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Steroidal Saponin Isolated from *Dioscorea alata L.* Extract Improve Digestive Tract Allergy in Balb/C Mice Induced by Ovalbumin

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ABSTRACT

Steroidal saponin compound in Dioscorea species is one of the crucial substances because it has some biological functions, such as immunomodulatory and anti-allergic agent. This study aims to analyze the effect of steroidal saponin of *Dioscorea alata L.* on CD4 T cells activation, cytokines IL-4, IL-5 and TGF-β. This study used primary lymphocyte cell which isolated from BALB/c mice spleen. Mice lymphocytes were cultured in the complete medium with presence of various concentrations (50, 25 and 12.5 µg/mL) of three isolate of steroidal saponin of D. alata for 24 h. Diosgenin was used as positive control. After 24 h of treatment, the activation of lymphocyte, IL-4 and IL-5 production was determined by Flowcytometry analysis. Data were analyzed using student t-test with $p < 0.05$ and $p < 0.01$. The results showed that the low concentration of Isolate 1 (12.5 µg/mL) was found to be effective to stimulate the activation of CD4 T cells, reduce the production of IL4 and IL-5 by CD4 T cells and increase TGF-β production. These results showed that a low concentration of steroidal saponin of D. alata could be considered as immunomodulatory and anti-allergic agent. Purification and identification of each isolate isolated from DAE is in progress.

Key words: allergy, inflammation, diosgenin, purple yam, steroidal saponin

INTRODUCTION

Allergies or hypersensitivity are immune failures so that the body becomes very sensitive when reacting immunologically with substances that are generally non-immunogenic. In this case, the human body overreacts to the substances that the body perceives as foreign or harmful (Abbas *et al*., 2019; Luis Muñoz-Carrillo *et al*., 2018). Substances that cause hypersensitivity are called allergens (Marwa & Kondamudi, 2021). The overreaction of the immune system in some cases lead to inflammatory response. All inflammatory responses have the same mechanism: 1) cell surface pattern receptors recognize noxious stimuli; 2) inflammatory pathways are activated; 3) inflammatory markers are released; and 4) inflammatory cells are recruited (Chen *et al*., 2018). One of immune cells, $CD4^+$ T helper (Th) cells, are well known for their role in infection, autoimmunity, and inflammation (Huber *et al*., 2017). Th type-2 (Th_2), which secrete interleukin (IL)-4, IL-5, IL-9 and IL-13, are thought to contribute to allergic reactions (Gour & Wills-Karp, 2015; Koyasu & Moro, 2011). In the other hands, Transforming Growth Factor Beta (TGF)-β play a pivotal role to suppress the Th_2 response (Macey *et al*., 2010). Thus, to improve homeostasis efficiently, the activities of $CD4^+$ T cells must be stabilized by modulating their cytokines.

A substance that affects the functioning of the immune system has been known as immunomodulator (Sharma *et al*., 2017; Shivhare *et al*., 2015). Many immunomodulators from synthetic compounds are currently under development for the therapy of allergic diseases targeting on cytokines (Casale & Stokes, 2008;

Catanzaro *et al*., 2018). But the problem is the synthetic compound has not easily absorbed in the body, and sometimes cause side effects. Saxena *et al*. (2012) explained that, side effects of prescription synthetic immunomodulator include nausea, vomiting, diarrhea, stomach ulcers, rash, malaise and liver inflammation (Saxena *et al*., 2012). There are also other side-effects, such as hypertension, dyslipidemia, hyperglycemia, peptic ulcers, liver, and kidney injury. Thus, it is important to develop immunomodulator substance from the natural product (Harun *et al*., 2020) because it is considered a safer alternative which have fewer side effects and less toxic to the body (Tarapdar *et al*., 2020). The discovery of immunomodulator from plant has potential to counteract the side effect and high costs of synthetic compounds (Jantan *et al*., 2015).

Purple yam (*Dioscorea alata* L.) is one of the staple foods derived from tubers. *D. alata* is classified as functional food because it has many benefits, especially in maintaining the body's defense system. However, it has not been much utilized optimally (Christina & Rifa'i, 2014). Our previous research proved that an ethanol extract of *D. alata* had hypo-allergenic and immuno-modulatory activity (Makiyah, 2018; Makiyah *et al*., 2014, 2016, 2015). Steroidal saponin is the most crucial compounds contained in *Dioscorea* species because it has some biological functions such as immunomodulatory and anti-inflammatory depend on its structure (Dong *et al*., 2008; Kaur *et al*., 2021; Wang *et al*., 2011). *D. alata* contains diosgenin (Cheng *et al*., 2007; D *et al*., 2019), the main aglycon steroidal saponin. The immunomodulatory activity of steroidal saponins from *D. alata* have not yet been much discovered up to date. Therefore, this research aims to analyze the immunomodulatory activity of *D. alata* extract and its steroidal saponin especially through measurement the relative number of cytokine IL-4, IL-5 and TGF-β in digestive tract allergy mice model.

MATERIAL AND METHODS

Preparation the ethanol extract of Dioscorea alata (DAE)

The simplicia of *D. alata* were extracted with maceration method used 70% ethanol solution (Chairunnisa *et al*., 2019). The pooled ethanol extracts were filtered and evaporated in Rotary Evaporator equipment. The resulting powder was used as a *D. alata* extract (DAE) for the subsequent assays.

Gas Chromatography-Mass Spectrophotometry (GC-MS)

GC-MS analysis of bioactive compounds from the N-hexane extract of *Dioscorea alata L.* tuber was carried out using the Agilent Technologies GC system with the model GC-7890A/MS-5975C (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5MS HP-Column. (30 m length x 250µm diameter x 0.25 m film thickness). Spectroscopic detection by GC-MS involves electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas at a flow rate of 1 mL/min. The initial temperature was set at 50 – 150°C with an increase rate of 3°C/min and a holding time of about 10 minutes. Finally, the temperature was raised to 300°C at 10°C/min. One microliter of 1% extract diluted with each solvent was injected in no-separation mode. The relative quantity of chemical compounds present in each N-hexane extract of *Dioscorea alata L*. tuber was expressed as a percentage based on the area of the peak produced in the chromatogram.

Isolation of steroidal saponin from DAE

The 10 g DAE was fractionated using column chromatography sequentially using chloroform 10x 100 mL (FA), chloroform:methanol [1:1] 10x 100 mL (FB) and methanol 10x 100 mL (FC). Each fraction was tested for its steroidal saponin content qualitatively by TLC with butanol:ethanol:water (BEA) eluent = 6:2:3 (Fischer *et al*., 2005). Liebermann–Burchad reagent, acetic anhydride and sulfuric acid (1:1), was added to the TLC results. The saponin preparations were detected by UV light, with a purple color indicating a positive result. The positive fraction contained steroidal saponins isolated by preparative TLC.

The TLC test results revealed the most active fraction among FA, FB, and FC. The most active fraction was then isolated with silica gel GF254 using hexane:chloroform in ratios of 6:4 (1000 mL), 7:3 (500 mL), 8:2 (1000 mL), 9:1 (500 mL), and 95:5 (1000 mL). The isolated fraction was tested for its saponin content by TLC, resulting in the steroidal saponin isolates, termed isolate 1 isolated from FB, isolate 2 isolated from FA, and isolate 3 isolated from FC.

Animal experiment

The experimental animal is 30 male Balb/C mice, body weight ± 20 grams, and aged ± 6 weeks (Animal Center, Experimental Animal Service Unit, Galaxy Science, Jember. East Java). The mice feed was standard feed (Galaxy Science, Jember). The

food and drink is provided *ad libitum* during research. They were acclimated for 10 days before the experiment. This research has received ethical clearance approval by the Research Ethics Commission from Brawijaya University Malang with a certificate number 144-KEP-UB.

Allergy induction with Ovalbumin

To create an allergy model, mice were sensitized and challenged intraperitoneally (i.p.) and orally with Ovalbumin (ova; Sigma Aldrich). The first is by i.p. injection with a dose of 0.0483 mg dissolved in an aluminum hydroxide on the 15th day and 0.0375 mg dissolved in a water on the 22nd day. Second, orally on 23rd and 28th day with a dose of 0.15 mg dissolved in a water (Fischer *et al*., 2005) with modifications by Diding (Diding *et al*., 2008).

Treatments

Each type of the mice was randomly assigned into 5 groups (6 mice/group) on Day 0. The groups were control, allergy (ova induction), DAE (ova+ DAE 170 mg/kg BW), AH (ova+ anti-histamine drug 0.4 mg/kg BW), and Diosgenin (ova+ diosgenin (Sigma-Aldrich USA D1634-5G) 200 mg/kg BW). The treatment was performed for 24 and 30 days. On the 25th (challenge phase) and 31st (allergy phase) day, three mice from each group were sacrificed by dislocation, the mice were dissected and their spleens were taken for lymphocyte isolation.

Lymphocytes cells isolation

Spleen then washed two times in sterile Phosphate Buffer Saline (PBS). Lymphocyte was isolated from spleen by crushing the spleen in PBS. The homogenate of lymphocyte cell was resuspended with 10 mL PBS in 15 mL polypropylene tube and then centrifuge at 2500 rpm, 4°C, for 5 min.

Lymphocyte cell culture

All culture procedure was conducted Laminar Air Flow. Healthy mice were dissected and the spleen was isolated. Cells was resuspended with 1 mL complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicilin-streptomycin). A total of 7.5 x 10^3 cells/ml was seeded in 24 well plates and then incubated for 24 h, 37°C in 5% CO_2 incubator. After 24 h of incubation, cells were then treated with DAE and three different isolate of steroidal saponin of *D. alata*: Isolate 1, Isolate 2, Isolate 3, and Diosgenin. After 24 h of treatment, cell were harvested by pipetting medium of each treatment and replaced into 15 mL polypropylene tube.

Immunostaining and flow cytometry analysis

The homogenate of lymphocyte isolated from spleen of in vivo experiment and/or harvested cells from in vitro experiment were centrifuged at 2500 rpm, 10°C for 5 min. Cells were resuspended with 1 mL of PBS and continued to immunostaining procedure. Pellet was stained with following extracellular antibody: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and phycoerythrin (PE)-conjugated anti-mouse CD62L. Pellet resuspended with 50 µl of antibodies in sterile PBS. Antibodies for intracelluler staining are PECy5-conjugated anti-IL4, PECy5-conjugated anti-IL5, and PECy5-conjugated anti-TGF-β. For intraselular staining, pellet were added with 50 µl cytofix-cytoferm and incubated for 20 min in darkroom. Then added with 500 µl washperm and centrifuged 2500 rpm, 4^{o}C, for 5 mi. Pellet was resuspended with 50 µl of intracellular antibodies in sterile PBS. Then, each sample was analyzed using flowcytometer (FACSCalibur; BD Biosciences, New Jersey, USA).

Data Analysis

Data were analyzed by BD CellQuest PRO software. The results presented in the figures are representative of at least three independent experiments that yielded similar results. The values are presented as the means ± standard deviation (SD). The differences were analyzed using Student's t-test. The statistical significance was set at $P < 0.05$ and $P < 0.01$.

RESULT AND DISCUSSION

Dioscorea alata extract (DAE) suppress the production of IL-4 and IL-5 cytokines in allergic model mice

IL-4 and IL-5 produced by T cells that belong to the Th_2 which thought to control the growth and effector function of those cell types that are involved in allergic inflammatory responses (Gour & Wills-Karp, 2015). In addition to driving the differentiation of Th_0 lymphocytes into the Th_2 phenotype, IL-4 is important in allergic immune responses owing to its ability to prevent apoptosis of T lymphocytes (Steinke & Borish, 2001). Thus in allergy condition, IL-4 and IL-5 cytokines will be increase. Indeed, in this study (Figure 1) IL-4 and IL-5 cytokines in allergy group were significantly increase in challenge and allergy phase compared to control. Interestingly, DAE has the great ability

to suppress those cytokines in allergy, as well as anti-histamine drug (AH), when compared to allergy group. While, diosgenin only showed tendency to reduced IL-4 and IL-5 cytokines.

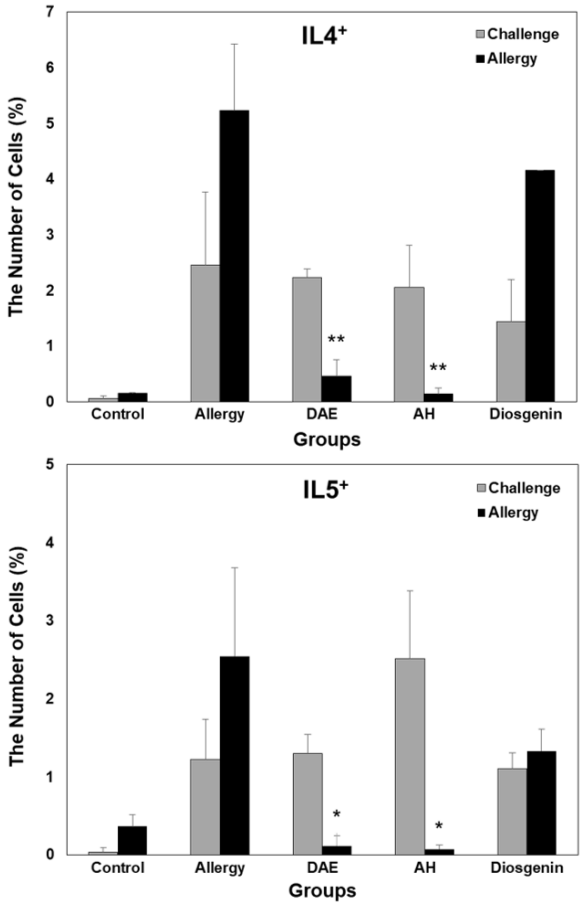

Figure 1. The Dioscorea alata extract (DAE) decrease the IL-4 and IL-5 cytokines in allergy phase. Cell extracts were prepared from the mouse spleen. The number of cytokines were analyzed by Flowcytometry. The data represent the means ± SD (n = 6 mice). * P < 0.05 and ** P < 0.01 versus allergy group.

The DAE improves the production of TGFβ cytokines in allergic model mice

The change in IL-4 and IL-5 cytokines during allergy condition is thought to be caused by a decrease in Th_2 response due to the increasing of another cytokine. TGF are cytokines that can act on mast cells during an immune response, especially during Th_2-driven allergic inflammatory response (Macey *et al*., 2010). It means that TGF-β can suppress the Th_2 response and their cytokines. In this result (Figure 2) the relative number of TGF-β produced by $CD4^+$ T cells significantly increase in allergy phase after administration of DAE, AH, and diosgenin compared to allergy group ($p < 0.01$). Whereas, in challenge phase, the number of these cytokine has not been affected yet.

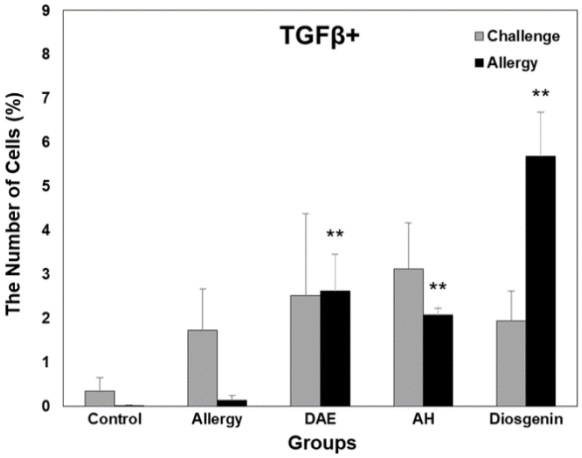

Figure 2. The *Dioscorea alata* extract (DAE) improves the TGF-β cytokines in allergy phase. Cell extracts were prepared from the mouse spleen. The number of cytokines were analyzed by Flowcytometry.
The data represent the means ± SD (n = 6 mice). * P < 0.05 and ** P < 0.01 versus allergy group.

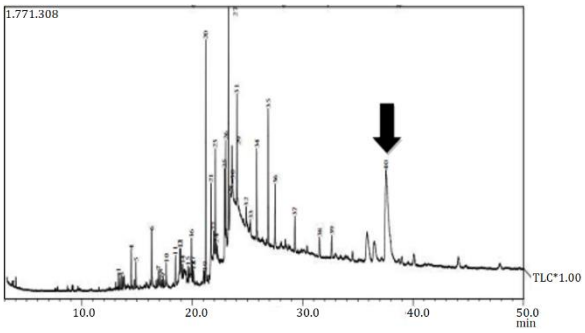

Figure 3. The GC-MS chromatogram of DAE

The DAE contain steroidal saponin detected by Gas Chromatography-Mass Spectrophotometry

The DAE is thought to contain steroidal saponin (Dong *et al*., 2008; Wang *et al*., 2011), thus we performed GC-MS analysis to determine the compounds contained in DAE. Gas Chromatography-Mass Spectrophotometry (GC-MS) results showed that DAE contain steroidal saponin (compound no.40) (Figure 3). The GC-MS results showed that there were 11 compounds that had the highest spectral peaks, namely compounds number 20, 21, 23, 25, 26, 27, 29, 31, 34, 35 and 40. These compounds were hexadecanoic acid methyl ester (methyl palmitic), hexadecanoic acid

(palmitic acid), alpha octadecene, hexadecadinoic fatty acids (palmitolinoleic acid), octadecanoic acid (methyl octadec-14 enoic, triglycerides), octadecanoic acid (oleic acid), eicosadinoic acid methyl ester, tricosanol (leicosanol), tetradecanol, benzenecarbolic acid (phthalic acid), γ-sitosterol (steroidal saponins).

Steroidal saponin isolated from DAE (SDA) potentially increase the proliferation and activation of CD4⁺ cells

Furthermore, we try to isolate the steroidal saponin from DAE to elucidate which constituents that responsible in immune system activation. The results showed that only Isolate 1 of steroidal saponin of DAE with a dose 12.5 µg/mL possessed a significant ($p < 0.01$) increase in CD4⁺ T cells activation compared to control and another isolates (Figure 4). The other isolates and diosgenin was not able to activate CD4⁺ T cells due to the same relative number of naive T cells and CD4⁺ T cells compared to control. Because only 12.5 µg/mL of isolate 1 significantly activated CD4⁺ T cells, we only focus on isolate 1 (hereinafter referred to as SDA) for further analysis.

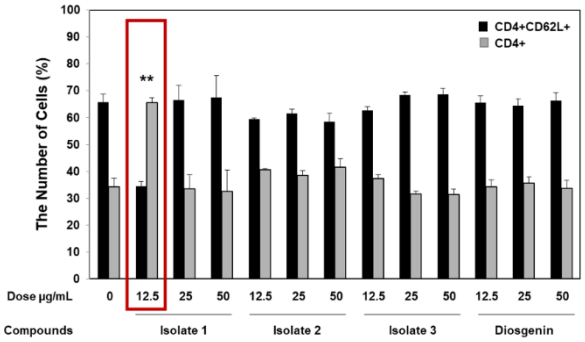

Figure 4. the impact of an alternative isolate of DAE's steroidal saponin on CD4+ T cell activation. For 24 h, the lymphocytes were grown in RPMI with/without isolates 1, 2, and 3 as well as diosgenin as a positive control. The number of cells was determined by flow cytometry, counted three times, and shown as means standard deviation (SD). **P 0.01 and *P 0.05 in comparison to control.

The SDA modulates cytokines produced by CD4⁺ T cells

This study demonstrated that Isolate 1 of steroidal saponin (SDA) from DAE is thought to be a compound that plays a role in modulating cytokines during the inflammatory allergic response. When SDA added to the culture medium, it decreased the relative number of IL-4 and IL-5 (Figure 5) produced by CD4⁺ T cells significantly, as well as DAE, compared to control group ($p < 0.05$). While, the number TGF-β cytokines was significantly increased (Figure 6).

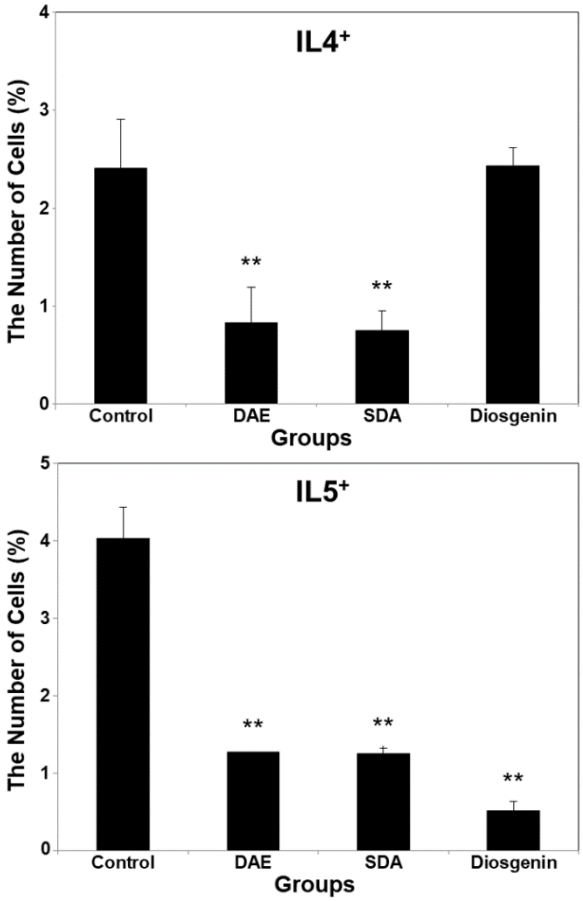

Figure 5. The SDA reduces the proportion of IL-4 and IL-5 cytokines in the body. For 24 hours, the lymphocytes were grown in RPMI either with or without isolate 1. The number of cytokines was analyzed by flowcytometry and measured in triplicate and are shown as a means ± standard deviation (SD). *Compared to the control, P0.05 and **P 0.01

Allergic reactions result from an excessive immune response to the allergens. The material used as an allergen in this study was ovalbumin. Ovalbumin is a protein derived from chicken egg whites. Allergen provocation will affect eosinophils as cells that have the ability to regulate immunity through modulation of T cell responses and local tissue inflammation (Jacobsen *et al*., 2007).

Exposure to antigen presenting cells by Ova will initiate the activation of Th cells. In the other hands, the activated of Th cells may lead to its differentiation to type 2 Th (Th_2). Several studies have documented increased numbers of activated Th cells in the lungs of subjects with asthma and in the majority of studies to date these cells were found to express cytokines characteristic of Th_2 cells (Georas *et al*., 2005; D. S. Robinson *et al*., 1993; Douglas S. Robinson *et al*., 1992).

In the other words, an enhanced Th_2 immune response and the elaboration of cytokines such as IL-4 and IL-5 contribute to the induction of allergy (Soyer *et al*., 2011). IL-4 is crucial for the differentiation of naïve Th cells into the Th_2 effector cells. IL-4 also has a central role in the pathogenesis of allergic inflammation (Gour & Wills-Karp, 2015; Magni *et al*., 2010). Similar to IL-4, IL-5 also plays an active role in the allergic responses. IL-5 act on mast cells to promote the histamine release seen in allergies (Greenfeder *et al*., 2001). Indeed, this research showed an increase of IL-4 and IL-5 cytokines in digestive tract allergy mice model both in challenge and allergy phase (Figure 1). While, *D. Alata* extract (DAE) administration in allergy mice model showed a decrease in the number of these cytokines, as well as anti-histamine drug (AH).

Beside IL-4 and IL-5, TGF-β is also a cytokine that plays a role during allergic responses. But its role is reversed with the previous two types of cytokines. When IL-4 and IL-5 act to enhance allergic responses, TGF-β inhibits them by suppressing the Th_2 response (Macey *et al*., 2010). These two groups of cytokines work in counterbalance to maintain immune system homeostasis. Digestive tract allergy mice model, the relative number of TGF-β was decrease in allergy phase compared to challenge phase (Figure 2). This condition is thought to be the main cause of the increase in IL-4 and IL-5 cytokines during the allergic response. In the other hand, in the allergy mice with DAE administration, the number of TGF-β cytokine was significantly increase, as well as AH and diosgenin administration. Thus, it may cause a decrease in IL-4 and IL-5 cytokines.

Many studies proved that steroidal saponin is the most active compounds contained in *Dioscorea* species because of its immune-modulatory and anti-inflammatory activity (Dong *et al*., 2008; Wang *et al*., 2011). Therefore, we tried to isolate the steroidal saponin form DAE. This study showed that the isolate 1 of steroidal saponin (SDA) from DAE was increase the activation of $CD4^+$ T cells *in vitro* (Figure 4). Naïve T cells had a variety of cell surface molecules, one of which is molecular adhesion CD62L. CD62L (L-selectin) was a T cell homing receptor playing an important role as a marker for the development of T cells expressing a naive T cell. The expression CD62L disappeared quickly as soon as T cells bound to the T cell receptor and turned T cells into $CD62L^-$ after activated (Sprent & Surh, 2011). It means that a decline in the number of CD62L on T cells, in this case $CD4^+$ T cells, indicated the activity of a naïve cell turned into a subset of $CD4^+$ T cells, that also known as T helper (Th) cells (Shivhare *et al*., 2015). Further we investigated the immunomodulatory activity of SDA on IL-4, IL-5 and TGF-β produced by Th cells. Interestingly, as shown in Figure 5, the SDA was significantly suppressed the relative number of IL-4 and IL-5 cytokines. While significantly enhanced the relative number of TGF-β (Figure 6).

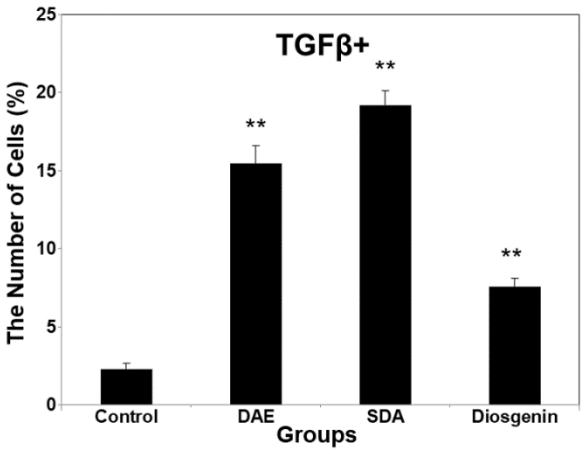

Figure 6. Shows that the SDA increases the proportion of TGF-β cytokines.. In RPMI with or without isolate 1, the lymphocytes were cultivated for 24 hours. The quantity of cytokines was counted by flowcytometry, quantified in triplicate, and represented as means standard deviation (SD). * P 0.05 and ** P 0.01 versus control.

Immunomodulators are substances that help to regulate or normalize the immune system. Immunomodulators works in 2 ways, repair weak immune systems and manage immune systems that are overactive. The main compounds contained in *D. Alata* is steroidal saponin. Steroidal saponins are glycosides consisting of an aglycone (diosgenin) and several glycosyl moieties. The most common sugars encountered in saponins are pentoses (arabinose, xylose, etc.), hexoses (glucose, galactose, etc.) and 6-deoxyhexose (rhamnose, etc.)

(Lin *et al*., 2007; Yuan *et al*., 2005). Steroidal saponins in the *D. alata* L. are thought to have anti-inflammatory and immunomodulatory activities (Cheng *et al*., 2007; Jayachandran *et al*., 2016; Jesus *et al*., 2016; Salehi *et al*., 2019). However, the mechanism of action of SDA to modulate the immune system during allergy has not been clearly understood. Purification and identification steroidal saponins isolated from DAE is in progress.

CONCLUSION

Steroidal saponin from *D. alata* extracts are potential as immunomodulatory agents by increasing the proliferation and activation of $CD4^+$ T cells. It also modulates cytokine by decreasing the number of cytokine IL-4 dan IL-5 trough increasing the number of TGF-β. Because Th_2 responses are the drivers of allergic responses, substance aimed at blocking key Th_2 cytokines such as IL-4 and IL-5 have been logical targets for the therapy of allergic diseases. Taken together, steroidal saponin compounds form *D. alata* extracts are thought to be responsible as the immunomodulatory and anti-allergic of *D. alata*.

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