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STEROIDAL SAPONIN IN ETHANOL EXTRACT TUBER OF PURPLE YAM (*Dioscorea alata L.*) AS ALLERGENIC AGENT ON SENSITIVITY PHASE BALB/c MICE MODEL ALLERGY

Sri Nabawiyati Nurul Makiyah[a*], Sri Tasminatun[b], Muhaimin Rifa'i[c], Widodo[c], Muhammad Sasmito Djati[c]

[a]*Department of Histology & Biology, Medical Science Study Programe Faculty of Medicine & Health Science, Universitas Muhammadiyah Yogyakarta; Jalan Lingkar Selatan Tamantirto Kasihan Bantul DIY, Indonesia 55183*
[b]*Pharmacy Study Programme, Faculty of Medicine & Health Science, Universitas Muhammadiyah Yogyakarta; Jalan Lingkar Selatan Tamantirto Kasihan Bantul DIY, Indonesia 55183*
[c]*Biology Department, Faculty of Mathematic and Sciences, Brawijaya University Jalan Veteran Malang East Java Indonesia*

Abstract

Purple yam (*Dioscorea alata L.*) is a source of biological tubers not been used optimally. Diosgenin is steroids saponin compounds that most important because it has multiple biological functions, such as allergenic activity. The objective of this research was to analyze the potential allergenic ethanol extract tuber of D. alata L. (EEDA) on BALB/c mice on sensitivity phase with measure the profile of transcription factor FoxP3 Treg cells and cytokine profile of Treg and IgE and IgG1 B cells. An experimental study using BALB/c mice divided into 7 groups: control group (I), the treatment group (II-V) ethanol extract of tuber *Dioscorea alata L.* dose of 0.00; 0.17; 2.01; 10.04 g/kg bw, the treatment group antihistamines drug and Diosgenin (VI-VII). For 17 consecutive days the group II-VII were treated in accordance with the group and with Ovalbumin induced allergy models. Mice were sacrificed on day 18. Spleen is removed, lymphocyte isolated and analyze the transcription factor FoxP3 Treg cells, cytokine profile of T reg cells and IgE and IgG1 B cells on spleen using Flowcytometry FACS Calibur. The results showed EEDA able to inhibit the production of B220IgE and B220IgG1, trigger Treg cells (CD4CD25) and the transcription factor FoxP3 (CD4CD25FoxP3) and profile of the cytokines produced by T reg cells. The conclusion is ethanol extract tubers of Dioscorea alata L (EEDA) does not trigger Treg (CD4CD25) and the transcription factor FoxP3 (CD4CD25FoxP3) and cytokine profiles produced by Treg cells namely CD4IL-10, CD8IL-10, CD4TGF-β and CD8TGF -β in the sensitivity phase.

Keywords: Saponin steroid of ethanol extract of the tubers of Dioscorea alata L.; allergenic agent; Treg cells; the transcription factor FoxP3; lymphocyte.

1. Introduction

Purple yam (*Dioscorea alata L.*) is a source of biological tubers that has not been used optimally. *D. alata L.* contains Diosgenin (Cheng *et al*., 2007) i.e. main steroidal saponin steroid aglicon as intermediate steroidal in pharmaceutical manufacture. Steroidal saponin is the most importance bioactive compound because it have biological function. The result of some research showed that steroidal saponin have allergenic activity (Zhang *et al*., 2012).

The latest studies of T regulatory (Treg) cells reveals the importance of effector function of this cell population in controlling allergic responses. It has been proven that an allergic person reduce the number and function of Treg (Shreffler et al., 2009; Palomares et al., 2010). Treg cells is able to inhibit the development of allergic Th2 response (Akdis et al., 2004) and plays an important role in allergen specific immunotherapy (Akdis et al., 1998; Jutel et al., 2003). FoxP3 transcription factor plays an important role in the maintaining self-tolerance and immune homeostasis (Sakaguchi et al., 2008). FoxP3 transcription factor important in the development of Treg cells and a marker of confirming the presence of Treg cells (Zheng & Rudensky, 2007; Sakaguchi, 2004).

Treg cells produce IL-10 and TGF-β cytokines which they inhibit the activation of lymphocytes and macrophages. Treg cells also directly interact and suppress other lymphocytes or antigen presenting cells (Abbas & Lichtman, 2011). Treg cells through its cytokines, IL-10 and TGF-β, capable monitoring the immune response, inhibits hyperpolarization Th1 (autoimmune disease) or Th2 (allergic diseases) and further regulate the balance of Th1/Th2 (Gourbeyre et al., 2011) as well as suppressing IgE released by B cells (Huang et al., 2009). TGF-β is an important factor in the regulation of T cell mediated immune response and plays a role in the induction of immune tolerance (Gorelik and Flavell, 2002; Chen & Wahl, 2002).

Allergic reactions involving IgE specific antibodies (Ewan, 1998). IgE is a key molecules that act as mediators of allergic responses (asthma, rhinitis, food allergies, atopic dermatitis, etc.) (Gould & Sutton, 2008). According to Saldanha et al. (2004), Ovalbumin as allergen sensitization in mice increases levels of IgE and IgG1 in blood sera.

The objective of this research was to analyze the potential allergenic ethanol extract tuber of Dioscorea alata L. (EEDA) on BALB/c mice on sensitivity phase with measure the profile of the transcription factor FoxP3 Treg cells and cytokine profile of Treg and IgE and IgG1 B cells.

2. Materials and Methods

Experimental animals are mice (*Mus musculus*) BALB/c strain, 7-8 weeks with a healthy condition. This research has got the certificate of eligibility of ethics (Ethical Clearance) of the Research Ethics Committee (Animal Care and Use Committee) UB No. KEP-144-UB.

Experimental research on BALB/c mice with *posttest only control group design*. Independent variable is ethanol extract of tubers Dioscorea alata L. dose 0.00 g/kg, 0.17 g/kg, 2.01 g/kg and 10.04 g/kg. Dependent variable is profile of transcription factor FoxP3, cytokine profile of T reg cells (IL-10, TGF-β) and IgE and IgG1 B cells on spleen.

Dose ethanol extract of the tubers of Dioscorea alata L. converted from dose human weighing 70 kg to 20 grams mice, multiplied by the conversion value 0.0026, so we get first dose of 0.17 g/kg, 2 doses of 2.01 g/kg and 3 doses of 10.04 g / kg.

Mice Model Allergic Digestive Tract on Sensitivity Phase. BALB/c mice intraperitoneally sensitized with Ovalbumin. Mice immunized with intraperitoneal injections on day 15 with 0.15 ml Ovalbumin in $Al(OH)_3$ which is made of 2.5 mg Ovalbumin dissolved in 7.75 ml of Alumunium Hydroxide (Fischer et al., 2005; Diding et al., 2008).

Twenty one male Balb C mice divided into seven groups: control (C), treatments with ethanol extract tubers of Dioscorea alata 0,00 g/kg, 0.17 g/kg, 2.01 g/kg, 10.04 g/kg (T I – T IV), treatment with antihistamine drug 0,4 mg/mice/day (T V) and treatment with Diosgenin 200 mg/kg (T VI) (Huang et al., 2010). For 17 consecutive days the T I- T IV groups were given ethanol extract of D. alata respectively with its dose, T IV group given antihistamine drugs and T V group given Diosgenin. On day 15, NC dan

T I – T V groups of mice were induced Ovalbumin 0,0483 mg/mice (Fischer *et al.,* 2005 with modification by Diding *et al.*, 2008). On day 18, three mice each group were sacrificed with cervical dislocation, mice were dissected and spleen is taken to isolated its lymphocytes and analyze the transcription factor FoxP3 Treg cells, cytokine profile of T reg cells and IgE and IgG1 B cells on spleen using Flowcytometry FACS Calibur.

Statistical analysis of the data. Data are presented as means ± SD. Comparison between groups was performed by the one-way analysis of variance continued with Tukey test. Considered a value of $p < 0.05$ was statistically significant.

3. Results and Discussion

Body weight of mice on sensitization phase weighed every day for 17 consecutive days. Figure 1 showed the weight of mice on sensitization phase after injected Ovalbumin intraperitoneally on day 15 experienced a weight loss of 5.44% on the 16th day and 4.15% on day 17. There is significant effect of Ovalbumin injection on weight loss ($p < 0.05$).

Weight parameter in this study need to be displayed because one of the marker of success murine model of allergic is the occurrence of diarrhea in mice is characterized by watery stools and weight loss. This study succeeded in making mice model of allergic through a slight drop weight after giving Ovalbumin allergen which is appropriate with Mollica et al. (2013) research. The diarrhea in mice is characterized by watery stool that also occurs in mice model of allergic gastrointestinal tract is shown with stools more watery and runny.

This research indicate that there is no difference in feed intake between groups of mice, but mice treated Ovalbumin lose a little of their body weight. It is caused by the occurrence of hypercatabolism which are caused by the production of inflammatory cytokines (Dourado et al., 2010). This study did not evaluate further whether weight loss in the treatment group Ova is caused by a decrease in feed consumption or water retention as Moreira (2006) research in mice were sensitized with Ovalbumin. Jan et al. (2007) research also showed that the body weight of mice which is treated Diosgenin dose 200 and 400 mg/kg, there was no significant difference in body weight of mice untreated control group.

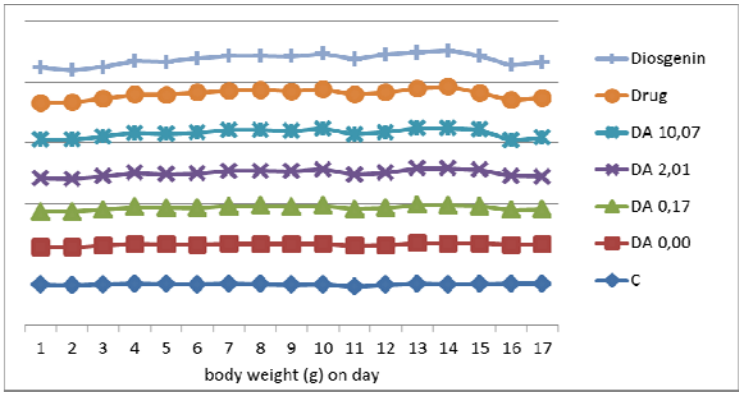

Figure 1. Comparison of body weight of mice (g) on the sensitivity phase in the control group (C) and treatment group with EEDA 0.00; 0.17; 2.01 and EEDA 10.04 g/kg, Antihistamines Drugs and Diosgenin.

According to Saldanha et al. (2004), Ovalbumin as allergen sensitization in mice increases levels of IgE and IgG1 in blood sera. IgE and IgG1 (Th2 response) versus IgG2a (Th1 response) is a good marker for the induction of allergic responses in mice (Adel-Patient et al., 2000). According Dourado et al. (2011), sensitization Ovalbumin in BALB/c mice increases levels of IgE and IgG1.

This study showed that it has been an increase in B220IgE and B220IgG1 cytokine profiles in treatment group induced Ovalbumin on EEDA 0.00; 0.17; 2.01; 10.04 g/kg; Anti Histamin Drugs and Diosgenin groups compared with the control group in the sensitization phase. The highest B220IgE and B220IgG1 cytokine profile EEDA 10.on04 g/kg group and the lowest B220IgE and B220IgG1 cytokine profile is in the control group as shown in Figure 2(a).

The results are consistent with Barwig et al. (2010), Aguilar-Pimentel et al. (2010) and Lee et al. (2013) that intraperitoneal injection of Ovalbumin with low doses as an antigen together with Aluminum Hydroxide as adjuvant result in increased formation of IgE antibodies with high levels. The results of this study demonstrate that Ovalbumin as allergens managed to create mice model of allergic proven by administering Ovalbumin can increase levels of IgE and IgG1 on BALB/c mice on sensitivity phase because one of the clinical symptoms of allergy are increased levels of IgE and IgG1 (Dourado et al., 2010, Makiyah et al., 2014).

Ovalbumin in this study can stimulate allergic reactions. IgE is a cytokine that was very crucial in the development of allergic reactions (Li-Weber and Krammer, 2003; Cookson, 2004; Diding et al., 2008). Haghighi et al. (2006) showed that administration of oral Ovalbumin in mice were able to induce the production of Th2 cytokines and IgE, which it will result in an immune response to the allergic-inflammatory.

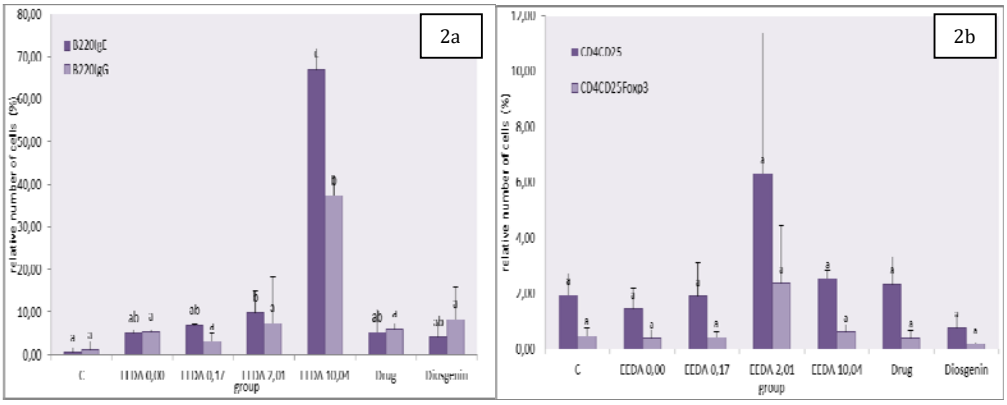

Figure 2 Comparison of relative number of cells of mice expressing B220IgE and B220IgG1 and **(b)** CD4CD25 and CD4CD25FoxP3 on the sensitivity phase. Description: a bar chart followed by the same letters between group means there is no significant difference (p> 0.05).

There are significant difference on B220IgE and B220IgG1 ($p < 0.05$) cytokine profile in sensitivity phase and Tukey's test showed there are significant difference B220IgE cytokine profile between EEDA dose 10,04 g/kg with the Control group. EEDA dose of 0.00; 0.17; 2.01 g/kg, Anti Histamine Drugs and Diosgenin while among the Control group, EEDA dose of 0.00; 0.17 g/kg, Anti Histamine Drug and Diosgenin showed there are no significant difference. Between EEDA dose of 0.00; 0.17; 2.01 g/kg, Anti Histamine Drug and Diosgenin groups showed no significant difference. Tukey test results showed significantly different between groups B220IgG1 cytokine profile of EEDA dose of 10.04 g/kg with the Control group. EEDA dose of 0.00; 0.17; 2.01 g/kg, Anti Histamine Drug and Diosgenin while among the Control group, EEDA dose of 0.00; 0.17; 2.01 g/kg, Anti Histamine Drug and Diosgenin showed there are no significant difference.

$CD4^{+}CD25^{+}$ cells is thought as conventional cells that express the CD25 surface mole-cules and regulatory T cells that will surely increase with the entry of foreign substances are inserted into the body. CD25 surface molecules known also as the IL-2α receptor, which may be expressed by conventional cells other than $CD4^{+}$ T cells (Lee et

al., 2006). CD4CD25 Treg cells were identified by expression of double-positive CD4 and CD25 markers specific membrane.

This study showed that on the sensitivity phase already increased CD4CD25 cytokine profile in the EEDA dose of 2,01; 10.07 g/kg and Anti Histamine Drug group, as also been has an increase in transcription factor CD4CD25FoxP3 profile in the EEDA doses of 2.01 and 10.04 g/kg group as shown in Figure 3. CD4CD25 cytokine profile and transcription factors CD4CD25FoxP3 on the sensitivity phase showed no significantly difference ($p > 0.05$).

Treg cells play an important role in immunological disorder underlying allergic disease (Wu et al., 2007). Treatment of asthma allergic diseases latest also focused on increasing the role of T reg cells to protect against severity of asthma (Nouri-Aria & Durham, 2008). Some studies showed the occurrence of a significant reduction of Treg cells $CD4^+CD25^+Foxp3$ in perifer blood and bronchoalveolar lavage fluid of patients with asthma (Ryanna et al., 2009). Some therapies in allergic disease is known to increase Treg cells (Nouri-Aria & Durham, 2008). Treg cells has been widely utilized as an experimental model for treating various diseases including allergic diseases (Kearley et al., 2008). Some research has also shown the role of FoxP3 and CD4CD25 Treg cells in allergic disease (Stock et al., 2006).

Susanti et al. 2013 research on children with asthma given a combination of adjuvant and *Nigella sativa* increase the percentage of $CD4^+CD25^+Foxp3^+$ Treg. Lopes et al. (2006) research proved that intracellular Foxp3 protein delivery inhibits the activation of T cells after stimulation of the T cell receptor (TCR) and the resulting suppressor function of T cells $CD4^+CD25^-$. Many recent studies have examined the role of Foxp3 Treg cells and $CD4^+CD25^+$ in allergic asthma and atopic patients (Stock et al., 2006). Peripheral blood mononuclear cells from patients with allergic rhinitis and asthma had fewer lymphocytes expression of $Foxp3^+$ and Foxp3 mRNA was significantly decreased (Hartl et al. 2007, Lee et al., 2007). Treg cells from atopic patients have less suppressor activity compared with Treg cells from non-atopic group (Ling et al., 2004), suggesting that Treg Foxp3 and has an important role in regulating the immune response in humans hypersensitive. Therefore, Foxp3 is a very important target for the development of immunosuppressive agents for both autoimmune diseases and allergies. Despite the importance of Foxp3 in imunotoleransi has been studied extensively, the clinical use of gene transfer of Treg or Foxp3 for this disease is still limited because of the difficulty of isolating the T reg cells human, transfection efficiencies are low, and the high risk gene viral-mediated delivery (Choi et al., 2010).

Treg cells eject function of its imunoregulatory through various mechanisms, among others, require CTLA-4, turn off the antigen presenting cell or T lymphocytes directly, using IL-2 cytokine, production immunosuppression cytokine such as IL-10, TGF-beta, IL-35 and galectin-1 (Vignali et al., 2008).

Treg cells secrete IL-10 and TGF-β in bulk (Shevach, 2009). IL-10 is a antiinflammatory cytokine produced Treg cells that control the peripheral immune response (Hawrylowicz, 2005) and plays a role in protection against allergic disease on individuals with mutations in FoxP3 (transcription factor Treg cells) in patients with food allergy (Hawrylowicz, 2005). Immu-notherapy to treat allergies have also been shown to increase levels of IL-10, which works by inhibiting the activity of Th2 cells (Akdis et al., 1998). Kearley et al. (2005) found a decrease in the production of Th2 cytokines in mice sensitized with Ovalbumin when granting Treg cells purified before exposure to antigen; they found that the production of IL-10 is not limited to Treg cells.

The result indicates that the sensitivity phase increased cytokine profile CD4IL-10 in the treatment group, namely Ovalbumin induced dose group EEDA 0.00; 0,17; 2,01; 10.07 g/kg, treatment with Anti Histamine Drugs and Diosgenin treatment, as well as an increase in CD8IL-10 cytokine profiles in the treatment group, namely Ovalbumin induced dose group EEDA 0.00; 0,17; 2,01; 10.07 g/kg and treatment with Anti Histamine Drug as shown in Figure 2(b).

CD4IL-10 and CD8IL-10 cytokine profile with one way Anova test showed no significant difference on CD4IL-10 cytokine profile (p>0.05) and significantly difference on CD8IL-10 cytokine profile (p<0,05). Tukey test results showed no significant difference CD8IL-10 cytokine profiles between treatment groups EEDA dose of 2.01 g/kg with Control, EEDA dose of 0.00; 0.17; 10.04 g/kg, Anti Histamine Drug and Diosgenin treatment. There was no significant difference between Control groups, EEDA dose 0.0 g/kg, Anti Histamine Drug and Diosgenin treatment. There was no significant difference between the Control groups with the EEDA dose of 0.00; 10.07 g/kg and Anti Histamine Drug treatment, there was no significant difference between EEDA 0.17 g/kg and 10.04 g/kg groups.

This study indicate that on the sensitivity phase increased CD4IL-10 cytokine profile in the treatment group induced with Ovalbumin namely EEDA dose 0.00; 0,17; 2,01; 10.07 g/kg, Anti Histamine Drug treatment and Diosgenin treatment, as well as an increase in CD8IL-10 cytokine profiles in the treatment group induced Ovalbumin namely EEDA dose 0.00; 0,17; 2,01; 10.07 g/kg and Anti Histamine Drug treatment as shown in Figure 3(a).

CD4IL-10 and CD8IL-10 cytokine profile in sensitivity phase showed no significant difference in CD4IL-10 cytokine profiles (p: 0.061) and significantly difference in cytokine profiles CD8IL-10 (p: 0.00). Tukey test showed significantly difference on CD8IL-10 cytokine profiles between EEDA dose of 2.01 g/kg treatment groups with Control, EEDA dose 0.00; 0.17; 10.04 g/kg, Anti Histamine Drug treatment and Diosgenin treatment. There is no significant difference between Control, EEDA dose 0,00 g/kg, Anti Histamine Drug treatment and Diosgenin treatment and there is no significant difference between the Control, EEDA dose 0,00; 10, 07 g/kg and Anti Histamine Drug treatment, no significant difference between groups EEDA dose 0.17 g/kg and EEDA dose10.04 g/kg.

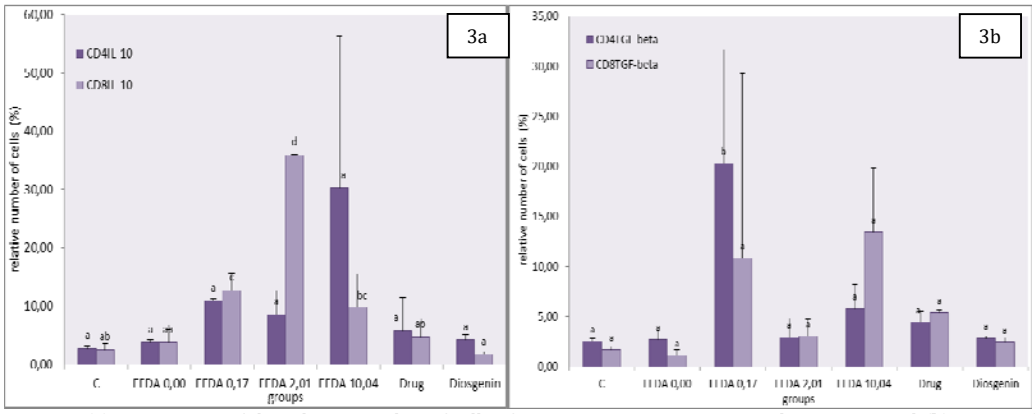

Figure 3. (a) Comparison of the relative number of cells of mice expressing CD4IL-10 and CD8IL-10 and **(b)** CD4TGFβ and CD8TGFβ on the sensitivity phase after given the ethanol extract tubers of Dioscorea alata L. (EEDA). Description: a bar chart followed by the same letters between group means there is no significant difference (p> 0.05).

The results of this study showed that the CD8TGFβ and CD4TGFβ cytokine profile in sensitivity phase increased in the treatment group namely EEDA dose of 0,17; 2,01; 10.07 g/kg, Anti Histamine Drug treatment and Diosgenin treatment.

There is significantly differences in CD4TGFβ cytokine profiles (p<0.05) and no significant differences in CD8TGFβ cytokine profiles (p>0.05) in the sensitivity phase. Tukey test showed there is significantly difference in CD4TGFβ cytokine profiles between treatment groups EEDA dose of 0.17 g/kg with Control group, EEDA dose 0,00; 2.01; 10.04 g/kg, Anti Histamine Drug treatment and Diosgenin treatment. There is no significant difference between Control groups, EEDA dose 0,00; 2.01; 10.04 g/kg, Anti Histamine Drug and Diosgenin treatment as shown in Figure 3(b).

Susceptibility to allergic diseases can not only be explained with a single through the impaired balance between Th1 and Th2 responses. Currently the immunoregulatory role of Treg cells has been known to suppress the adaptive immune response mediated by Th1 and Th2 (Herrick & Bottomly, 2003; van Oosterhout & Bloksma, 2005).

To develop immunosuppressive agents for treating autoimmune diseases or allergies, the regulation of T cell activation and function of Treg cells is an important target. Foxp3 has an important role not only as a key molecule in the development of regulatory T cells, but also as an inhibitor of T cell activation and repressor of transcription factors (Wan and Flavell, 2007; Campbell & Ziegler, 2007; Lopes et al., 2006).

This study showed that on the sensitivity phase, giving EEDA dose 2.01; 10.04 g/kg and Anti Histamin Drug increase CD4CD25 cytokine profile and giving EEDA 2.01; 10.04 g/kg increase transcription factors CD4CD25FoxP3, giving EEDA 0.17; 2.01; 10.04 g / kg; Anti Histamin Drug and Diosgenin increase cytokine profile CD4IL-10, giving EEDA 0.17; 2.01; 10.04 g/kg and Anti Histamin Drug increase cytokine profile CD8IL-10, giving EEDA 0.17; 2.01; 10.04 g/kg, Anti Histamin Drug and Diosgenin increase CD4TGF-β and CD8TGF -β cytokine profile.

The ethanol extract of Dioscorea alata L. with the active compounds contained it, one of which is a saponin steroids are useful as allergenic agent able to activate regulatory T cells so that the sensitization phase EEDA 2.01 and 10.04 g/kg increase CD4CD25 cytokine profile and transcription factors CD4CD25FoxP3. BALB/c mice which only induced Ovalbumin namely the EEDA 0,00 g/kg decreased the percentage of transcription factors CD4CD25FoxP3 on the sensitization phase. BALB/c mice which induced Ovalbumin and treated EEDA 0.17; 2.01; 10.04 g/kg, Anti Histamin Drug and Diosgenin are increase in the percentage of transcription factors CD4CD25FoxP3 on the sensitization phase.

Giving EEDA has no significantly effect on CD4CD25 cytokine profiles and the percentage of CD4CD25FoxP3 transcription factors in the sensitization phase. This shows that the possibility of increasing the activity of T reg cells and transcription factors CD4CD25FoxP3 triggered by other factors including through the profiles of cytokine produced by T reg cells: CD4IL-10, CD8IL-10, CD4TGF-β, CD8TGF-β. The results of this study according to Huang et al. (2009, 2010) which showed an increase in the number of cells of $IL\text{-}10^{+}FoxP3^{+}$ allergic of mice treated Diosgenin.

4. Conclusion

Ethanol extract tubers of Dioscorea alata L (EEDA) does not trigger Treg (CD4CD25) and the transcription factor FoxP3 (CD4CD25FoxP3) and cytokine profiles produced by Treg cells namely CD4IL-10, CD8IL-10, CD4TGF-β and CD8TGF -β in the sensitivity phase.

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