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Effects of Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp.) Leaves Extract on Lymphocytes Count and Spleen Index of Male Balb/C Strain Mice (*Mus musculus* L.)

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Abstract **Original Research Article**

Antibiotics are applied to directly kill pathogenic microbes as a causal agent of infection. The excessive use of antibiotics as therapeutical agents on pathogen infections could trigger microbial resistance inside human body and even cause new diseases. As an alternative to reduce the use of antibiotics, therapy with natural ingredients contained in medicinal plants can help body by stimulating immune cells to eliminate invading pathogens. These medicinal plants are utilized as immunostimulant. As a tropical country, Indonesia has a variety of plants that can be used as medicinal plants, which one of them is the ornamental plant Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp.). In this study, we tested the effect of Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp.) leaves extract on lymphocytes count and spleen index of Balb/C mice (*Mus musculus* L.) as test animals. Pucuk Merah leaves extract was obtained by maceration method using 96% ethanol as solvent. Phytochemical compounds were analyzed using qualitative biochemical test and Gas Chromatography-Mass Spectrophotometry (GC-MS). Three doses used in the administration of the extract are 0.127, 0.510 and 0.765 mg/g of body weight. The extract was administered orally using a cannula. Lymphocytes count of treated mice were mainly based on blood smear analysis. Spleen of tested animal were weighed to determine its organ index. Our results showed that Pucuk merah leaves extract contained flavonoid, steroid, and tannin as detected by biochemical assays. Phytochemical quantitative test using GC-MS instrument detected 15 compounds with 1,2,3-Benzenetriol (37,80 %) and caryophyllene (27,49 %) as dominant compounds. Lymphocyte count of mice was tended to be decreased on the treatment of low dose (0.510 mg/g of body weight or less) of Pucuk Merah extract, but high dose (0.765 mg/g of body weight) was observed to increase mice lymphocyte count. Spleen index of Pucuk Merah extract-treated mice was not correlated with its lymphocyte count.

Keywords: *Syzygium myrtifolium* (Roxb.) Walp., phytochemical compound, *Mus musculus* L, lymphocytes count, spleen index.

INTRODUCTION

Human body has its protection against pathogen in the form of immune cells namely leucocytes. In overwhelming situation of pathogen infection, therapeutical drugs such as antibiotics can directly kill microbes and help human immune cells to fight infection. Since its widely medical application in 1940's, the use of antibiotics may lead also to microbial resistance in human body (Ventola, 2015). As an alternative solution to reduce antibiotic adversed effect, the use of medicinal plants that contained active compound for modulating immune responses in infection scenario is also necessary to be done. Indonesia has a large variety of medicinal plants that can be used as immunostimulants through active compounds contained in these plants. One of active compounds sourced from plants is flavonoid that can induce proliferation and differentiation of T cell lymphocytes (Hendrajid, 2020; Gaudino & Kumar, 2019).

Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp are quite popular in Indonesia as ornamental plants. Known also as Red Lip, Pucuk Merah are easily grown without any special treatment. In addition to their function as ornamental plant, it was discovered that Pucuk Merah leaves contains phenols, flavonoids, and betulinic acid (Sembiring *et al*., 2015). In a previous study, Putra *et al*., (2020) stated that several active compounds found in plants, including flavonoids can increase the activity of immune system in the body.

Based on those backgrounds, this study aimed to identify active compounds contained in leaves extract of Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp and their potency in modulating immune response of test animals in the form of lymphocytes count and spleen index.

MATERIALS AND METHODS

Sample Collection

Pucuk Merah (*Syzygium myrtifolium* (Roxb.) Walp green leaves were collected from several places in Yogyakarta. Collected leaves were sundried for three days. Sun-dried leaves were further proceeded in oven drying with 50-60°C of temperature scale. The fully-dried Pucuk Merah leaves were grinded to produce fine powder using blender.

Sample Extraction

Pucuk Merah leaves powder was extracted with maceration method using 96% ethanol as solvent. Maceration was carried out for 5 days with 500 g of Pucuk Merah leaves powder was macerated in 1.5 liters of 96% ethanol. Obtained extract was evaporated using a rotary evaporator.

Determination of Yield

The percentage of extract yield was calculated using following equation:

$$Yield\ (\%) = \frac{Mass\ of\ dried\ extract}{Mass\ of\ extract\ powder} \times 100\%$$

Qualitative Biochemical Assays

a. Flavonoid

Zero point three gram (0.3 g) of extract on test tube was added with 3 ml of n-hexane and 20 ml of 80% ethanol. Solution was then divided to three different tes tubes and labelled as flavonoid A, flavonoid B, and flavonoid C. The flavonoid test was carried out with three methods as follows:

- Flavonoids A : Wilstatter's Test

Solution was added with 0.5 ml of concentrated HCl and 4 pieces of magnesium (Mg). After Mg was dissolved, color change should be observed, and addition of 1 ml of distilled water and 1 ml of butanol were done consequtively. A reddish orange color change indicates the presence of flavones, pale red indicates the presence of flavonols, and dark red indicates the presence of flavanones (Mondong *et al*., 2015).

- Flavonoids B: Bate-Smith Test

Solution was added with 0.5 ml of concentrated HCl and heated on a water bath. Color change to purple or red indicate the presence of leukoanthocyanin compounds (Mondong *et al*., 2015).

- Flavonoids C

Solution was added with 0.5 ml of concentrated H2SO4 and heated on a water bath. Solution color changes to yellowish green indicates the presence of flavonoids (Lisi *et al*., 2017).

b. Alkaloid

Zero point three gram (0.3 g) of extract was added with 5 ml of 2N HCl, heated on a water bath for 2-3 minutes and stirred slowly. Zero-point three gram (0.3 g) of NaCl was added to this warm solution, homogenized, and filtered using filter paper. Filtrate was divided into three parts and labelled as alkaloid A, alkaloid B, and alkaloid C. Three drops of distilled water were added to alkaloid A as a blank, three drops of Mayer's reagent were added to alkaloid B, and three drops of Wagner's reagent were added to alkaloid C. Solution color change and formation of aggregates (white or yellow aggregates) after addition of Mayer's reagent indicates the presence of alkaloid, while the presence of alkaloid after addition of Wagner's reagent is showed by brown precipitate formation (Dahanayake *et al*., 2019).

c. Steroid dan Terpenoid

Zero point three gram (0.3 g) of extract was dissolved in 15 ml of ethanol and solution were divided to three parts, namely solution A as a blank, solution B, and solution C. After addition of three drops of anhydrous acetic acid and 1 drop of concentrated H2SO4 to solution B, solution was shaken slowly to see any color changes as indicators for steroid and terpenoid compounds. Green color indicates the presence of steroidal saponins, red color indicates the presence of triterpenoid saponins, and light-yellow color indicates the presence of saturated sapogenins. On the other hand, 1-2 ml of concentrated H2SO4 was added to solution C through test tube wall. The presence of unsaturated steroids is indicated by the formation of a red ring (Dahanayake *et al*., 2019).

d. Saponin

Zero point three gram (0.3 g) of extract in a test tube was dissolved in 10 ml of hot distilled water, cooled, and shaken vigorously for 10 seconds. If the solution forms a stable 1-10 cm foam layer, it indicates saponin content (Lisi *et al*., 2017).

e. Tanin

Zero point three gram (0.3 g) of extracts was dissolved in 10 ml of hot distilled water, allowed to cool at room temperature, then 3-4 drops of 10% NaCl was added, and solution was stirred and filtered. The filtrate was divided into two parts, labelled as solution A as blank and solution B. Two or three drops of FeCl3 were added into solution B, and color change was observed. Color change to blackish blue or blackish green indicate tannin content (Makalalag *et al*., 2011).

Gas Chromatography-Mass Spectrophotometry (GC-MS)

Quantitative phytochemical tests were carried out by LPPT Universitas Gadjah Mada, Yogyakarta. The condition of the GC-MS when used was as follows: UHP (He) helium carrier gas, using an injector temperature of 260°C, split flow 50 ml/min, split ratio 50, front inlet flow 1.00 ml/min, MS transfer line temperature 250°C,

ion Source temperature 200°C, purge flow 3 ml/min, gas saver flow 5 ml/min, and gas save time 5 min.

Treatment on Test Animals

a. Origin of Test Animal

Balb/C male mice (*Mus musculus* L) were obtained from Tikus Lovers Breeder, Yogyakarta. Mice weight ranged from 25-30 g. Ethical Clearance for this study was issued by Health Research Ethics Commission, Faculty of Medicine, Universitas Kristen Duta Wacana, Yogyakarta, Number: 1316/C.16/FK/2021 dated July 2nd, 2021.

b. Acclimatization

Acclimatization was carried out for 4 until 7 days. Mice of positive control (+) and negative control (-) groups were acclimatized for 4 days, while mice treated with Pucuk Merah extract were acclimatized for 7 days. Difference acclimatization time between groups aimed to provide a time lag for surgical process to be more effective. During the acclimatization period, test animals were fed with 30 g of commercial feed and 80 ml of drinking water. Cages, which has dimension 25 x 35 x 10 cm, were cleaned every day to get rid of dirt produced by mice and its feed.

c. Treatment Formulation

Tested animal were grouped to 5 groups (Table 1) as follows: K- (negative control) animal were treated only with distilled water, K+ (positive control) animal were treated with a commercial herbal-based immunostimulant, treatment group 1 animal were treated with Pucuk Merah extract (dose of 0,127 mg/g of body weight), treatment group 2 animal were treated with Pucuk Merah extract (dose of 0,510 mg/g of body weight), and treatment group 3 animal were treated with Pucuk Merah extract (dose of 0,765 mg/g of body weight). Each treatment had 5 replicates.

Table 1: Treatment Description for Animal Experiment

Name of Treatment	Material	Dose	Number of replication
Negative control (K-)	Aquadest	0,2 ml	5
Positive control (K+)	Stimuno	0,2 ml (dose 25mg/5ml)	5
Treatment 1 (P1)	Leaves extract of Pucuk Merah (*S. myrtifolium* (Roxb.) Walp)	0,127 mg/g of body weight	5
Treatment 2 (P2)	Leaves extract of Pucuk Merah (*S. myrtifolium* (Roxb.) Walp)	0,510 mg/g of body weight	5
Treatment 3 (P3)	Leaves extract of Pucuk Merah (*S. myrtifolium* (Roxb.) Walp)	0,765 mg/g of body weight	5

Water, commercial immunostimulant and Pucuk Merah extract were orally administrated using syringe and cannula. The volume of liquid treatment material given to each test animals was 0.2 ml. Dose determination of Pucuk Merah leaves extract followed a formula described by Liu and Fan (2018) (Table 2):

$$\frac{\text{A Animal (mg/kg)}}{\text{B Animal mg/kg}} = \frac{\text{B Animal Km}}{\text{A Animal Km}}$$

Table 2: Conversion of Doses (Liu & Fan, 2018)

Species	Weight (kg)	BSA (m^2)	$K_m$factor
Human Adult	60	1.6	37
Human Child	20	0.8	25
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3

d. Lymphocytes count

To observe mice immune response after treated with Pucuk Merah leaves extract, lymphocytes from mice bloods were counted. Lymphocytes count were mainly based on the preparation of blood smear as described by Sholekha *et al*., (2018). Blood smears were prepered on day 0, day 5, and day 10. Blood sampling was carried out after 2 hours of treatment on mice. Blood was taken from caudal vein by cutting end section of mice tail. Two or three drops of blood were placed on object glass, then quickly wiped off using another object glass. After being wiped off, object glass contained blood on its surface was let to be air-dried. Object glass would be then put in methanol solution for 5 minutes as fixative. Object glasses were air-dried again before staining using Giemsa for 30 minutes. The blood smear

preparation was repeated 3 times in each treatment group. Calculation of lymphocytes was performed using the following formula (Arif, 2009):

$$total\ number\ of\ cells = \frac{Cell\ type}{100\ cel\ of\ leukosit} \times 100\%$$

Spleen Index

Mice were sacrificed on the day 10 using overdose chloroform. Before surgery, test animals were weighed using an analytical balance. Spleen was removed and weighed as well. Both body and organ weight data will be converted into spleen index data following formula described by Intan, *et al.*, 2017 as follows:

$$total\ organ\ index = \frac{organ\ weight}{test\ animal\ weight} \times 100\%$$

RESULTS AND DISCUSSION

Extract Yield

Maceration of Pucuk Merah leaves extract in 96% ethanol obtained a fair thick texture and reddish-brown extract. The simplicia powder of Pucuk Merah leaves used was 1,247 kg and yielded 296 g of extract. Percentage of extract yield was 23.736%. The obtained yield was high because the maceration process used a stable room temperature and long duration of maceration. Chairunnisa (2019) stated longer maceration duration will produce higher extract yield. In this study, the extraction duration was 5 days, therefore it could produce high yields effectively. Our result was relatively like what Juwita et al (2017) found. In their study, maceration of 200 g of Pucuk Merah green leaves yielded 45 g of extract with yield percentage is 22.5%.

Phytochemical Group Identified from Pucuk Merah Leaves Extract Using Qualitative Biochemical Assays

Haryati (2015) mentioned that qualitative biochemical assay can be employed to detect several groups of phytochemical compounds, such as alkaloids, triterpenoids, steroids, saponins, phenolics, and flavonoids. As showed in Table 3 and Figure 1, our qualitative biochemical assays showed the presence of flavonoids, steroids, and tannins in Pucuk Merah leaves extract.

Table 3: Phytochemical Group of Pucuk Merah Leaves Extract as Identified by Qualitative Biochemical Assays

Active compounds	Result
Alkaloid B	-
Alkaloid C	-
Flavonoid A	+
Flavanoid B	-
Flavonoid C	-
Steroid	+
Terpenoid	-
Saponin	-
Tanin	+

Note: The sign (+) indicates the presence of a compound contained in the sample, the sign (-) indicates the absence of the compound contained in the sample.

Figure 1: Color Indication of Phytochemical Group of Pucuk Merah Leaves Extract as Identified by Qualitative Biochemical Assays

Note: (from left to right) tannins, tannin blanks, steroid/terpenoid blanks, terpenoids, steroids, flavonoids A, flavonoids B, flavonoids C, alkaloids blanks, B alkaloids, and C alkaloids.

All three phytochemical compound detected in Pucuk Merah leaves extract, namely flavonoid, steroid and tannin have potential power to modulate immune responses in order to inhibit inflammation.

In this study, flavonoid compounds were tested with three methods. Of these three methods, only Wilstatter test detected the presence of flavonoids in Pucuk Merah extract as indicated by orange color formation. Flavonoid compounds are polar; thus, extraction of this compound need polar solvent such as ethanol (Minarno, 2015; Agustina *et al*., 2016). In addition, flavonoid compounds are phenolic compounds that have a polyphenolic structure and have many roles in plant growth and medicinal applications. Flavonoid compounds can act as anti-inflammatory because of this compound can inhibit formation of pro-inflammatory cytokines such as IL-1β, IL-8, IL-6, and TNF-α2 (Fitri & Putra, 2021). When experiencing fatigue or stress, human body will produce pro-inflammatory cytokines. If pro-inflammatory is not controlled, it can lead to a greater risk of infection in the body. That is why the formation of pro-inflammatory cytokines needs to be induced. Flavonoid compounds play an active role in controlling the immune system by increasing anti-inflammatory cytokines to prevent inflammation (Khoirunnisa & Sumiwi, 2019; Fitri & Putra, 2021).

The appearance of red color in biochemical assay indicated steroid content of Pucuk Merah leaves extract. Steroid compounds are a class of triterpenoid compounds. The use of steroids in medicine is intended to be used as a substance for inhibiting the expression of pro-inflammatory cytokines and prevent inflammation. In addition, steroid compounds are also used as drugs in patients with hormonal diseases (Ardyati *et al*., 2017; Nasrudin *et al*., 2017).

Tannin compounds are classified as phenolic compounds. The presence of tannin in Pucuk Merah leaves extract was detected by the formation of blackish blue color. Tannins are synthesized through glucose metabolism and act as antioxidants. In addition to its antioxidant's potency, tannin compounds play an active role as anti-diarrhea, and anti-bacterial (Malangngi, 2012).

Phytochemical Compound Identified from Pucuk Merah Leaves Extract Using Gas Chromatography-Mass Spectrometry (GC-MS)

Quantitative phytochemical tests using GC-MS instrument detected 15 compounds (Figure 2) with 1,2,3-Benzenetriol (37,80 %) and caryophyllene (27,49 %) present as dominant compounds. As a member of flavonoid compound, 1,2,3-Benzenetriol could play role as antioxidant, antifungal, antiseptic, antiviral, antidermatic, and antimutagenic due to the presence of pyrogallol as its component (Gupta *et al*., 2021). Caryophyllene, which is grouped as terpenoid, is also component present in essential oils (Parfati & Windono, 2016). Faris (2012) stated that essential oils can strengthen the immune response or reduce the excess of immune response in autoimmune conditions by reducing pro-inflammatory cytokines such as IL-1β, IL-6, IL-10, and TNF-α. The presence of essential oils in Pucuk Merah (*S. myrtifolum*) (Roxb.) Walp.) was also mentioned in the research of Suryanto *et al*., (2017).

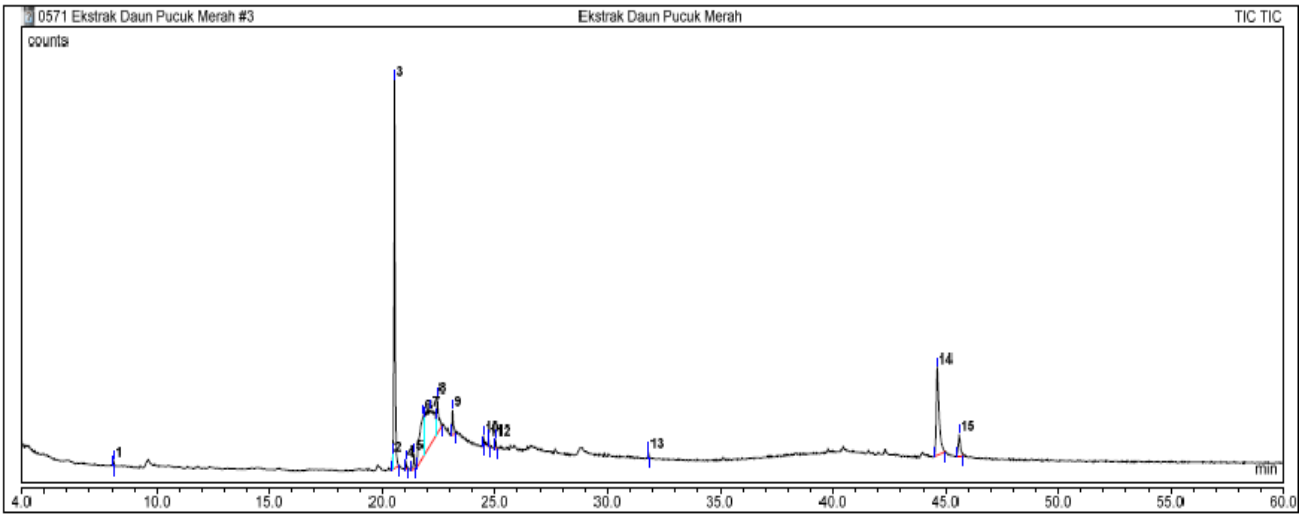

Figure 2: Phytochemical Compound of Pucuk Merah Leaves Extract Identified by Gas Chromatography-Mass Spectrophotometry

Lymphocytes Count in Pucuk Merah Extract-treated Mice

In this research, lymphocyte count was mainly based on blood smear preparation (Figure 3). The microscopic morphology of the lymphocyte on blood smear preparations showed a dark purple color, big round nucleus, and 7-10 micrometers cell diameter (Utami, 2020; Jacoby *et al*., 2002).

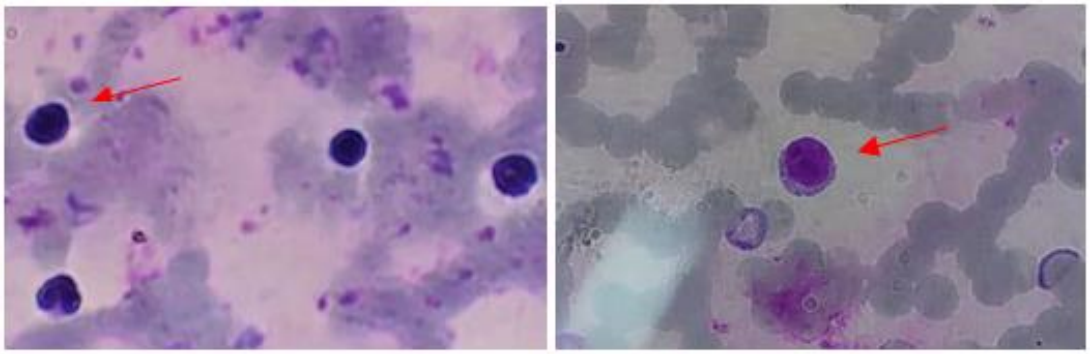

Figure 3: Mice lymphocyte morphology under microscope observation (100x magnification)

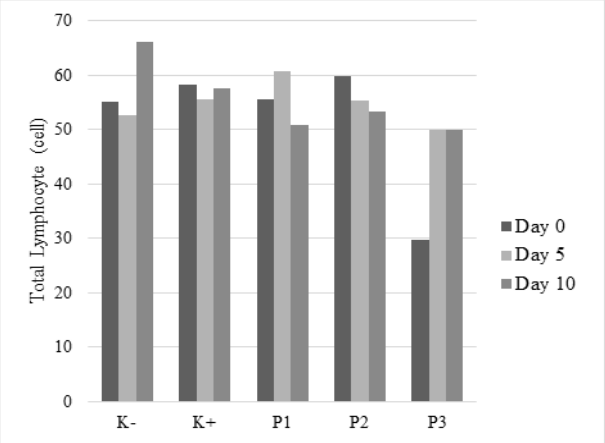

Figure 4: Mice Lymphocytes Count Using Blood Smear Method

Note: K- (Negative Control), K+ (Positive Control), P1 (extract dose : 0.127 mg/g of body weight), P2 (extract dose : 0.510 mg/g of body weight) and P3 (extract dose : 0.765 mg/g of body weight)

As shown in Figure 4, if compared to K- and K+ groups, lymphocytes count of Pucuk Merah extract-treated mice P1 (dose of 0.127 mg/g of body weight) and P2 (dose of 0.510 mg/g of body weight) were tended to be decreased at the end of experiment period (day 10). On the other hand, lymphocyte count of mice in P3 group (dose of 0.765 mg/g of body weight) showed to be increased in number during experiment period. An herbal-based commercial immunostimulant that was used as positive control (K+) in this research showed to sustain lymphocytes presence during experiment period. The number of lymphocytes on K+ group mice was relatively stable from day 0 to day 10. This herbal-based commercial immunostimulant contained Meniran (*Phyllanthus niruri* L.) leaves extract. A study from Rahmahani *et al*., (2016) stated that meniran leaves extract is potential as an immunostimulant.

Lymphocytes count of Pucuk Merah extract-treated mice might directed onto two ways. Lower dose of Pucuk Merah leaves extract (0.510 mg/g body weight or less) that was administrated orally for 10 days may decrease the presence of mice lymphocytes. The decreased lymphocytes count experienced by test animals can be seen as an indicator of immunosuppressant potency of the Pucuk Merah leaves extract. According to Lestariningrum (2012), immunosuppressive drugs can cause a temporary reversible decrease of lymphocytes number because of lymphocytes rapid response in the immune system. In other words, after experiencing a decrease, the number of lymphocytes will increase again. The decrease of lymphocyte number is not only affected by immunosuppressive compound. Number of lymphocytes can also be declined due to stress experienced by test animals when adapting to new environment and treatment condition. According to previous research conducted by Hendrajid (2020), decreased lymphocytes is caused by apoptosis progress of lymphoid follicles. Reduction in lymphoid follicles will reduce the activity of B cell lymphocytes and T cell lymphocytes.

Higher dose of Pucuk Merah leaves extract (dose of 0.765 mg/g of body weight) was observed to increase mice lymphocyte count. This phenomenon could indicate Pucuk Merah leaves extract potency as immunostimulant. Mataheru & Unitly (2020) states that the presence of flavonoid compounds can affect lymphocyte proliferation. Since Pucuk Merah extract also contained flavonoid as detected by biochemical assay and GC-MS, this immunostimulant potency could not be ignored. On the other hand, a higher dose of plant extract that was administrated to test animal could also lead to animal death because of their internal organ damage. Inflammation that involves immune cells such as neutrophil, monocyte and macrophage is a preliminary sign of internal organ dysfunction. Further inflammation will also recruit lymphocyte as component of adaptive immunity triggered by macrophage as antigen presenting cells. The increase of lymphocyte number after administration of plant extract could be related to the inflammation progress of internal organ, especially liver and kidney (Kaid *et al*., 2019; Chen *et al*., 2018).

Spleen Index

The spleen is an organ in vertebrates body that has a function as a place for blood reserves, site of lymphocyte production and antibodies formation. Spleen index which calculated based on comparison between spleen weight and body weight could be used as indicator of its function in immune response by producing lymphocytes (Wahyuningsih *et al*., 2017). As shown in Figure 5, Mice of K- group and most of Pucuk Merah extract-treated mice (P1 and P2 groups) showed higher spleen index than K+ group mice. It is assumed that higher index of spleen is related to the higher count of lymphocytes because spleen is an important site for lymphocytes production. Projection of spleen index to lymphocyte count, especially in day 10 (Figure 4) showed that spleen index is relatively correlated with lymphocyte count, as observed in K- group that has high spleen index and high lymphocyte count. Low doses of Pucuk Merah extract (dose of 0.510 mg/g of body weight or less) gave high spleen index of treated mice. On the other hand, high dose of Pucuk Merah extract (dose of 0.765 mg/g of body weight) resulted in low spleen index of treated mice. If we compare lymphocyte count and spleen index of K- group and extract-treated mice, it has no tendency that high spleen index will result in high count of lymphocyte and vice versa.

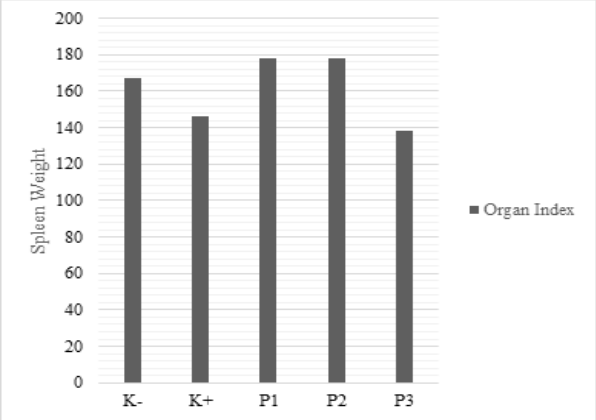

Figure 5: **Spleen Index of Treated Mice**

Note: K- (Negative Control), K+ (Positive Control), P1 (extract dose : 0.127 mg/g of body weight), P2 (extract dose : 0.510 mg/g of body weight) and P3 (extract dose : 0.765 mg/g of body weight). Spleen weight unit is miligram (mg)

Taken together, phytochemical compound contained in Pucuk Merah leave extract (*S. myrtifolium* (Roxb.) Walp) could be used as promising natural compound to modulate immune response, but method to test animal immune response is still needed to be developed to obtain clear immunomodulatory effect of this natural compounds.

CONCLUSION

Pucuk Merah leaves extract (*S. myrtifolium* (Roxb.) Walp) contained flavonoid, steroid, and tannin as detected by biochemical assay. Quantitative analysis using GC-MS identified 15 compounds with 1,2,3-Benzenetriol and caryophyllene were detected as dominant compounds. Modulation of mice immune response that was administrated with Pucuk Merah leaves extract depended on given dosage. Spleen index of Pucuk Merah extract-treated mice was not correlated with its lymphocyte count.

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