

Bioactivity of Purple Yam Tuber (*Dioscorea alata* L.) on the Level of CD8⁺ and CD8⁺CD462L⁺ T cells and Histology of Liver in BALB/c Mice Model of Digestive Allergy

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Abstract

Purple yam tuber (*Dioscorea alata* L.) is a family of Dioscoreaceae containing diosgenin which is known as immunomodulatory agent. This study aimed to understand the quantitative changes of naïve and activated memory of T cells on mice model of digestive allergy after orally treated with ethanol extract of purple yam tuber. In this experiment, architecture of hepar histopathology is also observed. Ethanol extract of purple yam tuber with three doses of 0.167 g/kg bw (U1), 2.008 g/kg bw (U2), and 10.039 g/kg bw (U3) are applied. Data were analyzed using One-way ANOVA ($p < 0.05$) and Tukey test using SPSS 16.0 for Windows. Ethanol extract of purple yam tuber triggers the immunocompetent activity of T cells in mice model of digestive allergy. The result showed that the number of memory type T cells in mice model of digestive allergy decreased in lower dose (0.167 g/kg bw (U1)). However, the number of naïve T cells, CD8⁺CD62L⁺ in mice with digestive allergy after administration of purple yam tuber ethanol extract increased significantly in lower dose (0.167 g/kg bw (U1)) compared with positive control (OVA). Dose variations of extract ethanol of purple yam tuber (0.167 g/kg bw) has a significantly effect to shift the T cell status from memory to naïve.

Keywords: Digestive allergy, *Dioscorea alata* L., histopathology, immunomodulatory, subset T cells

INTRODUCTION

Dioscorea species mostly produce tubers, which can be used for food or medicine traditional. Tuber contain amounts of carbohydrates, may be the manufacture starch or ethanol (alcohol). One of the species found in Indonesia is *Dioscorea alata* L. (purple yam, keribang, water yam) [1]. Utilization purple yam in Indonesia is still limited use as food and food coloring.

The analysis showed the content in purple yam tuber consists of 89.73% water, 0.62% ash, acid insoluble ash 0.55%, 0.67% fiber content, starch 10.93%, fat 0.82%, and 1.36% protein [2]. *D. alata* also contains diosgenin [3]. Diosgenin, a naturally-occurring steroid saponin is found abundantly in yams (*Dioscorea* sp.) [4]. Saponin diosgenin is similar to cholesterol, progesterone and DHEA (dehydroepiandrosterone) [5]. Saponin diosgenin is a precursor of various synthetic steroidal drugs that are extensively used in the pharmaceutical industry [4]. Recent studies has

indicated that diosgenin in *Dioscorea* species have a biological effects including anti-inflammatory, antitumor, estrogenic, hypocholesterolemic, and immunomodulatory activities [5]. According to Raju [4] research, saponin diosgenin suppresses cancer cell growth through multiple cell signaling events associated with proliferation, differentiation, apoptosis, inflammation and oncogenesis. Diosgenin decreased the elevated cholesterol in serum LDL and HDL fractions in cholesterol-fed rats [4].

Purple yam tuber has potential role as an anti allergenic agent is unknown. Allergy reactions occur when somebody is exposed to allergens that produce IgE antibodies (Immunoglobulin E) and then exposed again by the same antigen. Allergens trigger the activation of mast cells that bind to IgE on the network. IgE is an antibody that is often seen in the reaction against parasites, especially against parasitic worms that are generally prevalent in underdeveloped countries [8]. Allergy or hypersensitivity is too high sensitivity to antigens so that subsequent exposure to antigen will cause excessive immune response. Under normal circumstances there is a balance between Th1 and Th2, but under no circumstances will an increase in allergic Th2 and decreased Th1.

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Ethanol extract of purple tuber uwi very closely related to the digestive process in the body. It is based on the statement Gamiswarna *et al.* [9] that in the pharmacokinetics of each drug in the body undergoes the process of absorption, distribution, metabolism and excretion. Similarly, the purple yam tuber will be absorbed by the intestine, and is metabolized in the liver. Liver is the first organ that is achieved by drugs and other substances that are absorbed through the intestinal portal vein, so it is mentioned that the liver is the main place of drug metabolism and detoxification. The build up of toxic substances in the liver parenchymal cells can injure hepatocytes and causes histopathologic changes varied [10].

It is not known the effect of compounds contained in purple yam tubers (*Dioscorea alata* L.) to the digestive system, especially the liver. So it is necessary to investigate the effects of the ethanol extract of purple yam tuber (*Dioscorea alata* L.) with three doses in the level of CD8⁺ and CD8⁺CD62L⁺ T cells and histological analysis of the liver of mice (*Mus musculus* L.) strain BALB/c.

MATERIALS AND METHODS

This experiment was conducted in May 2013 until Januari 2014 in Laboratory of Animal Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang. The animal experiments were approved by the Animal Care and Use Committee of the Brawijaya University.

Ethanol Extract Preparation of Purple Yam Tuber

The preparation of ethanol extract of *Dioscorea alata* L. tuber were according to a previous study. 20.7 kg of *Dioscorea alata* tubers were peeled off, washed, and then dried underneath the sunlight. Crude was macerated in a glass jar with 70 % ethanol (crude : ethanol = 1:10) at room temperature for 5x24 hours. The ethanol extract then filtered and resoaked with 70 % ethanol (remaseration) for 2x24 hours. Material that has been filtered then evaporated at a temperature of 50°C using a vacuum pump evaporator.

Experimental Animals and Treatment

Twenty adult (3 months old) BALB/c 25-27 g male mice were used. The mice were randomly divided into five groups with each group consisting of 6 mice. The treatments were divided into 5 groups : control without treatment

(N), OVA-sensitized and challenged (OVA), dose 0.167 g/kg bw (U1), dose 2.008 g/kg bw (U2) and dose 10.039 g/kg bw. OVA were administered daily into mice by oral gavage throughout the experiment. Each mouse was sensitized with 0.15 ml of OVA in Al(OH)₃ by intraperitoneal injection on day 15 and later boosted on day 22 followed by repeated challenge with 0.15 ml OVA in aquadest, except for the mice in the N group. And the last injection on day 23 until 28 with OVA by oral injection. Ethanol extract of purple yam tuber were administered on day 1-28 in group U1, U2 and U3.

Flow cytometry Analysis

Flow cytometry analysis was to determine the cell number of CD8⁺ and CD8⁺CD62L⁺. The following purified antibodies were used for extracellular staining is fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and for intracellular staining is PE-conjugated anti-B220. For extracellular staining, pellet resuspended with 50 µl of antibodies in sterile PBS. For intracellular staining, pellet were added with 20 µl cytofix-cytoferm and incubated for 20 minutes, 4 °C. Then added with 500 µl washperm and centrifuged 2500 rpm, 4 °C, for 5 minutes. Pellet was resuspended with 50 µl of antibodies in sterile PBS. Then moved into the cuvette and mounted on the nozzle flowcytometer (BD FACS Calibur™). Do the settings on the computer with BD Cell Quest Pro software™ and carried connection with flowcytometer (acquiring mode).

Histological Examination

The liver specimens of each mice in all groups were fixed in 10% buffered formaldehyde for 24 hours and embedded into paraffin after 16 h of alcohol process. 5 µm thick sections were obtained from the paraffin blocks and stained with hematoxylin and eosin. Each slide was examined under a light microscope.

Statistic Analysis

Data were analyzed using SPSS 16.0 for Windows. One way ANOVA test was used to asses the statistical difference between the N control group, OVA group and the treatment of purple yam tuber ethanol extract groups ($p < 0.05$ was defined as statistically significant). If the obtained results are significant, then it is analyzed with Tukey test.

RESULT AND DISCUSSION

The Relative Number of CD8⁺

Ethanol extract of purple yam tuber was given in mice digestive allergy model showed immunomodulatory activity as immunosuppressant. This activity can be seen through the decrease in the cell number of CD8⁺ (T cytotoxic cells) in different doses. Based on the ANOVA, the relative number of CD8⁺ T cell in lower dose treatment shows significant difference compared to the positive control (OVA) ($p < 0.05$) on day 15 of OVA injection. OVA group showed the highest relative numbers of CD8⁺ T cells compared with negative control (N) with relative number 19.34%. The increase in the number of CD8⁺ cells is caused by OVA as antigen that trigger the immune cells move to sites of inflammation.

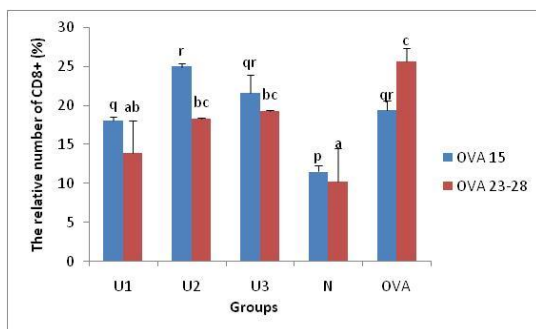


Figure 1. The relative number of CD8⁺ after injection OVA on day 15 and day 23-28.

Description:

N = Normal

OVA = OVA-sensitized and challenged

U1 = *D. alata* dose 1 (0.167 g/kg bw)

U2 = *D. alata* dose 2 (2.008 g/kg bw)

U3 = *D. alata* dose 3 (10.039 g/kg bw)

Dose of 0.167 g/kg bw showed a decrease of memory T cell significantly compared with positive control with relative number 18.01% (Figure 2). Dose of 2.008 g/kg and 10.039 g/kg bw still higher than the positive control (OVA) and gave no significant difference results ($p > 0.05$) with the relative number 24.95% and 21.63%. This suggests that dose of 0.167 g/kg was able to decrease the number of CD8⁺ T cells. CD8⁺ T cells needed to control the cytokines pro-inflammatory and helps the CD4⁺ T cells when the response of CD4⁺ T cells are not able to overcome the antigen in the body, resulting in the proliferation and differentiation of T cells to become cytotoxic T cells did not increase [11].

The decrease in CD8⁺ T cells also can be seen on days 23-23 of OVA injection (Figure 2) at all dose of ethanol extract. The effective dose to reduce the memory T cells is in the lowest dose

(0.167 g/kg bw). The number of CD8⁺ T cells in mice with digestive allergy at the last injection showed an increase of CD8⁺ T cells significantly compared to healthy mice ($p < 0.05$) with relative number 25.84% (Figure 2). These results indicate that at dose 0.167 g/kg with relative number 19.13% is more effective than dose 2.008 g/kg and 10.039 g/kg with relative number 18.28% and 19.31% (Figure 2). The decreasing in the number of CD8⁺ T cells after ethanol extract of purple yam tuber administration presumably because there is an activity of diosgenin in purple yam tuber ethanol extract.

Allergies are caused by systemic oral administration of ovalbumin. In addition, to CD4⁺ T cell population associated with the pathophysiology of digestive allergy, CD8⁺ T cells also play a role [12]. In mice exposed to digestive allergy increased IL-10 mRNA expression and production of IL-10 in MLN (mesenteric lymph node). Cytokine IL-10 is produced by CD8⁺ T cells [13]. Systemic allergen immunization can induce the development of CD8⁺ T cells [14]. Other helper T cell subset, known as Th1 secrete IL-2, TNF, and IFN- γ play a role in the hypersensitive response and inhibit the Th2 response [15]. IFN- γ is a Th1 cytokine that is responsible for inhibiting IL-4-mediated IgE response both in vitro and in vivo [13],[15].

Saponin in *Dioscorea alata* L. affects the activity of CD8⁺ T cells, so that the relative number of CD8⁺ in all treatment groups is significantly different. Mechanism of saponin diosgenin of *D. alata* as immunomodulator decreased the activity of IL-4 and proliferation of T cells. It is known that overproduction of IL-4 is associated with allergies. IL-4 is a cytokine that functions as one of the factor differentiated lymphocytes. IL-4 stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into plasma cells.

According to Huang (2010) research showed that the administration of diosgenin is able to reduce the expression of IL-4 and GATA-3 in intestinal Balb/C mice was sensitized by ovalbumin. Suppressive effect of diosgenin on allergen-induced Th2 response intestinal closely associated with upregulation of Treg cell immunity at the site of inflammation. Diosgenin has allergenic activity in Balb/C mice were sensitization and dichallenge ovalbumin demonstrated by the suppression of IgE production, infiltration and mast cell degranulation [5].

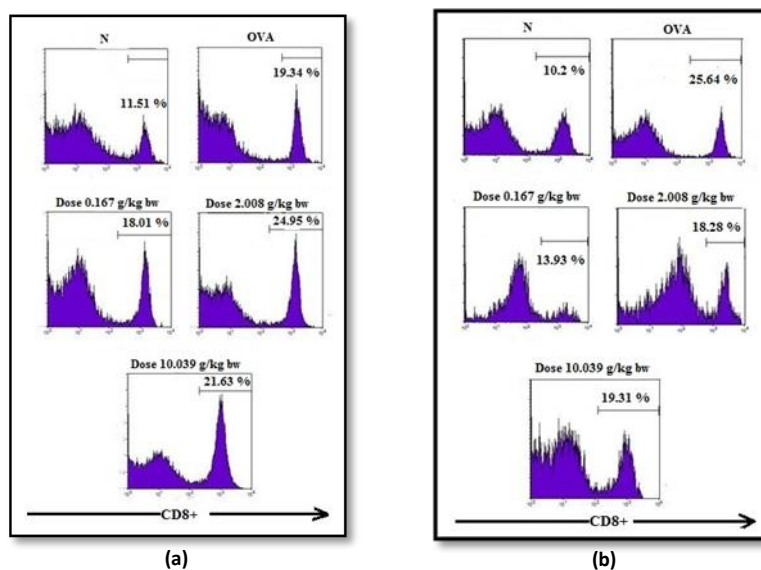


Figure 2. The percentage of relative number of CD8⁺ after injection OVA on day 15 (a) and day 23-28 (b)

The Relative Number of CD8⁺CD62L⁺

Ethanol extract of *Dioscorea alata* L. not only influence the decrease of CD8⁺ T cells in mice with digestive allergy, but also affect the proliferation of naïve T cells. The relative number of CD8⁺CD62L⁺ T cells in a positive control was 2.77 % compared with negative control were 16.49 % on days 15 OVA injection (Figure 4). Treatment with the ethanol extract of purple yam tuber showed the higher proliferation compared with all treatments.

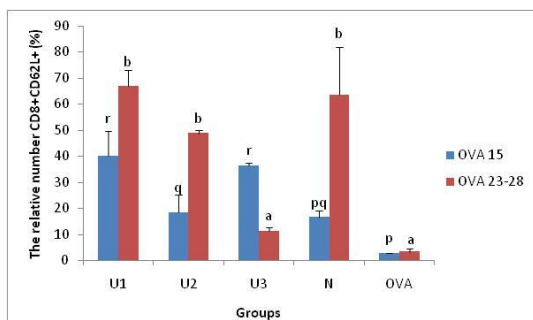


Figure 3. The relative number of CD8⁺CD62L⁺ after injection OVA on day 15 and day 23-28

Description
N = Normal
OVA = OVA-sensitized and challenged
U1 = *D. alata* dose 1 (0.167 g/kg bw)
U2 = *D. alata* dose 2 (2.008 g/kg bw)
U3 = *D. alata* dose 3 (10.039 g/kg bw)

Based on ANOVA, the relative number of naïve T cells in all dose treatment shows significant difference compared to the positive control ($p < 0.05$). However, dose 0.167 g/kg bw and 10.039 g/kg bw have no significant

difference ($p > 0.05$) with relative number 40.18 % and 36.4 % (Figure 4). The lowest dose (0.0167 g/kg bw) stimulate the highest cell proliferation of naïve T cells. This suggests that the active compounds in the extract ethanol of purple yam tuber can reduce the number of CD8⁺ T cells thereby increasing the number of naïve CD8 T cells.

The number of CD8⁺CD62L⁺ T cells in dose 0.167 g/kg bw on days 23-28 in mice digestive allergy showed a decreased significantly compared to positive control ($p < 0.05$) (Figure 3). Based on figure 4 is known that the relative number of CD8⁺CD62L⁺ in mice digestive allergy that fed by ethanol extract of purple yam tuber at dose 0.167 g/kg bw with relative number 67.04 % increased significantly compared with the dose 2.008 g/kg bw and 10.039 g/kg bw ($p < 0.05$) with relative number 48.77% and 11.2 %. This suggests that the treatment of purple yam tuber ethanol extract in small amounts can increase CD8⁺CD62L⁺ mice were exposed to digestive allergy. Allergic diseases are caused by uncontrolled Th2 cells based on the immune response to antigens from the environment. Several studies have shown that the likelihood of damage and weakness function of Treg cells in the pathogenesis of immune response against allergen [16]. CD62L is a marker of cell activation, resulting in a decrease in the number of CD8⁺CD62L⁺ naïve T cells indicate the activity is transformed into a CD8⁺ T cell subsets, such as regulatory T cells as a result of exposure to allergens into the body [6].

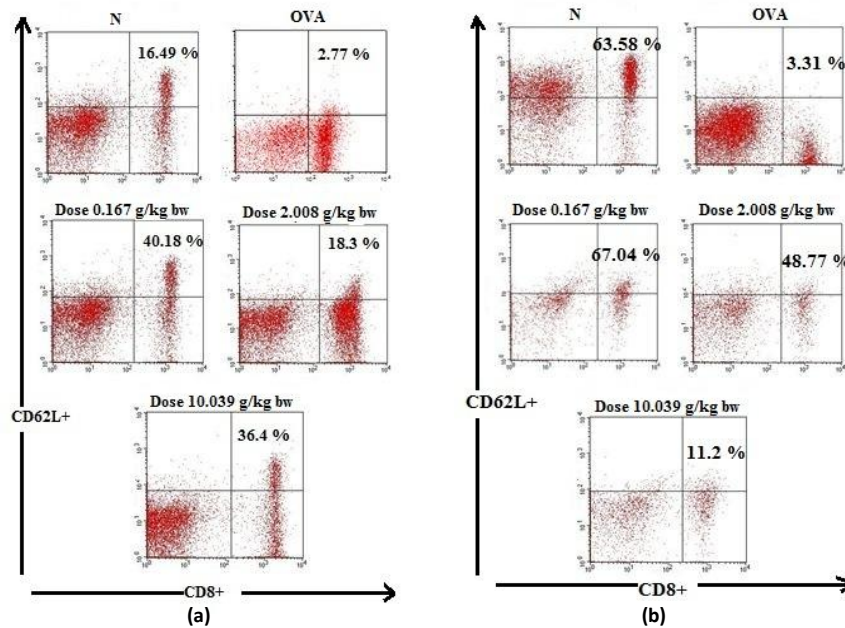


Figure 4. The percentage of relative number of CD8⁺ after injection OVA on day 15 (a) and day 23-28 (b)

Mechanism of action of CD4⁺ as a long-term modulation of the immune system, among others, through the activation of several cytokines that are able to facilitate the development and maturation of CD8⁺ T cells. Cytokines include IL-1, IL-2 and IFN γ . The increase in CD4⁺ T cells influence the activation of CD8⁺ T cells [17]. CD8⁺ response will be more active and function more optimally in the presence of various cytokines released by CD4⁺ T cells [17],[18].

Liver histology after administration of ethanol extract purple yam tuber (*Dioscorea alata* L.)

Histology analysis in mice with digestive allergy (OVA) showed the structure of hepatocytes in abnormal conditions, which is damaged hepatocytes and contained many infiltrating lymphocytes (Figure 5). OVA exposure to the mice caused structural damage of hepatocytes. Hepatocytes damage is shown by necrosis signs in the structure. Necrosis is the incidence of cell death induced by pathological processes. Some causes of cell necrosis are viruses, microorganisms, chemicals, or other dangerous agents [19]. Necrosis is characterized by the presence of DNA fragments which scattered in the cell. The structure in healthy mice with normal conditions (N group) is hexagonal shaped, nucleus in the middle of nuclei (Figure 5).

The structure of hepar cells after administration of OVA showed signs of necrosis, which is the nucleus shrinkage than other

hepatocytes and undergo pyknotic nuclei (Figure 5). Pyknotic is nucleus size shrinkage and nucleoli condensation, so that the nucleus appears solid purple with shrinkage. However, the ethanol extract of purple yam tuber able to reduce the cells undergo pyknotic and infiltration of mononuclear cells. Ethanol extract of purple yam tuber at dose 0.167 g/kg bw and 2.008 g/kg bw can reduce the distribution of lymphocyte infiltration. While the dose of 10.039 g/kg bw was obtained infiltration of mononuclear cells although the amount is not as much as the positive control digestive allergy.

Mononuclear cells, such as lymphocytes or neutrophils were present in the liver tissue structure, generally surrounding the necrotic cells. A collection of cells known as the necrotic foci. This is consistent with the study Huang et al. (2010), that diosgenin has allergenic activity in Balb/C mice were sensitized and dichallenge ovalbumin shown by the suppression of IgE production and infiltration of mast cell degranulation [5]. Necrotic foci in mice exposed to allergen showed the distribution of most digestion (Figure 5).

Liver damage due to toxic substances is influenced by several factors, such as the type of chemicals, doses administered, and the duration of exposure to substances such as acute, subchronic or chronic. The higher concentration of compound, the toxic response caused is greater. Liver damage can occur immediately or after a few weeks to several months. The damage can take the form of hepatocyte necrosis,

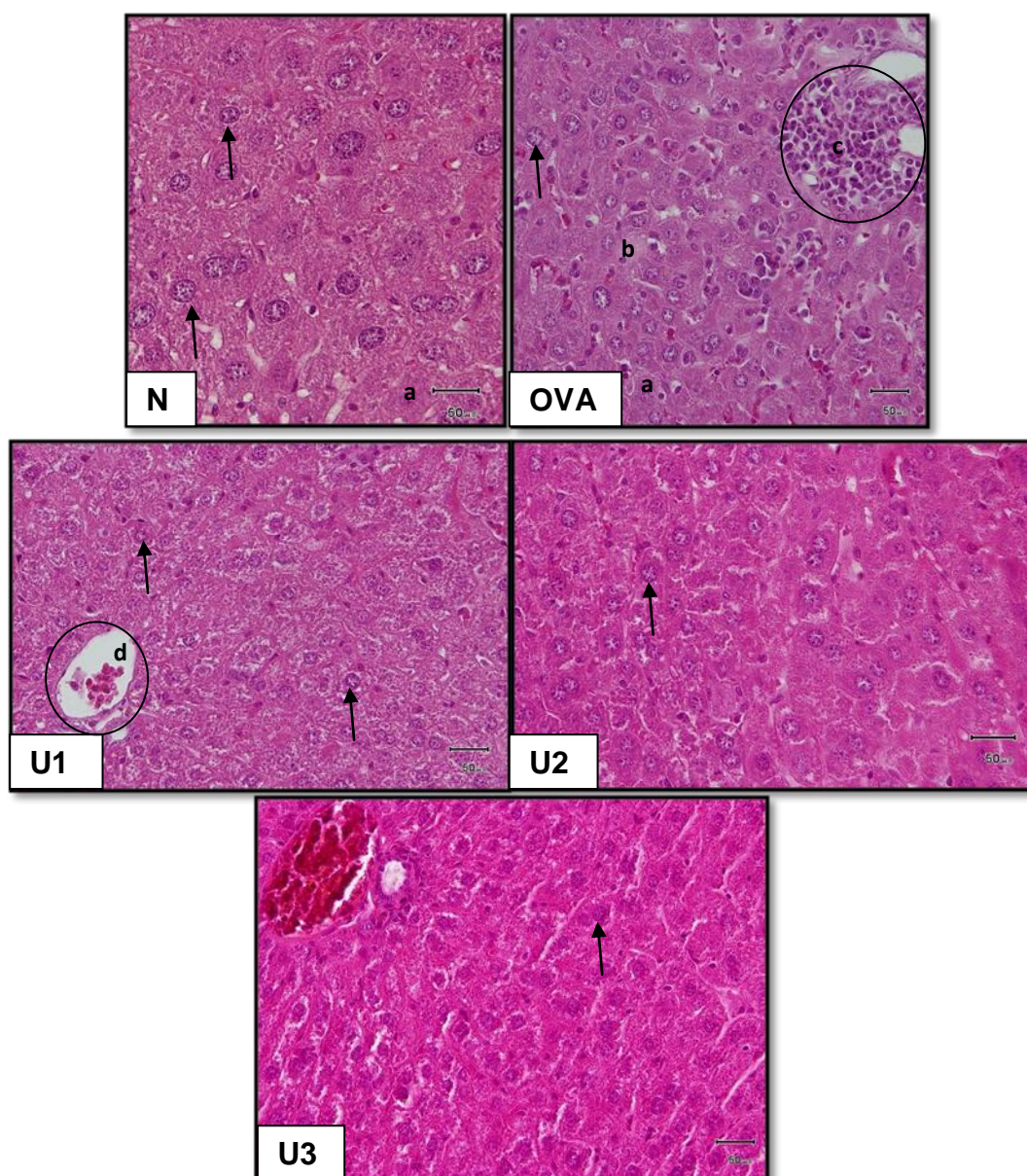


Figure 5. Histology of mice hepar after administration of purple yam tuber ethanol extract
Description; (N): negative control, OVA: positive control, U1 : dose 0.167 g/kg bw, U2 : dose 2.008 g/kg bw and U3 : dose 10.039 g/kg bw; 1 scale = 50 µm; → : normal hepatosit, a. Kariolysis, b. Picnosis, c. Necrotic foci, d. Vena Sentralis

cholestasis, hepatic dysfunction or onset slowly [20]. According to Robins and Kumar [20], liver damage due to chemical compounds characterized by lesions that provide a series of biochemical changes in function and structure. Some changes in the structure of the liver due to chemical compounds that can appear in such microscopic observation, inflammation, fibrosis, degeneration, and necrosis [21]. Although necrosis of liver cells also occurred in the control group but not included in the incidence of pathology because under normal circumstances

necrosis can also occur [22]. Ethanol extract of purple yam tuber reduced the nucleus pyknotic in hepatocytes cells and mononuclear cell infiltration in the liver tissue structure.

CONCLUSION

Ethanol extracts of purple yam tuber reduced CD8⁺ T cells in dose 0.167 g/kg BW and increased naïve T cells in mice digestive allergy. Ethanol extract of purple yam tuber reduced the nucleus pyknotic in hepatocytes cells and mononuclear cell infiltration in the liver tissue structure.

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