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Research Article

Alterations in Blood Profiles and Spleen Tissue Following Purple Sweet Potato Anthocyanin Extract Treatment in Restraint-Stressed Mice

Nia Kurnianingsih *, Dian Artamevia, Dian Hasanah, Novita Titis Harbiyanti, Retty Ratnawati

Department of Physiology, Faculty of Medicine, Universitas Brawijaya, 65145, Indonesia

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**Corresponding author:*
E-mail: nia_fkub@ub.ac.id

ABSTRACT

Stress is associated with broad changes in blood and immune cell dysfunction, which are correlated with the spleen as a secondary lymphoid organ. Anthocyanin, a plant bioactive compound from purple sweet potatoes, has previously been reported to have a beneficial effect on stress response behavior. However, the limited studies evaluated anthocyanin's effect on blood profiles and spleen tissue under stress. Therefore, this study aimed to explore the effect of total anthocyanin extract (ANC) from purple sweet potatoes (PSP) on the blood profiles and spleen tissue of restraint stressed mice. A total of 20 male adult BALB/c mice were divided into control (CTRL), stress (STR), stress + ANC 10 mg/kgBW (STR+ANC10), stress + ANC 20 mg/kgBW (STR+ANC20), and stress + ANC 40 mg/kgBW (STR+ANC10). Restraint stress was applied two hours/day for 14 days. An automatic blood analyzer was used for blood profiling. Immunoblotting of caspase-3 and hematoxylin-eosin-stained histological slides were observed for spleen tissue analysis. Combination of STR and ANC treatment reduced the erythrocyte count, hemoglobin level, and leucocyte count. The ANC-treated groups had a higher percentage of lymphocytes and a lower percentage of granulocytes. The expression of caspase-3 declined among ANC-treated groups. Spleen extramedullary erythropoiesis, vacuolization, and white pulp involution were demonstrated in stress and ANC-treated animals. In summary, restraint stress modified the blood cell profiles and spleen tissue in the mice model. Further research is necessary to evaluate the mechanism of ANC during stress on blood cells and splenic tissue repair enhancement.

Keywords: Anthocyanin, Blood, Caspase, Psychological stress, Purple potatoes, Spleen

Introduction

Stress is defined as nonspecific symptoms caused by stressful stimuli from external environment. Homeostatic response to stress is directed by the brain, and the adrenal is the most vital organ for stress regulation. Stress regulation involves orchestra collaboration between the autonomic nervous system and the hypothalamic-pituitary-adrenocortical (HPA) axis. However, prolonged stress disturbs these homeostatic stress responses, leading to homeostatic failure and diseases in the broad organs of the whole body [1–3].

The spleen, as an important lymphoid organ, has physiological roles in hematology and immune function regulation [4]. Stress can adversely affect the spleen function through the brain-spleen axis pathway [5]. Previous studies determined the effect of stress on hematology disorder as well as immune system suppression [6,7]. A broad range of hematological disorders including alteration in hemoglobin levels, red and white blood cell count were reported in stress-associated depression [8,9]. Immunological dysfunctions, such as functional immune defects on T regulatory cells, are also reported in stress-induced depression disorder [10,11].

Immune system depression during chronic stress is strongly suggested because of spleen apoptosis acceleration. Earlier studies demonstra-

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ted that stress exposure by food deprivation induced higher levels of serum corticosterone, followed by an increase in splenocyte apoptosis [12]. Inhibition of unfolded protein response and increased expression of phosphorylated-STAT3 in the endoplasmic reticulum was revealed as a potential pathway of splenocyte apoptosis during chronic stress exposure [13]. Previous studies showed chronic immobilization stress changes spleen structural tissue and blood leucocyte distribution [14,15]. Similarly, atrophy of the spleen's white pulp tissue is displayed in chronic corticosterone administration [16]. These findings collectively suggest that stress can induce spleen tissue apoptosis, morphology, and function disruption, potentially through various molecular pathways and stressors.

Apoptosis is programmed cell death initiated by an increase in mitochondrial outer membrane permeability. Sequentially, caspase cleavage and activation execute the pathway for cell death [17]. Chronic restraint stress increased the expression of cleavage caspase-3 and pro-caspase 8 in mice. Previous reports showed that lycopene as a plant metabolite compound alleviates the expression of this caspase in the spleen and relieves the systemic immunosuppression state in chronic restraint stress mice. However, the circulating blood profiles were not determined in that previous study [18].

Plant bioactive compounds such as anthocyanin from PSP demonstrated anti-inflammatory effects among restraint-stressed mice [19]. The advantages of anthocyanin from purple sweet potatoes reported on behavioral effects in the offspring of a prenatally restrained stress mother [20]. Anthocyanin is a red-blue plant metabolite active compound with anti-inflammatory and antioxidant properties [21]. Anthocyanin from PSP was computationally predicted as having antidepressant activity [22]. However, conflicting results stated the effect in different research fields. Anthocyanin reduced apoptosis in chemical toxicant-induced mice model by suppressing the caspase levels in testicular tissue. Conversely, anthocyanin induces apoptosis by stimulating the caspase level in the cancer model [23,24]. To date, there is a lack of exploration on anthocyanin roles in blood profiles and spleen tissue changes, particularly in caspase biomarkers in restraint stress mice model. Thus, this study aimed to analyze the effect of ANC from PSP on the blood profiles and spleen tissue of restraint-stressed mice.

Material and Methods

Plant materials

This study used purple sweet potatoes (Ipomoea batatas (L) Lam), a variety of Antin-3. The Antin-3 is an official Indonesian variety developed and planted by Balai Penelitian Tanaman Aneka Kacang dan Umbi (BALITKABI; Research Centre for Legume and Tuber Plants), East Java, Indonesia. The tuber was harvested at 4 months of planting age [20]. The geographical coordinates of the BALITKABI farm of purple sweet potato cultivation are 8.0472°S and 112.6255°E.

Total anthocyanin extraction

Fresh tuber roots (50 g) were ground and macerated in 100 ml methanol-HCL 1% (pH 4.5) at 25°C for 24 hours. The homogenates were filtrated using Whatman filter paper 0.45 µm. The filtrate evaporated at 50-60°C using a rotary evaporator (RE-25C 1L series). The extract then was kept at 4°C until further application [25].

Animals and treatment

All experimental procedures were previously approved by the Research Ethics Committee of Universitas Brawijaya, Indonesia (No:029-KEP-UB-2022). Animals were obtained from PT. Indoanilab (Bogor, Indonesia) then undergoes 7 days acclimatization period. Healthy mice were determined for inclusion by a veterinarian. A total of twenty adult males of BALB/c mice at 8 months of age were housed at ambient room temperature (22 ± 3°C), a room light: dark cycle was 12:12 hours, and standard laboratory food and water were provided available ad libitum [17]. Animals were randomly assigned into groups of control (CTRL), stress (STR); stress+ total anthocyanin extract 10 mg/kg BW (STR+ANC10); stress+ total anthocyanin extract 20 mg/kg BW (STR+ANC20) and stress+ total anthocyanin extract 40 mg/kg BW (STR+ANC40). The extract dose was selected based on our previous behavioral study. The extract was administered via oral route at once per day until 14 days in parallel with restraint stress exposure. Animal monitoring for physical illness and disability during the study was performed by a veterinarian to assess the exclusion criteria [19,26].

Stress application

Stress was applied as restraint stress. Mice were individually immobilized in a ventilated acrylic tube with a fitted diameter fitted to the body of the animals. The restraint stress procedure was performed for 2 hours each day for a duration of 14 days [27].

Blood collection and analysis

Blood was collected under general anesthesia by peritoneal injection of ketamine 80 mg/kgBW and xylazine 10 mg/kg BW [28]. Cardiac puncture was performed for blood collection [29]. The blood was released into an anticoagulant tube for hematological analysis using validated ABX Micros 60 analyzers (Horiba-ABX, Montpellier, France). The analysis involved erythrocyte count, hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red blood cell volume distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), percentage leucocyte count, and percentage of lymphocytes, granulocytes, and monocytes [30].

Spleen protein extraction and immunoblotting

Half portion of the spleen tissue was washed in phosphate buffer saline solution. Protein extraction and immunoblotting were performed as modifications of the previous method. Spleen tissue was ground in liquid nitrogen with mortar and pestle and then diluted in RIPA lysis and extraction buffer (G-Bioscience, no. catalogue:786-489). The homogenates were centrifuged for 15 minutes at 12.000 rpm to get the supernatant. The supernatant protein was measured using Bradford Assay. An equal concentration of extracted protein was diluted in reducing sample buffer (1:1) and then denatured at 95°C for 5 minutes. The solution was loaded to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred for 2 hours to nitrocellulose membrane using Wet Blotting System (Bio-Rad Laboratories. Inc). Blocking was performed using non-fat dry milk 10% (Rockland, no catalogue: B501-0500). The primary antibody (Caspase-3 Monoclonal Antibody, BIOSS, USA, no catalogue: bsm-33284M) was incubated overnight. A secondary antibody was applied for 1 hour. The protein band was visualized using chemiluminescence system. The blot band results were quantified by ImageJ software, and the relative expression levels were normalized to ß-actin [18].

Spleen histological examination

A half portion of spleen tissues were fixated in 4% of paraformaldehyde. The tissues were then processed for dehydration, clearing, infiltrating, embedding, and sectioning. Hematoxylin-eosin was used for tissue staining. The histological tissue sections were then carefully investigated under a light microscope (Olympus BX-53) at 100x magnification. The tissue observation was performed as a qualitative description [16], [23].

Statistical analysis

The data were statistically analyzed using GraphPad Prism 9 software. One-way ANOVA was used to determine the mean difference between groups. The significance threshold was set at p-value $p < 0.05$ [17].

Results and Discussion

The effect of restraint stress on blood profiles

The Hb level in STR group (12.30 ± 0.62) slightly declined by 2.5% compared to the CTRL group (12.63 ± 0.44) (Figure 1A). This study observed a tendency of erythrocyte count reduction in the STR group (6.54 × 106/mm3 ± 0.59) compared to the CTRL group (7.04 × 106/mm3 ± 0.45) (Figure 1B). The level of MCH in the STR group was not significantly different compared to the CTRL group (18.37 ± 0.37 pg vs18.65 ± 0.95 pg) (Figure 1C). Similarly, the level of MCV in STR group was not significantly different compared to CTRL group (56.25 ± 4.03 µm3 vs 55.75 ± 0.95 µm3) (Figure 1D). Stress had no significant different effect on MCHC (Figure 1E). The percentage of RDW in STR group was lower than CTRL (18.47 ± 0.38% vs 19.78 ± 1.22 % (Figure 1F).

Stress reduced leucocyte count (Figure 2A) with a higher percentage of lymphocytes, p = 0.003 (Figure 2B). In contrast to lymphocyte percentage, granulocyte percentage decreased in the STR group compared to control (15.33 ± 2.51 for STR vs 17.50 ± 3.31 for CTRL) (Figure 2C). The percentage of monocytes declined from 9.50 ± 1.73 in the CTRL group to 7.00 ± 2.00 in the STR group (Figure 2D). The platelet count and mean platelet volume (MPV) were not significant among CTRL and STR groups (Figure 2E, 2F)

The duration of stress exposure supposedly determined stress effect on hematological profiles.

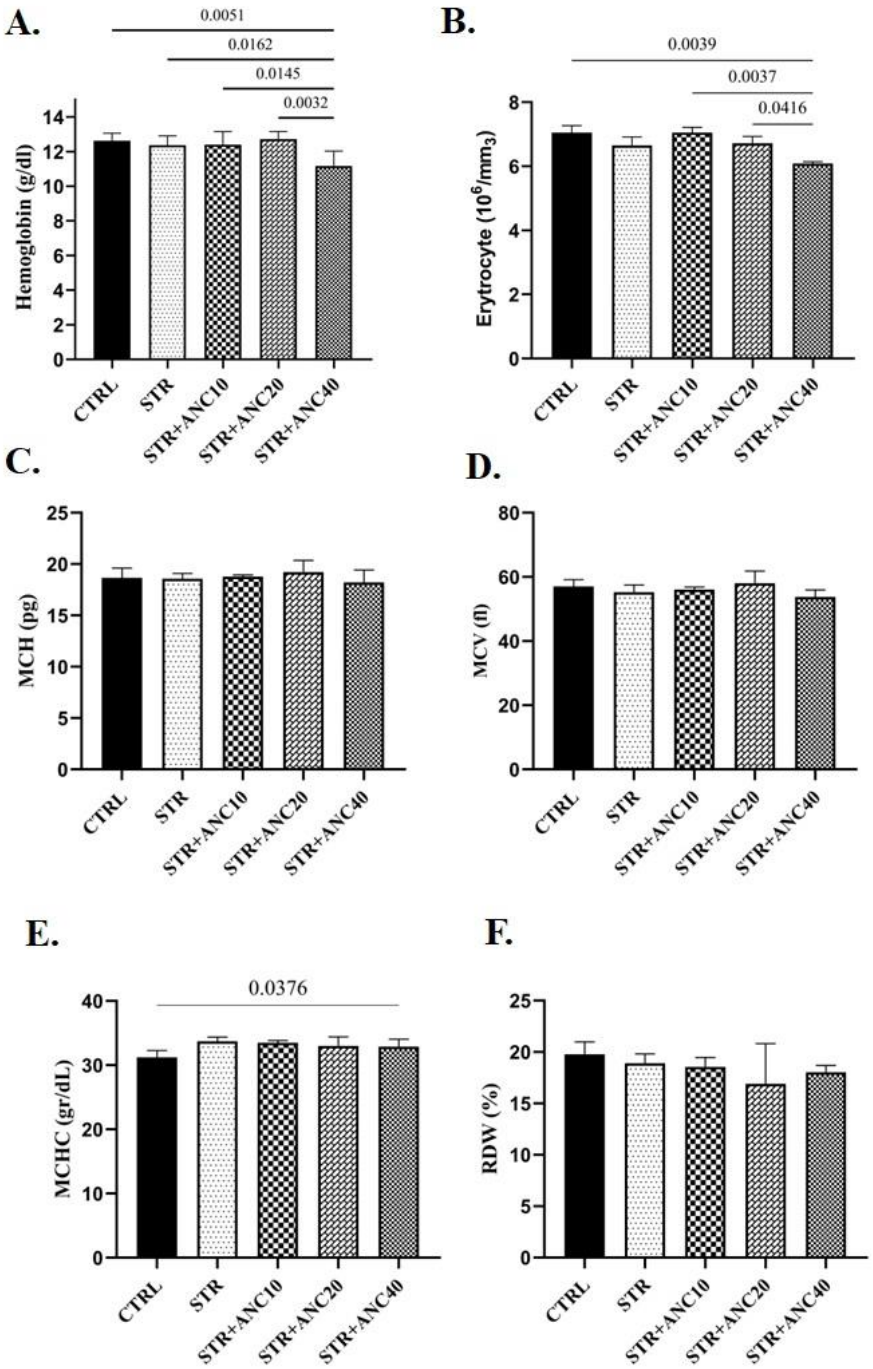

Figure 1. Blood profiles among mice groups. Decrease in hemoglobin level and erythrocyte counts in the STR+ANC40 group [A,B]. There was no significant difference between MCV, MCH and RDW levels among groups [C,D,F] but a difference was found for the MCHC level [E]. Results are shown as mean ± SD. Differences significant at $p < 0.05$ are noted above each bar of the groups.

Short acute stress has previously been reported to lower iron storage in the bone marrow and thus decrease the level of hemoglobin and erythrocytes in rats [31]. In contrast, chronic stress induced up-regulation of erythropoiesis through glucocorticoid stimulation on bone marrow proliferation [30]. Another study showed that unpredictable chronic stress induced normochromic and normocytic in mice [6]. A reduction of red blood cells was seen after 0.5 days of social defeat stress exposure, followed by longer stress repetition for 24 hours, which caused no significant difference in

red blood cell count. The activation of ß-adrenergic during social defeat stress is suggested to play a crucial role in reducing red blood cells [32].

Repeated social stress mobilized the myelopoiesis from the bone marrow into the spleen through sympathetic activation [32]. Stress also has biphasic effects on immune response depending on the duration of exposure. Acute stress enhances the immune system in response to sympathetic activity by epinephrine and adrenaline hormones that distribute the number of immune cells throughout the body. The release of glucocorticoids during acute stress activates the transcription of immune-related genes as a synergic mechanism of anti-inflammatory properties. Acute stress decreases the number of lymphocytes and monocytes and increases blood neutrophil levels [7]. Furthermore, it induces gene expression involved in cell migration and chemotaxis, especially neutrophils [33]. In chronic stress, glucocorticoid resistance modifies the negative feedback signal on glucocorticoid receptors and then shifts the situation into pro-inflammatory enhancement [7].

The effect of ANC on blood profiles of restraint stress mice

Total anthocyanin extract at the dose of 10 mg/kg BW increased Hb level to 12.40 ± 0.76. A higher dose of anthocyanin extract at 20 mg/kg BW increased the level of Hb to 12.73 ± 0.43. The Hb level declined to 11.18 ± 0.85 for a 40 mg/kg BW dose of total anthocyanin extracts ($p = 0.02$) (Figure 1A). The erythrocyte counts among anthocyanin groups were $7.05 \times 10^6/mm^3 \pm 0.32$; $6.71 \times 10^6/mm^3 \pm 0.43$ and $6.08 \times 10^6/mm^3 \pm 0.12$ for a dose of 10 mg/kg BW, 20 mg/kg BW and 40 mg/kg BW respectively ($p = 0.02$) (Figure 1B). Stressed mice treated with 40 mg/kg BW of total anthocyanin extracts had the lowest MCH level among groups (18.23 ± 2.21 pg) (Figure 1C). Similarly, the level of MCV in stress treated 40 mg/kgBW dose was the lowest (53.75 ± 2.22 μm^3) compared to the control (55.75 ± 0.95 μm^3), stress (56.25 ± 4.03 μm^3), stress+10 mg kg BW anthocyanin (56.00 ± 0.82 μm^3) and stress+20 mg/kg BW anthocyanin (58.00 ± 3.83 μm^3) groups (Figure 1D). The MCHC level in stress-treated 40 mg/kg BW was significantly different than the control group (Figure 1E). The RDW percentage was 19.78 ± 1.22 % for the control group, 18.47 ± 0.38% for the stress group, 18.55 ± 0.91% for a dose of 10 mg/kg BW, 16.90 ± 3.93% for a dose of 20 mg/kg BW and 18.03 ± 0.68% for dose 40 mg/kg BW with $p = 0.355$ (Figure 1F).

The average leucocyte count in anthocyanin-treated groups was 6.42 ± 1.88 $10^3/mm^3$, 4.90 ± 1.82 $10^3/mm^3$, and 3.75 ± 0.42 $10^3/mm^3$ for 10 mg/kg BW, 20 mg/kg BW and 40 mg/kg BW, respectively (Figure 2A). Anthocyanin treatment significantly increased the percentage of lymphocytes ($p = 0.003$) (Figure 2B). In contrast to lymphocyte percentage, the percentage of granulocytes decreased among treated groups ($p = 0.006$). The average granulocyte percentage was 17.50 ± 3.31, 18.00 ± 6.78, and 19.50 ± 1.73 for the anthocyanin-treated groups, respectively (Figure 2C). Anthocyanin decreased the percentage of monocytes to 6.00 ± 1.41 at the lowest dose and 6.75 ± 2.75 at the highest dose (Figure 2D). While differences in platelet count were insignificant between groups (Figure 2E), MPV was significantly different between groups at $p = 0.02$ (Figure 2F).

Plant antioxidants have protective effect against hemolysis and reduce oxidative stress in red blood cells [34]. The decrease of hemoglobin and erythrocyte count in the present study is interesting due to the inconsistency with a previous study that showed that a longer duration of anthocyanin treatment (28 days) as high as 1000 mg/kg improved the erythrocyte and hemoglobin levels in healthy rats [35]. The protective effect of anthocyanin from other plant sources, such as *Hibiscus sabdariffa*, has been previously demonstrated in the form of scavenging free radicals in 2,4 dinitrophenylhydrazine-induced hematotoxicity [36]. Anthocyanin also upregulated erythropoietin hormon in renal hypoxic mice [37]. Regardless of the level of erythrocytes, anthocyanin can interact with the erythrocyte membrane and induce changes in the morphological shape of erythrocytes without deep penetration into the lipid hydrophobic layer [38]. This generates a further path of investigation to reveal the details of the proposed mechanism of a combination of stress and anthocyanin treatment.

Previous studies revealed no significant difference in leucocyte count after administration of leaf extract from *Ipomoea batatas* [35]. A proliferation of peripheral blood mononuclear cells and modulation of human immune response were also reported after daily purple sweet potato leaf consumption [39]. However, other studies demonstrated that anthocyanin reduced monocyte/macrophage infiltration, and leucocyte adhesion

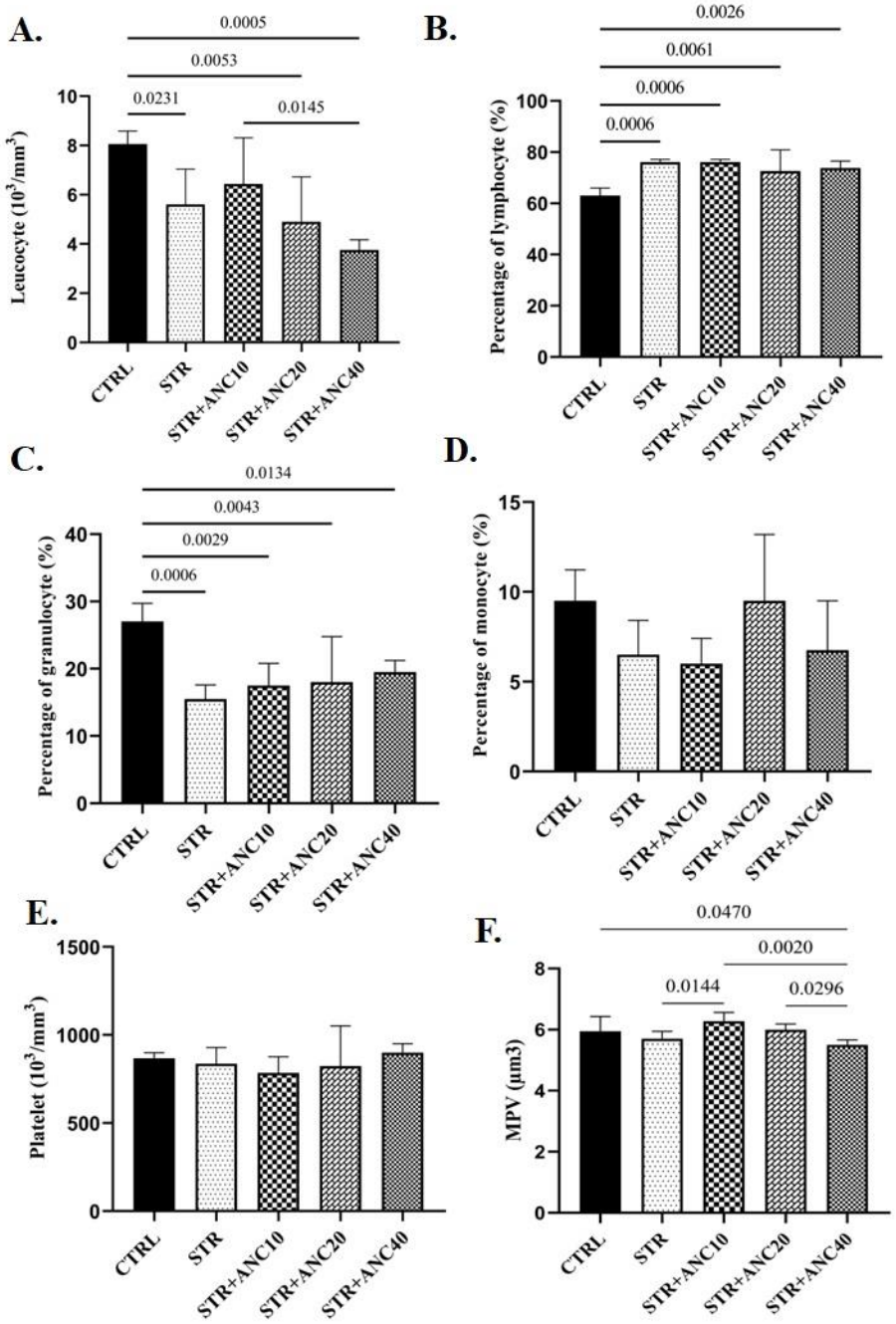

Figure 2. Leucocyte count and granulocyte percentage decreased in treatment groups [A, C]. In contrast, percentage of lymphocytes increased compared to the control group [B]. Percentage of monocytes decreased in treatment groups [D]. The platelet count showed no differences between groups [E]. MPV demonstrated significant differences among groups [F]. Results are shown as mean ± SD. Differences significant at $p < 0.05$ are noted above each bar of the groups.

increased Nrf2 gene transcription and supported DNA integrity in peripheral blood lymphocytes [40–42]. In dextran sulfate sodium-induced colitis mice, dietary red raspberry as an anthocyanin source reduced the infiltration of CD4 T cells by 50%. This promoted an anti-inflammatory effect on inflamed colon tissue [43]. Piekarsa *et al.* demonstrated the elevation of CD4 T cells, spleen lymphocyte proliferation, and leucocyte count after the administration of an iridoid-anthocyanin aqueous extract of cornelian cherry fruits in infected mice [44].

The effect of restraint stress on spleen tissue

Figure 3A shows that stress dramatically reduced spleen weight (0.15 ± 0.02 g) compared to the CTRL group (0.23 ± 0.03 g) (Figure 3A). The expression of spleen caspase-3 sharply increased in the stress group. The intensity of caspase-3 in the STR group was three times higher than the CTRL group (Figure 3B). We found a disarray of white pulp architecture in spleen tissue from stressed animals, accompanied by subcapsular vacuolization with a disarray of white pulp architecture and relatively increased erythrocyte

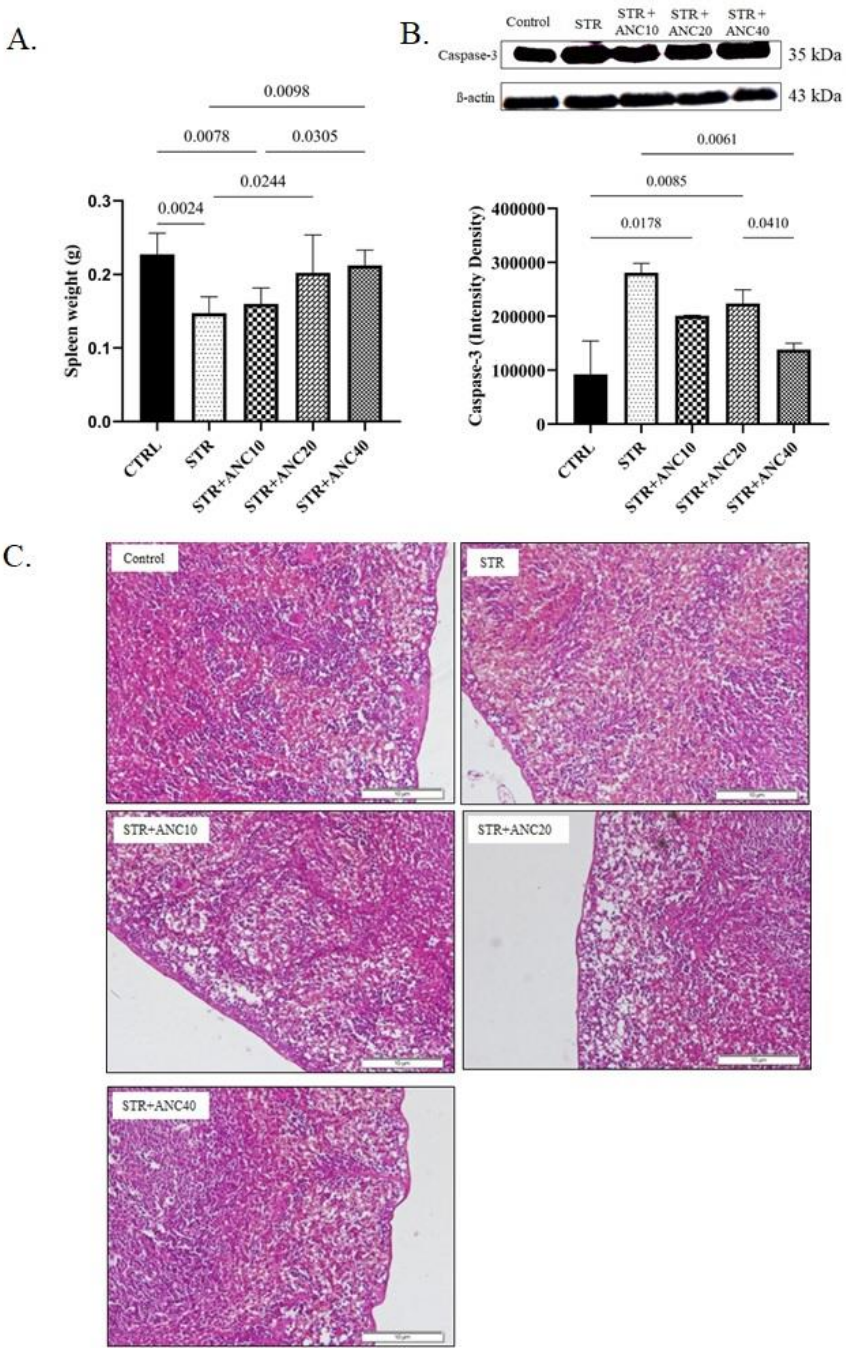

Figure 3. [A] Spleen weight reduced in stress group and increase following restraint application [B] The expression of caspase-3 in spleen tissue demonstrates using immunoblotting assay [C] Photomicrograph of spleen tissue sections from control, [B] treated with restraint stress 2 hours/day for 14 days, stress+total anthocyanin extracts 10 mg/kgBW, stress+total anthocyanin extracts 20 mg/kgBW, stress+total anthocyanin extracts 40 mg/kgBW (hematoxylin and eosin stain; 100× magnification).

number (Figure 3C). The spleen is a major lymphoid organ that plays a significant role in leucocyte distribution during chronic stress. Splenectomy before restraint stress application changed the ratio of CD4/CD8 lymphocytes [15]. Stress induces the activation of the brain-spleen axis, thus increasing the inflammatory pathway by stimulating immune cells. Furthermore, stress affects both the morphology and function of the spleen. Previous reports found splenomegaly during depression and stress in mice. Interestingly, antidepressant treatment in major depressive disorder normalized the spleen morphology [5]. Our restraint stress application dramatically increased the expression of caspase-e. Chronic stress was reported to increase caspase-3 in the abdominal aorta of rabbits [45]. Immobilization stress induced involution of white pulp, expansion of red pulp, and loss of the marginal zone [14]. Stress changed the histological features of spleen tissue, and we propose that the administration of anthocyanin did not provide improvements in stress effects in spleen tissue. However, a previous study showed a protective effect of anthocyanin-rich extract from lingonberry in higher doses towards irradiation stress [47].

The effect ANC on spleen tissue of restraint stressed mice

Anthocyanin treatment increased the weight of the spleen to 0.16 ± 0.02 g, 0.21 ± 0.05 g and 0.21 ± 0.02 g for doses of 10, 20 and 40 mg kg BW, respectively. Anthocyanin from PSP significantly reduced the expression of caspase 3. The dose of 40 mg/kg BW had the most significant reduction in the STR group (Figure 3B). This study supported the previous report that found anthocyanin from purple sweet potato alleviated the caspase-3 of lead-induced toxicity in mice—the inhibition of JNK signaling pathway proposed as potential mechanism of previous finding [23]. The opposite effect as apoptosis induction in cancer cells was shown by a malvidin-3-O-galactoside fraction of anthocyanin from blueberry [46]. We assumed different plant origins and anthocyanin fractions might raise different effects on apoptosis. Thus, further investigation is necessary to evaluate the total anthocyanin extract from purple sweet potatoes in the spleen of the stressed-treated mouse model.

Conclusion

This study established that the total anthocyanin extracts of purple sweet potatoes alter erythrocyte count, hemoglobin, MCHC, leucocyte count, MPV, and percentage of lymphocytes and granulocytes. Histological changes and apoptosis indicators in spleen tissue existed under stress in the absence and presence of anthocyanin extract administration.

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