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Polyherbal EMSA ERITIN Promotes Erythroid Lineages and Lymphocyte Migration in Irradiated Mice

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Abstract: Radiotherapy is commonly used to kill malignant cells, but it can significantly deplete hematopoietic and splenic erythroblasts. Radioprotective agents are therefore very important in clinical radiotherapy. We examined the effect of poly-herbal EMSA ERITIN on immunological responses when administered to sublethally irradiated mice with the aim of highlighting promotes erythroid lineages and lymphocytes migration in irradiated mice with the parameter are TER119$^+$CD123$^+$ in bone marrow and SDF-1 in bone marrow and spleen organ. Normal BALB/c mice were sublethally irradiated with 600 rad. EMSA ERITIN was administered orally at different doses: (1.04, 3.125 and 9.375 mg/g body weight) for 15 days. On day 16 erythroid lineages (TER-119$^+$CD123$^+$) were observed in bone marrow and lymphocytes migration by the production of SDF-1 in spleen and bone marrow. Lymphocytes migration was indicated by the production of SDF-1 in spleen and bone marrow using flow cytometry analysis. EMSA ERITIN increased the generation of erythroid lineage cells marked by TER-119$^+$CD123$^+$ and promoted lymphocyte migration by increasing SDF-1 production in bone marrow and spleen. EMSA ERITIN appears to be a powerful medicinal herb with potential as a food supplement to normalize homeostasis and erythropoiesis after radiation.

Keywords: CD123, erythropoiesis, irradiation, polyherbal, SDF-1, TER-119

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1 Introduction

Radiotherapy is commonly used to kill malignant cells. Radiotherapy also used to prepare patients for bone marrow transplant, in a process called total body irradiation (TBI) [1]. Bone marrow suppression is caused by radiation in moderate or high doses during TBI. In addition, radiation also disrupts the hematopoietic system when administered for long periods. The resulting damage includes renewal of hematopoietic stem cell defects (HSC), loss of the ability of self-renewal [2], neutropenia and thrombocytopenia, increasing morbidity and complications, such as infection and bleeding [1,3]. The balance between the total dose of radiotherapy and the tolerable threshold of normal tissue around the cancer cells is very important. The normal tissue should be given protection against radiation injury. Radioprotective agents are therefore very important in clinical radiotherapy [4]. Various studies have shown that exposure to sublethal doses of TBI in mice increased levels of reactive oxygen species (ROS). Increased oxidative stress is associated with DNA damage, p16 expression, and inhibition of HSC function, as well as induction of HSC senescence. Increased oxidative stress is not associated with HSC apoptosis [3,5]. In addition, oxidative stress is also responsible for the loss of self-renewal primer [6].

We report a novel study about the potential of a polyherbal product in the treatment of radiation. EMSA ERITIN is a polyherbal composed of red rice, soybeans, and coconut water extract. EMSA ERITIN is rich in beneficial natural compounds such as genistein, cytokinin, nicotinic acid, pantothenic acid, biotin, riboflavin, folic acid, thiamine, vitamin C, pyridoxine, daidzein, phenolic acids, and anthocyanins. Genistein has been proven to prevent blood cell damage and increase hematopoiesis, while other isoflavones and anthocyanins function as radioprotective agents by acting as antioxidants and by increasing the activity of antioxidant enzymes [7,8]. This study aimed to investigate the effect of EMSA ERITIN to

promote erythroid lineages and lymphocyte migration in irradiated mice, assessed using the parameters $TER119^{+}CD123^{+}$ in bone marrow and SDF-1 in bone marrow and spleen organ.

2 Methods

This research was conducted from January 2015 to April 2015 in the Laboratory of Animal Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya.

2.1 Mice

7-8 week old normal BALB/c mice were used and maintained in a pathogen-free facility. Total number of mice was 24 (4 mice in each group) each weighing at least 25 g. The experimental protocol was approved by the Research Ethics Committee (Animal Care and Use Committee), University of Brawijaya No. 255-KEP-UB.

2.2 Total Body Irradiation

Total Body Irradiation (TBI) was performed using Co-60 Teletherapy NPICEM with a sublethal dose of 6 Gy. The dose was measured according to IAEA protocols from the middle of the field for 50 × 50 mm^2 at 80 cm source to surface distance (SSD) for machine GWXJ80 (NPIC, China) installed at Dr. Saiful Anwar Hospital, Malang.

2.3 EMSA ERITIN and Hemapo Epoetin alfaTM treatment

This study was divided into 6 groups of treatment include negative control (normal mice), positive control (irradiated mice), EPO (Hemapo Epoetin-α™ at a dose of 0.21 mg/g body weight), low dose of EMSA ERITIN (1.04 mg/g body weight), normal dose of EMSA Eritin (3.125 mg/g body weight), and high dose of EMSA Eritin (9.375 mg/g body weight). Determination of EMSA ERITIN doses for in vivo experiments were based on human consumption (60 kg body weight consuming as much as 15 g of EMSA ERITIN). EPO or Erythropoietin-α™ was used in this study as a comparison with EMSA ERITIN treatment. EMSA ERITIN was administered to mice orally starting in 24 hours after radiation exposure, for two weeks. EPO was injected intraperitoneally twice a week.

2.4 Lymphocytes Isolation and Flow Cytometry Analysis

The mice were sacrificed after two weeks of treatment. Spleen was isolated and crushed clockwise with syringe base. Lymphocyte homogenates were transferred into new propylene tubes and 10 ml PBS was added. Bone marrow was isolated by flushing out the femur and tibia of mice into 50 mL Falcon tubes by inserting a 26-gauge needle, attached to a 20 mL syringe filled with PBS at the knee side of both types of bone. PBS was passed through the bone until the color of the bone turned from red to white indicating that all the marrow had been expelled. The filtrate was centrifuged at 2500 rpm 4°C for 10 minutes.

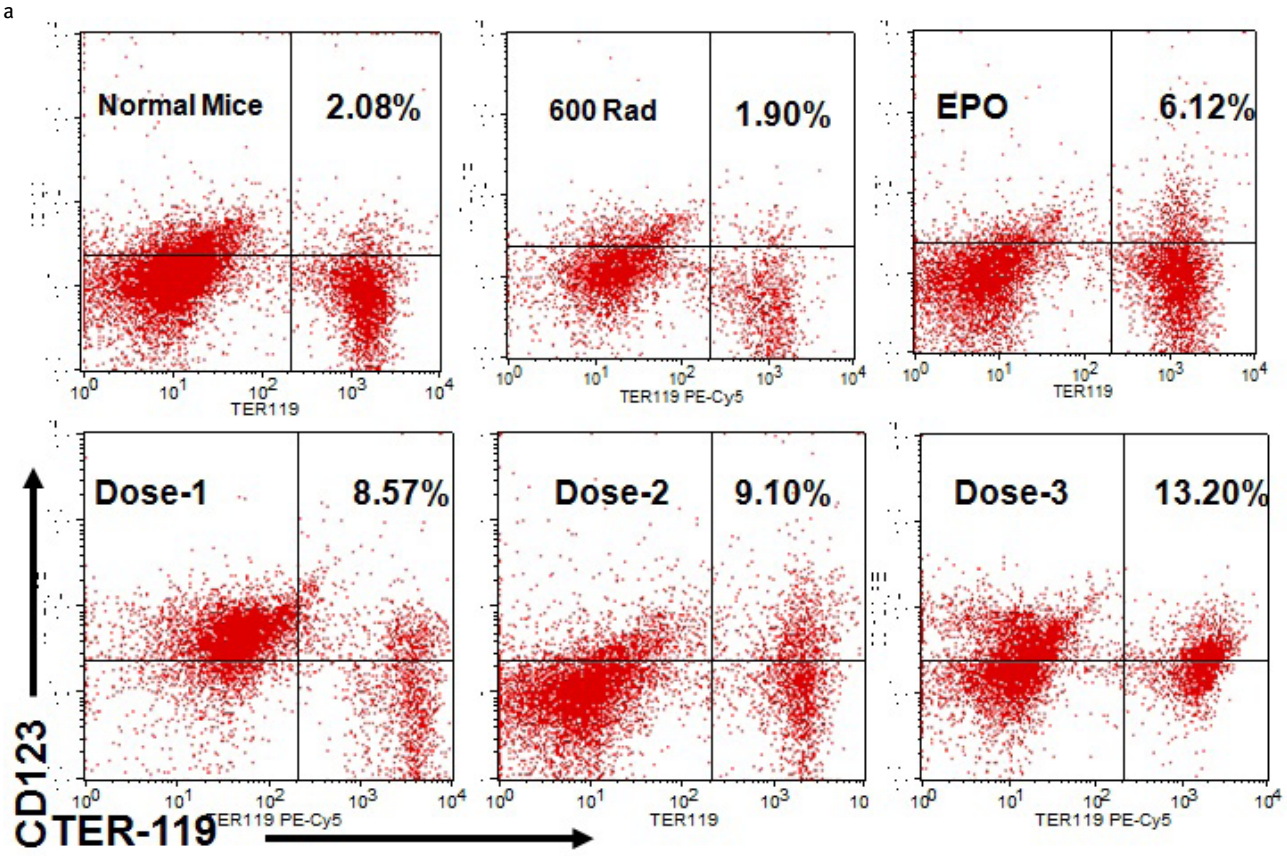
The supernatant was discarded, washed and then centrifuged again to obtain a pellet of bone marrow cells, which was incubated with monoclonal antibodies: phycoerythrin (PE)/Cy5 anti-mouse TER-119/Erythroid Cells (clone LotB169021), and PE anti-mouse CD123 (clone 5B11), for 15 minutes. Antibodies for intracellular staining were PE-Cy5 conjugated anti-mouse CXCL12 (SDF-1α) (clone LotQXB0213092). For intracellular staining, 50 µL cytofix-cytosperm was added to the pellet and incubated for 20 minutes at 4°C. Then 500 µL washperm was added and centrifuged at 2500 rpm, at 4°C, for 5 min. The pellet was resuspended with 50 µL of antibodies in sterile PBS. Next, the pellet was resuspended in 500 µL PBS and accessed via a BD FACS Calibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The data was then processed using the BD Cell Quest Pro™ software.

2.5 Statistic Analysis

Data were analyzed using SPPS 16.0 for Windows. One way ANOVA test was used to assess the statistical difference between the N control group, positive control, radiation group and the different EMSA ERITIN treatment groups. $p < 0.05$ was defined as statistically significant. Significant results were analyzed with Tukey Test.

3 Results

Erythroid-lineage expressing CD123 molecules ($TER119^{+}CD123^{+}$) showed depletion in the number of $TER119^{+}CD123^{+}$ cells in the bone marrow of sublethally irradiated (600 rad) mice compared to normal mice (2.08% vs. 1.90%) (Fig. 1). The number of $TER119^{+}CD123^{+}$ cells in irradiated mice treated with EMSA ERITIN increased from low dose to high dose respectively (8.57%, 9.10%,



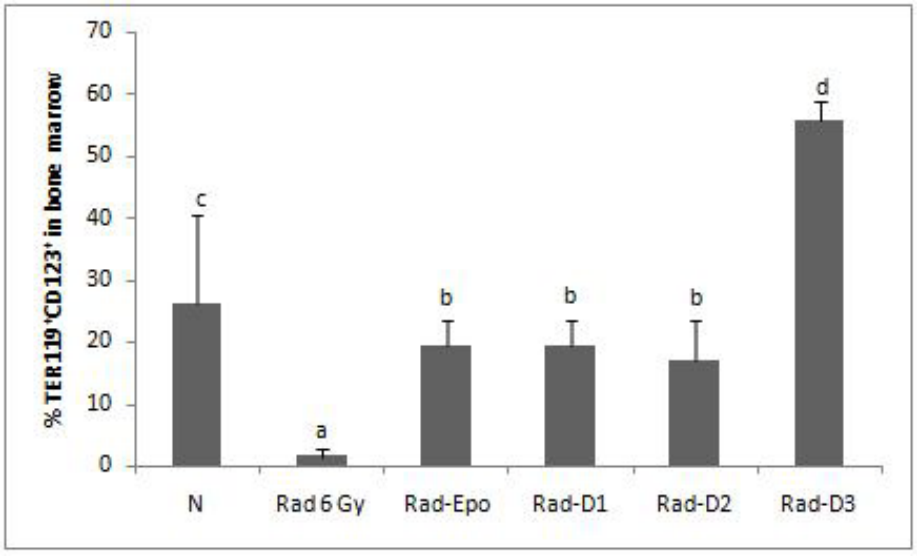


Figure 1. EMSA ERITIN accelerates growth and differentiation of erythroid lineages. (a). The relative number of TER-119^{+}CD123^{+} cells in bone marrow of mice after two weeks of treatment. (b). On day 15, mice were terminated and analyzed using flow cytometry and tabulated into Microsoft Excel. Data are mean ± SD values of 4 mice in each group. N: normal mice (un-irradiated mice); Rad 6 Gy: Positive control (irradiated mice with dose of 600 rad); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW), D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW).

13.20%) compared with Hemapo Epoetin alfa™ treatment (6.12%). In this experiment, we used Hemapo Epoetin alfa™ as a comparison with EMSA ERITIN to promote the erythropoiesis in irradiated mice. As far of our knowledge, erythropoietin (EPO) is the most famous drug to drive hematopoietic stem cell to differentiate rapidly and become mature blood cells. Based on ANOVA (Fig. 1), the highest dose of EMSA ERITIN showed more effective in promoting erythropoiesis than the other doses.

As we know, SDF-1 is strongly chemotactic for lymphocytes. During embryogenesis, it directs the migration of hematopoietic cells from foetal liver to bone marrow and the formation of large blood vessels. EMSA ERITIN promoted lymphocytes migration by increasing SDF-1 production in the spleen. It can be seen in figure 2 that the number of SDF-1 in spleen was decreased significantly ($p < 0.05$) in irradiated mice compared to the normal mice (1.91% vs. 6.84%) (Fig. 2). After administration with EMSA ERITIN, the number of SDF-1 increased from the normal to high dose respectively (12.63%, 20.78%), see Figure 2. The low dose did not significantly increase the number of SDF-1 (4.45 %). The high dose appeared more effective than the normal dose in increasing the production of SDF-1 in irradiated mice. Treatment with Hemapo Epoetin alfa™ did not significantly increase the production of SDF-1 in irradiated mice 7.45%. It was proved that EMSA ERITIN in irradiated mice can increase the production of SDF-1 and promote lymphocyte migration in the spleen.

The expression of SDF-1 in bone marrow was analyzed with flow cytometry assay. Based on the result (Figure 3), the level of SDF-1 in irradiated mice was decreased as much as 4.10% compared to normal mice (7.49%), indicating that radiation blocks B cell migration. Treatment with EMSA ERITIN for two weeks promotes the production of pre-B-cell growth-stimulating factor (SDF-1) in bone marrow. EMSA ERITIN increases B cell expressing SDF-1 molecules ($SDF\text{-}1^{+}$) significantly compared to EPO treatment. SDF-1 in irradiated mice treated with EMSA ERITIN increased from low to high dose respectively (5.04%, 9.09%, 34.85%) compared with Hemapo Epoetin alfa™ treatment (5.36%). It was indicated that EMSA ERITIN more effective than Hemapo Epoetin alfa™ treatment in promoting B cell migration by increasing SDF-1. The high dose of EMSA ERITIN appeared to be more effective than other doses.

4 Discussion

Radiation causes DNA damage and then performs repair mechanisms. One of the most important effects of radiation is induced DNA strand breaks (DSB). P-53 is a tumor suppressor protein that is needed to induce apoptosis and the stress-induced senescence mechanism. Despite the avoidance of HSC apoptosis induced by high lethal dose of radiation, many HSC lose their ability to proliferate and undergo senescence. Loss of the ability of HSC proliferation is a critical stage of the long-term survival of organisms exposed to radiation. Total body irradiation dose of 0.5 Gy was able to suppress the formation of blood cells, so that the number of blood cells will decrease. In normal conditions, the relative populations of granulocytes are around 70-72% of total leukocytes. Meanwhile, the lymphocyte population are 25-35% of the number of leukocytes. Induction of radiation can cause a temporary increase in granulocytes (a few days) which immediately drops to very low levels for a few weeks, returning to normal after a few months [9].

CD123 is the IL-3 receptor α chain (IL-3Rα) that associates with β subunit (CD131/common β-chain) to form the functional IL-3 receptor complex. CD123 is a member of the cytokine receptor superfamily (I3) [10]. CD123 is expressed on a subset of peripheral blood dendritic cells, myeloid precursor, basophils, mast cells, macrophages, megakaryocytes, and also lymphocytes. CD123 or IL-3Rα plays a significant role in hematopoietic progenitor cell growth and differentiation from multiple hematopoietic lineages. IL-3 not only stimulates the proliferation of hematopoietic progenitor cells but also helps to maintain cellular viability via the suppression of apoptosis [11]. In our study, we observed the expression of CD123 in erythrocytes that expressed TER119 antigen. TER119 is commonly used as a lineage marker of erythroid cells from early proerythroblast to mature erythrocyte stages in adult blood, spleen, and bone marrow. TER119 has been reported to react with mature erythrocytes; 20-25 % of bone marrow cells and 2-3% of spleen cells were reactive with TER119 in adult mice [12]. The TER-119 antigen is not expressed by cells of earlier erythroid development at BFU-e (blast-forming unit erythroid) stage or CFU-e (colony-forming unit erythroid) stage [13]. From the obtained result, EMSA ERITIN can accelerate growth and differentiation of erythroid lineages by increasing $TER119^{+}CD123^{+}$ production in irradiated mice. EMSA ERITIN increased the level of CD123 that plays in important role for hematopoietic lineages.

SDF-1 is expressed in several organs such as the lungs, liver, skin, and bone marrow [14]. There are two main forms of SDF-1, namely SDF1-α and SDF1-β. These are expressed in the highest amount in the liver, pancreas, and spleen [15]. Stromal cell-derived factor-1 (SDF-1) belongs to a group α-chemokines which bind to the transmembrane protein receptor CXCR4. SDF1 expressed in the spleen, liver, lung,

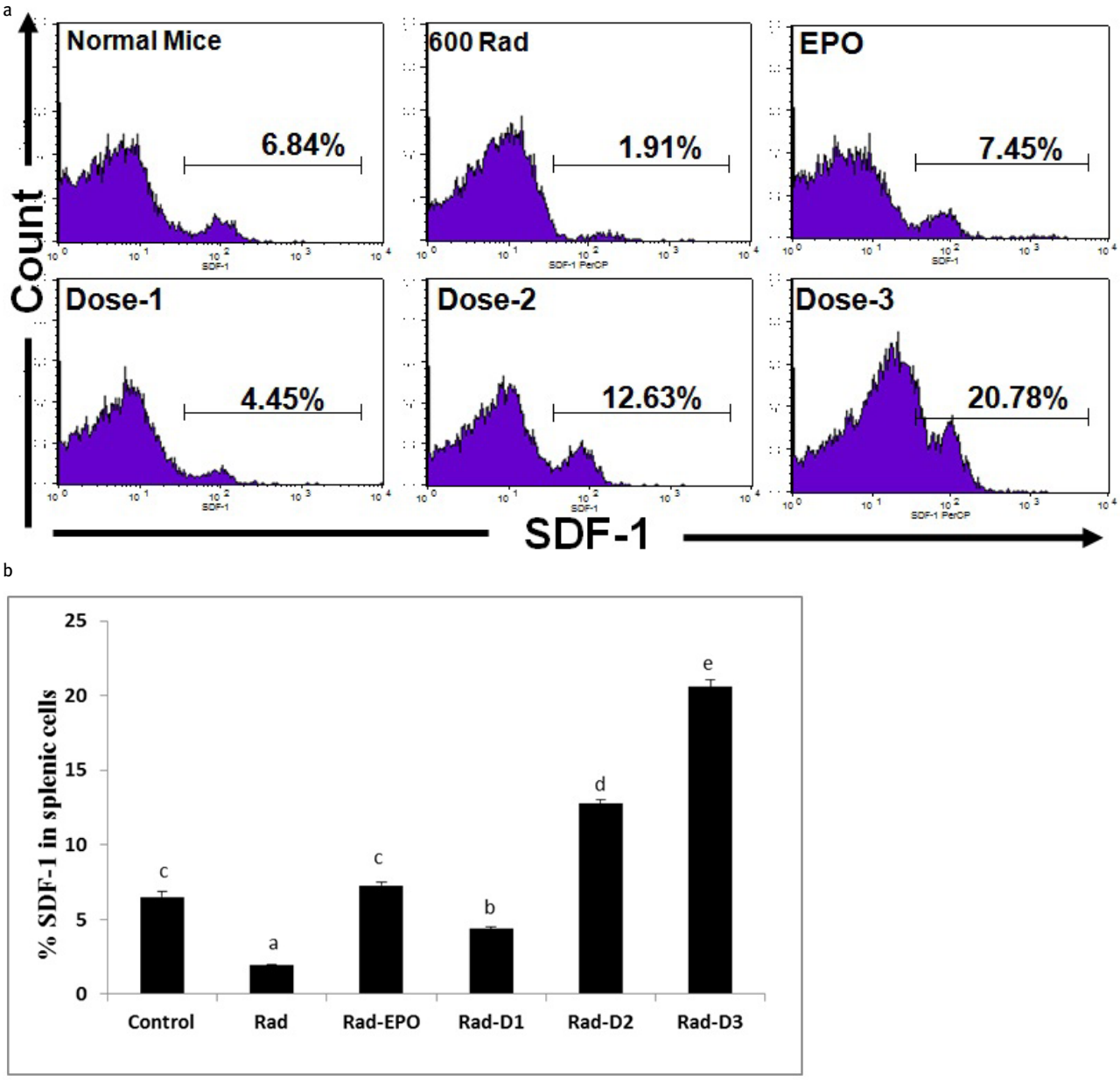


Figure 2. EMSA ERITIN promotes lymphocytes migration by increasing SDF-1 production in spleen. (a). The relative number of SDF-1$^+$ cells in spleen of mice after two weeks of treatment. (b). On day 15, mice were terminated and analyzed using flow cytometry and tabulated into Microsoft Excel. Data are mean ± SD values of 4 mice in each group. N: normal mice (un-irradiated mice); Rad 6 Gy: Positive control (irradiated mice with dose of 600 rad); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW), D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW).

thymus, and brain [16], stromal cells [17], osteoblasts [18], immune cells, blood vessels, and heart. The main physiological function of the interaction SDF1/CXCR4 is set homing, storage, and defense of hematopoietic stem cells and progenitor cells that are still primitive [19]. From the obtained result, we found that EMSA ERITIN promote the production of pre-B-cell growth-stimulating factor, SDF-1, in bone marrow. The expression of SDF-1 after treatment with EMSA ERITIN is higher than after treatment with EPO in irradiated mice.

EMSA ERITIN normalizes homeostasis and erythropoiesis after radiation because of its combination of three herbal ingredients i.e soy, coconut water and red rice extract. EMSA ERITIN contains beneficial natural compounds such as genistein, cytokinin, nicotinic acid, pantothenic acid, biotin, riboflavin, folic acid, thiamine,

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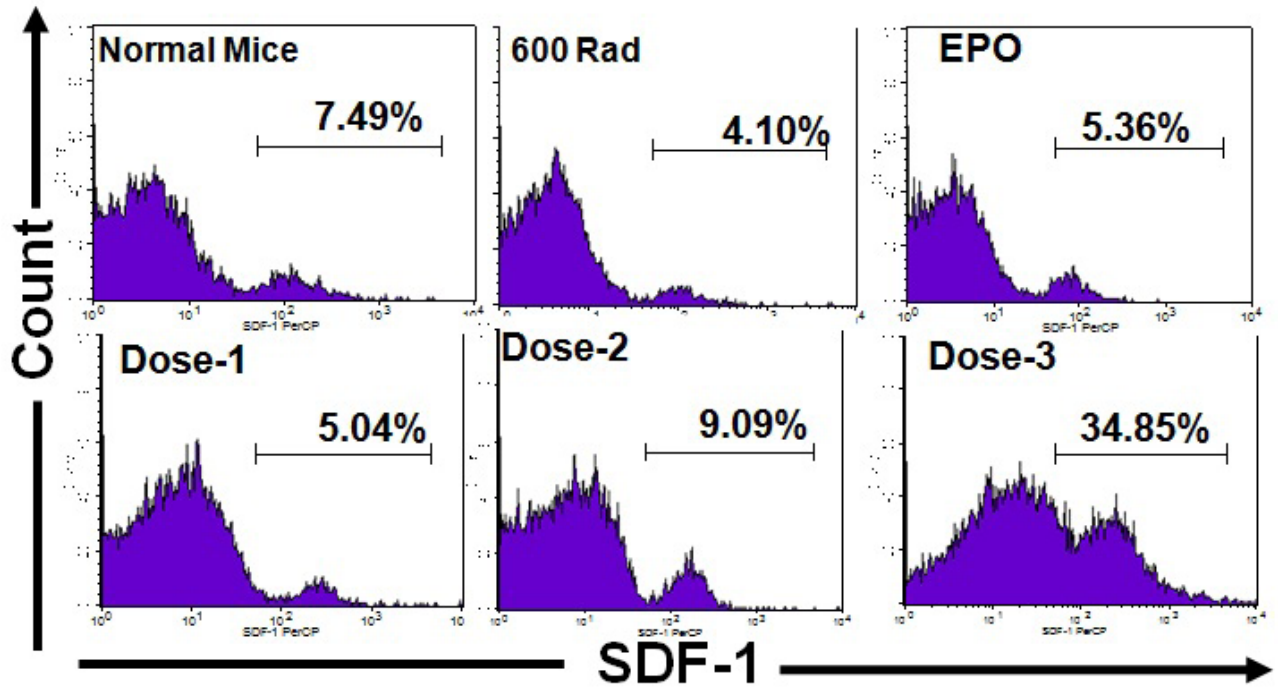

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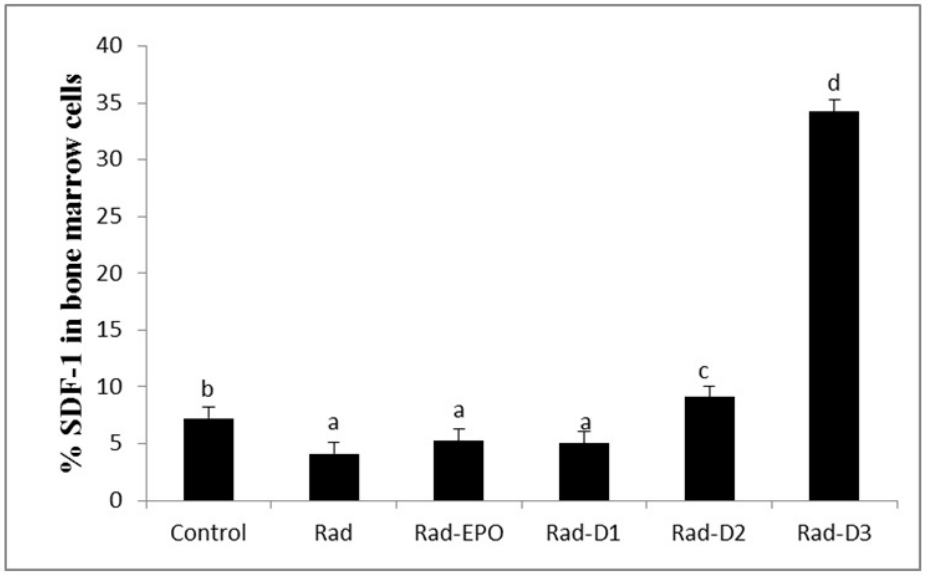

Figure 3. EMSA ERITIN promotes the production of pre-B-cell growth-stimulating factor, SDF1 in bone marrow. (a). The relative number SDF-1^{+} cells in bone marrow of mice after two weeks of treatment. (b). On day 15, mice were terminated and analyzed using flow cytometry and tabulated into Microsoft Excel. Data are mean ± SD values of 4 mice in each group. N: normal mice (un-irradiated mice); Rad 6 Gy: Positive control (irradiated mice with dose of 600 rad); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW), D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW).

vitamin C, pyridoxine, daidzein, phenolic acids, and anthocyanins. Isoflavones are suitable candidates as radiation protective agent because isoflavones are effective antioxidants which help to eliminate free radicals and increase the activity of antioxidant enzymes [7]. Several mechanisms such as the prevention of damage, free radical scavenging, DNA and membrane repair, renewing cells and stimulating the activity of immune cells are a few points to consider in explaining the radio-protective effect [20]. Free radicals induced by radiation cause damage to DNA and

other cellular macromolecules. Molecules that function as free radical scavenger can prevent radiation damage.

Genistein in soy is a powerful antioxidant and is able to activate the antioxidant system in order to reduce levels of free radicals from lipid peroxidation products, and stabilize the structure of the cell membrane [21,22]. Wei et al. (2002) mentions that genistein provides protection against non-ionizing radiation of ultraviolet-B and against reactive oxygen species (ROS), indirectly acting as an anti-inflammatory when genistein was applied to the skin of mice 1 hour before exposure to radiation. Arora et al. (2000) found that soy isoflavonoids may inhibit the diffusion of free radicals and lower the reaction kinetics of free radicals thus stabilizing the structure of cell membranes. Damage to normal tissue can be controlled using radioprotective agents. Radio-protective agents developed from natural plant products could potentially counteract the free radicals caused by radiation, and function to prevent and treat some diseases. One radio-protector known is the use of anthocyanins in red rice pigment [24].

Extracts of red rice and black rice is proven to reduce oxidation of the indicator and have a total antioxidant activity known through increased antioxidant enzymes such as superoxide dismutase (SOD) and catalase [25]. Kinetin contained in coconut water can reduce the formation of reactive oxygen species that initiate neurotoxic processes in Alzheimer's disease and Parkinson's disease. Kinetin acts as an anti-aging agent, although the mechanism of kinetin's effect is unclear. It has been shown in astrocyte cultures that the mechanism of kinetin in antioxidant activity is through interaction with rRNAse and tRNAse to stimulate the synthesis of SOD and GSH-Px. Kinetin can also slow the aging of endothelial cells by increasing their capacity for cell proliferation and cell metabolism. Additionally, kinetin is able to stimulate nuclear chromatin in cultured human fibroblasts [26]. This study indicates EMSA ERITIN that containing soy, coconut water and red rice extract could be used as a radioprotective agent. EMSA ERITIN is an alternative treatment to stabilize the immune system after radiation.

5 Conclusions

EMSA ERITIN administered to irradiated mice increased the generation of erythroid lineage cells marked by TER-119 in bone marrow. We conclude that EMSA ERITIN is a powerful medicinal herb with potential as a food supplement to normalize homeostasis and erythropoiesis after radiation treatment.

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Conflict of interest: Authors declare nothing to disclose.

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