

## The Potential of Purple Sweet Potato-based Food Bar as an Immunomodulator and Hepatoprotector Against Diethylnitrosamine-induced Mice

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### Abstract

The food bar's effect as an immunomodulator and hepatoprotector *in vivo* was tested on male BALB/c mice induced with diethylnitrosamine (DEN). The experimental animals were treated using the Post-test Control Group Design method. Six groups of 30 mice each made up the sample: The negative control group of healthy mice fed with a normal diet (P0); the group of healthy mice given the food bar (P1); the positive control group of mice induced with DEN and given a normal diet (P2); the group of mice induced with DEN and given a normal diet + food bar at 11,700 mg/kg (P3); the group of mice induced with DEN and given a normal diet + food bar at 23,400 mg/kg (P4); and the group of mice induced with DEN given the food bar (P5). The observations conducted included the spleen, which was used for the analysis of immunomodulators on T CD4+CD25+, CD4+, and CD8+ cells with cytokines TNF- $\alpha$ , IFN- $\gamma$ , and IL-10, blood serum for the analysis of SGPT and SGOT, and liver organ for the analysis of SOD, MDA, and histopathology. The results of the immunomodulator testing showed that the administration of the food bar (P5) significantly ( $p < 0.05$ ) resulted in the highest percentage of CD4+ T cells at 2.93 % and CD8+ T cells at 5.30 %, a lower percentage of CD8+ T cells expressing proinflammatory cytokines (TNF- $\alpha$  at 0.27 % and IFN- $\gamma$  at 0.29 %), a higher percentage of CD4+ T cells expressing CD25+, and CD25+ T cells expressing anti-inflammatory cytokines (IL-10) at 2.83 %. This group also exhibited the lowest levels of SGPT and SGOT, a higher percentage of SOD, and a lower percentage of MDA compared to other groups. The food bar, based on the mix of purple sweet potato, mung beans, moringa leaves, and strawberries, also reduced liver damage, as demonstrated through histopathological testing.

**Keywords:** Immunomodulator, Hepatoprotective, Purple sweet potato, Moringa, Mung bean, Diethylnitrosamine

### Introduction

Breakfast food bars are popular in several developed countries and are made from wheat, corn, and rice cereals. However, breakfast food can also be made from tubers as a source of carbohydrates, combined with legumes as a source of protein, and mixed with fruits as a source of fiber and vitamins. The selection of ingredients for the composite formulation is crucial to produce a good product with functional properties. Several research results have shown that purple sweet potatoes, moringa leaves, green beans, and strawberries, besides containing complex nutrients and bioactive compounds, also exhibit potential health benefits as antioxidants, anti-inflammatory agents, hepatoprotectors [1-3], and immunomodulators. Immunomodulators are substances derived from biological or synthetic sources capable of modulating one or more immune system components, including the adaptive immune response. The immune system of a healthy organism produces various metabolites to maintain the body's homeostasis. The immune system can recognize and eliminate pathogens through innate and adaptive immunity mechanisms. The immune system, which is responsible for protecting the host from various pathogenic microorganisms, also controls the immune response and prevents excessive reactions from the body's own cells. A decrease in body resistance can affect the body's ability to fight infections or other diseases. Therefore, the presence of

immunomodulatory compounds that can enhance immune responses to disease or infection is a vital component of the immunological system [4].

The immunomodulatory effect of anthocyanin compounds in purple sweet potatoes has been shown to suppress TNF- $\alpha$ , providing anti-inflammatory benefits [5]. Purple sweet potatoes can reduce the inhibition of T and B cell proliferation and balance Th1 and Th2 cytokines, indicating that immune dysfunction can be improved by modulating antioxidant activities [6]. The mung bean exhibited the most significant immunomodulatory activity in promote the production of nitrit oxide and immune reactive molecules (IL-1 $\beta$ , IL-6 and interferon- $\gamma$  (IFN- $\gamma$ ) [7]. Moringa leaves contain bioactive components with immunomodulatory potential, inhibiting nitric oxide and proinflammatory cytokine production in macrophages [8]. As was already mentioned, one of *Fragaria*'s traditional applications is as an anti-inflammatory. The presence of anthocyanins, which are known to have anti-inflammatory potential and are most represented in the form of pelargonidin and cyanidin derivates, was shown to have anti-inflammatory characteristics both *in vitro* and *in vivo* [9]. The immune system's defense effectively works when it can confront foreign substances as soluble mediators, integrated with its ability to differentiate between self (own cells and tissues) and non-self (foreign microbes and molecules) [10].

Diethylnitrosamine (DEN) is known as both a hepatotoxin and hepatocarcinogen. It is a substrate of CYP2E1 and plays an active role in ROS generation. DEN concentrations and animal ages result in varying efficacy and efficiency. Severe inflammatory damage (necrosis), liver cirrhosis, and disease have been associated with impaired liver immune function, leading to liver defense [11]. Liver damage can be prevented by utilizing compounds with hepatoprotective properties, where these compounds can mitigate the effects of toxic substances that may damage the liver or even repair damaged liver tissue. Some bioactive plant compounds responsible for hepatoprotective effects include polyphenols, flavonoids, tannins, ascorbic acid, and polysaccharides [12].

No specific research has been conducted on immunomodulatory and hepatoprotective food bars. Therefore, the purpose of this study was to investigate the potential of purple sweet potato-based food bars as immunomodulators and hepatoprotectors in the form of practical, energy-dense, and nutritionally rich functional food products. The study will use *in vivo* methods, employing male mice induced with DEN as the experimental model.

## Materials and methods

### Tools and materials

The tools and materials used in this research were a stove, digital scale, food dehydrator, dry blender, oven, knife, food bar mold, mice cages (plastic box with wire lid), water bottles, feed containers, scoop, and gloves. The equipment used for analysis included surgical board, forceps, surgical instruments, centrifuge, spectrophotometer, flow cytometry, and microscope.

The materials used in this research were purple sweet potato paste, mung bean flour, moringa leaf flour, and dried strawberries. Other ingredients used in the food bar preparation were eggs, margarine, and stevia. The optimal food bar formula obtained from the results of data processing using the Minitab extreme vertices mixture design in the previous study is 54.84 % purple sweet potato paste, 33.95 % mung bean flour, 6.21 % moringa flour, and 5.00 % dried strawberries. Making food bars is done by stirring margarine, stevia, and eggs, then adding purple sweet potato paste, mung bean flour, moringa leaf flour, and dried strawberry pieces. The dough is molded in a pan and then baked in a 105 °C oven for 45 min. The dough was removed from the pan to be cut into pieces and then baked again in the oven for 6 min to be fully cooked [13].

For testing purposes, wood powder and modified pellet feed, drinking water, pellet with food bar formula sample (for mice maintenance), chloroform, 70 % alcohol, 4 % formalin, and distilled water (for mice surgery and liver and spleen organ extraction), 2 % FBS in PBS, non-sterile PBS, and Ice gel (for lymph and liver cell isolation), hematoxylin and eosin (for liver histopathology testing), FACFlow BD, non-sterile PBS, monoclonal extracellular antibodies CD25+, CD4+, CD8+, intracellular monoclonal antibodies IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 (for flow cytometry testing), SGPT and SGOT reagent kit (for SGPT and SGOT testing), male BALB/c mice (test animals), ketamine and xylazine (anesthetic agents), and DEN (inducing agent) were used.

### Preparation of experimental animals

The preparation of experimental animals' housing began with the construction of rectangular plastic cages with dimensions of 45×35.5×14.5 cm<sup>3</sup> and wire mesh as cage covers. In addition, drinking bottles, feeding dishes, spray equipment, sawdust, modified pellet feed preparation, 70 % alcohol, and male

BALB/c mice (*Mus musculus*) aged 6 - 8 weeks, weighing approximately 20 - 30 g each, were prepared. The mice were acclimatized for 1 week before the experiment and divided into 6 treatment groups.

### Research design

This study is experimental research using a Completely Randomized Design (CRD). The post-test-only control group design, also known as a true experimental design, compares the experimental group to the control group to evaluate the treatment's impact on that group. The study consists of 6 treatments, with each treatment involving 5 mice, resulting in a total of 30 mice. The explanations for each treatment are as follows:

1) P0 (negative control + STD): Healthy mice as the negative control group, not induced with DEN, and provided with a normal/standard diet for 42 days.

2) P1 (healthy mice + FB): Healthy mice treated with ad libitum food bar consumption for 42 days.

3) P2 (positive control DEN + STD): Mice were induced with DEN on day 28 of the experiment and provided a normal/standard diet for 42 days as the positive control group.

4) P3 (DEN-induced mice + STD + FB1): Mice were induced with DEN on day 28 of the experiment and provided with a normal/standard diet + food bar consumption of 11,700 mg/kg body weight for 42 days.

5) P4 (DEN-induced mice + STD + FB2): Mice were induced with DEN on day 28 of the experiment and provided with a normal/standard diet + food bar consumption of 23,400 mg/kg body weight for 42 days.

6) P5 (DEN-induced mice + FB): Mice induced with DEN on day 28 of the experiment and provided ad libitum food bar consumption throughout the study (42 days)

Standard diet (STD) modified made on an isocaloric and isoprotein basis referring to the American Institute of Nutrition/AIN-93M [14]. The dose of the food bar given was based on the conversion from the human dose (weight of 70 kg), where each bar is 30 g, and humans consume 3 - 6 bars per day. The calculated conversion of 30 g of food bar for mice resulted in a dose of 3,900 mg/kg of mice body weight. Thus, the treatment involving 3 bars per day was given at a dose of  $3,900 \times 3 = 11,700$  mg/kg, while the treatment involving 6 bars per day was given at a dose of  $3,900 \times 6 = 23,400$  mg/kg. The food bar used in this study is based on purple sweet potatoes, mung beans, moringa leaves, and dried strawberries. The food bar treatment was administered for 42 days. On the other hand, the dose of DEN induced in the experimental animals weighing approximately 20 - 25 g was modified [15] to be 50 mg/kg of mice body weight.

### Flow cytometry analysis

Flow cytometry assay was used to identify CD4+, CD8+, CD4+CD25+, CD25+IL-10, CD8+TNF- $\alpha$ , and CD8+IFN- $\gamma$  cell populations. Spleen organs were taken from mice that had been sacrificed and then cleaned with phosphate-buffered saline (PBS). Lymphocyte cells were isolated from the spleen by squeezing the spleen organ with the tip of a syringe, placing it in a petri dish, and then grinding and suspending it with 3 mL PBS. Furthermore, the cells were obtained using a wire, producing a homogenate. Then the homogenate obtained was centrifuged at 2500 rpm at 10 °C for 5 min. The supernatant obtained was discarded, the pellet was resuspended with 1 mL PBS then vortexed, and 50  $\mu$ L was taken. After that, the suspension was put into a microtube, and added 50  $\mu$ L extracellular antibody then vortexed and incubated in an icebox at 4 °C for 20 min then added 50  $\mu$ L fixative and then resuspended, incubated again at 4 °C for 20 min added 500  $\mu$ L wash-perm, vortexed and incubated at 4 °C for 20 min, then centrifuged at 2500 rpm at 10 °C for 5 min to get pellets added with 50  $\mu$ L intracellular antibody and vortexed then incubated at 4 °C for 20 min in low light conditions. After that, 400  $\mu$ L of PBS solution was added, vortexed, and transferred to a flow cytometry cuvette. Finally, running flow cytometry was carried out [16]. Flow cytometry was also performed on the liver for SOD (Superoxide Dismutase) MDA (Malondialdehyde) analysis.

### SGPT (Serum Glutamic Pyruvic Transaminase) and SGOT (Serum Glutamic Oxaloacetic Transaminase) assay

Mice were dissected, intracardial blood was taken and then centrifuged to obtain blood serum. Blood serum was taken as much as 50  $\mu$ L mixed in 1000  $\mu$ L of mixed reagents on SGOT and SGPT. Then the absorbance was read on a spectrophotometer  $\lambda$  340 nm for 3 min. The absorbance results were then calculated to determine the enzyme levels in the form of U/L units with the formula [17].

### Histopathological examination

The liver was removed from the dissected mice and cleaned, then submerged in PBS. The livers were prepared for paraffin embedding by first being preserved in a 10 % buffered formalin solution for 24 h. Sections of approximately 5 mm were cut and stained with hematoxylin and eosin. Histological changes of the liver were observed under a light microscope at 100× - 400× magnification. Necrotic cells or damaged cells were counted at 10 fields of view. Necrotic cell counts were used as qualitative data [18].

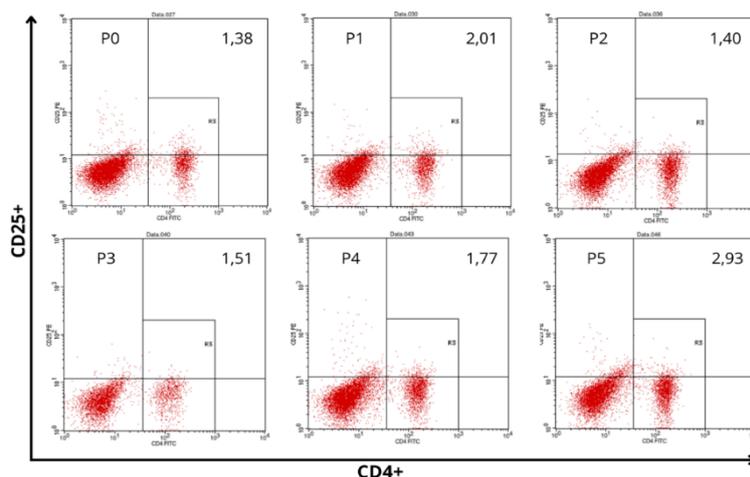
### Statistical analysis

The data in this study included quantitative data analyzed statistically using the Minitab 19.0 program. Data analysis in this study used comparative analysis between groups with the ANOVA test. Suppose there is a significant difference ( $p < 0.05$ ); the test is continued with Fisher's LSD test with a confidence level of 95 % to see more clearly the location of differences between treatment groups.

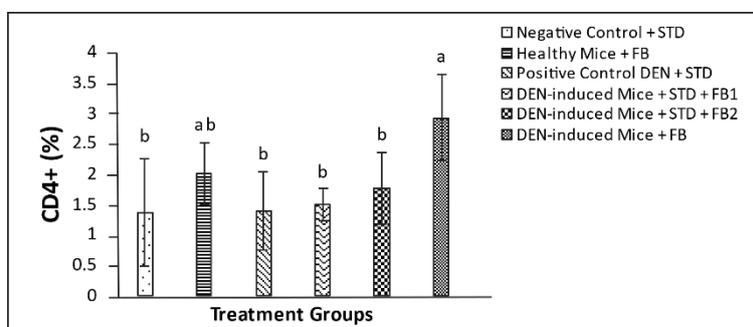
### Results and discussion

#### CD4+ cell population in the spleen

CD4 is glycoproteins involved in T cell antigen recognition and activation with function as T cell 'coreceptors' for antigen bind to MHC-II molecules, which serve as antigen for the main T cell population and involved in early signaling following T cell antigen recognition [19]. CD4+ flow cytometry analysis results in the spleen can be seen in **Figure 1**. Based on the graph of the average percentage of CD4+ cells in the spleen (**Figure 2**), it is evident that the P5 group had a significantly higher average percentage of CD4+ cells in the spleen compared to the P2 group.



**Figure 1** CD4+ flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN+ STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



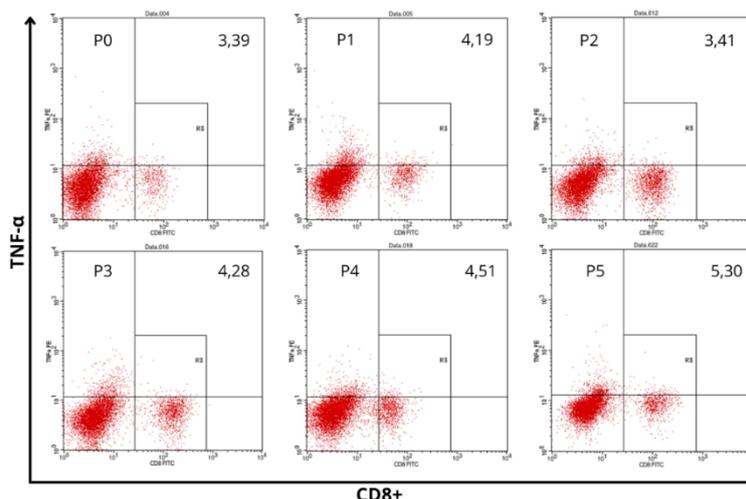
**Figure 2** CD4+ analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

Compared to group P0, group P2’s average proportion of CD4+ cells in the spleen was larger (**Figure 2**). It was believed to be caused by the immune response to DEN induction, which played a role in the CD4+ T cell’s ability to detect and clear pre-cancerous hepatocytes while preserving altered cells. DEN increased CD4+ T cells and activated B cells, exerting suppressive functions on inflammation up to liver cancer. CD4+ T cells represent helper T cells that assist lymphocytes in differentiating and producing antibodies [20].

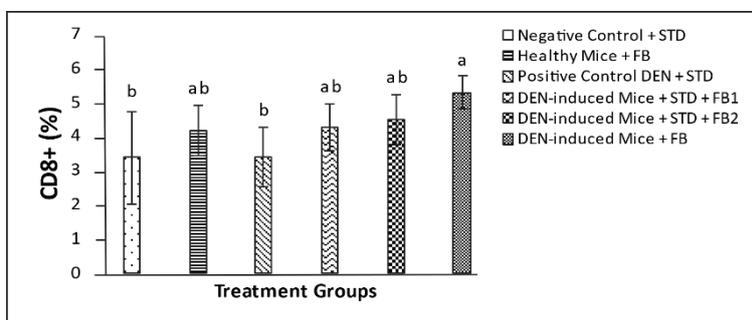
In groups P1 and P5, as well as groups P3 and P4, there was an increase in the average CD4+ T cell count compared to groups P0 and P2. It was due to the role of purple sweet potato carbohydrates, which interacted with dendritic cells and were then transmitted to CD4+ T cells, activating the transforming growth factor  $\beta$  (TGF- $\beta$ ) growth factor and enhancing the function of FoxP3+ CD25+ regulatory cells with anti-inflammatory properties and IL-10 expression [21].

### CD8+ cell population in the spleen

CD8+ is a glycoprotein present on approximately one-third of T cells in lieu of CD4+. Similar to CD4+, it binds to a monomorphic region of MHC, although it binds to class I rather than class II antigens. CD8+ defines cytotoxic effector cells and perhaps subsets of natural killer and regulatory cells [22]. CD8+ flow cytometry analysis results in the spleen can be seen in **Figure 3**. Based on the average graph of CD8+ percentage in the spleen (**Figure 4**), it is shown that the P5 group had a significantly higher average CD8+ percentage in the spleen compared to the P2 group.



**Figure 3** CD8+ flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN+ STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



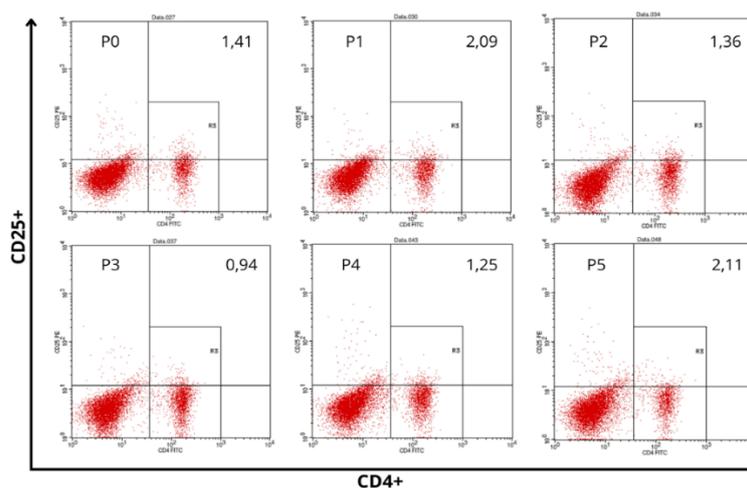
**Figure 4** CD8+ analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher’s LSD test.

Compared to the negative control group (P0), the P2 group's average proportion of CD8<sup>+</sup> cells inside the spleen was higher (**Figure 4**), suggesting an immune response to the induction of DEN, leading to the activation of CD8<sup>+</sup> T cells to eliminate the entering antigen. In the adaptive immune cells, a significant increase in CD8<sup>+</sup> T cells was detected in the DEN-induced liver, causing early-stage inflammation of the tumor [23].

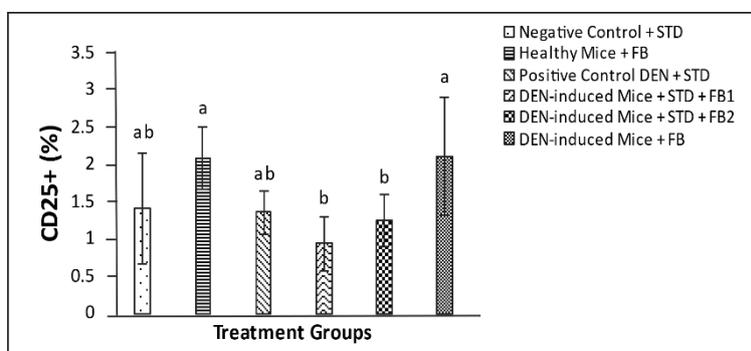
In the P1 and P5 groups, as well as the P3 and P4 groups, there was an increase in the average CD8<sup>+</sup> T cell count compared to the control groups (P0 and P2). This increase is attributed to the presence of antioxidant compounds that enhance CD8<sup>+</sup> T cell proliferation. Arrest of oxidative reactions with antioxidants with CD8<sup>+</sup> T cells that block differentiation allows proliferation and generation of long-lived T-cells with characteristics like cells at metabolic rates [24].

#### CD4+CD25<sup>+</sup> cell population in the spleen

T cells from the CD4 population expressing CD25 (CD4+CD25<sup>+</sup>) are known as immune cells capable of controlling other cells called regulatory cells. These regulatory cells function as suppressors that prevent inflammation. CD4+CD25<sup>+</sup> regulatory T cells are natural suppressor cells that play a crucial role in controlling diseases such as inflammation, with their suppressive mechanisms seemingly multifactorial, including contact-dependent cell interactions or under the effect of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$  [25]. CD4+CD25<sup>+</sup> flow cytometry analysis results in the spleen can be seen in **Figure 5**. The average percentage graph of CD4+CD25<sup>+</sup> in the spleen (**Figure 6**) indicates that both groups P1 and P5 have a significantly higher average percentage of CD4+CD25<sup>+</sup> cells in the spleen compared to the positive control group or the group without food bar administration, which only received normal diet (P2).



**Figure 5** CD4+CD25<sup>+</sup> flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN+ STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



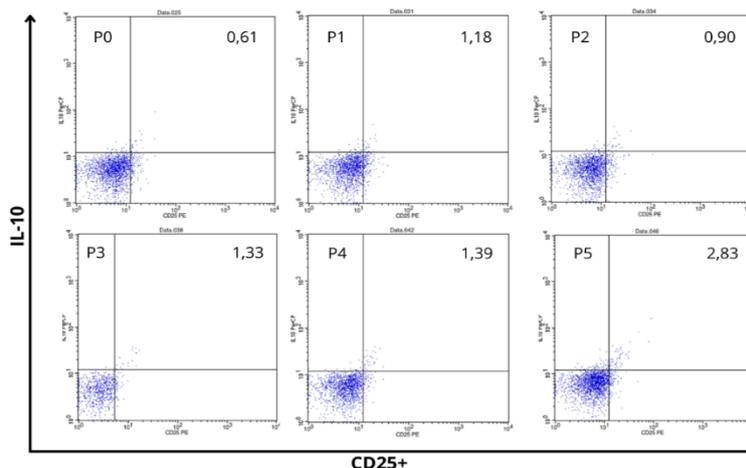
**Figure 6** CD4+CD25<sup>+</sup> analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

The average percentage of CD4+CD25+ in the spleen for the P2 group was lower compared to the P5 group (**Figure 6**). It was suggested to be due to the occurrence of inflammation, as the percentage of anti-inflammatory cytokines did not differ significantly from the normal diet, which was induced by the DEN in the immune response experiencing infection with cancer-related inflammation controlled by CD4+CD25+ cancer regulatory T cells and circulating cells that suppress the activity and proliferation of CD4+ effector T cells. Regulatory T cells are the dominant cell type that releases cancer inflammatory immunity, leading to disturbed lymphocyte infiltration, i.e., tumor lymphocytes and TILs [26].

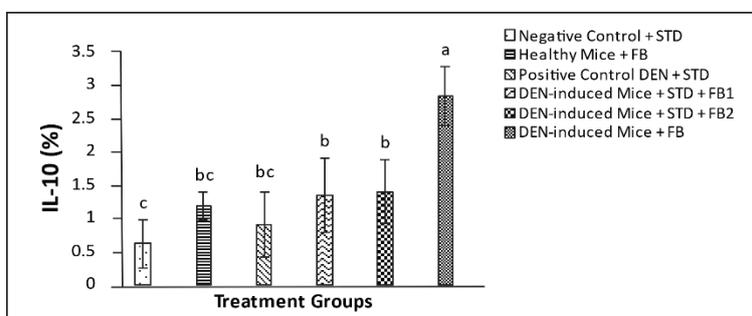
In the food bar ad libitum administration groups (P1 and P5), there was an increase in the average CD4+CD25+ compared to the control group (P0 and P2). It was because of the protein content in moringa leaves, which enhanced CD25+ expression. It is consistent with a study by Chandrasekaran *et al.* [27] stating that polyphenolic compounds also help reduce the production of specific IgE antigens originating from B cells by increasing CD4+CD25+ regulatory T cells in the spleen, resulting in reduced inflammatory responses.

### CD25+IL-10 cell population in the spleen.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that prevents inflammatory and autoimmune pathologies [27]. The production of IL-10 from CD25+ cells assists in controlling immune responses and managing certain chronic infections. IL-10 also plays a significant role in downregulating inflammation and preventing carcinogenesis in mice models, and it functions as an antitumor agent that suppresses effector T cells [27]. CD25+IL-10 flow cytometry analysis results in the spleen can be seen in **Figure 7**. Based on the graph of the average percentage of CD25+IL-10 cells in the spleen (**Figure 8**), it is evident that the P5 group exhibited a significantly higher average percentage of CD25+IL-10 cells in the spleen compared to the P2 group.



**Figure 7** CD25+IL-10 flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN+ STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



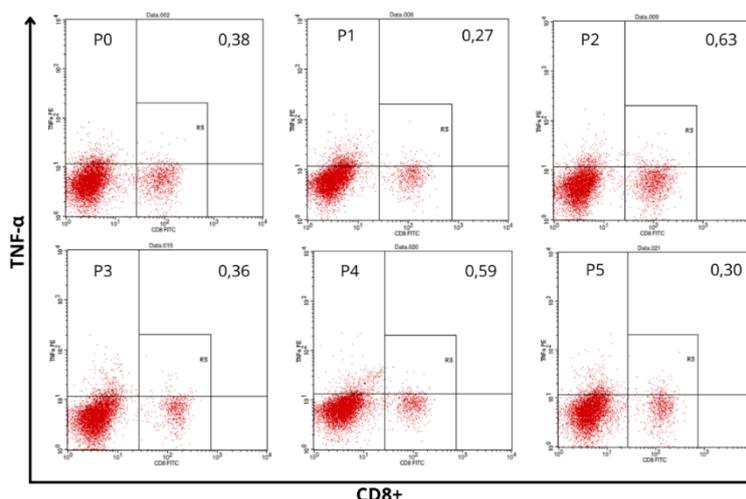
**Figure 8** CD25+IL-10 analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

The average percentage of CD25+ IL-10 in the spleen for the P2 group was lower compared to the P5 group (**Figure 8**), suggesting that it was caused by inflammation from the carcinogen compound DEN due to the low percentage of anti-inflammatory cytokine IL-10. IL-10 plays a role in the proliferation and development of cancer cell survival [27].

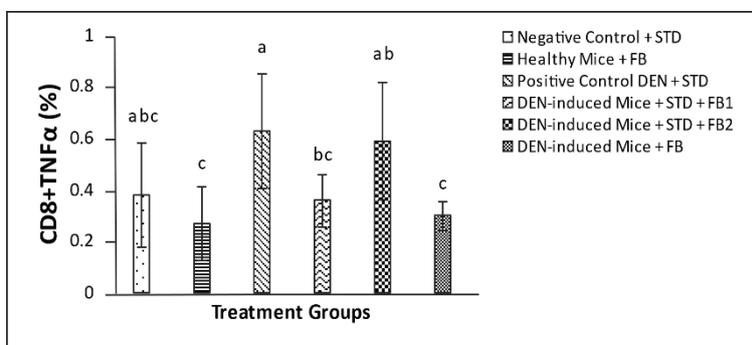
In the groups receiving ad libitum food bars (P1 and P5) and the groups receiving a normal diet added with food bars at doses 1 and 2 (P3 and P4), there was an increase in the average CD25+ IL-10 compared to the control groups (P0 and P2). This is due to food-bar antioxidant compounds that increase IL-10 cytokine expression. The increase in IL-10 was also due to the carbohydrates in purple sweet potatoes, which inhibited the synthesis of other cytokines and could reduce excessive proinflammatory cytokines such as IL-6 and TNF- $\alpha$ . Macrophages produce IL-6 and TNF- $\alpha$  cytokines during the early stages of inflammation, while the anti-inflammatory cytokine IL-10 is produced during the later stages of inflammation [28].

#### CD8+ TNF- $\alpha$ cell population in the spleen

Tumor necrosis factor-alpha (TNF- $\alpha$ ) is a major cytokine in the acute inflammatory response to microbes and pathogens, triggering the production of TNF- $\alpha$  in large quantities and causing systemic reactions. The primary sources of TNF- $\alpha$  are mononuclear phagocytes and antigen-activated T cells, mast cells, and Natural Killer (NK) cells. CD8+ TNF- $\alpha$  flow cytometry analysis results in the spleen can be seen in **Figure 9**.



**Figure 9** CD8+ TNF- $\alpha$  flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN+ STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



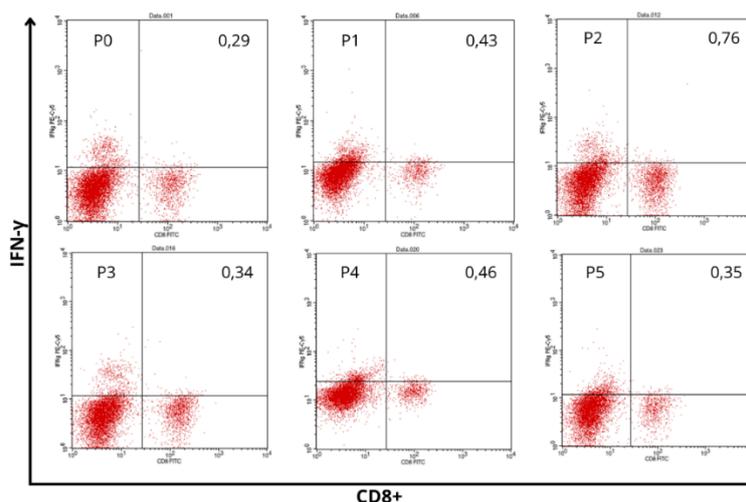
**Figure 10** CD8+ TNF- $\alpha$  analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

Based on the average percentage graph of CD8+ TNF- $\alpha$  in the spleen (**Figure 10**), it shows that the P2 group exhibited a significantly higher average percentage of CD8+ TNF- $\alpha$  in the spleen, which was significantly different ( $p < 0.05$ ) compared to the group of mice induced with DEN and provided with ad libitum food bar (P5). The average percentage of CD8+ TNF- $\alpha$  in the spleen for the P2 group was higher than the P1 group indicating the occurrence of inflammation due to an increase in proinflammatory cytokines caused by the induction of DEN. TNF- $\alpha$  is a significant component that triggers inflammation, including cytokine induction after liver damage caused by DEN [29].

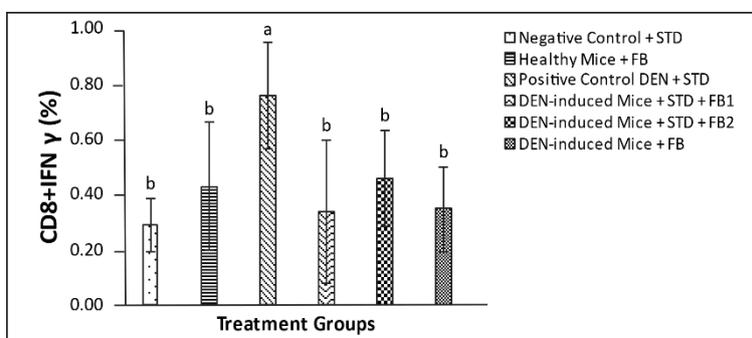
In the P3, P4, and P5 groups, a lower average percentage of CD8+ TNF- $\alpha$  was observed compared to the P2 group. This is due to flavonoid compounds in the moringa leaves, which inhibit TNF- $\alpha$  secretion. Moringa leaves, a source of flavonoids and phenols, can suppress TNF- $\alpha$  due to the interaction of its immunostimulant compounds with the immune system, initiating various cellular and molecular events. The moringa leaf polysaccharides significantly stimulate macrophage proliferation through ROS and TNF- $\alpha$  secretion, ultimately activating the immune system and inhibiting the proliferation of cancer cells induced by DEN [30]. Moringa leaves contain quercetin, caempferol,  $\beta$  sitosterol and caffeoylquinic acid significantly inhibit edema and the expression of proinflammatory cytokines namely CD11b+ TNF- $\alpha$ + [31].

### CD8+ IFN- $\gamma$ cell population in the spleen

Interferon (IFN- $\gamma$ ) or Interferon (IFN) type II is a subgroup of cytokines that function in activating macrophages and other types of cells [10]. The production of IFN- $\gamma$  is mainly regulated by Natural Killer (NK) cells in innate immunity. In contrast, CD8+ T cells and CD4+ T cells are the main sources of IFN- $\gamma$  during the adaptive immune response [32]. CD8+ IFN- $\gamma$  flow cytometry analysis results in the spleen can be seen in **Figure 11**.



**Figure 11** CD8+ IFN- $\gamma$  flow cytometry analysis results in the spleen (P0 = negative control + STD, P1 = healthy mice + FB, P2 = positive control DEN + STD, P3 = DEN-induced mice + STD + FB1, P4 = DEN-induced mice + STD + FB2, P5 = DEN-induced mice + FB).



**Figure 12** CD8+ IFN- $\gamma$  analysis graph results in the spleen. Data are means  $\pm$  SD of 4 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

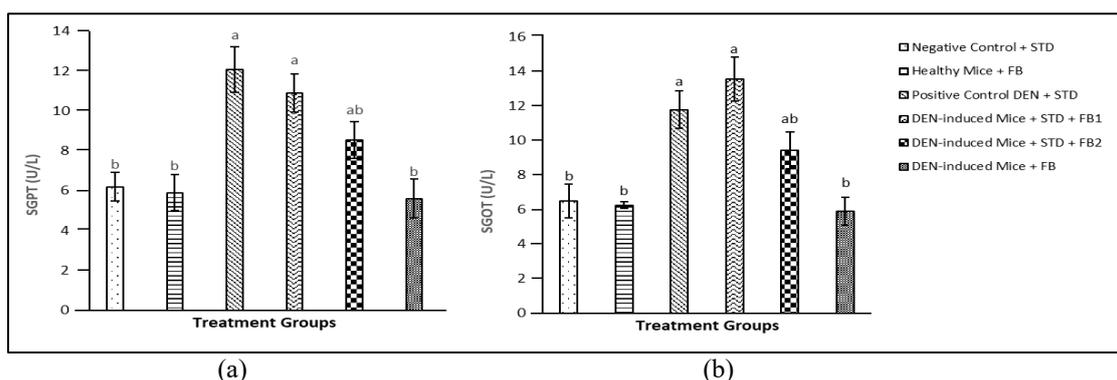
Based on the average percentage graph of CD8+ IFN- $\gamma$  in the spleen (**Figure 12**), it is shown that in groups P3, P4, and P5, the average percentage of CD8+ IFN- $\gamma$  is lower compared to the P2 group. The average percentage of CD8+ IFN- $\gamma$  in the spleen for the P2 group was higher compared to the P0 group, presumably due to an increase in proinflammatory cytokines caused by the carcinogenic compounds from DEN. IFN- $\gamma$  may have contributed to the activation of monocytes/macrophages observed during the hepatocarcinogenesis process, and it may have also contributed to increased oxidative damaged DEN-induced hepatocarcinogenesis process [33].

In groups P3, P4, and P5, the average percentage of CD8+ IFN- $\gamma$  was lower compared to group P2. This is thought to be because purple sweet potatoes have antioxidant compounds that can suppress the production of IFN- $\gamma$  to prevent it from obstructing antiviral and anti-inflammatory activities in the immune regulation process. Purple sweet potatoes could also reduce Th1-type cytokines (IL-2 and IFN- $\gamma$ ) due to immune system dysfunction [6].

### SGPT and SGOT enzyme activities

The difference in the average levels of SGPT and SGOT in mice under each treatment was due to the hepatoprotective activity of the food bar, as observed in **Figure 13**. In the figure, it was generally seen that groups P2, P3, and P4 had higher levels of SGPT and SGOT in the blood serum compared to groups P0, P1, and P5. Given the ad libitum food bar (P5), the DEN-induced group exhibited the lowest SGPT and SGOT activities. It did not differ significantly ( $p < 0.05$ ) from the negative control + normal diet group (P0) and the healthy mice + FB group (P1). The SGPT values decreased in the DEN-induced mice groups given the combination of a normal diet and food bar at 11,700 mg/kg (P3), DEN-induced mice + combination of a normal diet and food bar at 23,400 mg/kg (P4), and DEN-induced mice + ad libitum food bar (P5).

The negative control + STD (P0) and healthy mice + ad libitum food bar (P1) treatment groups had low SGPT levels compared to the other treatments because these groups did not experience DEN induction which caused an increase in blood SGPT levels. The induction of DEN may cause mice to experience stress that leads to oxidative damage. DEN causes the formation of reactive oxygen species (ROS), resulting in oxidative stress and cellular injury [34]. Hepatocellular ROS accumulation can stimulate pathogenic redox signaling that can cause oxidative cellular damage and various disease conditions [35]. Damage to many important cellular components, such as mitochondria, DNA, lipids, and proteins, will result in cell damage and death. Intracellular enzymes such as SGPT and SGOT are helpful indicators to assess liver function, where an increase in these enzymes indicates liver damage. Both enzymes are the most sensitive markers for diagnosing liver damage as they are located in the cytoplasm and released into the bloodstream after cell damage [36]. This allows the enzymes to be detected in the serum analysis of mice. SGPT is a specific marker for acute hepatocellular damage, whereas SGOT indicates chronic liver damage. It resulted in the DEN-induced group having high SGPT and SGOT activities.



**Figure 13** Analysis graph of SGPT (a) and SGOT (b) activities in mice serum. Data are means  $\pm$  SD of 3 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

Changes in SGPT and SGOT activity values in the blood serum of mice in each treatment can be seen in **Table 1**. Compared to the positive control + normal diet group (P2), the DEN-induced treatment group given the food bar (P5) experienced a decrease of 53.66 % in SGPT and 50.00 % in SGOT levels. This indicates that the food bar can protect the liver and reduce the release of SGPT and SGOT. The compounds with antioxidant properties in the extract can stabilize free radicals and prevent cell membrane damage [37].

The hepatoprotective activity of the food bar is believed to be attributed to the bioactive compounds present in the ingredients used.

**Table 1** The difference in SGPT and SGOT activity in mice blood serum.

Treatment	Different of SGPT (%)**	Different of SGOT (%)***
Negative control + STD (P0)	0.00	0.00
Healthy mice + FB (P1)	4.76	30.00
Positive control DEN+ STD (P2)	0.00 <sup>b</sup>	0.00 <sup>b</sup>
DEN-induced mice + STD + FB1 (P3)	9.76 <sup>b</sup>	15.00 <sup>b</sup>
DEN-induced mice + STD + FB2 (P4)	29.77 <sup>ab</sup>	20.00 <sup>ab</sup>
DEN-induced mice + FB (P5)	53.66 <sup>a</sup>	50.00 <sup>a</sup>

\* Different letters indicate significant differences in Fisher's LSD test (95 % confidence interval)

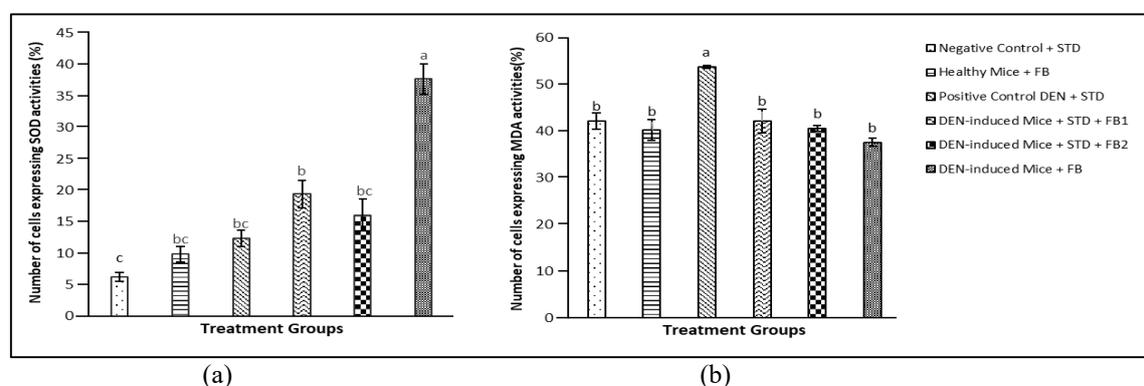
\*\* SGPT difference of P1 calculated against P0; SGPT difference of P3, P4, and P5 calculated against P2

\*\*\* SGOT difference of P1 calculated against P0; SGOT difference of P3, P4, and P5 calculated against P2

The food bar based on purple sweet potato, mung bean flour, moringa flour, and dried strawberries contains bioactive compounds that act as antioxidants. Anthocyanins could attenuate liver injury induced by dimethylnitrosamine by promoting antioxidant enzyme defense via the nuclear erythroid 2-related factor 2 (Nrf2) pathway and attenuate inflammation via NF- $\kappa$ B [38]. Green bean seeds, sprouts, and skin contain a substantial amount of macronutrients (protein, polypeptides, oligosaccharides, and polysaccharides) and micronutrients (flavonoids, phenolic acids, organic acids, sterols, triterpenes, aldehydes) that provide strong antioxidant properties. These antioxidant properties are observed in their ability to inhibit the formation of free radicals by chelating metal ions or inhibiting key enzymes (protein kinase, xanthine oxidase, GSH, lipoxygenase, cyclooxygenase, NADH oxidase, and GST) involved in the radical formation mechanism [39]. This result is also in line with the research of Oshiobugie *et al.* [40], where treatment with moringa significantly reduced liver damage, demonstrated by a decrease in SGPT and SGOT activity levels.

### SOD and MDA levels

Oxidation of fatty acids, lipids, and proteins is a characteristic feature of oxidative stress caused by DEN [41]. The liver is an organ responsible for detoxification and xenobiotic metabolism, making it the most susceptible to oxidative stress compared to other organs. One of the liver's adaptations is the production of superoxide dismutase (SOD) [42]. The level of changes in antioxidant protein/SOD can indicate the presence of oxidative stress in the liver. One response to oxidative stress is lipid peroxidation, where reactive oxygen species (ROS) cause oxidation of lipids with carbon-carbon double bonds in the membrane lipid bilayer [43]. Malondialdehyde (MDA) is the end product of lipid peroxidation which is considered a biomarker of lipid peroxidation and an indicator of cell membrane damage [44]. The high amount of free radicals can trigger the formation of oxidative stress by an increase in MDA levels as the result of lipid peroxide and a decrease in systemic endogenous antioxidant capacity in the form of enzymes such as SOD and glutathione peroxidase (GPx) [45].



**Figure 14** The number of cells expressing SOD (a) and MDA activities (b). Data are means  $\pm$  SD of 3 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

The hepatoprotective effect of the food bar on the activity of increasing the number of cells expressing SOD and decreasing the number of cells expressing MDA is shown in **Figure 14**. Based on these results, there were differences between the treatment groups. The lowest SOD level was observed in the negative control group induced with DEN + normal diet (P0), while the highest SOD level was observed in the DEN-induced group treated with the ad libitum food bar (P5). The lowest MDA level was observed in the DEN-induced group treated with the food bar (P5), whereas the highest MDA level was observed in the positive control treatment group induced with DEN and given a normal diet (P2).

The number of cells expressing SOD in the negative control + normal diet (P0) group was very low, probably because the negative control + normal diet (P0) group did not experience DEN induction. DEN affects cell mitochondrial metabolism, disrupting the balance between reactive oxygen species (ROS) and antioxidants, causing oxidative stress [46]. Without oxidative stress that produces ROS, endogenous SOD in the body will not be induced, causing low levels of SOD in the negative control group + normal diet (P0) and healthy mice group + ad libitum food bar (P1).

**Table 2** Difference value of SOD and MDA in mice liver cells per treatment.

Treatment	Different of SOD (%)**	Different of MDA (%)***
Negative control + STD (P0)	0.00	0.00
Healthy mice + FB (P1)	58.20	9.87
Positive control DEN+ STD (P2)	0.00 <sup>b</sup>	0.00 <sup>b</sup>
DEN-induced mice + STD + FB1 (P3)	56.85 <sup>b</sup>	21.57 <sup>a</sup>
DEN-induced mice + STD + FB2 (P4)	29.73 <sup>b</sup>	24.58 <sup>a</sup>
DEN-induced mice + FB (P5)	204.11 <sup>a</sup>	30.13 <sup>a</sup>

\* Different letters indicate significant differences in Fisher's LSD test (95% confidence interval)

\*\* SOD difference of P1 calculated against P0; SOD difference of P3, P4, and P5 calculated against P2

\*\*\* MDA difference of P1 calculated against P0; MDA difference of P3, P4, and P5 calculated against P2

As shown in **Table 2**, the DEN + ad libitum food bar group (P5) increased SOD by 204 % from the positive control group DEN + normal diet (P2). The increase in SOD activity may be due to bioactive compounds in food bars with antioxidant activity, such as phenols and flavonoids. Flavonoid content can reduce the adverse effects of free radicals by inhibiting lipid peroxidation by activating peroxidase enzymes which are endogenous (enzymatic) antioxidants, such as SOD and catalase [47]. The higher the dose of food bar given, the tendency of SOD level to increase.

DEN-induced mice experience oxidative stress, as evidenced in **Figure 14(b)**, where there is the highest level of malondialdehyde (MDA) in the positive control treatment of DEN induction + normal diet (P2). That means that the P2 group experienced the highest oxidative stress. This oxidative stress is triggered by the presence of DEN, which has oxidizing properties, thus causing oxidative stress that can damage liver cells by increasing the activity of toll-like receptor 4 (TLR4) so that inflammation increases [48]. Increased lipid peroxidation is one of the hallmarks of oxidative stress [49]. MDA is produced at high levels during lipid peroxidation, so that it is commonly used as a measure of oxidative stress [50]. That caused the percentage level of MDA to be higher in the group given DEN + normal diet (P2).

In 3 DEN induction groups that were given food bars either 11,700 mg/kg (P3), 23,400 mg/kg (P4), and ad libitum food bars (P5), there was a significant decrease in MDA levels ( $p < 0.05$ ) from the positive control group + normal diet (P2) with a percentage decrease of 21.57, 24.58 and 30.13 % respectively. Still, there was no significant difference between the 3 food bar treatment groups (P3, P4, P5). It indicates that providing food bars can normalize liver function like normal liver conditions by reducing the percentage level of MDA. The hepatoprotective role of the food bar is indicated by an increase in the level of SOD and a decrease in the level of MDA in the liver cells of mice. The bioactive compounds in the food bar will help significantly modulate DEN metabolism by decreasing cytochrome P450 activity or neutralizing free radicals formed during DEN hepatotoxicity [51]. One of these bioactive substances is anthocyanin, which can inhibit lipid peroxidation, neutralize free radicals, and control the release of pro-inflammatory mediators [52].

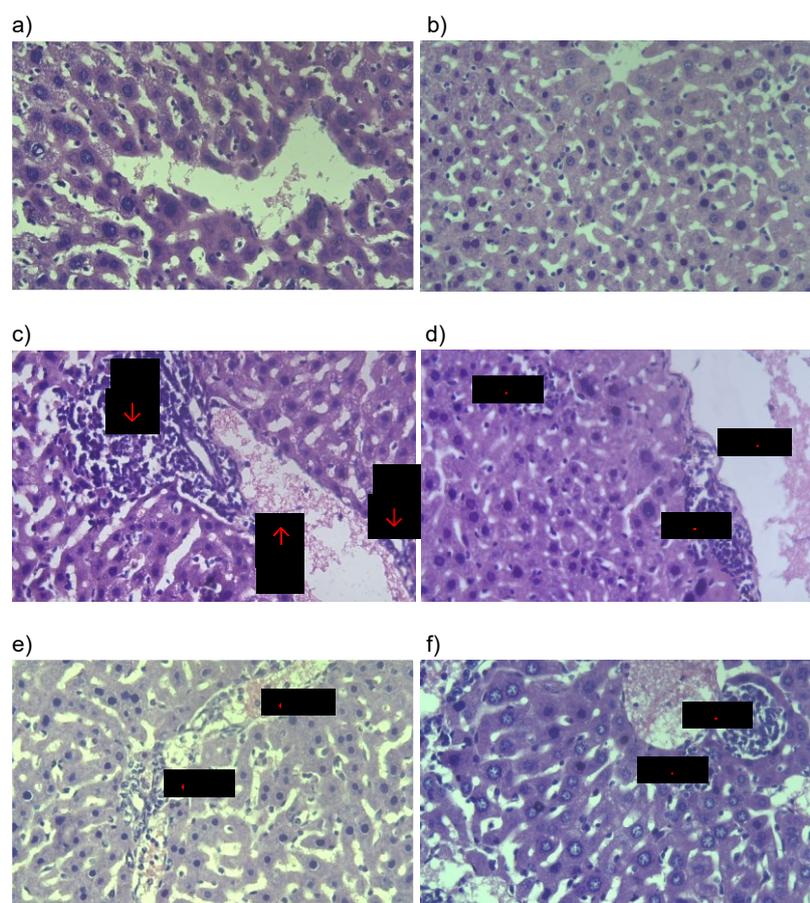
### Mice liver histopathology

Liver histopathology was analyzed to assess the condition of liver cells. Normal liver cells were characterized by central veins, hepatocytes, and sinusoids, whereas damaged liver cells exhibited signs of inflammation, necrosis, and fibrosis. Additionally, the number of damaged liver cells was quantified for each treatment group.

**Figure 15** shows that each treatment resulted in different histopathological outcomes. In the normal liver (**Figure 15(a)**), the negative control group, cells stained with hematoxylin and eosin exhibited purple cytoplasm and dark purple nuclei with clearly visible central venous blood vessels, as reported in the study [53]. **Figure 15(b)** also observed this liver histopathology, representing the healthy mice group given ad libitum food bars. It indicates that consuming food bars in a certain amount can maintain the normal condition of the mice's liver due to the presence of clearly visible hepatocyte cells and nuclei.

In the positive control group (**Figure 15(c)**), some cells were found to be degraded and turned into inflammatory cells. Cell degradation was due to cell death, or necrosis, with signs of reduced cell nuclei, possible plasma membrane rupture, and nuclear changes leading to cell death. The inflammatory process was involved in triggering cellular and molecular events that led from liver damage to fibrosis and, eventually, liver cancer [54].

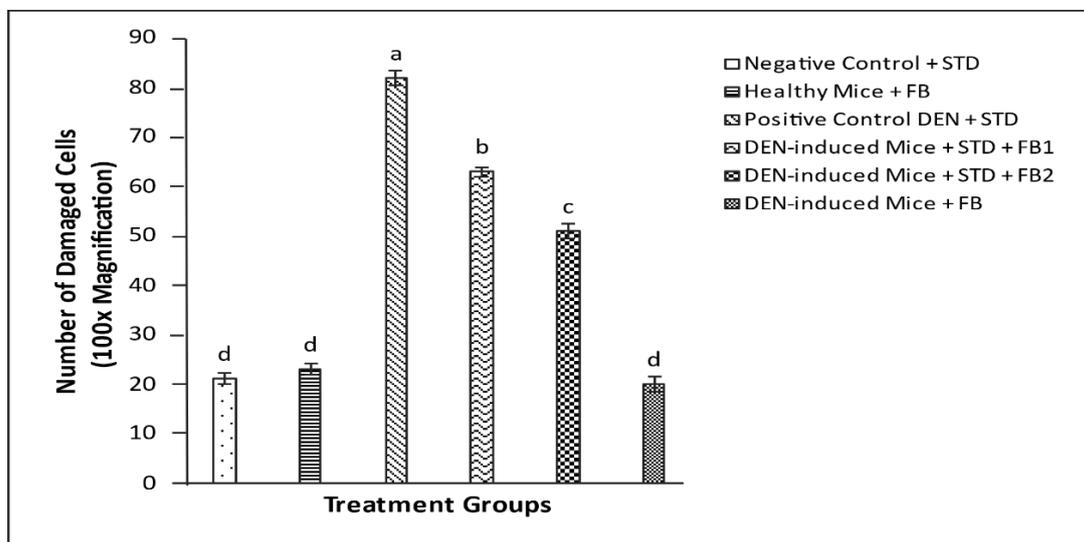
**Figures 15(d) - 15(f)** showed that the diet interventions improved the liver cell condition due to DEN induction with food bar administration. In the DEN-induced mice + food bar dose 11,700 mg/kg group (**Figure 15(d)**), have fibrosis and inflammation but the rate of inflammations in the group is lower than in the positive control group induced by DEN. In the DEN-induced mice + food bar dose 23,400 mg/kg group (**Figure 15(e)**), fibrosis and inflammation were also present but in a smaller amount than the group given food bar dose 11,700 mg/kg because dose 23,400 mg/kg of food bar administration was higher. This phenomenon is referred to as spotty necrosis, which depicts the necrosis of a small group of hepatocytes typically associated with lymphocytes [55]. The DEN-induced mice + ad libitum food bar (P5) group (**Figure 15(f)**) showed inflammation in the central vein area. However, the inflammation level experienced by the DEN-induced mice + ad libitum food bar (P5) group was lower than that of the food bar dose 11,700 and 23,400 mg/kg groups. Many normal hepatocytes were present in the intact DEN + food bar group, indicating that the cells had not undergone acute damage caused by DEN.



**Figure 15** Histopathological appearance of mice liver with inflammation (I) and fibrosis (F), a) P0 = negative control + STD, b) P1 = healthy mice + FB, c) P2 = positive control DEN+ STD, d) P3 = DEN-induced mice + STD + FB1, e) P4 = DEN-induced mice + STD + FB2 and f) P5 = DEN-induced mice + FB.

### The number of damaged liver cells

**Figure 16** illustrates that groups P5 and P1 had the least cell damage, followed by P3 and P4. This indicates that the liver's ability to protect itself from food bar products is better than when only given a normal diet or a normal diet + food bar. The amount of cell damage in group P5 was almost the same as in group P1. This suggests that the purple sweet potato, mung bean, moringa leaf, and strawberry-based food bar can preserve liver cells and reduce damage caused by DEN, restoring liver cells to their normal state.



**Figure 16** Number of cells showing damage observed under 100× magnification. Data are means ± SD of 3 replicates in each experiment. Different lower-case letter(s) indicate significant difference at  $p < 0.05$  significance level according to the Fisher's LSD test.

**Figure 16** displayed the highest number of damaged cells in the negative control group. DEN compound can cause inflammation, necrosis, and fibrosis. DEN contains carcinogenic compounds mediated by cytochrome P450 enzymes to induce DNA damage. The relationship between increased ROS and histopathological changes/necrosis is compatible with impaired immune cell response to DEN over time [46]. In the histopathological study during DEN induction, lipids occupied the cytoplasm, pushing the nucleus to the periphery, and the hepatocyte cytoplasm filled with small lipid droplets. Inflammation also emerged, consisting of inflated inflammatory cells (lymphocytes, neutrophils, eosinophils, and Kupffer cells). Further inflammation led to pathological changes in blood pressure, resulting in liver cirrhosis. Following the inflammatory response, fibrosis gradually emerges and becomes a pathological picture [56]. Hepatocellular fibrosis begins with exposure that can cause damage or apoptosis of the hepatocytes. Damage from the hepatosis causes the release of pro-inflammatory cytokines such as *Tumor Necrosis Factor* (TNF-) and *Reactive Oxygen Species* (ROS) [57].

### Conclusions

Based on the results of data analysis and research findings, it can be concluded that the administration of the food bar demonstrates effectiveness as an immunomodulator by increasing CD4+ and CD8+ T-cell populations, enhancing CD4+ T-cells expressing CD25+, elevating CD25+ expressing anti-inflammatory cytokine (IL-10), and reducing CD8+ T-cells suppressing the expression of proinflammatory cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) in the spleens of DEN-induced mice. The provision of the food bar as the main diet resulted in reduced levels of SGPT, SGOT, and hepatic MDA in DEN-induced mice and increased SOD activity in mice induced with 50 mg/kg of DEN. The food bar based on purple sweet potato, green beans, moringa leaves, and strawberries was able to protect and reduce liver cell damage caused by DEN, as demonstrated by histopathology tests.

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