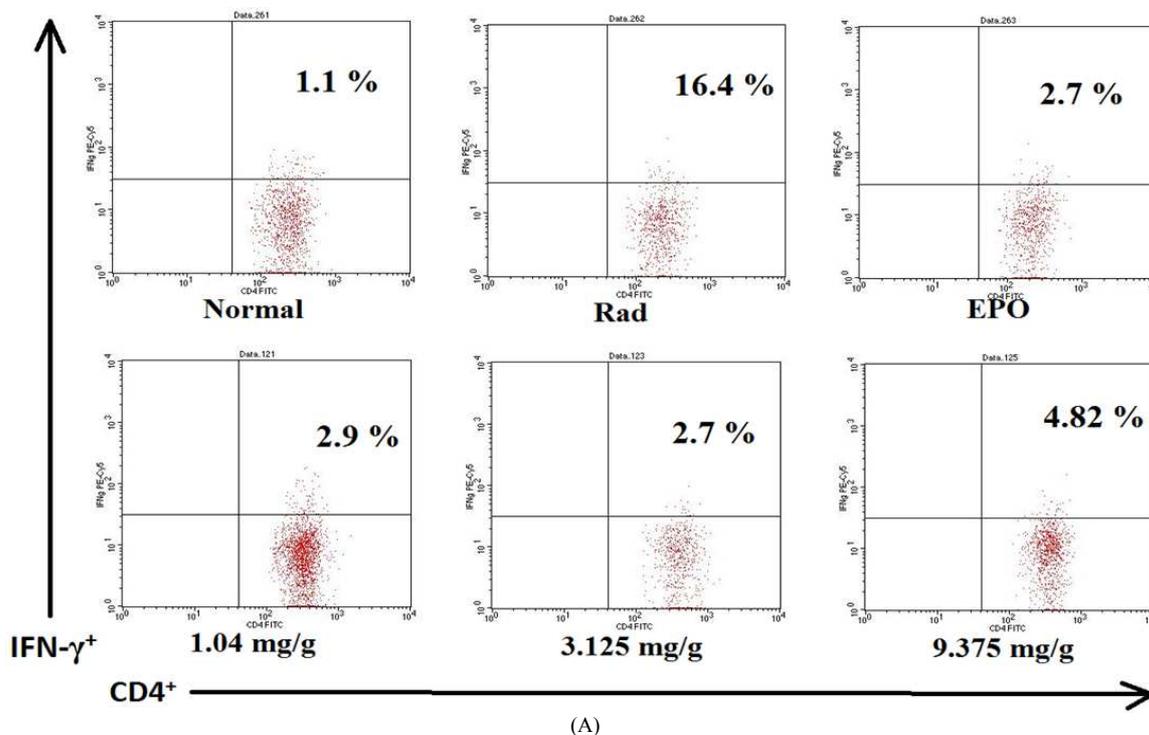


Fig. 4. Proinflammatory cytokines of IFN- γ produced by CD4 T cells after EMSA ERITIN treatment. (A). Flow cytometry analysis showed the expression of IFN- γ was decreased in all EMSA ERITIN treatments and EPO treatment. (B). The bars are calculation of the number of CD4 T cells expressing positive IFN- γ in the spleen cells of mice that had been received with EMSA Eritin. All dose of EMSA ERITIN showed the same result to suppress the level of IFN- γ . Treatment with EPO can suppressed the level of IFN- γ . Data are mean of \pm SD values of 3 mice in each group. (N: Normal mice, Rad: Irradiated mice, EPO: Hemapo Epoetin alfa $^{\text{TM}}$ treatment, Rad+D1 (low dose): $1.04 \text{ mg g}^{-1} \text{ BW}$, Rad+D2 (normal dose): $3.125 \text{ mg g}^{-1} \text{ BW}$ and Rad+D3 (high dose): $9.175 \text{ mg g}^{-1} \text{ BW}$)

EPO or Hemapo Epoetin alfa™ is a glycoprotein hormone that commonly used in human for treatment after radiation. EPO used to stimulate the level of erythrocytes that decreased after radiation also make the immune system balanced. So in this study we compared between EMSA ERITIN and EPO treatment which more effective to balanced the immune system by suppression of transcription factor of inflammation and pro-inflammatory cytokines. Supression of inflammation marker caused by the active compound in EMSA ERITIN. EMSA ERITIN is a product that consist of soy, coconut water and red rice extract. In this study we proved that the administration of EMSA ERITIN in vivo was able to suppressed the expression of TNF- α and IFN- γ from CD4 T cells and also suppressed the NF- κ B that are produced by CD4 and CD8 T cells in mice after radiation.

Discussion

The effects of radiation on the immune cells will reduce the quality of immune surveillance cancer that mainly do by CD8⁺ T cells or Cytotoxic T cells or (CTL), Natural Killer Cell (NK cells), macrophages and Lymphokine Activated Killer cells (LAK). Decreasing of immune function due to radiotherapy causes oxidative stress, an increase in the concentration of reactive oxygen metabolic and free radicals in immune cells that will lead to an increase in production of inflammatory cytokines. Tumor Necrosis Factor Alpha (TNF- α) is the most inflammatory cytokines play a role in the process inflammatory and is used as an indicator for cells undergoing oxidative stress, apoptosis or necrosis. Levels of TNF- α released by macrophages or inflamed lymphocytes increases (Heimdall *et al.*, 2000).

In giving radiation, the most widely established is a radical OH. Free radicals that caused by ionizing radiation can activated the genes of Nuclear Factor-Kappa B (NF- κ B) which causes stimulation of inflammatory cytokines (IL-1, IL-6, IL-8 and TNF- α) (Agroyannis *et al.*, 1992; Heimdall *et al.*, 2000). Lymphocytes is the most radiosensitive cells and first disappeared from circulation, followed by granulocytes. Radiotherapy can causes a decrease in total lymphocyte count and quality. After radiotherapy, a decline in the total number of lymphocytes by 50-60% compared with prior radiotherapy. Besides the T lymphocytes, radiotherapy also caused a decrease in lymphocyte count B and NK cells peripheral blood (Maity *et al.*, 1994).

Free radicals are formed by the ionization of water will cause stress (exhaustion stage) in immunocompetent cells, especially macrophages that cause decrease in the activity of immunological cells that play a role in the Th1 response. Every normal tissue has tolerance doses of radiation that not cause necrosis. Tissue with high mitotic index such as bone marrow, gastrointestinal mucosa, the basal layer of the

epidermis, lymphocytes, thymus, hair follicles, eyepiece, spermatozoa and ovum) are very sensitive to radiation. While tissue with slow mitotic index such as nerve and muscle cells are radioresistant. Dose between 5 Gy-50 Gy caused in a decrease in the rate of DNA synthesis as well as a block in movement cells from G1 to S phase (Maity *et al.*, 1994).

EMSA ERITIN showed anti-inflammatory activity when administred to the lymphocytes in BALB/c mice after radiation. EMSA ERITIN reduced the level of NF- κ B that produced by CD4 and CD8 T cells. NF- κ B is widely used by eukaryotic cells as regulator genes to control cell proliferation and cell survival. Active NF- κ B keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis (Prajapati *et al.*, 2010). The composition of polyherbal EMSA ERITIN showed effective to suppress the transcription factor of inflammation. EMSA ERITIN consists of soy extract, coconut water extract and red rice extract. As we know that soybean contains of many active compound. One of the active compounds is genistein. Genistein is an isoflavone isolated from soybean, is a potent antioxidant with both antiproliferative and anti-inflammatory effects (Wei *et al.*, 2002). Under 6 Gy of TBI dose in BALB/c mice and genistein treatment given orally for seven days showed the process of recovery and survival of hematopoietic system (Zhou and Man-Tian, 2005).

Another study found that soy isoflavonoids may inhibit the diffusion of free radicals and lower the reaction kinetics of free radicals that can stabilize the structure of cell membranes (Arora *et al.*, 2000). EMSA ERITIN also contain of red rice and coconut water extract. Pigment in red rice has potential as a free radical scavenger, including radicals of oxygen and nitrogen derivatives (Hu *et al.*, 2003). Coconut water contains various cytokinins such as kinetin and trans-zeatin. Kinetin in coconut water can reduce the formation of reactive oxygen species. Kinetin was shown to act as a strong antioxidant both under *in vitro* and *in vivo*. Moreover, kinetin riboside also has significant on inhibiting the growth of human heptamoa and mammary carcinoma (Liu *et al.*, 2011). So, under *in vitro* treatment we know that poly-herbal EMSA ERITIN can reduce significantly the level of NF- κ B.

TNF- α is a cytokine secreted during the initial phase of tumor response and it has a strong inflammatory and pro-apoptotic activity (Sologuren *et al.*, 2014; Levy *et al.*, 2013). This cytokine can inhibit tumor angiogenesis working together with radiation treatment (Desai *et al.*, 2013). It is clearly indicated that all dose treatment of EMSA ERITIN can suppressed the level of TNF- α on cell culture of lymphocytes mice BALB/c. Here we proved that in vivo treatment with EMSA ERITIN can reduced the marker of inflammation. It is useful for treatment after radiation to stabilize the

immune responses. *In vivo* results raise the question of whether these observations are relevant to suppressing the inflammation *in vitro*.

IFN- γ is a potent pro-inflammatory cytokine, critical in tumor immunity. This cytokines mainly produced by T helper (Th) 1, Natural Killer (NK) and Natural Killer T (NKT) cells. IFN- γ amount released on the tumor area induces local chemokines production, which helps to recruit more cells of the innate immune system in the tumor. After radiation, IFN- γ produced within the tumor microenvironment, also leads to infiltration of T cells (Sologuren *et al.*, 2014). The key role of IFN- γ in the anti-tumor response is highlighted by the fact that one of the mechanisms that allow tumors to escape from elimination by the immune response is the development of IFN- γ insensitivity. This result indicated that EMSA ERITIN can be use as radioprotective agent from the effect of radiation through suppression of pro-inflammatory cytokines of IFN- γ . Wei *et al.* (2002), mentions that genistein on soy act as an anti-inflammatory when applied to the mice skin for 1 hour before exposure to radiation. Genistein's antioxidant properties and anti-proliferative effects may be responsible for its anti-carcinogenic effect. Its high content in soybeans and relatively high bioavailability favor genistein as a promising candidate for the prevention of human cancers (Wei *et al.*, 2002).

Conclusion

EMSA ERITIN can decrease proinflammatory cytokine TNF- α and IFN- γ , also transcription factor of NF- κ B. The increasing of dose in EMSA ERITIN showed more effective than lower doses. So, EMSA ERITIN is potential herbal plant used as a preventive agent or radio-protective after radiation.

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Author's Contributions

Yuyun Ika Christina: Wrote the manuscript, coordinated the data-analysis and participated in all experiment.

Mansur Ibrahim: Discussed and participated in study design.

Muhaimin Rifa'i: Revised the manuscript and the study design. All authors read and approved the final manuscript.

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.

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