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***THE EFFECT OF KESAMBI BARK EXTRACT ON MICE
LYMPHOCYTE COUNT AND SPLEEN INDEX***

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Abstract

Kesambi is a plant widely used by people of Timor Island. Flavonoid content of Kesambi is potential to be used as immunomodulators. This study aimed to study the effect of Kesambi bark extract on lymphocyte count and spleen index of male mice as an indicator of their immune responses. Research included identification of phytochemical compounds macerated from Kesambi bark, lymphocytes count and spleen index calculation. Mice injected with sheep erythrocytes were treated orally with three doses of Kesambi extract (0.113 mg/g BW, 0.491 mg/g BW, and 0.712 mg/g BW) for 10 days. Lymphocyte count using blood smear method was carried out on day 0, 5 and 10, while lymphocyte count using hematology analyzer and spleen index measurement were performed after mice sacrificed. Kesambi bark contains alkaloids, flavonoids, tannins, and steroids. Treatment with Kesambi bark extract reduced mice lymphocyte count. Administration of 0.491 mg/gr BW dose reduced the mice lymphocytes count close to the number of lymphocytes in the negative control group. The spleen index of mice had no correlation with changes in the number of lymphocytes. This preliminary study shows a potential use of Kesambi bark extract as a natural immunosuppressant.

Keywords: *Kesambi; Lymphocytes; Spleen Index; Mice*

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INTRODUCTION

Kesambi is a commonly found plant in Timor Island and traditionally used as medicinal plant (Anonymous, 2015).

Several active compounds such as triterpenoids, phenolic, alkaloids, and flavonoids contained in kesambi bark supported its potency to be used as traditional medicines. Kesambi bark also has antibacterial activity against *Staphylococcus aureus* (Puspita *et al.*, 2019). Holil & Griana (2020) stated that flavonol as active compound of kesambi bark extract is potential to induce immunomodulation effect. Flavonol, which is a flavonoid compounds, is able to stimulate phagocytic cells to carry out phagocytic responses as an effort to increase the immune response, possessed antioxidant activity that can suppress tumor growth and adjust immune system through the proliferation and activation of T and B lymphocytes (Haeria *et al.*, 2017).

Though it has many benefits, research on Kesambi, especially on its bark, is still limited and rare. Some of the tests carried out were only limited to the crude extract and not yet specific. Based on Kesambi beneficial potency, this research aimed to study the effect of Kesambi (*Schleichera oleosa* (Lour.) Oken) bark extract on lymphocyte count and spleen index of male

mice (*Mus musculus* L) as indicators of their immune responses.

MATERIALS AND METHODS

Extract Preparation and Yield Calculation

Four hundred gram of Kesambi bark simplicia powder was placed into macerator, then dissolved in 4 L of 70% ethanol, stirred until homogeneous and kept in a light-proof room for 5x24 hours while stirring occasionally. On day 5, solution was filtered using filter paper and evaporated using a rotary evaporator to obtain a thick extract (Tenda *et al.*, 2017). The yield of kesambi bark extract was calculated using formula as follows:

$$\text{Yield} = \frac{\text{extract weight}}{\text{simplicia weight}} \times 100\%$$

Qualitative Biochemical Test

Alkaloid test

A total of 5 mL of 2N HCl was added to 0.3 g of kesambi bark extract in a test tube and heated for 2-3 minutes while stirring. After cooling, 0.3 g of NaCl was added to the test tube and filtered. The filtrate was added with 5 mL of 2N HCl and divided into 1A, 1B, and 1C solutions. Solution 1A was used as a blank. Solution 1B was added with Mayer's reagent. Appearance of cloudy solution after Mayer's reagent addition indicated the

presence of alkaloid compounds. Solution 1C was added with Wagner's reagent and formation of precipitate indicates the presence of alkaloid compounds (Marliana, 2011).

An amount of 0.3 g of kesambi bark extract was dissolved in 3 mL of n-hexane, added with ethanol and divided into 3 parts of solutions 2A, 2B, and 2C. Solution 2A was used as a blank. Solution 2B was added with 0.5 mL of HCl and heated while the color change was observed. The slowly formation of a bright red or purple color indicates the presence of leucoantocyanin compounds. Solution 2C was added with 0.5 mL of concentrated HCl and 4 pieces of magnesium and color change was observed. The 2C solution would then be diluted using distilled water, added with 1 mL of butanol, homogenized and observed for color changes. Color change to red orange, pale red, or dark red indicates the presence of flavonoid compounds (Marliana, 2011).

Tannin test

As much as 0.3 g of kesambi bark extract was dissolved in 10 mL of hot distilled water and 3-4 drops of 10% NaCl were added. The homogeneous solution was then filtered and divided into 2 parts, namely 3A and 3B. Solution 3A was used as a blank, while into solution 3B a little of

gelatin solution and 5 mL of 10% NaCl solution were added to observe the changes that occurred. The presence of a white precipitate indicates that it contains tannin compounds (Marliana, 2011).

Steroid test

Fifteen milliliters of ethanol was added to 0.3 g of kesambi bark extract and divided into 3 parts of solution 4A, 4B, and 4C. Solution 4A was used as a blank, solution 4B was added with 3 drops of H₂SO₄ then shaken slowly to produce a green-blue-red-purple-light yellow color indicating a positive result. In the 4C tube, 1-2 mL of concentrated H₂SO₄ was added. If a red ring is formed, it indicates the presence of steroid compounds (Marliana, 2011).

Saponin test

A total of 0.3 g of kesambi stem bark extract was dissolved in 5 mL of distilled water and then vortexed for 30 seconds. Saponin is indicated by the formation of stable foam with a height of 3 cm above the surface of the liquid for more than 1 minute (Marliana, 2011).

Preparation of Sheep Erythrocytes Suspension

Sheep blood was divided into 4 falcon tubes and centrifuged at 3000 rpm for 10 minutes. Supernatant was discarded, Phosphate Buffer Saline (PBS) was added

as much as three times of pellets volume and then centrifuged again. This process was repeated for 3 times in order to obtain pure erythrocytes free from blood plasma proteins. After the last centrifugation, supernatant was discarded and PBS was added as much as the volume of pellets so that 50% of erythrocytes was obtained. From the existing 50% of erythrocytes, 0.4 mL was taken and diluted in 9.6 mL of PBS to obtain 2% erythrocytes.

Gas Chromatography-Mass

Spectroscopy (GC-MS) Analysis

Quantitative phytochemical assays were carried out at the LPPT of Universitas Gadjah Mada Yogyakarta using Gas Chromatography-Mass Spectroscopy (GC-MS) with following equipment conditions: UHP Helium carrier gas (He), injector temperature 260 °C, split flow 50 mL/minute, split ratio 50, front inlet flow 1.00 mL/minute, MS transfer line temp 250°C, ion source temp 200°C, purge flow 3

mL/minute, gas saver flow 5 mL/minute, gas saver time 5 minutes.

Acclimatization and Treatment of Test Animals

Male Balb/c mice (*Mus musculus* L) as test animals were treated according to ethical standards stated by Medical Research Ethics Commission, Faculty of Medicine, Universitas Kristen Duta Wacana in the Statement of Ethical Eligibility number 1331/C.16/FK/2021 which was issued on August 11, 2021.

Before being treated, 25 male Balb/c mice were adapted and fed for seven days. After the acclimatization process, mice were randomly taken and divided into 5 groups. Each group consisted of 5 male mice. Kesambi bark extract doses to be applied to mice were calculated using formula used by Wahyu *et al* (2021):

$$\frac{A \text{ (animal)}}{B \text{ (human)}} = \frac{Km B}{Km A}$$

Treatment of each group of test animals is shown in Table 1 as follows:

Table 1. Description of treatment and dose to mice

Group	Treatment
Control- (K-)	Aquades injection + 0.2 ml of distilled water orally
Control + (K+)	HRD 2% injection + 0.2 ml of distilled water orally
EKBK 12.5	HRD 2% injection + extract 0.113 mg/kg BW orally
EKBK 50	HRD 2% injection + extract 0.491 mg/kg BW orally
EKBK 75	HRD 2% injection + extract 0.712 mg/kg BW orally

Each preparation is given once a day for 10 days.

Lymphocyte Cell Count

On day 0, day 5 and day 10, blood smear preparations were made to count the number of mice lymphocytes. The percentage of lymphocytes from a total of 100 leukocyte cells was calculated using formula according to Hartika *et al.*, (2014):

$$\% \text{ Lymphocyte} = \frac{Z \text{ Lymphocyte}}{100} \times 100\%$$

On the 10th day, mice were anaesthetized using chloroform, dissected and 1 mL of blood was taken from the heart. Blood samples were inserted into the EDTA vacutainer tube for routine hematological analysis at Sadewa Clinical Laboratory Yogyakarta.

Spleen Index Calculation

Spleen organ was collected from dissected mice on day 10. Mice weight before sacrifice was also documented. Both data were used on spleen index calculation. Spleen index was calculated using formula described by Fatirah *et al.*, (2019) as follows:

$$\text{organ index} = \frac{\text{organ weight (g)}}{\text{animal weight (g)}}$$

RESULTS AND DISCUSSION

Kesambi Bark Extract

Simplicia powder of Kesambi bark extracted using maceration method produced 295.38 g of extract. This

ethanolic extract has a paste form, blackish brown color, and aromatic odor. The average yield value of 96% ethanol kesambi bark extract was 19.69%. This study used a non-stratified maceration method, so it was gained a lower yield when compared to research conducted by Istiqomah *et al.*, (2021) which used stratified maceration method. In their study, Istiqomah *et al.*, (2021) gained 25.86% of kesambi bark extract yield. Based on Permadi *et al.*, (2015), stratified maceration method produces a higher yield value when compared to non-stratified maceration.

Identification of Phytochemical Groups of Kesambi Bark Extract with Qualitative Biochemical Tests

As shown in Table 2, we identified the presence of alkaloids, flavonoids, tannins and steroids on Kesambi bark extract. Istiqomah *et al.*, (2021) showed that ethanol extract of kesambi bark contained alkaloids, flavonoids, and tannins. In contrast to mentioned studies, the results of phytochemical tests in Mariyah's research (2020) stated that alkaloid compounds were not identified in the kesambi peel extract. Differences in phytochemical test results can be influenced by environmental conditions where plants grow and the age of the

sample used (Supriatna *et al.*, 2019).

Table 2. Results of identification of groups of phytochemical compounds

Test	Results
Alkaloids	+
Flavonoids	+
Tannins	+
Steroids	+
Saponins	-

Note:

+: Detected group of compounds tested,

-: No class of compound was detected

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

Compound analysis using GC-MS succeeded in detecting 13 compounds with 4 main compounds having the highest peaks. First highest peak at retention time 43.33 minutes and area percentage 7.60% was detected as 7,8-Epoxy lanostan-11-ol, 3-acetoxy which is a triterpenoid steroidal derivative compound. This group of compounds has pharmacological activity in the form of anti-inflammatory which can reduce and inhibit the inflammatory process (Iswary *et al.*, 2019). Second highest peak in retention time 43.44 minutes and the area percentage 6.26% was detected as 2,4,6,8,10-Tetradecapentaenoic acid, 9a-(acetyloxy)-1a,1b,4,4 a derivative compound of terpenoids (Abdel-Rahman *et al.*, 2019). This compound has anti-inflammatory properties which can both inhibit production of TNF- α and expression of

cyclooxygenase-2 which can affect the formation of prostaglandins during inflammatory process (Tanjaya, 2015). The third highest peak at retention time of 45.71 minutes and area percentage of 35.50% was identified as Methyl glycocholate, a derivative of 3-Trimethylsilyl (TMS). The fourth highest peak at retention time of 54.87 minutes and area percentage of 4.81% was identified as 3-Pyridinecarboxylic acid, 2,7,10-tris(acetyloxy)-1,1a,2,3,4,6,7,1.

Effects of Kesambi Bark Extract on Lymphocyte Count of Male White Mice

Lymphocytes count using blood smear method (Figure 1) showed the mean value of lymphocytes in the negative control treatment group (-) on day 0 was the lowest one when compared to positive control (+), low dose (0.113 mg/gBW), medium (0.491 mg/gBW), and high doses (0.712 mg/gBW) treatment groups. The administration of sheep erythrocytes as an antigen affected the changes on mice lymphocyte count. Sheep erythrocytes were injected 2 times into mice within period of 7 days. The first dose was injected on H-7 and the second dose was injected on H-0 of treatment.

Figure 1. Number of mice lymphocytes on the application of Kesambi bark extract for 10 days as counted with blood smear method

Note:

K-: Negative Control (-), K+: Positive Control (+), P1:Low Dose (0.113 mg/gBW), P2: Medium Dose (0.491 mg/gBW), P3: High Dose (0.712 mg/gBW)

Lymphocytes count from sheep erythrocytes-treated mice on day 0 using blood smear method made 2 hours after injection showed average cell count above 50 cells. On the other hand, negative control (-) treatment group which was only injected with distilled water had the lowest lymphocyte count with an average value of 34.8 cells. This observation supported the idea that sheep erythrocyte as an antigen was able to stimulate mice immune system by increasing the number of lymphocytes. Situngkir study in 2021 obtained the same result. They mentioned an increase of mice immune response when induced with 2% sheep erythrocytes. Sheep erythrocytes is known to have strong negative charge that will bind to antibodies. Stronger immune response will be induced by more foreign antigen (Novitae, 2013).

As observed on day 5, there was an increase in the number of mice lymphocyte in negative control (-) and positive control (+) treatment groups, while number of lymphocyte in all Kesambi bark extract treatment groups mice were decreased.

Mice lymphocyte count of negative control (-), positive control (+), and low dose (0.113 mg/gBW) groups on day 10 increased and was inversely proportional to the number of lymphocytes in the medium dose (0.491 mg/gBW) and high dose (0.712 mg/gBW) treatment groups. High dose of Kesambi bark extract was less able to inhibit antigen effect in mice circulatory and resulted in the accumulation of leukocytes. The results of this study were in accordance with a study conducted by Dani in 2012 which showed a change in the number of leukocytes that was marked by a decrease of lymphocyte

number in the granulation tissue of male wistar rats.

Based on the study of Puspita *et al.*, (2019), flavonoids were the most dominant compounds possessed by Kesambi, all in its bark, leaves, fruits and seeds. Flavonoid content of Kesambi organ extract acts as an anti-inflammatory which can block the release of arachidonic acid and the secretion of lysozyme to inhibit the proliferative and exudative phases of cells involved in inflammation (Rinidar *et al*, 2018). This inhibitory activity results in reduced arachidonic substrate for the cyclooxygenase and lipoxygenase pathways so it can suppress the amount of prostaglandins, prostacyclins, endoperoxides, hydroxatetraenoic acid, and leukotrienes. Reduction of these various compounds can affect inflammation process and leukocyte migration which resulted in the suppression of increasing lymphocytes number (Aria *et al*, 2020; Attiq *et al*, 2018).

Inhibition of prostaglandin pathway by flavonoids was able to reduce vasodilation and local blood flow so that migration of leukocytes to the site of inflammation decreases (Hämäläinen *et al*, 2011).

The mean of lymphocyte as counted using hematology analyzer performed on day 10 (Figure 2) showed a relatively similar pattern to the lymphocyte count using blood smear method. However, calculations using hematology analyzer obtained higher mean number of lymphocytes than blood smear method. Calculation using hematology analyzer produce more accurate result of leucocyte differential calculation and easier to evaluate the data. Calculation using simpler blood smear method has limitations due to the uneven distribution of leukocyte cells on the slide, resulting in differences in the number of counted cells in each preparation (Sugiarto *et al*, 2011).

Figure 2. Number of mice lymphocytes on day 10 counted using hematology analyzer.

Note:

K-: Negative Control (-), K+: Positive Control (+), P1: Low Dose (0.113 mg/gBW), P2: Medium Dose (0.491 mg/gBW), P3: High Dose (0.712 mg/gBW)

Morphology of mice lymphocytes as nuclei and surrounded by cytoplasm observed on blood smears preparation (Utami *et al.*, 2020). (Figure 3) showed dark purple cells, round

Figure 3. Morphology of mice lymphocytes under microscopy observation (100x magnification)
Image source: personal documentation

Spleen Index

The spleen is an organ that contains white blood cells including lymphocytes which have important roles on body immune response. Nugroho (2018) stated that the increase in the weight of spleen is directly proportional to the increased number of leukocytes, and increasing

weight of spleen indicated increasing number of leukocytes it contained. Morphology of fresh spleen taken from mice used in this study showed no significant visual difference between each treatment groups. Figure 4 indicates the appearance of mice spleen after being preserved in a fixative for 1 month.

Figure 4. Representative images of mice spleen from various treatments groups

Note :

K-: Negative Control (-), K+: Positive Control (+), P1:Low Dose (0.113 mg/g BW), P2: Medium Dose (0.491 mg/gBW), P3: High Dose (0.712 mg/gBW)

Mice spleen index that is calculated in the negative control group. Statistical analysis of spleen index data of test animals on day 10 showed that mice from high dose treatment group had the highest spleen index (Table 3). Mice from medium dose treatment (P2) showed low spleen index in all treatments showed no significant difference in the weight of the mice spleen which was close to the spleen index of mice in each treatment group.

Table 3. Spleen index of mice in 5 treatment groups

Treatment	Average Spleen Weight (g)	Spleen Index
K-	0.269	125.5
K+	0.207	162.2
P1	0.160	164.5
P2	0.193	136.4
P3	0.148	202.5

Note :

K-: Negative Control (-), K+: Positive Control (+), P1:Low Dose (0.113 mg/gBW), P2: Medium Dose (0.491 mg/gBW), P3: High Dose (0.712 mg/gBW)

Figure 5. Correlation between mice spleen index and lymphocyte count

Based on the results of simple linear regression analysis in Figure 5, the coefficient of determination is 0.0835, which means that the influence of the spleen index on the number of lymphocytes is 8.35%. The significance value obtained is 0.627 ($P > 0.05$), so it can be concluded that the spleen index did not have a significant effect on the number of lymphocytes.

CONCLUSION

Ethanollic extract of kesambi bark (*Schleichera oleosa* (Lour.) Oken) contained phytochemical groups of alkaloids, flavonoids, tannins and steroids which were detected with qualitative biochemical tests. Kesambi bark extract has the potency to reduce number of lymphocytes in mice-induced sheep erythrocytes. The most effective dose of

Kesambi bark extract to reduce mice lymphocytes was 0.491mg/grBW, resulted in 48.67 cells as the lowest lymphocyte count. Decreased number of mice lymphocytes observed in this study was not correlated with their spleen index.

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