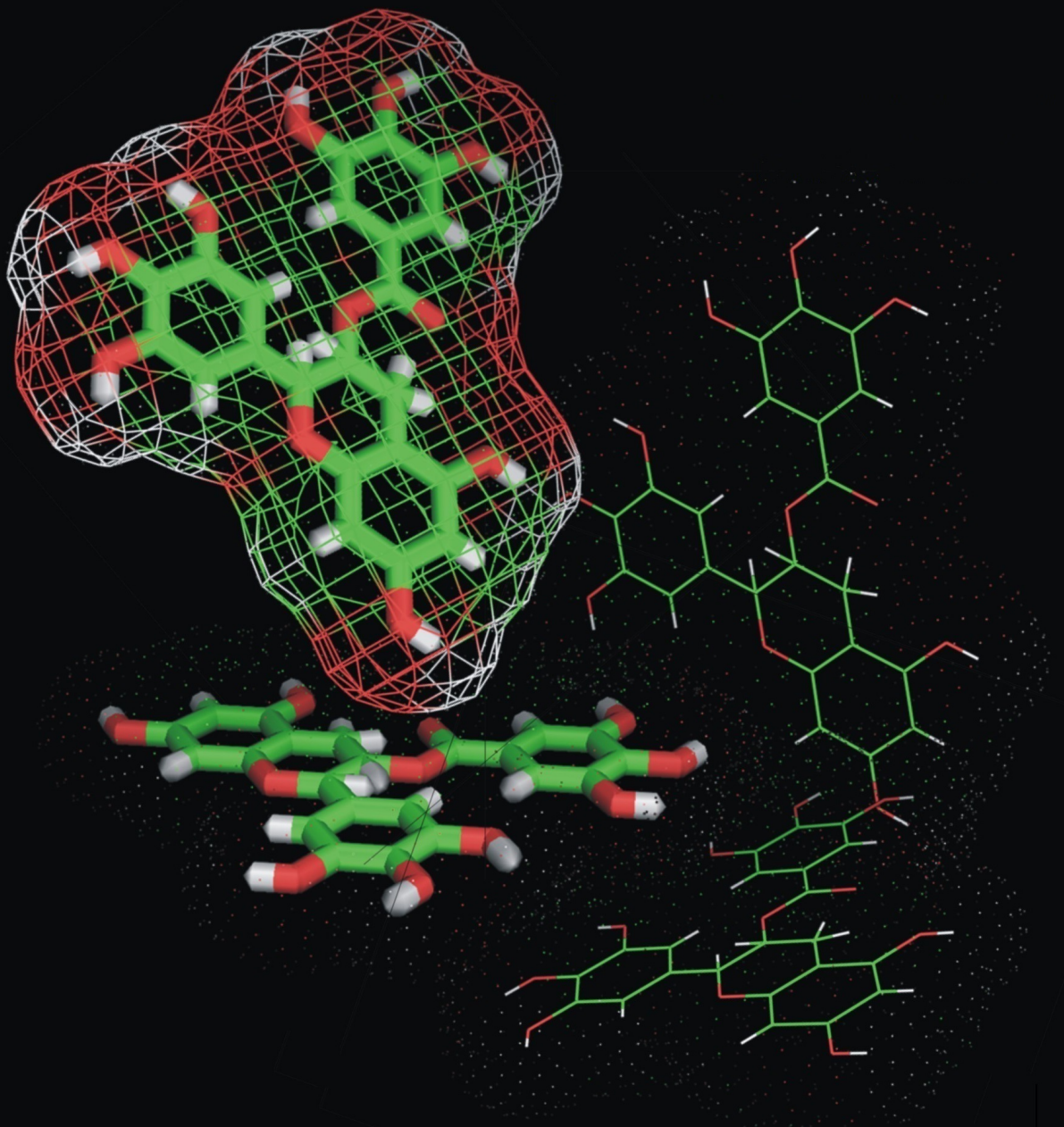
ISSN 2087-2852

The Journal of Experimental Life Science

Discovering Living System Concept through Nano, Molecular and Cellular Biology



J. Exp. Life. Sci. | Vol. 4 | No. 2 | page. 27-55 | Desember 2014

Published by

Graduate Program, University of Brawijaya
in Cooperation With
Masyarakat Nano Indonesia (MNI)

ISSN. 2087-2852
E-ISSN. 2338-1655

The Journal of Experimental Life Science

Discovering Living System Concept through Nano, Molecular and Cellular Biology

Editorial Board

Address
The Journal of Experimental Life Science
Building E, 2nd Floor, Graduate Program, University of Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145
Telp: (0341) 571260 ; Fax: (0341) 580801
Email: jels@ub.ac.id
Web: http://www.jels.ub.ac.id

ISSN. 2087-2852
E-ISSN. 2338-1655

Table of Content

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

Yuyun Ika Christina[1] and Muhaimin Rifa'i[2]*

[1]Graduate Program of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

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.

* Correspondence author:
Muhaimin Rifa'i
Email : rifa123@ub.ac.id
Address : Laboratory of Animal Physiology, University of Brawijaya, Jl. Veteran, Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

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 intracelluler staining is PE-conjugated anti-B220. For extracelluler staining, pellet resuspended with 50 µl of antibodies in sterile PBS. For intraselular staining, pellet were added with 20 µl cytofix-cytoferm and incubated for 20 minutes, 4 ^oC. Then added with 500 µl washperm and centrifuged 2500 rpm, 4 ^oC, 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 hoursand 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 SPPS 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.

ISSN. 2087-2852
E-ISSN. 2338-1655

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 immunosupresant. 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.

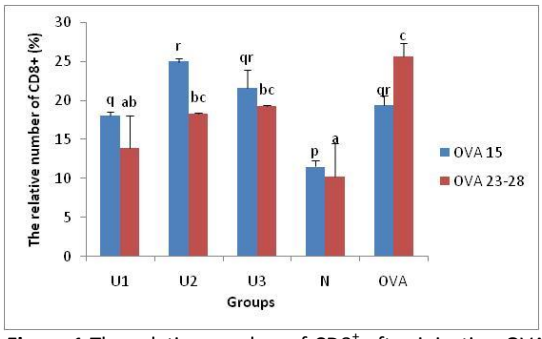


Figure 1. The relative number of $CD8^+$ after injection OVA on day 15 and day 23-28.
Description:
N = Normal
OVA = OVA-sensitized and challenged
U1 = *D. alata* dose 1 (0.167 g/kg bw)
U2 = *D. alata* dose 2 (2.008 g/kg bw)
U3 = *D. alata* dose 3 (10.039 g/kg bw)

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].

ISSN. 2087-2852
E-ISSN. 2338-1655

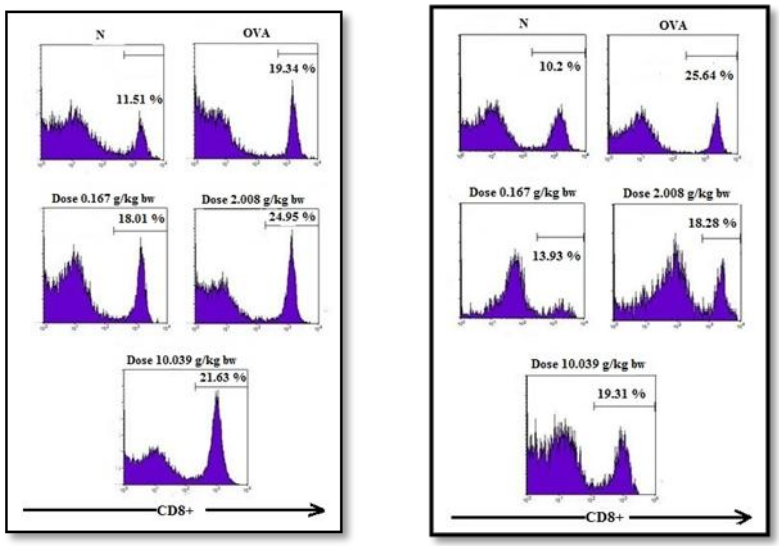


Figure 2. The percentage of relative number of $CD8^+$ after injection OVA on day 15 (a) and day 23-28 (b)

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.

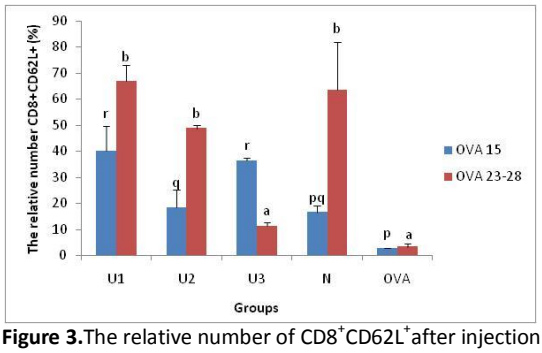


Figure 3. The relative number of $CD8^+CD62L^+$after injection OVA on day 15 and day 23-28

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 positif 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].

ISSN. 2087-2852
E-ISSN. 2338-1655

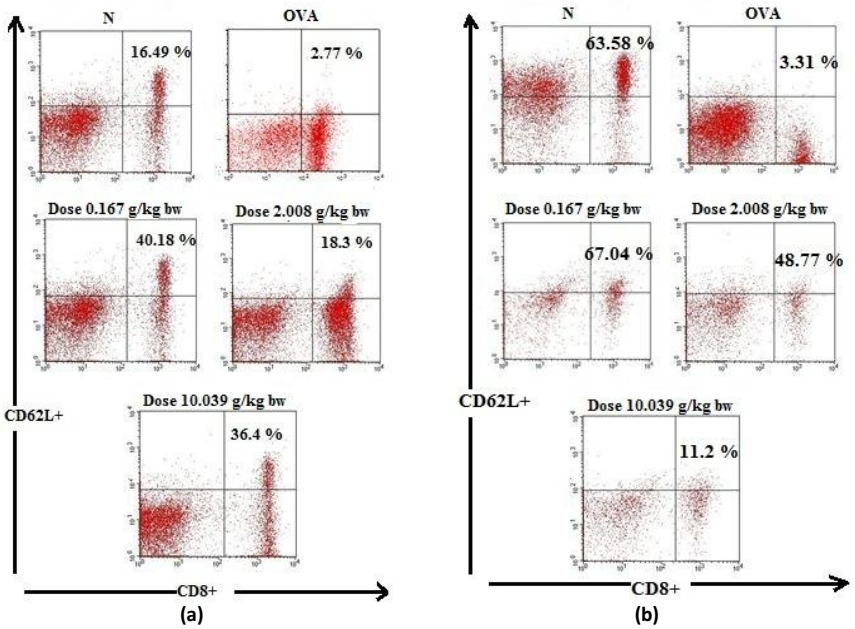


Figure 4. The percentage of relative number of $CD8^+$ after injection OVA on day 15 (a) and day 23-28 (b)

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,

ISSN. 2087-2852
E-ISSN. 2338-1655

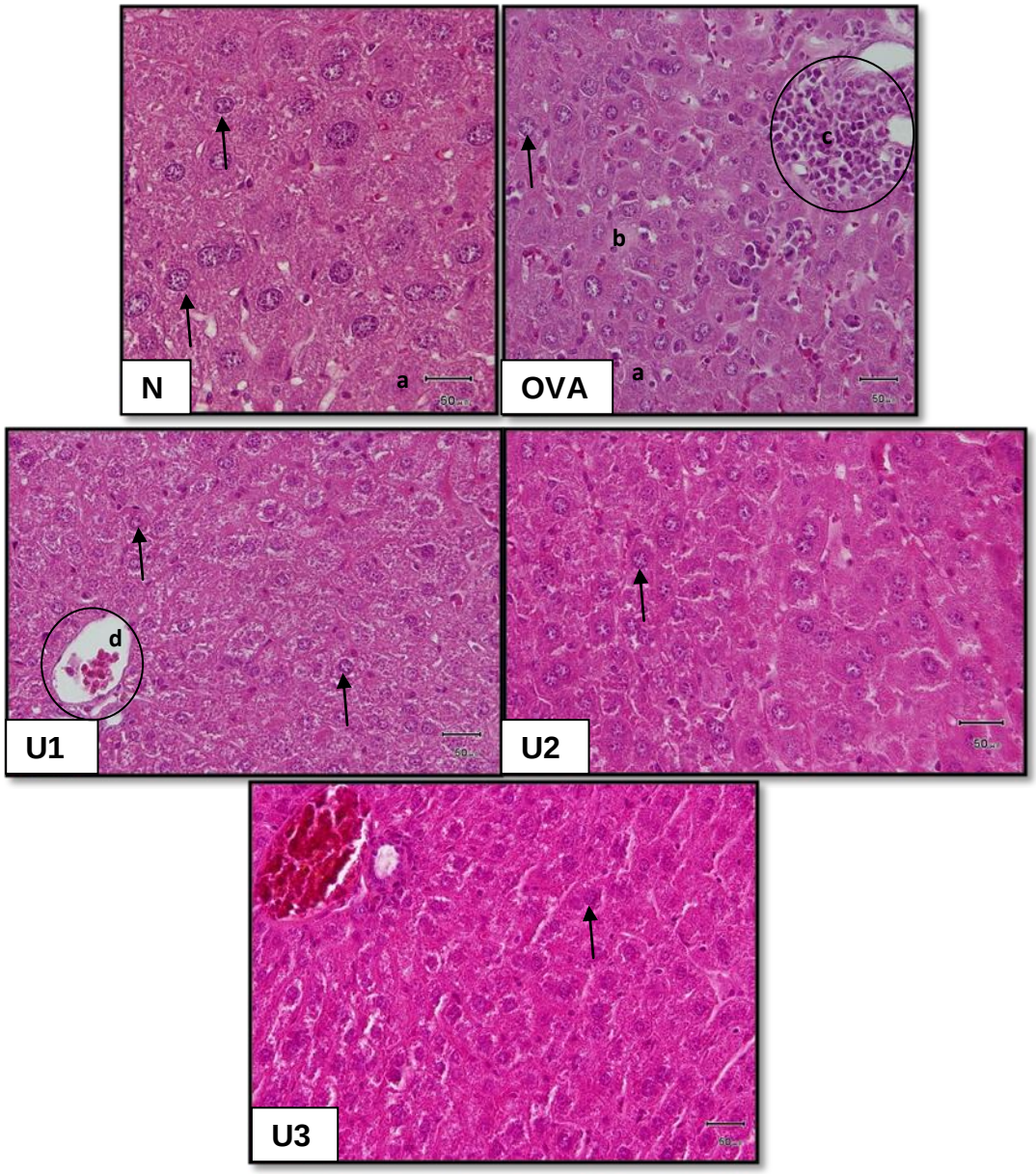


Figure 5. Histology of mice hepar after administration of purple yam tuber ethanol extract
Description; (N): negative control, OVA: positive control, U1 : dose 0.167 g/kg bw, U2 : dose 2.008 g/kg bw and U3 : dose 10.039 g/kg bw; 1 scale = 50 µm; → : normal hepatosit, a. Kariolysis, b. Picnosis, c. Necrotic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

ISSN. 2087-2852
E-ISSN. 2338-1655

ACKNOWLEDGEMENT

The author would like to thank to Mrs. Sri Nabawiyati Nurul Makkiyah who has fund this research.

REFERENCES

[1] Purnomo, B.S. Daryono, Rugayah, I. Sumardi. 2012. Studi Etnobotani *Dioscorea* spp. (Dioscoreaceae) dan kearifan budaya lokal masyarakat di sekitar Hutan Wonosadi Gunung Kidul Yogyakarta. *Jurnal Nature Indonesia.* 14(3). 191-198.

[2] Lubag, A. 2008. Antioxidants of purple and greater yam (*Dioscoreaalata* L.) varieties from the Philippinesmore. *Philippine J. of Sci*. 137(1).61-67.

[3] Cheng, W.Y., Y.H. Kuo, C.J Huang. 2007. Isolation and identification of novel estrogenic compounds in yam tuber (*Dioscorea alata* Cv. Tanjung No. 2). *J. Agric. Food Chem*. 55 (18). 7350-7358.

[4] Raju, J., C.V. Rao. 2012. Diosgenin, a steroid saponin constituent of yams and fenugreek: emerging evidence for applications in medicine, bioactive compounds in phytomedicine. In: Rasooli, I. (Ed.). InTech.

[5] Huang, C.H., C.Y. Ku, T.R Jan. 2010. Diosgenin, a plant-derived sapogenin, enhances regulatory T-cell immunity in the intestine of mice with food allergy. *J. Nat. Prod*. 73. 1033-1037.

[6] Jan, T.R., S.P. Wey, C.C. Kuan, M.H. Liao, H.Y. Wu. 2007. Diosgenin, a plant-derived sapogenin, enchanges regulatory T-cell immunity in the intestine of mice with food allergy. *J. Planta Med*. 73. 421-426.

[7] Benghuzzi, H., M. Tucci, R. Eckie, J. Hughes. 2003. The effects of sustained delivery of diosgenin on the adrenal gland of female rats. *J. Biomed. SciInstrum*. 39. 335-340.

[8] Rifa'i, M. 2011. Alergi dan Hipersensitif. Department of Biology. Faculty of Science. Brawijaya University. Malang.

[9] Gamiswarna, S.G., S. Rianto, F. D. Suyatna, Purwantyastuti, Nafrialdi. 1995. Farmakologi dan terapi. Edisi 4. Department of Pharmacology. Faculty of Medical. University of Indonesia. Jakarta.

[10] Himawan, S. 1992. Patologi. Penerbit Buku Kedokteran EGC. Jakarta.

[11] Wahyuni, N. 2009. Perubahan Kadar Interleukin-2, Jumlah Relatif Sel T CD4+dan CD8+, dan Histopatologi Usus Halus Mencit (BALB/c) Splenectomy paparan Salmonella typhi. Department of Biology. Faculty of Science. Brawijaya University. Malang.

[12] Yamada, A., Y. Ohshima, M. Yasutomi, K. Ogura, S. Tokuriki, H. Naiki, M. Mayumi. 2009. Antigen-primed splenic $CD8^+$ T cells impede the development of oral antigen-induced allergic diarrhea. *J. Allergy Clin. Immunol*. 123 (4). 889-894.

[13] Seymour, W.P., L.J. Gershwin, R.L. Coffman. 1998. Aerosol-induced Immunoglobulin (Ig)-E Unresponsiveness to Ovalbumin Does Not Require $CD8^+$ or T Cell Receptor (TCR)-γ/ T Cells or Interferon (IFN)-gin a Murine Model of Allergen Sensitization. *J. Exp. Med*. 187 (5). 721-731.

[14] Orihara, K., S. Nakae, R. Pawankar, H. Saito. 2008. Role of regulatory and Proinflammatory T-Cell populations in allergic diseases. *WAO Journal*. 9-13.

[15] Rifa'i, M., Z. Shi, S.Y. Zhang, Y.H. Lee, H. Shiku, K. Isobe, H. Suzuki. 2008. CD8+CD12+ regulatory T cells recognize activated T cells via conventional MHC class I–αβTCR interaction and become IL-10-producing active regulatory cells. *Int. immunol.* 20 (7). 937-947.

[16] Dewi, L.K., S. Widyarti, M. Rifa'i. 2012. Pengaruh pemberian ekstrak Etanol daun Sirsak (*Annona muricata* Linn.) terhadap peningkatan jumlah sel T CD4+ dan CD8+ pada Timus Mencit (*Mus musculus*). Department of Biology. Faculty of Science. Brawijaya University. Malang.

[17] Rifa'I, M., Y. Kawamoto, I. Nakashima, H. Suzuki. 2004. Essential roles of CD8+CD122+ regulatory T cells in the maintenance of T cell homeostasis. *The J. of Exp. Med*. 200 (9). 1123-1134.

[18] Junqueira, L., C.J. Carneiro, R.O. Kelley. 2005. Histologi Dasar. 10^{th} Ed. Tambayong, J. (Transl.). Penerbit Buku Kedokteran EGC. Jakarta.

[19] Amalina, N. 2009. Uji toksisitas akut ekstrak Valerian (*Valeriana officinalis*) terhadap hepar Mencit Balb/C. Scientific Papers. Faculty of Medical, Diponegoro University. Semarang.

[20] Robbins, S. L., V. Kumar. 1992. Buku ajar patologi 1. Penerbit Buku Kedokteran EGC. Surabaya.

[21] Kasno, P.A. 2008. Patologi hati dan saluran empedu ekstra hepatik. Balai Penerbit Universitas Diponegoro. Semarang.

ISSN. 2087-2852
E-ISSN. 2338-1655

Adaptive Immune Response Stimulation on Nephrolithiasis Mice Model after Treatment of Tempuyung (*Sonchus arvensis* L.) Leaf Extract

Khoirin Maghfiroh[1], Muhaimin Rifa'i[2], Sri Widyarti[2*]

[1]Master Program of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Calcium crystal accumulation on kidney can cause kidney stone (nephrolithiasis). The oxalate calcium crystal which is deposite on the kidney can trigger inflammation on the epithelial that is able to induce cells death (necrosis). The necrosis is able to cause inflammation and it will affect the body's immune system. Infection agent that comes to the body will be responded by the innate immunity which will be responded later by adaptive immunity. One of herbal agent that is expected to be used to stimulate adaptive immunity response is Tempuyung (*Sonchus arvensis)* leaf extract. The aim of this study is to find out the change of relative amount of $CD4^+$ T cells, $CD8^+$ T cells, and B (B220) cells on nephrolithiasis mice model after *S.arvensis* leaf extract. The mice are divided into six groups; control group, placebo, nephrolithiasis, *S.arvensis* leaf extract for 7 days, nephrolithiasis then it is continued with *S.arvensis* leaf extract for 7 days, and simultaneous (nephrolithiasis and *S.arvensis* leaf extract for 3 months). The amount of relative T lymphocyte cells is measured by using BD FACSCalibur Flowcytometer™. The data is analyzed by using ANOVA one way ($p<0.05$) using SPSS 16.0 software for Windows. The result shows that there are changes of relative $CD4^+$ T cells, $CD8^+$ T cells, and B (B220) cells on nephrolithiasis mice model after the giving of *S.arvensis* leaf extract. The treatment of *S.arvensis* leaf extract on the nephrolithiasis mice model can stimulate the homeostatic activity by suppressing the B cells. Compound of *S.arvensis* leaf extract that can inhibit of Th1 cells and the increase of Th2 cells by proliferation cells activity. The treatment of *S.arvensis* leaf extract for 7 days can suppress $CD4^+$. The *S.arvensis* leaf extract can stimulate adaptive immune response which is caused by immunomodulatory active component.

Keywords: Nephrolithiasis, *Sonchus arvensis* L., leaf extract, adaptive immune response.

INTRODUCTION

Kidney stone is formed by calcium crystallization inside urinary tract and it usually causes pain, urine stoppage, and kidney damage [1]. The crystal formation inside the kidney is normal and is not dangerous if it is not excreted by urine. Any concentration in the urine inside the kidney will maintain water and nutrition and also eliminate excretion residue. Around 1,5 L excreted blood will become urine and there is urine supersaturation alongside two types of salt: calcium phosphate (CaP) and calcium oxalate (CaOx) which form crystal sediment on the process [2]. Adhesion of calcium oxalate crystal causes inflammation through cells death (necrotic) on kidney tubules proximal. Besides, the calcium crystal is also able to induce reactive oxygen species on kidney tubules observation [3].

The change on blood vessel can be overtaken by inflammation process which stimulates leukocyte activation. The inflammation is a response to protect and trigger tissue recovery, but it can also stimulate tissue damage. Natural immunity response is a defense form of body towards inflammation agent of epithelial barrier, phagocyte cells (neutrophils and macrophage), NK (natural killer) cells and cytokine which control and coordinate various activities of the default immunity cells. Innate immune response will trigger specific immune response activity which is played by lymphocyte T and lymphocyte B cells. T cells controls antigen and is essential for adaptive immunity. T cells and B cells are produced in bone marrow while undergoing maturity while T cells has their own in thymus. When inflammation response happens, the antigen which is brought by macrophage will activate $CD4^+$ T cells. The activated $CD4^+$ T cells can activate $CD8^+$ T cells, B cells, macrophage and NK cells. $CD4^+$ T cells stimulate IL-4 secretion so that it can activate B cells and then the cells will differentiates to become plasma cells which produce antibody as a response towards specific immunity [4].

B cells also has signal receptor which is B220 (CD45) [5]. B220 is a B cells marker that comes

* Correspondence author:
Sri Widyarti
Email :swid@ub.ac.id
Address : Dept. of Biology, Faculty of Mathematic and Natural Sciences, University of Brawijaya, Jl. Veteran, Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

from glycoprotein on cells surface of lymphoid. The B220 marker is only found in B cells and bone marrow subset which covers B cells precursor in the progress and B cells differentiation [6].

Immune response modulation is an immuno-modulator activity through response suppression which is known as immunosuppress-ant and immune response increase is known as immunostimulant [7]. The work of immunosti-mulant includes augmentation of anti-inflammation immunity by the cells form of the body immune system which covers lymphocyte subset, macrophage, dendritic and natural killer (NK) cells. The next mechanism occurres through competent cells induction which is involved in adaptive immunity [8]. Immunosuppressant activity through cells suppression which produce cytokine pro inflammation so that it can prevent T cells activation [9]. Wahyudi shown that *S.arvensis* as herbal medicine is the basic material which is proven to be able to destroy kidney stone [10]. Up to now the mechanism of *S.arvensis* in modulating immune response is still unknown; that is why scientific study about the plant's immunomodulator activity is needed.

MATERIALS AND METHODS

Ethical Clearence

All experimental procedures were performed with the approval of the ethic committee of research of the Brawijaya University, ethical clearance no.127-KEP-UB.

Mice

Specific pathogen free 2-months-old male BALB/c mice were obtained from the Galaxy Science, Wringinangung Jombang Jember, Indonesia. Mice were acclimated for 7 days before treatment. Mice were maintained in animal chamber pathogen free.

Induction of Nephrolithiasis

Nephrolithiasis model mice were made by administrated with 6 mg/100g BW/day of porang tuber (*Amorphopallus muelleri)* powder orally for 3 months [11].

Treatment of Tempuyung (*Sonchus arvensis*) Leaf Extract

Giving 3.3 mg/g BW/ day of *S.arvensis* leaf extract orally for 7 days. As a placebo, hydrochlorothiazide administrated orally with 0.00142 mg/g BW / day for 6 days [12].

Lymphocytes Cells Isolations

Experimental animals were killed by neck dislocation, sectioned using standard method. The spleen was removed and washed three times with sterile PBS, transferred another petri dish which containing PBS. The spleen crushed by syringe base and then suspension are filtered with BD cells strainer and transfered into propylene tube. Then added PBS until 10 ml and centrifuged by 2500 rpm for 5 minutes in 4°C. Pellet resuspended in 1 ml PBS. Cells suspension is taken 200 µl and transferred into micro tube and added with 500 µl PBS, and then it is centrifugated by 2500 rpm for 5 minutes in 4°C. The pellet is incubated with antibodies : washperm 1 : 200 [13].

Antibody Staining and Flow Cytometry Analyze

Antibody are FITC – conjugated anti-mouse CD4, PE – conjugated anti-mouse CD8 and PE-conjugated anti-B220. Analytical flow cytometry were performed by using FACS Calibur flow cytometer (BD Bioscience). Preparative cells sorting were performed by using FACS Vatage cells sorter (BD Bioscience).

Statistical Analysis

The data is analyzed by using one way ANOVA (Analysis of Variance) ($p<0,05$) with SPSS (Statistical Product and Service Solution) 16.0 software, followed by Tukey HSD.

RESULT AND DISCUSSION

$CD4^+$ and $CD8^+$ T cells Profiles

The nephrolithiasis treatment can increase significantly $CD4^+$ T cells profile by 20.29±0.89%. Calcium crystal can stimulate secretion of pro inflammation cytokine through NLRP3 (NOD like receptor). Activation of NLRP3 can causes recruitment to the inflammasome triggers caspase-1 for cleves pro inflammatory cytokine into their active and secreted forms. The pro inflammatory cytokine secretion is stimulated by $CD4^+$ T cells activation [14,15,16]. $CD4^+$ T cells profile is T helper (Th) cells which has matured because of activation of antigen exposure from the outside. Macrophage brings antigen and it is brought to be represented through MHC II. The process is adaptive immunity response as a response towards antigen exposure [7].

$CD4^+$ T cells activity increase after *S.arvensis* leaf extract treatment and nephrolithiasis induction is not different significant. But, there is $CD4^+$ T cells profile decrease for 18.77±0.85%. The research in line also argued that flavonoid

ISSN. 2087-2852
E-ISSN. 2338-1655

can suppress $CD4^+$ T cells and $CD8^+$ T cells activation through activation suppression of nuclear factor kappa beta (Nf-Kβ) [17]. $CD4^+$ T cells activity increase after *S.arvensis* leaf extract treatment and nephrolithiasis induction is not different significant. But, there is $CD4^+$ T cells profile decrease for 18.77±0.85%. The research in line also argued that flavonoid can suppress $CD4^+$ T cells and $CD8^+$ T cells activation through activation suppression of nuclear factor kappa beta (Nf-Kβ) [17]. The treatment of *S.arvensis* leaf extract after nephrolithiasis induction is not different significantly from placebo group with average 7.52±0.65%. The 7 days treatment of *S.arvensis* leaf extract is different significantly in decreasing $CD4^+$ T cells profile for 7.58±0.14%.

Flavonoid is able to obstruct Nf-kβ and MAPK (mitogen activated protein kinase) activation. Nf-kβ transcription factor obstruction through ERK1/2 (extracellsular signal-regulated kinase) regulation decrease, JNK (Jun N-terminal kinase), and protein p38 signaling pathways [18]. MAPK obstruction is related to the activation of macrophage activity which synthesizes cytokine pro inflammation. Besides, it is effectively able to decrease MCP-1 (monocyte chemoattractant protein) and ICAM-1 (intracellsular adhesion molecule) regulation which have important role to activate lymphocyte [7,19]. Treatment of *S.arvensis* leaf extract simultaneously on the average 14.37±0.27% of $CD4^+$ T cells. This were not significant difference.

The activated $CD4^+$ T cells can stimulate cytokine IL-2 secretion to activate $CD8^+$ T cells. The treatment of *S.arvensis* leaf extract for 7 days and it significantly can decrease the $CD8^+$ T cells profile for 10.14±0.17% (Fig.1). Activity of compound flavonoid in the plants extract can suppress $CD8^+$ T cells activity. Nephrolithiasis group with average 16.13±0.37% of $CD8^+$ T cells is not significantly different from control gorup. The treatment of the *S.arvensis* leaf extract after nephrolithiasis induction 11.23±1.16% is not significantly different from nephrolithiasis group and control group but there is a decrease of $CD8^+$ T cells profile. The treatment of the extract simultaneously on average 10.97±0.74% is not significantly different in affecting $CD8^+$ T cells profile change. Quercetin activity from flavonoid can suppress $CD8^+$ T cells activity through TCF 1 (T cells Factor) or β catenin obstruction via P13K/AKT/ERK pathway [20]. TCF-1 has a role in expanding selection positive in Thymus [21]. $CD8^+$ T cells proliferation is stimulated by IL-2 secretion. Flavonoid can decrease IL-2 secretion so that it also can decrease $CD8^+$ T cells activity [22].

B Cells Profile

B cells profile indicates the amount of B cells which has matured to produce antibody. Based on the data in Figure 2, it is shown that nephrolithiasis significantly can decrease B cells profile by 50.34±0.66%. However, it is not significantly different from placebo group. The treatment of *S.arvensis* leaf extract after nephrolithiasis induction significantly can decrease B cells profile by 43.77±1.50%. B cells profile significantly increases 69.88±0.58% in the 7 day treatment. B cells profile increase significantly in the 7 days treatment for the *S.arvensis* leaf extract seems to be able to increase the Th2 proliferation and prevent Th1 cells activation through TGFβ stimulation.

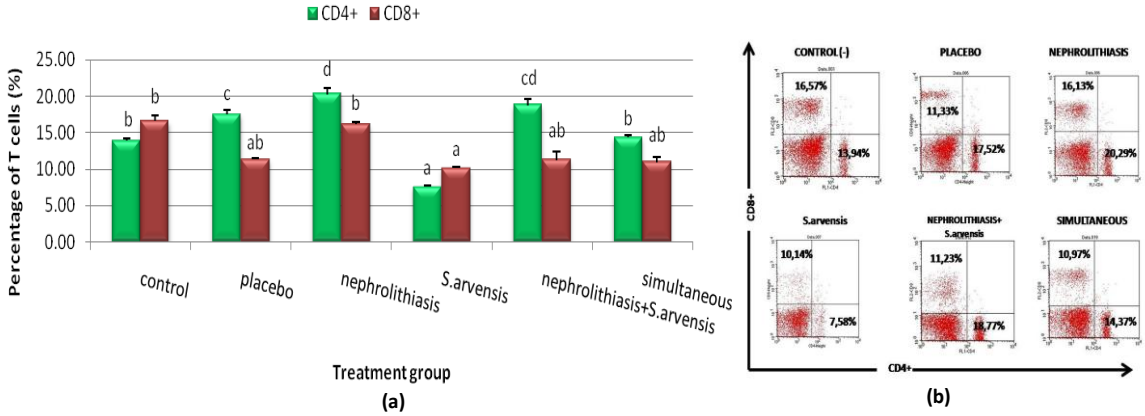


Figure 1. $CD4^+$ T cells and $CD8^+$ T cells in BALB/c mice spleen. (a) Relative numbers of each $CD4^+$ T cells and $CD8^+$ T cells subpopulation derived from BALB/c mice determined by FACS analysis. Data are mean ± SD values of three mice in each group. (b) Spleen cells were obtained from 13-wk-old BALB/c mice, stained with indicated fluorescence-conjugated antibodies, and analyzed by flow cytometry. Percentages of $CD4^+$ T cells and $CD8^+$ T cells are shown in each panel.

ISSN. 2087-2852
E-ISSN. 2338-1655

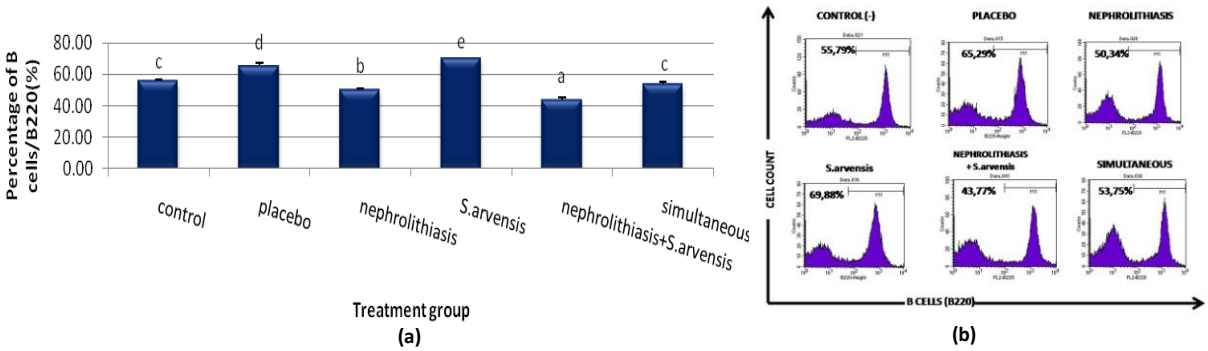


Figure 2. B cells in BALB/c mice spleen. (a) Relative numbers of each B cells subpopulation derived from BALB/c mice determined by FACS analysis. Data are mean ± SD values of three mice in each group. (b) Spleen cells were obtained from 13-wk-old BALB/c mice, stained with indicated fluorescence-conjugated antibodies, and analyzed by flow cytometry. Percentages of B cells are shown in each panel.

Flavonoid in the *S.arvensis* leaf extract suggest can stimulate TGF-β. The treatment of *S.arvensis* leaf extract after nephrolithiasis is significantly decrease B cells profile. It is estimate that *S.arvensis* leaf extract can modulate immune response through increasing Th1 and Th2. Th1 and Th2 become the down-regulator and up-regulator each other to keep the homeostatic [23]. Treatments of nephrolithiasis induction can suppression significantly B cells. It is suspect that proliferation after CaOx accumulation in nephrolithiasis and suppression of Th2 proliferate can cause suppression B cells production.

Nephrolithiasis followed by *S.arvensis* leaf extract treatment can suppression significantly B cells profile. It is suspect that *S.arvensis* leaf extract can modulate immunity response through increase homeostatic of Th1 and Th2[24]. Flavonoid is suspected can stimulate of proliferation Th-2 to suppress of Th-1. The Th-2 cytokine is role plays stimulatory B cells for antibody secretion[8].

CONCLUSION

S.arvensis leaf extract can stimulate adaptive immunity response by suppressing $CD4^+$ T cells and $CD8^+$ T cells activation. Besides, the suppression of B cells can induce the homeostatic activity. The *S.arvensis* leaf extract can increase immunomodulator activity in nephrolithiasis.

ACKNOWLEDGEMENT

This work was supported by grants from herbal company PT. Perusahaan Jamu Tradisional DR. Sardjito in Yogyakarta, Indonesia.

REFERENCES

[1] Yang, X. 2011. Effects of traditional Hmong herbal tea on urinary parameters associated with kidney stones. *UW-L J. of Under-graduate Res. XIV*.

[2] Verkoelen, C.F. 2006. Crystal retention in renal stone disease: a crucial role for the Glycosaminoglycan Hyaluronan?. *J. Am. Soc. Nephrol.* 17. 1673-1687.

[3] Schepers, M.S., E.S. van Ballegooijen, C.H. Bangma, C.F. Verkoelen. 2005. Crystals cause acute necrotic cell death in renal proximal tubule cells, but not in collecting tubule cells. *Kidney Int.* 68(4). 1543-53.

[4] Abbas, A.R., A.H. Lichman, J.S. Pober. 2000. Cellular and Molecular Immunology, 4th Ed. WB Saunders Company.

[5] Rodig, S.J., A. Shahsafaei, B. Li, D.M. Dorfman. 2005. The CD45 isoform B220 identifies select subsets of human B cells and B-cell lymphoproliferative disorders. *Hum Pathol.* 36(1). 51-57.

[6] Bell, J., D. Gray. 2003. Antigen-capturing cells can masquerade as memory B cells. *J. Exp. Med*. 197. 1233–1244.

[7] Janeway, C.A.Jr., T. Paul, W. Mark, J.S. Mark. 2001. Immunoglobuly, 5th Edition The immune system in health and disease. Garland Sciences, New York.

[8] Nijkamp, F.P., J.P. Michael. 2005. Principle of Immunopharmocology. 2nd revised and extended. Birkhauser Verlag, Germany.

[9] Noh, C., D.C. Woon, S. Sancheti, M.L. Jeong, J.L. Young, G.S. Hyung, H.C. Jung. 2012. Anti-inflammatory and immunosuppressive activity of mixture of Trachelospermum asiaticum and Paeonia suffruticosa extracts (novel herbal formula SI000902). *J. of Med.l Plants Res.* 6(25). 4247-4254.

[10] Wahyudi, B. 1986. Efek menghambat pembentukan batu kandungan kemih buatan dari infus tempuyung (*Sonchus*

ISSN. 2087-2852
E-ISSN. 2338-1655

arvensis L.) pada tikus putih. *Medika.* 12 (10).

[11] Rosyida, A., S. Widyarti, S. Rahayu. 2013. Accumulation of calcium crystal in Rat (*Rattus norvegicus*) kidney. *J. of Trop. Life Sci*. 3 (1).

[12] Yendt, E.R., M. Cohanim. 1978. Prevention of calcium stones with thiazides. *Kidney Int.* 13. 397—409.

[13] Rifa'i, M. 2007. Essential roles of $CD4^+CD25^+FOXP3^+$ Regulatory T Cells in the Allogeneic Bone marrow Transplantation. *J. of Indonesia Sci*. 1. 347-352.

[14] Mulay, S.R., O.P. Kulkarni, K.V. Rupanagudi, A. Migliorini, M.N. Darisipudi, A. Vilaysane, D. Muruve, Y. Shi, F. Munro, H. Liapis, H.J. Anders. 2013. Calcium oxalate crystals induce renal inflammation by NLRP3-mediated IL-1 β secretion. *J. Clin. Invest.* 123(1). 236–246.

[15] Elyaman, W., P. Kivisa kk, J. Reddy, T. Chitnis, K. Raddassi, J. Imitola, E. Bradshaw,V.K. Kuchroo, H. Yagita, M.H. Sayegh, and Samia J. Khoury. 2008. Distinct functions of Autoreactive memory and effector CD4+ T Cells in experimental Autoimmune Encephalomyelitis. *The Am. J. Pathol.* 173 (2).

[16] Anders, H.J., A.M. Daniel. 2011. The Inflammasomes in Kidney disease. *J. Am. Soc. Nephrol.* 22. 1007–1018.

[17] Song, B., S. Guan, J. Lu, Z. Chen, G. Huang, G. Li, Y. Xiong, S. Zhang, Z. Yue, X. Deng. 2013. Suppressive effects of fisetin on mice T lymphocytes in vitro and in vivo. *J. Surg. Res.* 185(1). 399-409.

[18] Hong, G.E., J.A. Kim, A. Nagappan, S. Yumnam, H.J. Lee, E.H. Kim, W.S. Lee, S.C. Shin, H.S. Park, G.S. Kim. 2013. Flavonoids identified from Korean *Scutellaria baicalensis* Georgi inhibit Inflammatory signaling by suppressing activation of NF-kβ and MAPK in RAW 264.7 Cells. *Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine.* Volume 2013, Article ID 912031.

[19] Sheryanna, Abdulmunem, G. Bhangal, J. McDaid, J. Smith, A. Manning, B.M.J. Foxwell, M. Feldmann, H.T. Cook, C.D. Pusey, F.W.K. Tam. 2007. Inhibition of p38 Mitogen-Activated Protein Kinase Is effective in the treatment of experimental Crescentic Glomerulonephritis and suppresses Monocyte Chemoattractant Protein-1 but Not IL-1 or IL-6. *J. Am. Soc. Nephrol* .18. 1167–1179.

[20] Lee, Do Y., Beom K. Choi, Don G. Lee, Young H. Kim, Chang H. Kim, Seung J. Lee, Byoung S. Kwon. 2013. 4-1BB Signaling Activates the T Cell Factor 1 Effector/β-Catenin Pathway with delayed Kinetics via ERK signaling and delayed PI3K/AKT activation to promote the proliferation of $CD8^+$ T Cells. *Plos One,* 3 (7).

[21] Schilham, W. Marco, A. Wilson, P. Moerer, J.B.T. Barry, A. Cumano, H.C. Clevers. 1998. Critical involvement of Tcf-1 in expansion of Thymocytes. *The J. Immunol*. 161. 3984 – 3991.

[22] Cano, F.J.P., M.C. Malen, F. Àngels, C. Cristina, C. Margarida. 2013. The effects of cocoa on the immune system. *Frontiers in Pharmacol.* 4. Article 71.

[23] Kishore, R., J.M. Tebo, M. Kolosov, T.A. Hamilton. 1999. Cutting edge: clustered AU-rich elements are the target of IL-10-mediated mRNA destabilization in mouse macrophages. *J. Immunol.* 162.2457–61.

[24] Rifa'i, M. 2011. Autoimmune and Bioregulator. UB Press, Malang.

ISSN. 2087-2852
E-ISSN. 2338-1655

Bioactivity of Ethanolic Extract of Propolis (EEP) in Balb/C Mice's $CD4^+CD25^+$ and $B220^+$ Lymphocyte Cells

Aisyah Zahroh Aden, Muhaimin Rifa'i*

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

This experiment was aimed to determine the bioactivity of ethanolic extract of propolis (EEP) against the changes in the quantity of $CD4^+CD25^+$ and $B220^+$ lymphocytes and determine the optimal dose of EEP to increase the number of $CD4^+CD25^+$ and $B220^+$ cells in Balb/c mice. Balb/c mice were divided into four treatment groups: control treatment, treatments of EEP at a dose of 50 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 200 $mg.kg^{-1}$ body weight of mice. All mice were dissected after two weeks post treatment. Profiles of lymphocytes from the spleen expressing $CD4^+CD25^+$ and $B220^+$ cells were analyzed by flowcytometry using CellQuest software. Data was analyzed by Kruskall Wallis and Mann Whitney statistical test with $P<0.05$ using SPSS 16.0 for Windows. The results showed that the treatment of dose of 50 $mg.kg^{-1}$ of EEP can increase relative number of $CD4^+CD25^+$ cells significantly, but those cells decrease significantly when we apply the dose of 100 and 200 $mg.kg^{-1}$. The relative number of $B220^+$ cells increase in the dose of 50 and 200 $mg.kg^{-1}$ and decrease in the dose of 100 $mg.kg^{-1}$ compared to the control. This experiment suggest that EEP has bioactivity to modulate the quantity of $CD4^+CD25^+$ and $B220^+$ in dependent manner.

Keywords: $B220^+$, $CD4^+CD25^+$, lymphocyte, propolis

INTRODUCTION

Propolis is a natural product from a mixture of plant derived products, which modified and used by bees for various needs. Bees use propolis in nature as antibiotics and protective materials from the threat of drought to their nests [1]. Propolis contains resin, fatty acids, waxes, proteins, polysaccharides, hydrocarbons, and various other organic compounds [2,3]. Propolis as a traditional medicine used by people as an anti-inflammatory, antibacterial, antiparasitic, antifungal, antitumor, antioxidant, and have immunomodulatory effects [1].

Immunomodulator is one mechanisms that needed in the body's immune system. Immunomodulatory activity is a form of biological or pharmacological effects on various factors in the immune response. The purpose of immunomodulatory itself is to modulate the homeostasis of immune system. Immunomodulatory activity also used to treat and prevent various diseases originating from any occurred imbalance. The activities of immunomodulators are immunosuppressants and immunostimulant [4]. Immunomodulatory effects of propolis was known better compared to other immunomodulatory substance that has only flavonoid components as main substances [5]. A number of clinical studies have proved that propolis has the ability to activate the body's immune system in mice and humans. This is supported by experiment data that showed an increase in the secretion of IL1, IL2, and IL4, increased antibody responses, cell proliferation of T lymphocytes, increased ratio of CD^+/CD^+, and activation of macrophages [1].

The effect of propolis on CD4 T cell differentiation process has not known yet [6]. CD4 T cell activation is also known to be influential in the process of B cell proliferation [7]. This study was conducted to determine the bioactivity of EEP on production of $CD4^+CD25^+$T cells and $B220^+$ B cells in the spleen organ of Balb/c mice.

MATERIALS AND METHODS

This experiment was conducted on September 2013 until June 2014 at Laboratory of Animal Physiology and Animal Room, Department of Biology; Biomedical Laboratory and Laboratory of Pharmacology, Faculty of Medicine, University of Brawijaya, Malang. There are four treatment groups that used in this experiment i.e. negative control, EEP dose 50, 100, and 200 $mg.kg^{-1}$ body weight of mice.

Materials and Equipments

The materials which were used in this experiment are ethanolic extract of propolis

* Correspondence author:
Muhaimin Rifa'i
Email : rifa123@ub.ac.id
Address : Dept. of Biology, Faculty of Mathematic and Natural Sciences, University of Brawijaya, Jl. Veteran, Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

(EEP), Balb/c mice, mineral water, pellets BR-1, sterile distilled water, 70% alcohol, sterile PBS, and the monoclonal antibody (rat anti-mouse CD-4, CD-25, and B220). The equipments which were used in are cage of mice, masks, scales, spatulas, erlenmeyer glass, oral administration tool, sectio sets, surgical board, petri dish, micropipette, mikrotube, propylene tubes, yellow and blue tip, centrifugation, flowcytometry cuvette, and FACS Calibur™ flowcytometry.

Animal

Mice (*Mus musculus*) strains Balb/c 8 weeks old in healthy condition with ± 25 g body weight. Mice were purchased from mice farmers in Jember with pathogens free certification. Acclimatization mice were performed for seven days before giving the treatment.

Propolis Extraction

Propolis obtained from local bee keepers in Lawang, Malang, Indonesia. Propolis extraction process performed by maceration method. Clumps of propolis were collected from the hiveside. The propolis weighed as much as 200 grams and then maserated with 1 L of absolute ethanol. The solution of propolis maceration was filtered and evaporated for ± 1.5-2 hours. The resulting ethanolic extract of propolis was filtered and evaporated in a heated dish in the oven. The EEP is placed in a bottle and stored in a refrigerator with temperature of 4°C.

Oral Administration of EEP in Balb/c Mice

Each of mice in the treatment groups were given EEP with various doses (doses of 50, 100, and 200 $mg.kg^{-1}$ body weight of mice) for 14 days. The EEP diluted with mineral water in accordance with the need of each dose then given to mice orally. Group of control mice was not given oral administration of EEP treatment.

Animal Dislocation and Spleen Organ Isolation

Mice were killed by neck dislocation. Mice that had been killed was dissected in their dorsal part to isolate the spleen organ. Spleen which has been isolated then was washed twice and soaked in sterile PBS.

Flowcytometry Analysis

Spleen organ was crushed in a sterile petri dish containing sterile PBS. Spleen homogenates were filtered using wire and placed in propylene was then centrifuged at 2500 rpm speed, with the temperature of 4°C for 5 minutes. Pellets were separated from the supernatant was then added with 1 ml of sterile PBS and homogenized. The second homogenates were then taken for 50 mL and placed in mikrotube containing 500 mL of sterile PBS. Homogenate in mikrotube was centrifuged again at 2500 rpm speed, with the temperature of 4°C for 5 minutes. Pellets then added and incubated with solution of 50 mL monoclonal antibodies (rat anti-mouse CD4, rat anti-mouse CD25, and rat anti-mouse B220) respectively, which is specific to cells expressing $CD4^+CD25^+$ and $B220^+$. The number of lymphocytes cells expressing $CD4^+CD25^+$ and $B220^+$ was then calculated by inserting the cuvette containing pellet and antibodies was resuspended with 300 mL PBS into flowcytometry tool (FACS Calibur™ flowcyto-metry) that has been setting before.

Data Analysis

Data were analyzed with CellQuest software and were further tested with Kruskall Wallis test ($p<0.05$). The data show significant test results continue tested by Mann Whitney test. Data analysis was done using SPSS 16.0 for Windows.

RESULT AND DISCUSSION

Bioactivity of EEP on $CD4^+CD25^+$T Cells

Changes in the relative number percentage of $CD4^+CD25^+$ T cells to the lymphocyte cells population due to the effect of EEP between treatment groups generally show a decline pattern based on the results of flowcytometry analysis (Figure 1). The EEP in the treatment of D1 group is not giving significant effect on changes in the relative number of $CD4^+CD25^+$ T cells to the lymphocyte cells population when compared with the control group. The relative number percentage of $CD4^+CD25^+$ T cells in the control group by 0.97% is not different if it's compared to the group of D1 by 0.98%.

Various doses of EEP can give significant effect ($P<0.05$) on decrease in the relative number of $CD4^+CD25^+$ T cells population between D1, D2, and D3 treatment groups based on the Mann Whitney test (Figure 2). The lowest relatif number of $CD4^+CD25^+$ T cells affecting by giving EEP was seen in the D3 treatment group with a percentage of 0.56%. These results showed that the EEP doses of 100 and 200 $mg.kg^{-1}$ body weight of mice can effect of reducing the relative number of $CD4^+CD25^+$ T cells whereas a dose of 50 $mg.kg^{-1}$ influential in increasing the relative number of $CD4^+CD25^+$ T cells even though it is not significantly when compared to the control.

ISSN. 2087-2852
E-ISSN. 2338-1655

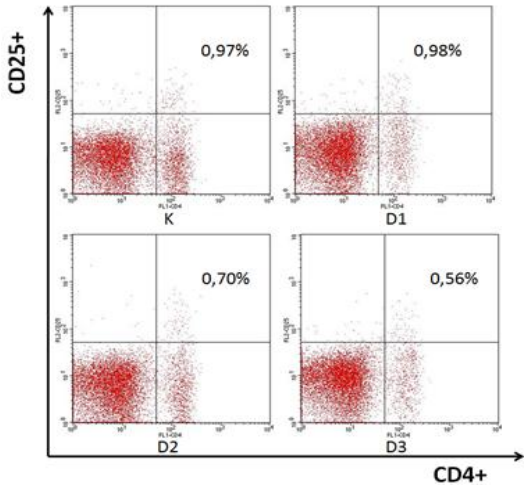


Figure 1. Profile of relative number percentage of $CD4^+CD25^+$ T cells to the lymphocyte cells population flowcytometry analysis results between groups (K= control, D1 = EEP at dose of 50 $mg.kg^{-1}$, D2 = EEP at dose of 100 $mg.kg^{-1}$, and D3 = EEP at dose 200 $mg.kg^{-1}$).

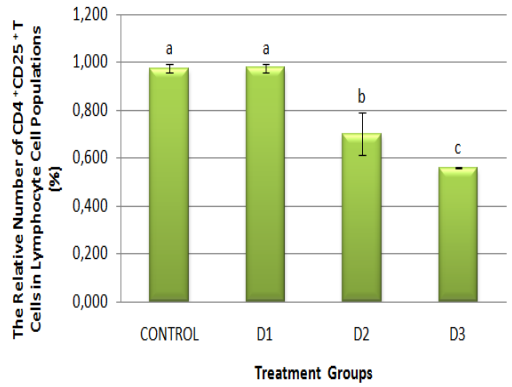


Figure 2. The relative number of $CD4^+CD25^+$ T cells to the lymphocyte cells population between treatment groups of mice after the oral administration of EEP for two weeks.

Increased doses of EEP which is gived in mice based on this experiment may be will boost the number of other immunocompetent cells than the number of $CD4^+CD25^+$ T cells population. The total activation of other immunocompetent cell that occurs in the cell population of lymphocytes can be greater than the number of $CD4^+CD25^+$ T cells were activated itself. T reg cells beside derived from CD4 T cell populations are also known can be derived from the population of $CD8^+$ cells by $CD8^+CD122^+$ marking. According to the experiment by Rifa'i *et al.* [8] $CD8^+CD122^+$ cells have the ability to control the activation of $CD8^+$ and $CD4^+$ T cells through the study of in-vivo and in-vitro were performed. $CD8^+CD122^+$ population thus able to be used to control the presence of abnormal T cells.

The content of compound in propolis there is known as an immunosuppressant. One such of that compound is Artepilin C (Art-C). Immuno-suppressive function of Art-C are acting in suppress the percentage of $CD4^+CD25^+$ T cells and IL-10 cytokine production in cultured lymphocytes in-vitro. The function of T reg cells $CD4^+CD25^+FOXP3^+$ mediated through the production of IL-10 and TGFβ [9]. Cytokine TGF-β and IL-10 are known have a role on the process of differentiation of Treg cells [10]. Cytokine IL-10 may play a role in autocrine against Treg cells mainly serves to maintain FoxP3 expression [11]. Significant decrease in the relative number of $CD4^+CD25^+$ T cells in this experiment thus can also occur due to the content of Art-C compounds in propolis that has function as immunosuppressive as where the results of experiment conducted by Cheunga *et al.* [10].

Giving Brazilian EEP at a dose of 200 $mg.kg^{-1}$ for 14 days in mice are known to inhibit the production of IL-1, IL-6, IFN-γ, IL-2 and IL-10 by spleen cells [1,12]. CAPE in propolis compounds known to inhibit IL-2 gene transcription and expression of IL-2R (CD25), and T cells ploriferation in humans. The process can occur by working CAPE compounds that inhibit the transcription factor NFAT and NF-Kb [13]. IL-2 is a cytokine that is able to stimulate the activation and differentiation of T reg cells [14]. T reg cells that stay in peripheral areas will be maintained through the presence of IL-2, although IL-2 does not play an important role against the development of T reg cells in the thymus [15]. Therefore, if the amount of IL-2 was reduced following by administration of EEP then it can be closely related to a decrease in the number of Treg cells in the lymphocyte cells population. Effect of EEP in mice against suppression to the relative number of $CD4^+CD25^+$ T cells in the lymphocyte cells population thus can be due to a content of compounds that are CAPE as imunosupresor.

Treg cells are a population of lymphocytes that are characterized by the high expression of $CD25^+$ molecules surface markers. Another molecules marker but also provide a more specific characteristic for T reg cells [16]. Specific Treg cells can be identified by the presence of FoxP3. Molecule transcription factor FoxP3 in Treg cells is a molecule that plays a key role in the development of $CD4^+CD25^+$ T cells as well as transcription molecule that induces the

ISSN. 2087-2852
E-ISSN. 2338-1655

expression of $CD25^+$ [17]. Effect of EEP in this experiment but only up to the marking of $CD4^+CD25^+$ T cells and was not done until the identification of the presence of FoxP3. Therefore, the results of in experiment have not been able to explain in more specific about how the effect of EEP to change in the real relative number Treg cells $CD4^+CD25^+FOXP3^+$ in lymphocyte cells population.

Bioactivity of EEP on $B220^+$ Cells

Treatment of EEP in mice provide a diverse effect on the profile of the relative number percentage of $B220^+$ cells in each treatment group (Figure 3). Effect of EEP was significantly ($P<0.05$) differences in the relative number of $B220^+$ cells seen in mice in the treatment D1, D2, and D3 group when compared with the relative number of $B220^+$ cells in the control treatment group (Figure 4).

The increase in the relative number of $B220^+$ cells was significantly affected by EEP with D1 dose (50 $mg.kg^{-1}$) and D3 dose (200 $mg.kg^{-1}$). The relative number of $B220^+$ cells on D1 increased compared to the control group, aproximately 59.90%. It is also not different from the relative number of $B220^+$ cells on D3 group with a percentage of 60.02%. The increase in the relative number of $B220^+$ cells due to the effect of EEP but did not different significantly between D3 group with D1 group. Effect of EEP in reducing the relative number of $B220^+$ cells was significantly seen in D2 group with the relative number of $B220^+$ cells was only aproximately 49.61%.

The results of this experiment showed that ther are instability of the patterns of change in the relative number of $B220^+$ cells due to the influence of EEP. The increase in the relative number of $B220^+$ cells in the lymphocyte population in the D1 and D3 treatment group showed that the content of compounds in propolis at that dose range that is assumed to be able to increase the relative number of $B220^+$ cells in the lymphocyte cell population. Effect of EEP in decrease the relative number of $B220^+$ cells significantly that occur in the D2 group indicate the presence of a suppressor mechanism that occurs in there.

Assumptions that can be awakened to explain this phenomenon is in the dose of 100 $mg.kg^{-1}$ body weight of mice is a possible dose that can maximize suppressor molecules activity to reduce the relative number of $B220^+$ cells in the lymphocyte cells population.

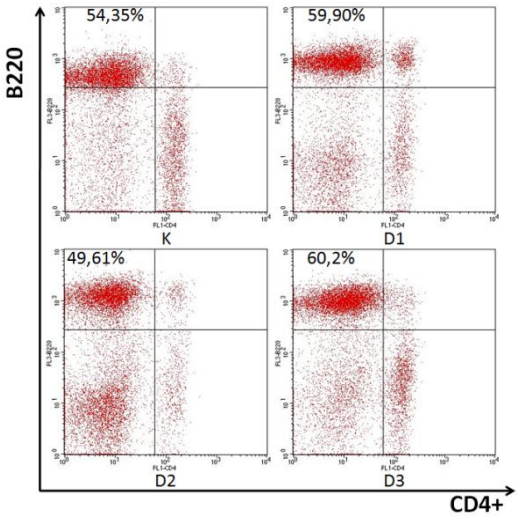


Figure 3. Profile of relative number percentage of $B220^+$ cells flowcytometry analysis results between groups (K= control, D1 = EEP at dose of 50 $mg.kg^{-1}$, D2 = EEP at dose of 100 $mg.kg^{-1}$, and D3 = EEP at dose 200 $mg.kg^{-1}$).

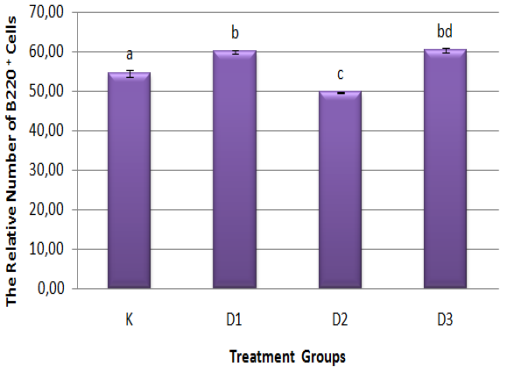


Figure 4. The relative number of $B220^+$ cells between treatment groups of mice after the oral administration of EEP for two weeks.

Experiment conducted by Draganova *et al.* [18] showed that the ethanol extract of Bulgarian propolis at low concentrations 1 and 2.5 $mg.L^{-1}$ was able to increase the proliferation of B cells in PBMC (Peripheral Blood Mononuclear Cells) cell culture. The use of high concentration 10 $mg.L^{-1}$ on the other hand can induce apoptosis in B lymphocyte cells up to 56.08%. Ethanolic extract of propolis with low concentrations preferable for the entire population of cells to support proliferation activities and it will also provide protection for B lymphocytes cell. Therefore it can be assumed that the EEP with a low dose (D1) is the best dose to increase the relative number of $B220^+$cells.

Propolis is known to have the ability to enhance the activation of macrophages and T lymphocytes. Activation of macrophage cells will

ISSN. 2087-2852
E-ISSN. 2338-1655

produce various kinds of cytokines, there are IL-1, IL-6, IL-12, and TNF-α. Cytokine product was produced by macrophages at the same time will stimulate T cell activation. Increased T cell activation will give possibility to improve the production of IFN-γ. Activation of $CD4^+$ T cells will have an impact on the process of differentiation of $CD4^+$ T cells into Th1 cells. Th1 cells it has contributed to the production of IL-2 and IFN-γ [12,19,20]. IFN-γ products are known to have activity in suppressing the B cell differentiation process [5]. The number of IFN-γ produced by macrophages it can inhibit the proliferation of B and T cells if it is too much [21]. Therefore, the content of compounds in propolis production is assumed to be able to suppress the humoral immune response as a result of increased production of cellular imun response.

CONCLUSION

Giving ethanolic extract of propolis (EEP) with dose of 50 mg/kg, 100 mg/kg, and 200 mg/kg body weight of mice thus significantly influence changes in the quantity of $CD4^+CD25^+$ T cells and $B220^+$ cells, with a dose of 50 mg/kg as the optimal dose to increase the relative number of $CD4^+CD25^+$ T cells and $B220^+$ B cells in Balb/c mice.

ACKNOWLEDGEMENT

The author would like to thank Dr. Ir. M. Sasmito Djati, MS. and Drs. Aris Soewandono, MS. as the reviwer; the entire team of Laboratory of Animal Physiology, Department of Biology, University of Brawijaya, Malang. This experiment was made possible through funding by Program of *Penelitian Unggulan Madya*.

REFERENCES

[1] Fatahinia, M., A.R. Khosravi, H. Shokri. 2012. Propolis efficacy on TNF-α, IFN-γ and IL-2 Cytokines production in old mice with and without Sistemic Candidiasis. *J. Mycol.* 22. 237-242.

[2] Jaya, F., L.E. Radiati, K.U.A. Awwaly, U. Kalsum. 2005. Effect of Propolis extract on the Cellular Immune System in Rats (Rattus norvegicus) of Wistar Strain. Faculty of Animal Husbandry, University of Brawijaya. Malang.

[3] Lopes, A.A., S.F. Thiago, T.N. Renata, L. Mannuella, M.P.P. Karla, M.S. Ari, M.B. Ricardo, J.R.S. Antonio, S.V. Samuel, C.P. Luís. 2014. Antioxidant action of Propolis on mouse lungs exposed to short-term cigarette smoke. *Bioorg. Med. Chem.*

[4] Nazir, N. 2014. Imunomodulatory activity of Isoflavones isolated from Iris Kashmiriana: Effecton T-Lymphocyte Proliferation and Cytokine Production in Balb/c Mice. *J. Biomed. Prev. Nutr.* 3. 151–157.

[5] Mustafiah, S.E., F. Dina, Y. Iwang. 2011. Peritoneal Macrophage Phagocytic Power Index after administration of Propolis on Mice (*Mus musculus*). Faculty of Medicine, Islam University of Sultan Agung. Semarang.

[6] Okamoto, Y., H. Takazumi, E. Tatsuya, F. Takashi, M. Toshiyuki. 2014. Brazilian Propolis Ameliorates Trinitrobenzene Sulfonic Acid-Induced Colitis in Mice by Inhibiting Th1 differentiation. *J. Int. Immunopharmacol.* 16. 178–183.

[7] Daniel, R.P. 2006. B Cells as Antigen Presenting Cells. Rev. Cell. Immunol. 238. 67–75.

[8] Rifa'i, M., Y. Kawamoto, I. Nakashima, H. Suzuki. 2004. Essential roles of $CD8^+CD122^+$ Regulatory T Cells in the maintenance of T Cell Homeostasis. *J. Exp. Med.* 9. 1123-1134.

[9] Lee, Y.H., M. Rifa'i. 2011. $CD4^+CD25^+$ $FOXP3^+$ Regulatory T Cells in Allogeneic Hematopoietic Cell Transplantation. *J. Trop. Life Sci.* 2. 69-75.

[10] Cheunga, K.W., M.Y.S. Daniel, K.C. Wing, D. Rui-Xia, T. Wenwei, C.F.C. Godfrey. 2011. Brazilian green Propolis and it's constituent, Artepillin C inhibits Allogeneic Activated Human CD4 T Cells expansion and activation. *J. Ethnopharmacol.* 138. 463–471.

[11] Murai, M., T. Olga, K. Gisen, M. Rajat, L.K. Christopher, C. Hilde, K. Mitchell. 2009. Interleukin 10 acts on Regulatory T Cells to maintain expression of the Transcription Factor FoxP3 and suppressive function in Mice with Colitis. *Nat. Immunol*. 11. 1178–1184.

[12] Rifa'i, M. $CD4^+CD25^+$. 2013. Regulatory T Cells prevent detrimental autoimmune reactions International Journal. *The Open Autoimmun. J.* 5. Bentham Open Publisher.

[13] Marquez, N., R. Sancho, A. Macho, M.A. Calzado, B.L. Fiebich, E. Mu˜noz. 2004. Caffeic Acid Phenethyl Ester Inhibits T-cell Activation by Targeting Both Nuclear Factor of Activated T-cells and NF-kB Transcription Factors. *J. Pharmacol. Exp. Ther.* 308. 993–1001.

ISSN. 2087-2852
E-ISSN. 2338-1655

[14] Nelson, H. Brad. 2004. IL-2, Regulatory T Cells and Tolerance. *J. Immunol.* 172. 3983-3988.
[15] Maloy, K.J., P. Fiona. 2005. Fueling Regulation: IL-2 Keeps $CD4^+$ Treg Cells Fit. *Nat. Immunol*. 6. 1071 – 1072.
[16] Campbell, D.J., S.F. Ziegler. 2007. FOXP3 modified Thephenotypic and functional properties of Regulatory T Cells. *Nat. Rev. Immunol*. 7. 305–310.
[17] Askenasy, N., K. Ayelet, Y. Shai. 2008. Mechanisms of T Regulatory Cell Function. *Autoimmun. Rev.* 7. 370–375.
[18] Draganova, M.F., M. Nikolova, A. Mihova, L. Peychev, V. Sarafian. 2010. A pilot study on the Immunomodulatory effect of Bulgarian Propolis. *Biotechnol. Biotechnol.* 24. 119-124.
[19] Rifa'i, M., Z. Shi, S.Y. Zhang, Y.H. Lee, H. Shiku, K. Isobe, H. Suzuki. 2008. $CD8^+CD12^+$ Regulatory T Cells Recognize Activated T Cells via Conventional MHC Class I–αβTCR Interaction and become IL-10-Producing Active Regulatory Cells. *Int. Immunol*. 20. 937-947.
[20] Sforcin, J.M., B. Vassya. 2011. Propolis: is there a potential for the development of new drugs? *Rev. J. Ethnopharmacol.* 133. 253–260.
[21] Baratawidjaja, KG., R. Iris, 2010. Basic Immunology, 9th edition. Faculty of Medical, University of Indonesia. Jakarta.

ISSN. 2087-2852
E-ISSN. 2338-1655

Dried *Skeletonema costatum* in Feed Formulation for the Growth of Vaname Shrimp (*Litopenaeus vannamei*)

Dewi Putri Lestari[1*], Arning W. Ekawati[2], Maftuch[2]

[1] Graduate Program, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia
[2] Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia

Abstract

The aim of this study was to determine the effect and the best dosage used by the *Skeletonema costatum* in feed formulation on the growth of Vaname shrimp. This experiment used Completely Randomized Design (CRD) with four treatments and three replications. Treatment was given by using dried *S. costatum* in feed formula (iso protein 37% and iso energy 3.6 kkal/g feed). Feed treatment was the substitution of fish meal protein A = 0%, B = 2.5%, C = 5% and D = 7.5% with dried *S. costatum* protein. The observed parameters include Survival Rate (SR), Survival Growth Rate (SGR), Feed Efficiency Ratio (FER), and Protein Efficiency Ratio (PER). The results showed that the use of dried *S. costatum* in feeding are effective in increasing the specific growth rate, feed efficiency ratio and the ratio of protein efficiency than controls. The best dosage in feeding formula is ranged from 4.39% to 4.75%.

Keywords: feeding formula, *Skeletonema costatum,* Vaname Shrimp

INTRODUCTION

Vaname shrimp (*Litopenaeus vannamei* Boone) is one of the leading commodities of Indonesian fishing industry. This shrimp has high productivity in intensive pond thus many farmers cultivate its as the substitute of tiger shrimp. Vaname shrimp is widely known for its advantages of faster ability in growing than Black Tiger and *Stylirostris* shrimp. Vaname shrimps also can be stocked at high densities, resistant to wide range of salinity, need lower protein and resistance to diseases [1,2,3].

Diatoms contain essential nutrients and also have an appropriate size as feed for Vaname shrimp. One of a diatoms which commonly used for feed is *Skeletonema costatum* (Greville) Cleve. The use of diatoms as a natural feed for Vaname shrimp can accelerate its growth in larval stadia. *Skeletonema costatum* is used as a supplementary food for basic nutrition in cultured *Artemia*, shrimp larvae and shellfish spat. Nutritional content of *S. costatum* has been widely studied, which is consisted of 31% protein, 21.5% carbohydrate and 1.3% total fat. In addition, *S. costatum* also contain fatty acid composition, free amino acids, ß-1, 3 glucan and cell wall polysaccharides [4,5,6].

However, the use of diatom *S. costatum* in dry form has not been widely used. The glucan which also found in diatom acts as an immunostimulant which will enhance the body's resistance of the shrimp. Thus the aim of adding dried *S. costatum* on Vaname shrimp's feed formulations are to enhance the growth and productivity by observing the parameter which measures the growth rate of shrimp, e.g. survival, specific growth rate, feed efficiency ratio, and protein efficiency ratio. Observed parameter choosen because related to the feed utilization and growth of the shrimp. This study aimed to determine the best used dose of dried *S. costatum* in the feed formulation on the growth of Vaname shrimp.

MATERIALS AND METHODS

Preparation of Dried *S. costatum*

Skeletonema costatum was obtained from Center of Brackish Aquaculture Situbondo, from seawater which were purified repeatedly with pure subsequently cultured in a laboratory-scale stage, semi mass scale and mass scale. Furthermore, *S. costatum* were dried using an oven in temperature 60°C for 24 hours. The dried *S. costatum* analyzed proximately for its drying content using the oven method and its crude protein concentration using the micro kjedahl.

Preparation of feed

Before determining the formula, other feed's constituent material (fish meal, shrimp meal, soybean flour, tapioca flour, fish oil, vitamin, min mix and CMC) had been prepared and analyzed the proximate data according to AOAC [7].

* Correspondence author:
Dewi Putri Lestari
Email : dewiputrilestari1@gmail.com
Address : Faculty of Fisheries and Marine Sciences,
University of Brawijaya, Jl. Veteran, Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

Table 1. Composition of experiment feed ingredients for vaname shrimp (*Litopenaeus vannamei*)

Ingredient	Dried Content (%)*	Protein (%)*	Fat (%)*	Ash Content (%)*	Rugged fiber (%)*	BETN**	Energy (kkal/gr)***
Fish meal*	92.19	56.86	6.12	28.7	4.91	3.41	296.16
Shrimp meal*	92.20	50.36	4.33	1.33	8.94	35.04	380.55
S. costatum meal*	87.38	24.70	2.22	55.55	1.04	16.49	184.74
Soybean flour*	91.74	45.65	0.14	7.90	4.23	42.08	352.18
Tapioca flour*	87.19	0.09	0.02	0.06	1.01	98.82	398.82

Description :
* : Result analysis of Laboratory Nutrition and Feed, Faculty of Animal Husbandry, University of Brawijaya
** : BETN = 100 – Protein – Fat – Ash content – Rugged fiber
*** : Energy = (4 x %Protein) + (9 x %Fat) + (4 x %BETN)

Table 2. Experiment feed formula of Vaname shrimp (*Litopenaeus vannamei*)

Ingredient (%)	Treatment Dose			
	A	B	C	D
Fish meal	24.73	23.10	21.47	19.85
Shrimp meal	26.45	26.45	26.45	26.45
Soybean meal	21.07	21.07	21.07	21.07
S. costatum meal	-	3.74	7.49	11.23
Tapioca meal	8.31	7.78	7.25	6.72
Fish oil	9	9	9	9
Vit + Min Mix	5	5	5	5
CMC	5.44	3.86	2.27	0.68
Total	**100**	**100**	**100**	**100**

Description :
A = *S. costatum* meal protein substitution on fish meal protein 0%
B = *S. costatum* meal protein substitution on fish meal protein 2.5%
C = *S. costatum* meal protein substitution on fish meal protein 5%
D = *S. costatum* meal protein substitution on fish meal protein 7.5%

Based on the proximate data of feed's constituent material (Table 1), the feed formulation is determined by worksheet method. Feed formulations based on the isoprotein 37% and isoenergi 3.6 kcal.g^{-1}. The treatments were protein substitution of fish meal to *S. costatum* meal (Table 2). All the materials were weighed according to the formulation and mixed until it reached its homogeneity. Homogenate were molded and dried in an oven on the temperature of 30°C for 1 day. Feeds of Vaname shrimp were made in pellets form in 0.55 mm size.

Biological Trials (In vivo)

Biological test of feeding experiments on Vaname shrimp (*Litopenaeus vannamei*) were conducted for 30 days. Each treatment was repeated three times. Control diet without using *S. costatum* meal, compared with three feed formulas that use *S. costatum* meal as the independent variable. The four treatments of feed formulations observed to assess its effect on survival rate (SR), specific growth rate (SGR), feed efficiency ratio (FER), and protein efficiency ratio (PER), which is referred as dependent variable.

This study used Vaname shrimp (*Litopenaeus vannamei*) in juvenile stadia with weight 6.41±0.55 g per shrimp, derived from Center of Brackish Aquaculture Situbondo. The Vaname shrimps were maintained in an aquarium sized 45x45x45 cm^3 volume of 71 liters filled with sea water and the range of the salinity for 33-34 ppt. The outer surface of aquarium was covered with black plastic to imitate the real habitat of the shrimps. The aeration was also included in the aquarium.

Shrimp was acclimatized in the experimental container for 7 days toward the environment and feed. The day before the study was conducted, the shrimp were not feeded and weighed it right after fasting to determine the initial weight. Shrimp were stocked with initial weights uniformly with density of 12 shrimp per aquarium. Feed trials were given as much as 10% of the biomass weight, 4 times daily; as much as 20% at 07.00, 20% at 11.00, 20% at 17.00 and 40% at 21.00 of the amount given per day. Shrimp were weighed every 10 days. Removal of remains feed were done every morning before feeding and the water replaced as much as 20-30% of the total volume.

ISSN. 2087-2852
E-ISSN. 2338-1655

Measurement of water quality during the study include temperature, DO (Dissolved oxygen), pH and salinity which was done every day in the morning and evening. Otherwise, amonial content was measured at the beginning and end of the study.

Data analysis

Data were analyzed using SPSS 21.0 version with one way ANOVA test. The analysis was used to examine the effect of treatment, continued to Least Significant Difference (LSD) test with Duncan test method. This test followed by the analysis of orthogonal polynomials to get the best treatment value.

Survival Rate (SR)

Survival of Vaname shrimp (*L. vannamei*) is calculated using the formula [8]:

$$SR = \frac{Nt}{No} x100\%$$

Description:
SR = Survival Rate
Nt = Final number of shrimp
No = Initial number of shrimp

Survival Growth Rate (SGR)

Observations growth of vannamei shrimp (*L.vannamei*) was done by weighing once every 10 days. Specific growth rate [9] can be calculated using the formula:

$$SGR = \frac{\ln \overline{Wt} - \ln \overline{Wo}}{t} x100\%$$

Description:

SGR = Survival Growth Rate (%BW/day)
$\overline{Wt}$ = Average final weight of individual (gram)
$\overline{Wo}$ = Average initial weight of individual (gram)
t = Time (days)

Feed Efficiency Ratio (FER)

Feed Efficiency Ratio was calculated by comparing the average final weight after reducing the initial average weight of shrimp compared to the amount of feed (dry weight of feed) using the formula [10]:

$$FER = \frac{\overline{(Wt} - \overline{Wo)}}{F}$$

Description:
FER = Feed Efficiency Ratio
F = the amount of given feed (gram)
$\overline{Wt}$ = Average final weight of individual (gram)
$\overline{Wo}$ = Average initial weight of individual (gram)

Protein Efficiency Ratio (PER)

Protein Efficiency Ratio [9] calculated using the formula:

$$PER = \frac{\left(\overline{Wt} - \overline{Wo}\right)}{(FxPf)}$$

Description:
PER = Protein Efficiency Ratio
F = the amount of given feed (gram)
$\overline{Wt}$ = Average final weight of individual (gram)
$\overline{Wo}$ = Average initial weight of individual (gram)
Pf = Protein content in feed (%)

RESULT AND DISCUSSION

The Survival Rate (SR), Specific Growth Rate (SGR), Feed Efficiency Ratio (FER) and Protein Efficiency Ratio (PER) on Vaname shrimp can be seen in Table 3. The results of one-way ANOVA showed that survival rate of Vaname shrimp is not significantly different between treatments (P>0.05). It indicates that the use of *S. costatum* meal in the feed formulation does not cause a negative response to the survival rate of Vaname shrimp.

Factors affecting survival rate are abiotic factors such as the adaptation ability to the environment, human handling, stocking density, competitors, age and the presence of predators. High stocking density would result lower survival due to cannibalism. The range of the water quality values showed in Table 4.

Table 3. Value parameters each treatment

Parameters	Treatment			
	A	B	C	D
SR (%)	77.78±4.81[a]	86.11±4.82[a]	88.89±4.82[a]	88.89±4.82[a]
SGR (%BW.day^{-1})	0.71±0.01[a]	1.09±0.01[c]	1.21±0.05[d]	0.93±0.01[b]
FER (%)	8.22±0.35[a]	12.88±0.26[c]	14.74±0.76[d]	11.09±0.01[b]
PER	0.22±0.01[a]	0.34±0.01[c]	0.40±0.02[d]	0.30±0.00[b]

ISSN. 2087-2852
E-ISSN. 2338-1655

Table 4. Water Quality of Vaname shrimp's Aquaculture during the study

Treatment	Water Quality Paramaters				
	pH	Temperature (°C)	DO (mgL^{-1})	Salinity (ppt)	Ammonia (mgL^{-1})
A (0.0%)	7.0 – 7.1	30.00 – 31.44	7.01 – 7.23	33 – 34	0.008 – 0.012
B (2.5%)	7.0 – 7.1	30.10 – 31.32	7.09 – 7.30	33 – 34	0.004 – 0.009
C (5.0%)	7.0 – 7.1	30.06 – 31.28	7.11 – 7.25	33 – 34	0.001 – 0.007
D (7.5%)	7.0 – 7.1	30.11 – 31.31	7.06 – 7.20	33 – 34	0.001 – 0.018

Table 5. Water Quality References

Parameters	Water Quality Value	
	Study	References
pH	7.0 – 7.1	6.8 - 8.7 [15]
Temperature (^0C)	30.00 – 31.44	27 - 32 [16]
DO (mgL^{-1})	7.01 – 7.30	> 3 [16]
Salinity (ppt)	33 – 34	33-35 [17]
ammonia (mgL^{-1})	0.001 – 0.018	> 0.01 [18]

Based on statistical analysis the water quality does not significantly affect the value of each treatment. The values of the parameters of water quality during the maintenance are in the normal range, accordance with other studies in Table 5.

Other factors that may increase the survival rate during the culture is Vaname shrimp's feed quality and feeding level so that the feed requirements can be met without any competition. Frequency of feeding behavior of vaname shrimp was also noted by de Lima *et al.* [11] indicating that feeding can affect the activity of shrimp itself. In the natural ecosystem, they generally actively feed at night, therefore the shrimp included in the group of nocturnal animals. This is supported by Kordi [12], feeding were given gradually, but more frequent. Thus the shrimp are not quickly satisfied and the shrimp's appetite is maintained.

The relationship between the amounts of substitution *S. costatum* meal protein to fish meal protein (x) with survival (y_1) showed in the equation:

$$\mathbf{y_1 = -0.333x^2 + 3.944x + 77.91;\ R^2 = 0.57}.$$

The highest known value is 88.98% survival rate with the best dose of 5.92% *S. costatum* meal to fish meal in the feed formula. According to Suwoyo and Mangampa [13], the increase of average weight of biota which is kept in line with the duration of maintenance can lead to the decrease of the individual specific growth rate. The difference in the growth rate could be due to the differences in the initial size and density of shrimp which were stocked, quality and quantity of feed, maintenance duration and cultivation containers which were used.

The relationship between the amounts of substitution *S. costatum* meal protein to fish meal protein (x) with survival growth rate (y_2), showed in the equation:

$$\mathbf{y_2 = -0.026x^2 + 0.231x + 0.698;\ R^2 = 0.98}$$

The best value obtained from the treatment at a dose of *S. costatum* meal protein substitution by 4.44% in feed formula with a specific growth rate of 1.21% $BW.day^{-1}$. Feed efficiency ratio indicates that better utilization of feed and absorbed feed by the body increase the growth. The increasing feed efficiency values were caused by high nutrient that can be optimally utilized by the body.

The relationship between the amounts of substitution *S. costatum* meal protein to fish meal protein (x) with feed efficiency ratio (y_3) showed in the following equation:

$$\mathbf{y_3 = -0.008x^2 + 0.076x + 0.215;\ R^2 = 0.95}$$

The best dosage is 4.39% with a value of feed efficiency ratio 14.47%. The degree of protein efficiency ratio proteins is influenced by the quality of the feed. The higher the protein efficiency ratio values of feed protein quality the better it will be to increase the growth rate of shrimp. According to Buwono [14], protein quality of food is determined by the particular amino acid content of essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine).

The relationship between the amounts of substitution *S. costatum* meal protein to fish meal protein (x) with protein efficiency ratio (y_4) showed in the equation below. The best dose is 4.75% with a protein efficiency ratio 0.40.

$$\mathbf{y_4 = -0.332x^2 + 2.912x + 8.082;\ R^2 = 0.96}$$

ISSN. 2087-2852
E-ISSN. 2338-1655

CONCLUSION

The use of *Skeletonema costatum* meal protein to substitute fish meal protein in feed formula of Vaname shrimp affect the specific growth rate, feed efficiency ratio and protein efficiency ratio. The best dosage of *S. costatum* meal to increase the production of Vaname shrimp renged 4.39% - 4.75% in feed formula.

REFERENCES

[1] Briggs, M., S.F. Smith, R. Subanghae, M. Phillips. 2004. Introduction and movement of *Penaeus vannamei* and *P. stilyrostis* in Asia and Pacific. FAO. 40p.

[2] Widodo, R.H., A.S. Dian. 2005. Shrimp vannamei: cultivation and market prospects disease resistance of white shrimp. Penebar Swadaya. Jakarta. 75p.

[3] Nur'aini, Y. L., H. Bambang, S. Subyakto, T. Gemi., 2007. Active surveilance of infectious myonecrosis virus (IMNV) in pond-cultured white shrimp (*Litopenaeus vannamei*) in East Java and Bali. *Jurnal Perikanan UGM*. IX (1). 25-31.

[4] Granum, E., S., Kirkvold, S. M. Myklestad. 2002. Cellular and extracellular production of carbohydrate and amino acid by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. *Mar. Ecol. Prog. Ser*. 242. 83-94.

[5] Monkonsit, S., S. Powtongsook, P. Pavasant. 2011. Comparison between airlift photobio-reactor and bubble column for Skeletonema costatum cultivation. *Eng. J.* 15(4). 53-64.

[6] Widiastuti, R., J. Hutabarat, V.E. Herawati. 2012. Effect of feeding different natural (*Skeletonema costatum* and *Chaetoceros gracilis*) to absolute biomass growth and nutrient content of local *Artemia* sp. *J. Aquacult. Manage. Technol*. 1 (1). 236-248.

[7] AOAC. 1995. Official methods of analysis 12th Ed. Association of Official Analytical Chemists. Washington DC. 114p.

[8] Degani, G., D. Levanon. 1983. The influence of low density on food adaptation, cannibalism and growth of eels (*Anqullia anquilla*). Migal. Galilee Technological Center, Kiriat Shmona. Israel. 8p.

[9] De silva, S.S., T.A. Anderson. 1994. Fish nutrition in aquaculture. Chapman and Hall. London, UK. 319p.

[10] Li, M.H., E.H. Robinson, C.S. Tucker, B.B Manning, L. Khoo. 2009. Effect of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*. *Aquaculture.* 292. 232-236

[11] de Lima, P.P., C.S. Pontes, M. de Fátima Arruda. 2009. Activity pattern of the marine shrimp *Litopenaeus vannamei* (Boone 1931) in laboratory as a function of different feeding frequencies. *Aquac. Res.* 1-8.

[12] Kordi, M.G.H. 2010. Cultivation of catfish in tank Tarpaulins. Andi Publisher. Yogyakarta. 114p.

[13] Suwoyo, H.S., M. Mangampa. 2010. Probiotics application with different concentration on maintenance Vaname Shrimp (*Litopenaeus vannamei*). Proceeding of Forum on Aquaculture Technology Inovation. 239-247.

[14] Buwono, I.D. 2000. Essential need of Amino Acid in fish Ransum. Kanisius. Yogyakarta. 56p.

[15] Ramanathan, N., P. Padmavathy, T. Francis, S. Athithian, N. Selvaranjitham. 2005. Manual on polyculture of tiger shrimp and carps in freshwater. Tamil Nadu Veterinary and Animal Science University, Fisheries College and Research Institute. Thothukudi. 1-161.

[16] Suprapto. 2005. Vannamei shrimp (*Litopenaeus vannamei*) cultivation techniques manual. CV Biotirta. Bandar Lampung. 25 p.

[17] Komaraeidjaja, W. 2003. Effect of application of microbial consortium of nitrifying the ammonia concentration (NH3) in the case of pond water: In Grinting village of Brebes. *J. Tech. Env. P3TL-BPPT*. 4(2). 62-67.

[18] Taqwa, F.H., M. Fitrani, B.T. Esto. 2012. Performance of post-larval shrimp vaname (*Litopenaeus vannamei*) in various long period of adaptation with the addition of a low salinity decrease Sodium, Potassium, and Calcium. Proceedings of 2nd SEMNAS on Fisheries and Marine Sciences Research. Faculty of Fisheries and Marine Sciences, Diponegoro University. Semarang. 3. 52-59.

ISSN. 2087-2852
E-ISSN. 2338-1655

Effect of Reducing Sugar and Total Nitrogen to Ethanol Production from Molasses by *Saccharomyces cerevisiae*

Ekwan Nofa Wiratno[1]*, Tri Ardyati[1], Agustin Krisna Wardani[2]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2] Department of Agricultural Product Technology, Faculty of Agriculture Technology, University of Brawijaya, Malang, Indonesia

Abstract

Indonesia's oil production has declined, while demand for derivative products is increasing. Objective of this research are to understand effect of reducing sugar and total nitrogen variation to ethanol production and fermentation efficiency, cell viability, acidity, temperature, dissolved oxygen with molasses by *Saccharomyces cerevisiae* (SAF Instant). Step of this research consist of determination of reducing sugar, ethanol fermentation, total nitrogen determination, ethanol determination and data analysis. Treatment of reducing sugar (GR) and total nitrogen (N) ($g.L^{-1}$) that are GR 100 N 0, GR 100 N 6, GR 100 N 10, GR 125 N 0, GR 125 N 6 and GR 125 N 10. Fermentation was carried out for 72 hours with three replications. Observation parameters every 24 hours are ethanol and reducing sugar concentration, temperature, acidity and dissolved oxygen. Highest ethanol resulted from GR 125 N 6 (3.68 $g.L^{-1}$) and GR 100 N 6 (3.53 $g.L^{-1}$). Low reducing sugar consumption inhibited by by-product of yeast metabolism and molasses chemical compound, lead leaves high sugar concentration (> 80 $g.L^{-1}$). GR 100 N 6 and GR 125 N 6 have highest fermentation efficiency (69 and 57 %). There was no increase in temperature and decrease in pH significantly (α>0.05). Dissolved oxygen decreased significantly (α>0.05) at the early of fermentation and decrease until the end of fermentation. Total nitrogen 6 g $g.L^{-1}$ has the highest fermentation efficiency.

Keywords: ethanol, molasses, reducing sugar, *Saccharomyces cerevisiae*, total nitrogen

INTRODUCTION

Indonesia's oil production has declined, while demand for derivative products is increasing. Comparison of ethanol and the need for production in 2012 reached 15: 1 [1]. One of the solutions is use ethanol for replacing fossil fuel. The advantages of ethanol use for transportation purpose that it can be produced every time and low of CO^2 emission [2].

Saccharomyces cerevisiae is active microbe in the conversion of glucose to ethanol. Yeast growth is influenced by nutritional factors and environmental factors. The main nutrients that are important in the life of yeast cells are source of carbon, nitrogen, oxygen, and hydrogen. Other materials needed in small amounts for the cell (element phosphorus, sulphur, potassium and magnesium). The critical environmental parameters are temperature, pH, and oxygen and air pressure [3].

The major raw material for ethanol production in Indonesia is molasses. Molasses is a sugar cane factory waste and is not widely used as a staple food as in the case with starchy materials [4]. Molasses has been used because this material does not require pre-treatment processes, so microbes can use it directly for metabolism [5].

However, the use of the concentration of reducing sugar in the molasses as a carbon source and additional nutrients in the form of urea as a nitrogen source was varied in each industry that use it. This research is important to understand the variation on the use of reducing sugars and total nitrogen in increasing ethanol production which is expected to encourage energy independence and reduce reliance on fossil energy sources.

MATERIALS AND METHODS

Total Nitrogen and Calcium (Ca) Concentration Measurement

Total nitrogen measured use the Kjeldahl method [6]. Calcium concentration of molasses use Indonesian standard method/SNI [6].

Measurement of Reducing Sugar Concentration

Concentrations of reducing sugar of pure molasses and molasses was measured for every 24 hours in the fermentation process using 3,5-dinitrosalicylic (DNS) method [7].

* Correspondence author:
Ekwan Nofa Wiratno
Email : ekwannwiratno@gmail.com
Address : Faculty of Mathemathics and Natural Sciences, University of Brawijaya, Jl. Veteran, Malang, 65145

Ethanol Fermentation

The molasses was diluted with distilled water to make a reducing sugar concentration 125 g.L^{-1} and urea has added 0.37 g.L^{-1} (pH 5.5). Molasses was boiled at a temperature of 100°C for 10 minutes. The fermentation processes was consist of enrichment, pre-fermentation and fermenta tion.

Total of 20 ml of sterile molasses was added with 2 g of SAF Instant Yeast Gold Label and homogenized at a speed of 120 rpm for 3 hours at 30°C (enrichment). These results was poured into a sterile molasses to the volume of 200 ml and incubated at a speed of 120 rpm for 12 hours at 30°C (pre-fermentation). After it was moved to the sterile molasses with reducing sugar concentration and nitrogen setting (Table 1) to 1000 ml volume and incubated at a speed of 300 rpm for 72 hours.

Table 1. Treatment of Total N and reducing sugar concentration

No	Treatment	
	Reducing Sugar (g.L^{-1})	Total Nitrogen (g.L^{-1})
1	100	2.7
2	100	6
3	100	10
4	125	3.3
5	125	6
6	125	10

Ethanol Measurement

Ethanol concentration was measured using Dichromatic Acid Method [8].

Fermentation Efficiency

Fermentation efficiency was estimated using theoretical and real concentration of ethanol [9].

$$TE = SK \times V \times 0.511$$

$$FE = \frac{\text{Ethanol total} - \text{Initial ethanol}}{\text{End sugar} - \text{Initial sugar}}$$

$$FE = \frac{\text{Ethanol } \textit{Yield} \text{ (g)}}{\text{Theoretical ethanol}} \times 100\%$$

Description:

TE = theoretical ethanol
SK = sugar concentration
V = volume of fermentation medium
FE = fermentation efficiency

Fermentation Parameter

Parameters was measured consisted of acidity, temperature and dissolved oxygen (DO). Acidity was measured using pH meter, the temperature measured by the thermometer, while DO was measured with DO meter.

RESULT AND DISCUSSION

Ethanol Production and Reducing Sugar Concentration

Highest ethanol concentration was produced is GR 100 N 6 (35.3 g.L^{-1} or 3.53 %) (Fig. 1) and GR 125 N 6 (36.85 g.L^{-1} or 3.68 %) (Fig. 2). Increasing of ethanol concentrations was significantly (α> 0.05) occurred at 24 hours. Largest ethanol production occurs in the first 24 hours in the fermentation process [10]. This is caused by an increase in the fermentation process up to 10 % in the early phase of fermentation [11].

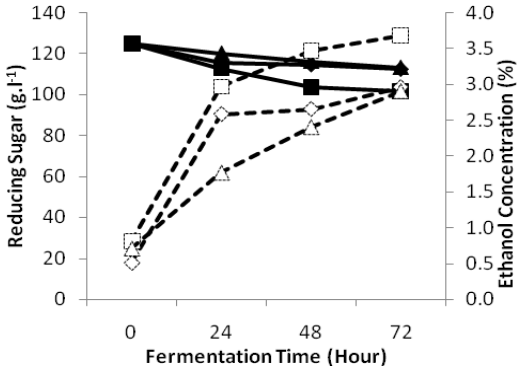


Figure 1. Effect of reducing sugar initial concentration 125 g.L^{-1} to reducing sugar concentration (close symbol) and ethanol concentration (open symbol)
Total nitrogen 0.27 & 0.33 g.L^{-1} (♦ & ◊), 6 g.L^{-1} (■ & □), 10 g.L^{-1} (▲ & Δ)

Result of ethanol fermentation with treat-ment of GR 100 N 6 and GR 125 N 6 were significantly differences with the other (α = 0.05). Reducing sugar concentration of 125 g.L^{-1} resulted in higher ethanol concentrations than reducing sugar 100 g.L^{-1}. Ethanol yield is influenced by the concentration of the substrate, the higher the substrate (not more than 15 %), produced the higher ethanol yield [12].

Treatment of total nitrogen 6 g.L^{-1} influence the concentration of produced ethanol. Addition of urea for increasing nitrogen concentration which produced the maximum ethanol concentration was 6 g.L^{-1}. Nitrogen is a major nutrient that influence fermentation efficiency and at the same time also become a limiting factor to the fermentation process. Limitations of the nitrogen will lead to a decrease in sugar transport activity in the stationary phase resulting in a decrease in the rate of

ISSN. 2087-2852
E-ISSN. 2338-1655

fermentation. The presence of nitrogen triggers the rate of metabolism of sugar and ethanol production [13].

Reducing sugar in all treatments decreased slightly so that a reducing sugar is still remains in a high concentration. This indicates that the fermentation process is not running optimally. Inhibition of this process of reducing sugar consumption may occur due to the influence of chemical compounds inside the compound of molasses and yeast cell metabolism. One of the compounds derived from molasses is sulphur dioxide [14].

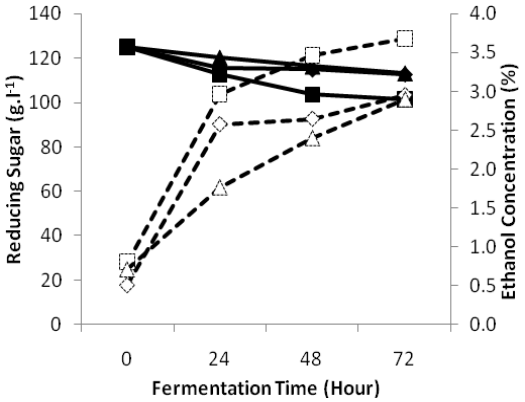


Figure 2. Effect of reducing sugar initial concentration 100 $g.L^{-1}$ to reducing sugar concentration (close symbol) and ethanol concentration (open symbol)
Total nitrogen 0.27 & 0.33 $g.L^{-1}$ (♦ & ◊), 6 $g.L^{-1}$ (■ & □), 10 $g.L^{-1}$ (▲ & Δ)

Sulphur dioxides have antimicrobial effects by means of penetrating cell membranes and interfere with the activity of enzymes and proteins in the cell. When sulphur dioxide is added to the fermentation process, there will be a decrease in the rate of fermentation and increased production of acetaldehyde. Sulphur dioxide inhibits an enzyme that inhibits the SH-group and NAD [15].

Some of the yield compounds of yeast cell metabolism include furfural, 5-hydroxymethyl-furfural (HMF), acetic acid [15] and propionic acid [16]. Furfural and HMF at concentrations more than 0.5 % led to a decrease in sugar consumption and a decrease in the concentration of ethanol (7.5% and 26%). While the acetic acid concentration more than 0.15% causes reduction of ethanol concentration and productivity [17]. Furfural was caused to the accumulation of reactive oxygen species (ROS), mitochondrial membrane damage, and damage to the vacuole membrane, nucleus and actins chromatin damage [18]. Concentration of 0.68 % propionic acid is toxic for *Saccharomyces cerevisiae* [17].

Effect of Reducing Sugar and Total Nitrogen to Fermentation Efficiency

The highest fermentation efficiency is GR 100 N 6 treatment (69%). This was followed by treatment of the GR 125 N 6 by 57%. This fermentation efficiency showed that the total nitrogen treatment at 6 $g.L^{-1}$ was able to improve the efficiency of fermentation. Fermentation media in the form of molasses had a fermentation efficiency of 68.5% [11]. The decline in the efficiency of the fermentation was due to the high concentration of reducing sugars and other components that were toxic [19].

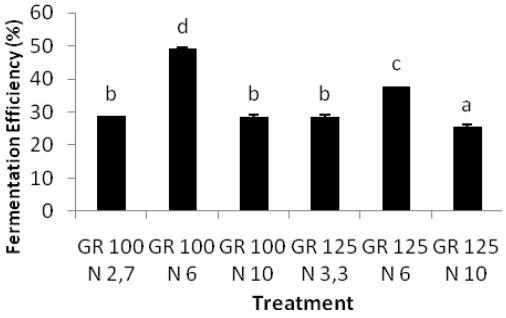


Figure 3. Ethanol Fermentation Efficiency after 72 hour

Efficiency of ethanol fermentation decreases with increase in sugar concentration resulting in increased osmotic pressure of the media or the number of cells that over capacity due to the high concentration of substrate. The high concentration of substrate inhibits the growth of yeast cells that lead to high osmotic pressure and low water concentration. This causes the yeast cells become dehydrated [20]. N concentration of 6 $g.L^{-1}$ led to the increased cell viability so that the process of carbohydrate metabolism (ethanol fermentation) increased [21].

Effect of Reducing Sugar and Total Nitrogen to Fermentation Parameters (T, pH and DO)

An increase on temperature of all treatments during the initial fermentation process (24 hours) were not significant (α>0.05) (Fig. 4). It indicates the process of carbohydrate metabolism. The increase on temperature in the initial phase of fermentation (first 24 hours) caused by exothermic metabolic activity (heat generating) by the microbial population [11].

Fermentation temperature at initial phase occurred at yeast fermentation of glucose (9 g or

ISSN. 2087-2852
E-ISSN. 2338-1655

1.7 kcal). The energy is sufficient to raise the temperature of 17.9°C medium. Increased of the 17.9°C temperature was not observed either because there is a process made possible release into the environment temperature [12].

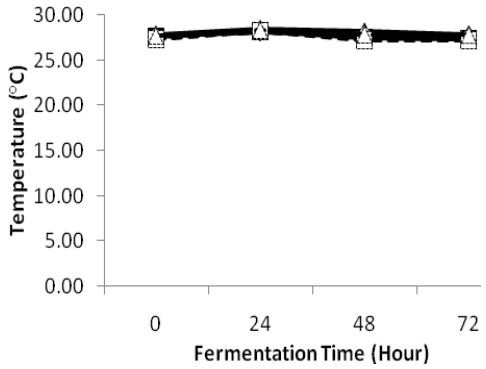


Figure 4. Effect of treatment to temperature during ethanol fermentation
GR 100 N 0.27 (♦), GR 125 N 0.33 (◊), GR 100 N 6 (■), GR 125 N 6 (□), GR 100 N 10 (▲), GR 125 N 10 (Δ)

In general, a decrease of the acidity (pH) in all treatments of the fermentation process, were not significant (α>0.05) (Fig. 5). According to Khongsay *et al.* [22], when the fermentation process using a batch system, decrease the acidity of the medium to 0.25 in the early phase of fermentation (24 hours), then a decrease in pH occurs slowly.

When the yeast to metabolize carbohydrates also produced acetic acid and formic acid, has cause decrease of the acidity of the growth medium. Decrease of the acidity is closely linked to the consumption of nitrogen and release of H^+ ions. The acid is lipo-soluble can enter the cell through the plasma membrane and intracellular acidity affect [23].

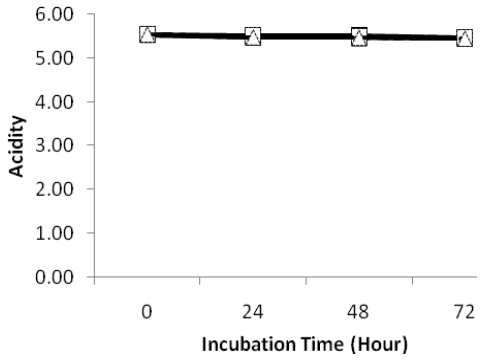


Figure 5. Effect of treatment to acidity during ethanol fermentation
GR 100 N 0.27 (♦), GR 125 N 0.33 (◊), GR 100 N 6 (■), GR 125 N 6 (□), GR 100 N 10 (▲), GR 125 N 10 (Δ)

Increasing the concentration of acetic acid causes a decrease in carbohydrate consumption. It is resulting in a decrease in the rate of ethanol production [24].

There was a decrease of dissolved oxygen during the process of fermentation in all treatments (Fig. 6). Decrease of the DO was significantly (α>0.05) occurred in the treatment of GR 100 N 6, GR 100 and GR 125 N 10 N 10 This indicated that the concentration of total nitrogen (6 and 10 $g.l^{-1}$) caused an increase in oxygen consumption by yeast cells.

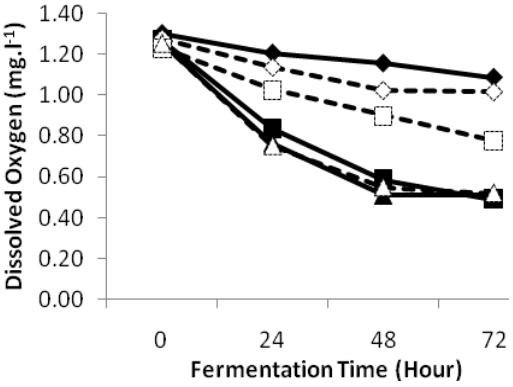


Figure 6. Effect of treatment to dissolved oxygen during ethanol fermentation
GR 100 N 0.27 (♦), GR 125 N 0.33 (◊), GR 100 N 6 (■), GR 125 N 6 (□), GR 100 N 10 (▲), GR 125 N 10 (Δ)

Yeasts used oxygen in the process of cell reproduction that would correlate with the number of cells [25] and keep the cell concentration remains high [26]. The presence of oxygen will inhibit the fermentation process (Pasteur Effect) [25]. The function of oxygen is to produce ATP in glycolysis and oxidative phosphorylation. The process of oxidative phosphorylation is a process that is most prominent in the production of ATP. If there is no oxygen (anaerobic), NADH in the mitochondria cannot be re-oxidized in the citric acid cycle, resulting in the formation of ATP and nutrient solution is interrupted [21]. In addition of nitrogen, other factors that affect the process of respiration are the presence of salt. The addition of 1.0 M KCl or NaCl causes an increase in the rate of respiration and cell metabolism [27].

CONCLUSION

Highest ethanol concentration resulted GR 125 N 6 (3.68 g.L-1) and GR 100 N 6 (3.53 g.L-1) treatment. Low reducing sugar consumption inhibited by by-product yeast metabolism and

molasses chemical compound, lead remains high sugar concentration (> 80 g.L-1). GR 100 N 6 and GR 125 N 6 treatment have highest fermentation efficiency (69 and 57%). There was no increase in temperature and decrease in pH significantly (α>0.05). DO decreased significantly (α>0.05) at the early of fermentation and slowed until the end of fermentation. Total nitrogen 6 g.L-1 had the highest fermentation efficiency.

REFERENCES

[1] ESDM. 2012. Produksi, konsumsi, ekspor dan impor minyak Indonesia 2009. http://www.migas.esdm.go.id/wap/?op=Berita&id=2787. Accessed June 26th 2012.

[2] Jeon, Y., S.J. Kim, D.H. Kim, B.K. Na, D.H. Park, H.T. Tran, R. Zhang, D.H. Ahn. 2007. Development of a serial bioreactor system for direct etanol production from starch using *Aspergillus niger* and *Saccharomyces cerevisiae*. *Biotechnol. Bioprocess Eng.* 12. 566-573.

[3] Loureiro, V., M. Malfeito-Ferreira. 2003. Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.* 86 (1-2). 23-50.

[4] Kurniawan, Y., A. Susmiadi, A. Toharisman. 2005. Potensi pengembangan industri gula sebagai penghasil energi di Indonesia. Research Centre of Sugar Plantation Indonesia (P3GI). Pasuruan.

[5] da Silva, G.P., E.F. de Araújo, D.O. Silva, W.V. Guimarães. 2005. Ethanolic fermentation of Sucrose, Sugarcane Juice and Molasses by *Escherichia coli* Strain Ko11 and *Klebsiella oxytoca* Strain P2. *Braz. J. Microbiol.* 36. 395-404.

[6] Sudarmadji, S., Haryono, B. Suhardi. 1996. Analisa bahan makanan dan pertanian. Liberty. Yogyakarta.

[7] Miller, G.L.. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry 3 (31): 426-248.

[8] Caputi, A. Jr, M. Ueda, T. Brown. 1968. Spectrophotometric determination of ethanol in wine. *Am. J. Enol. Viticult.* 3 (19). 160-165.

[9] Zhang, K., H. Feng. 2010. Fermentation potentials of *Zymomonas mobilis* and its application in ethanol production from low-cost raw sweet potato. Afr. J. Biotechnol. 9 (37). 6122-6128.

[10] Balakumar, S., V. Arasaratnam. 2009. Comparison of industrial scale ethanol production from palmyrah-based carbon source by commercial yeast and a mixed culture from palmyrah toddy. *J. Instr. Brewer.* 115 (2). 105-109.

[11] Ingledew, M.W. 1993. Yeasts for production of fuel ethanol. *The Yeasts.* 5. 245-286.

[12] Rodrigues, F., P. Ludovico, C. Leao. 2005. Sugar metabolism in yeasts: an overview of aerobic and anaerobic glucose catabolism. University of Minho. Braga.

[13] Ghosh, S., R. Chakraborty, U. Raychaudhuri. 2010. Modulation of palm wine fermentation by the control of carbon and nitrogen source on metabolism of *Saccharomyces cerevisiae*. *J. Food Technol.* 8 (5). 204-210.

[14] Foster, F. 2012. Harmful chemicals in Molasses. http://www.rawfoodexplained.com/junk-foods-molasses/harmful-chemicals-in-molasses.html. Accessed October 3rd 2012.

[15] Henderson, P.. 2009. Sulfur Dioxide, science behind this anti-microbia, anti-oxidant, wine additive. *Pract. Winery Vineyart J.* 1-6.

[16] Al-Jasass, F.M., S.M. Al-Eid, S.H.H. Ali. 2010. A comparative study on date syrup (dips) as substrate for the production of baker's yeast (*Saccharomyces cerevisiae*). *J. Food, Agr. Environ.* 8 (2). 314-316.

[17] Wikandari, R., R. Millati, S. Syamsiyah, R. Muriana, Y. Ayuningsih. 2010. Effect of Furtural, Hydroxymethylfurfural and Acetic Acid on indigeneous Microbial Isolate for Bioethanol production. *Agr. J.* 5 (2). 105-109.

[18] Allen, S.A., W. Clark, J. M. McCaffery, Z. Cai1, A. Lanctot1, P.J. Slininger, Z.L. Liu, S.W. Gorsich. 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. *Biotechnol. Biofuels.* 3. 2-10.

[19] Abubaker, H.O., A.M.E. Sulieman, H.B. Elamin. 2012. Utilization of *Schizosaccharomyces pombe* for Production of ethanol from Cane Molasses. *J. Microbiol. Res.* 2 (2). 36-40.

[20] Soni, S.K., N. Bansali, R. Soni. 2009. Standardization of conditions for fermentation and maturation of wine from Amla (*Emblica officinalis* Gaertn.). *Nat. Product Radiance.* 8 (4). 436-444.

[21] Supriyanto, T., Wahyudi. 2010. Proses produksi etanol oleh *Saccharomyces cerivisiae* dengan Operasi kontinyu pada kondisi vakum. University of Diponegoro. Semarang.

[22] Khongsay, N., L. Laopaiboon, P. Jaisil, P. Laopaiboon. 2012. Optimization of agitation and aeration for very high gravity ethanol fermentation from sweet sorghum juice by *Saccharomyces cerevisiae* using an orthogonal array design. *Energ.* 5. 561-576.

[23] Maurice, M.L. 2011. Factors effecting ethanol fermentation via simultaneous saccharification and fermentation. Shanghai Jiao Tong University. Shanghai.

[24] Casey, E., M. Sedlak, N.W.Y. Ho, N. S. Mosier. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 10. 385–393.

[25] Walker, G.M. 1998. Yeast: physiology and biotechnology. John Wiley and Sons. Chichester.

[26] Tao, F., J.Y. Miao, G.Y. Shi, K.C. Zhang. 2003. Etanol fermentation by an Acid-tolerant *Zymomonas mobilis* under non-sterilized condition. *Process Biochem.* 40. 183-187.

[27] Montiel, V., J. Ramos. 2007. Intracellular Na^+ and K^+ distribution in *Debaryomyces hansenii*. Cloning and expression in *Saccharomyces cerevisiae* of DhNHX1. *FEMS Yeast Res.* 7. 102–109.

ISSN. 2087-2852
E-ISSN. 2338-1655

MANUSCRIPT SUBMISSION

FOCUS AND SCOPE

Journal of Experimental Life Science (JELS) is scientific journal published by Graduate Program of Brawijaya University as distribution media of Indonesian researcher's results in life science to wider community. JELS is published in every four months. JELS published scientific papers in review, short report, and life sciences especially nanobiology, molecular biology and cellular biology. JELS is scientific journal that published compatible qualified articles to academic standard, scientific and all articles reviewed by expert in their field.

Journal of Experimental Life Science (JELS) have vision to become qualified reference media to publish the best and original research results, and become the foundation of science development through invention and innovation on cellular, molecular, and nanobiology rapidly to community.

Journal of Experimental Life Science (JELS) have objectives to published qualified articles on research's results of Indonesian researchers in life science scope. JELS encompasses articles which discuss basic principles on nature phenomenon with cellular, molecular, and nanobiology approach.

PEER REVIEW PROCESS

Publication of articles by JITODE is dependent primarily on their validity and coherence, as judged by peer reviewers, who are also asked whether the writing is comprehensible and how interesting they consider the article to be. All submitted manuscripts are read by the editorial staff and only those articles that seem most likely to meet our editorial criteria are sent for formal review. All forms of published correction may also be peer-reviewed at the discretion of the editors. Reviewer selection is critical to the publication process, and we base our choice on many factors, including expertise, reputation, and specific recommendations. The editors then make a decision based on the reviewers' advice, from among several possibilities:

Accepted, with or without editorial revisions
Invite the authors to revise their manuscript to address specific concerns before a final decision

Rejected, but indicate to the authors that further work might justify a resubmission

Rejected outright, typically on grounds of specialist interest, lack of novelty, insufficient conceptual advance or major technical and/or interpretational problems

PUBLICATION FREQUENCY

JELS publish 2 Issues per year.

OPEN ACCESS POLICY

This journal provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.

COPYRIGHT NOTICE

Authors who publish with this journal agree to the following terms:

Authors retain copyright and grant the journal right of first publication with the work simultaneously licensed under a Creative Commons Attribution License that allows others to share the work with an acknowledgement of the work's authorship and initial publication in this journal.

Authors are able to enter into separate, additional contractual arrangements for the non-exclusive distribution of the journal's published version of the work (e.g., post it to an institutional repository or publish it in a book), with an acknowledgement of its initial publication in this journal.

Authors are permitted and encouraged to post their work online (e.g., in institutional repositories or on their website) prior to and during the submission process, as it can lead to productive exchanges, as well as earlier and greater citation of published work (The Effect of Open Access).

PRIVACY STATEMENT

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

ETHICS PUBLICATION

Research that using animal, human, and clinical testing is should already have ethical clearance certificate from authorized institution.

Title Typed in Bold, Capitalize each First Letter of Each Word, Except Conjunctive, *Scientific name* should not be Abbreviated (Calibri 14 Bold Center, should not exceed 12 words, except conjuctive)

First Author[1]*, Second Author[2], Third Author[3] (Calibri 12 Center, without title)

[1]First Author Affiliation, Correspondence author should be indicated by * symbol (Calibri 9 Center)
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[3]Laboratorium of Physiology, Faculty of Medicine, University of Brawijaya, Malang, Indonesia

Abstract (Calibri 9 Bold Center)

This article illustrates preparation of your paper using MS-WORD (.doc or .rtf). Manuscript was numbered consecutively. Main text typed in two columns (67 characters), except title and abstract in one column. The manuscript should be written in English. The length of manuscript should not exceed 10 pages including table and figure in this format using A4 paper single space. The text should be in the margin of 3 cm up, down and left side, 2.5 cm on right side. Abstract includes the research purposes, research method and research results in one paragraph of *essay,* not *enumerative*. No citation in abstract. Abstract should not exceed 200 words. Keywords typed after abstract. (Calibri 9 Justify).

Keywords: manuscript, English, format, 5 words maximum (Calibri 9 Left)

INTRODUCTION*(Calibri 10 Bold, Left, Capslock)

All submitted manuscripts should contain original research which not previously published and not under consideration for publication elsewhere. Articles must be written in ENGLISH and manuscripts may be submitted for consideration as research report articles, short reports or reviews.

The introduction explains the background of the problem, the study of literature and research purposes. Some initial introduction paragraphs explain the problem and background to these problems [1]. The next few paragraphs explain the study of literature that contains recent knowledge development which is directly related to the issues. The last paragraph of the introductory section contains a description of the purposes of the study. (Calibri 10 Justify)

MATERIAL AND METHOD(Calibri 10 Bold, Left, Capslock)

This section describes the types of methods (qualitative, quantitative or mixed-method) with details of methods of data collection and data analysis [2]. This section also describes the perspective that underlying the selection of a particular method. (Calibri 10 Justify)

Correspondence address: (Calibri 8 Bold, Left)
Full name of correspondence author
Email : sapto@jurnal.ub.ac.id
Address : afiliation address include post code

Data Collection (Calibri 10 Bold, Left)

Explain the data collection methods, i.e. surveys, observations or archive, accompanied by details of the use of such methods. This section also describes the population, sampling and sample selection methods. (Calibri 10 Justify)

The use of English language should followed proper grammar and terms. Name of organism shoul be followed by its full scientific name in the first mention, in *italic* [3]. Author of the scientific name and the word of "var." typed regular. Example: *Stellaria saxatillis* Buch. Ham. First abbreviation typed in colon after the abbreviated phrase.

Author must use International Standard Unit (SI). Negative exponent used to show the denominator unit. Example: g l^{-1}, instead of g/l. The unit spaced after the numbers, except percentage [4]. Example: 25 g l^{-1}, instead of 25gl^{-1}; 35% instead of 35 %. Decimal typed in dot (not coma). All tables and figures should be mentioned in the text.

RESULT AND DISCUSSION (Calibri 10 Bold, Left, Capslock)

This section contains the results of the analysis and interpretation or discussion of the results of the analysis. Describe a structured, detailed, complete and concise explanation, so that the reader can follow the flow of analysis and thinking of researchers [5]. Part of the results study should be integrated with the results of the

analysis and the results and discussion are not separated.

Table

Table should be submitted within the manuscript and in separated file of *Microsoft Excel* (xls.). Table whould not exceed 8 cm (one column) and 17 cm (two columns). Table should be embedded in different page after references.

Table should be numbered in sequence. Table title should be brief and clear above the table, with uppercase in initial sentence. Vertical line should not be used. Footnote use number with colon and superscripted. Symbol of (*) or (**) was used to show difference in confidence interval of 95 and 99%.

Table 1. Example of the Table (Calibri 8.5 Left)

No	Point (Calibri 8.5 Justify)	Description
1		
2		
3		
4		
5		

Sources: Journal of PPSUB (Calibri 8.5 Left)

Figures

Figures should be in high resolution and well contrast in JPEG or PDF with the following conditions:

- Monochrome image (line art), figures of black and white diagram (solid/no shades of gray), resolution 1000-1200 dpi (dot per inch).
- Combination Halftone, combine figure and text (image containing text) and coloured graphic or in grayscale format. Resolution 600-900 dpi.
- Halftone, coloured figure or grayscale format without text. Resolution 300 dpi.
- Black and white figure should be in the grayscale mode, while coloured figures should be in RGB mode.
- Figure should not exceed the width of 8 cm (one column), 12.5 cm (1.5 columns) or 17 cm (two columns).
- Figures title typed clearly below the figure.
- Figure with pointing arrow should be grouped (grouping).
- Figures were recommended in black and white.
- Legend or figure description should be clear and complete. If compressed, the figure should be readable.
- Statistic graphic should be supplemented with data sources.
- If the figures come from the third party, it should have the copyright transfer from the sources.

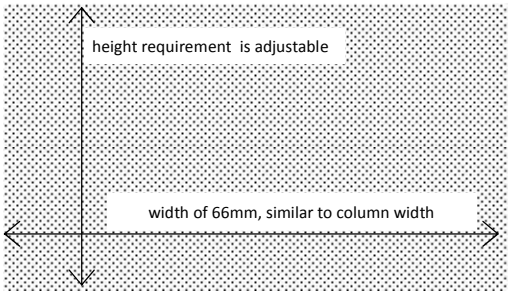


Figure 1. Illustration of Dimensional Figure of one column width. Figure dimension adjusted to the width of one column. Name the figure (diagram) written below the image. (Calibri 8.5 Justify)

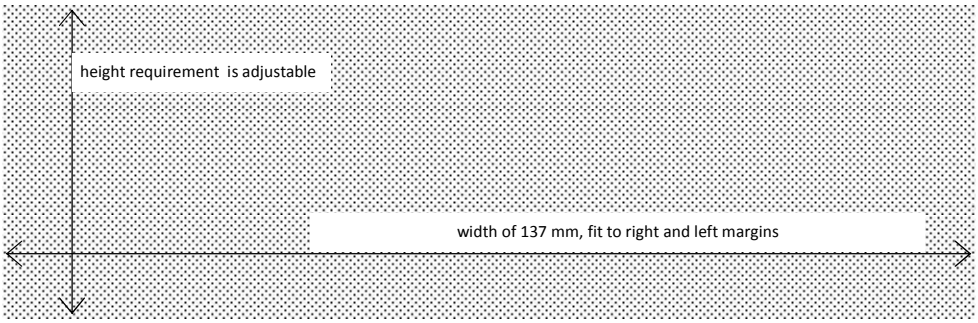


Figure 2. . Illustration of Dimensional Figure of two column width. Figure dimension adjusted to the width of two columns (137 mm). Figure were align top or bottom of the page. (Calibri 8.5 Justify)

References

1. Primary references include journal, patent, dissertation, thesis, paper in proceeding and text book.
2. Avoid self citation.
3. Author should avoid reference in reference, popular book, and internet reference except journal and private ana state institution.
4. Author was not allowed to use abstract as references.
5. References should been published (book, research journal or proceeding). Unpublished references or not displayed data can not be used as references.
6. References typed in numbering list (format number 1,2,3,…), ordered sequentially as they appear in the text (system of Vancouver or author-number style).
7. Citation in the manuscript typed only the references number (not the author and year), example: Obesity is an accumulation of fat in large quantities which would cause excessive body weight (overweight) [1]. Obesity is a risk factor of diabetic, hypertension dan atherosclerosis [2].

CONCLUSION (Calibri 10 Bold, Left, Capslock)

Conclusion of the study's findings are written in brief, concise and solid, without more additional new interpretation. This section can also be written on research novelty, advantages and disadvantages of the research, as well as recommendations for future research.(Calibri 10 Justify)

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially. (Calibri 10 Justify)

REFERENCES (Calibri 10 Bold, Left, Capslock)

[1].(Calibri 10 Justify, citation labelling by references numbering)

[2].Vander, A., J. Sherman., D. Luciano. 2001. Human Physiology: The Mecanisms of Body Function. McGraw-Hill Higher Education. New York. (Book)

[3].Shi, Z., M. Rifa'i, Y. Lee, K. Isobe, H. Suzuki. 2007. Importance of CD80/CD86-CD28 interaction in the recognation of target cells by $CD8^{+}CD122^{+}$ regulatory T cells. *Journal Immunology*. 124. 1:121-128. (Article in Journal)

[4].Syafi'i, M., Hakim, L., dan Yanuwiyadi, B. 2010. Potential Analysis of Indigenous Knowledge (IK) in Ngadas Village as Tourism Attraction. pp. 217-234. In: Widodo, Y. Noviantari (eds.) Proceed-ing *Basic Science National Seminar 7* Vol.4. Universitas Brawijaya, Malang. (Article within conference proceeding)

[5].Dean, R.G. 1990. Freak waves: A possible explanation. p. 1-65. *In* Torum, A., O.T. Gudmestad (eds). Water wave kinetics. CRC Press. New York. (Chapter in a Book)

[6].Astuti, A.M. 2008. The Effect of Water Fraction of *Stellaria* sp. on the Content of TNF-α in Mice (*Mus musculus* BALB-C). Thesis. Department of Biology. University of Brawijaya. Malang. (Thesis)

Cover Images:

3D Structure of EGCG (*Epigallocatechin-3-Gallate*)
Green Tea Component

Created by:
Widodo, S.Si.,M.Si.,Ph.D MED Sc.

Address:

Building E, 2nd Floor, Graduate Program, University of Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145
Telp: (0341) 571260 ; Fax: (0341) 580801
Email: jels@ub.ac.id
Web: jels.ub.ac.id

9 772087 285007