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Yufri Aldi*, Elidahanum Husni, Relin Yesika

ABSTRACT

Introduction: Kincung Flower (*Etlingera elatior* (Jack) R.M.Sm.) is a native herbal plant in Southeast Asia that traditionally used to many diseases, especially in Indonesia. **Aim:** This study was conducted to determine the activity of kincung (*Etlingera elatior* (Jack) R.M.Sm.) on the total number of leukocytes and differential leukocyte cells in allergic mice. **Material and Methods:** The semi-solid extract of Kincung flower (*Etlingera elatior* (Jack) R.M.Sm.) was made by the maceration method using 70% ethanol solvent. The animals used were 20 male white mice that have allergies of skin that treated with 20% albumen antigens given on the first day 0.2 mL/20 g intraperitoneally, then on the seventh day are given antigens with the same dose subcutaneously. Allergic mice indicated by redness at the injection site. It divided into four groups: the negative control group and three dose groups (100; 300; and 1000 mg/kg). On the seventh day after administration of the extract, observed the value of total leukocytes and differential leukocyte cells in mice. **Results:** The results after administration of extracts in 3 dose groups (100; 300; and 1000 mg / kg) and the negative control group showed sequentially the total number of leukocytes was: 3.95; 4.73; 6.01; and 3.6 x10^3/µL and the percentage of leukocytes consisting of lymphocytes: 67.6%; 62.0%; 56.8% and 70.0%, neutrophils: 22.4%; 29.2%; 36.8% and 20.0%, eosinophils: 6.4%; 5.8%, 4.2% and 6.6%, monocytes: 3.6%; 3.0%, 2.2% and 3.4%, and basophils: 1.8%; 1.4; 0.8% and 2.0%. It concluded that kincung flowers could increase total leukocytes significantly ($p<0.05$), decrease lymphocytes, eosinophils, basophils significantly ($p<0.05$), increase neutrophils significantly ($p<0.05$), and reduce monocytes insignificantly ($p>0.05$). **Conclusion:** Kincung flowers (*Etlingera Elatior* (Jack) R.M.Sm.) can be used as an immunomodulator and decreasing the percentage of basophil cells, and eosinophils can used as an anti-allergic drug.

Key words: Allergies, *Etlingera elatior* (Jack) R.M.Sm, Kincung Flower, Leukocyte Percentage, Mice, Total Leukocytes.

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INTRODUCTION

The immune system includes all structures and processes for the body's defence against disease. It cannot separate from the role of leukocytes which consist of several cells that have various types and functions that can respond to the presence of foreign objects that enter the body that can cause inflammation and infection.[1] The immune system divided into two parts called the natural or innate (non-specific immune system) and the adaptive (specific immune system). The natural immune system will destroy all foreign objects that enter into the body even though it has never been exposed before while the adaptive immune system is specific to certain antigens that have previously been exposed that includes the process of recognizing specific antigens, then the formation of antibodies or T lymphocytes which will only react to specific antigens.[2-4]

Kincung Flower (*Etlingera elatior* (Jack) R.M.Sm.) is a herbal plant native to Southeast Asia which can found in several countries such as Indonesia, Malaysia, and southern Thailand. In Indonesia, it has been traditionally used to eliminate bad breath and body odour by brewing or boiling flower buds for drinking water.[5]

Preliminary tests conducted stated that *Etlingera elatior* (Jack) R.M.Sm. has a phagocytic activity of macrophages against male white mice. It also has activity in inhibiting the active cutaneous anaphylactic reaction in mice,[6] and also inhibit degranulation of mast cells in mice.[7] The last study also stated that *Etlingera elatior* (Jack) R.M.Sm. has properties such as anti-cancer and tumors.[8-10] and showed anticancer activity against cervical cancer cells,[11] activity against skin cancer[12] has high antioxidants[13,14] and antibacterial to gram-positive bacteria *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*,[15,16] antidiabetic and anti-inflammatory,[17] whitening and antiaging.[18]

Etlingera elatior (Jack) R.M.Sm. contains many secondary metabolites such as alkaloids, flavonoids, saponins, tannins, terpenoid.[19-21] Identification with GC-MS, *Etlingera elatior* (Jack) R.M.Sm. has compounds such as 1-dodecanol, dodecanal, 17-pentatriacontane,[22] cyclododecane dan 1,1-dodecanediol diacetate.[23]

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Based on the presence of kincung flower activity in inhibiting cutaneous-active hypersensitivity and macrophage phagocytosis, the researchers are interested in continuing research related to kincung activity on the number of leukocytes and the percentage of leukocytes in male mice hypersensitivity.

MATERIAL AND METHOD

Place and time

The research conducted in two months in April-September 2019. Preparation and extraction of *Etlingera elatior* (Jack) R.M.Sm. also determinating the characterisation from kincung flower's extract were conducted in three weeks at Central Laboratory in Faculty of Pharmacy Universitas Andalas.

Tools and materials

The equipment used in this study were the evaporator (Buchi® R-210 Rotavapor), UV-vis spectrophotometer (Thermo Scientific GENESYS 10S UV-Vis), beaker glass (Pyrex), erlenmeyer (Pyrex), Object glass microscope slides, pipette volume (Pyrex), digital analytical balance (Ohaus), Silica gel 60 F254 (Merck), desiccator, spatula, dark bottle, TLC vessel, mortar and mortar, sonde instrument, surgical instrument, filter paper, animal cage, haemocytometer, microscope.

The materials used in this study were kincung flower (*Etlingera elatior* (Jack) R.M.Sm.), aqua dest (Bratachem), ethanol 70%, ethanol p.a (Merck), formic acid (Merck), Ethyl acetate (Merck), wright stain (B-Jes), Giemsa (B-Jes), Turk (B-Jes), EDTA, emersion oil, physiological NaCl (Widatra Bhakti), albumen, rutin comparison (Merck), methanol (Merck), ethanol 80%, aluminium chloride (Merck), sodium acetate (Merck),

Extracting Kincung Flower (*Etlingera elatior* (Jack) R.M.Sm)

As much as 2 kg *Etlingera elatior* (Jack) R.M.Sm. was dried until it becomes dry simplicia, then proceed to make powder and sifted with sieve number 60. As much as 250 g powder was macerated using 70% ethanol solvent (1:10), soaked for six first hour, stirring occasionally, then let stand for 18 hours. Twice repetition using the same type and amount of solvent. Filter using filter paper and the results of the mass are collected and then evaporated on a rotary evaporator until it becomes a thick extract (35.276 g).

The TLC and total flavonoid test

The some of parameters of standardisation of extract *Etlingera elatior* (Jack) R.M.Sm. were total flavonoid test and thin layer chromatography test (TLC) that based Indonesian Pharmacopea Herbal.

TLC test was from the routine that dissolved with ethanol P, and the extract dissolved with methanol P. Put into the TLC plate (Silica gel 60 F25) into a chromatographic vessel which containing a mobile phase solution consisting of ethyl acetate P, formic acid P and water (100: 15:17). Dry the TLC plate, then look under the UV light.[24]

Total flavonoid test was extract of *Etlingera elatior* (Jack) R.M.Sm. and routine solution dissolved in ethanol 80%, then put extract solution in centrifuge. Then pipette 0.5 mL supernatant and routine solution, add 1.5 mL of ethanol, 0.1 mL of $AlCl_3$10%, 0.1 mL of Na acetate 1 M and 2.8 mL aqua dest. Shake and put aside for 30 minutes at room temperature. Measure the absorption at the maximum absorption in wavelength 418nm.[24]

Calculate the percentage of leukocyte cells

After seven days of preparation, the blood smear was made to count leukocytes by taking blood from mice from the vein, then drop the blood on the slide and use another slide to flatten, wait to dry. After dry fixation with methanol and wait for it to dry. Drops of diluted wright solution with Aquadest (1:20) to see basophil cells and 10% Giemsa solution to see other leukocyte cells (neutrophils, monocytes, lymphocytes, eosinophils) then let stand for 20 minutes. Wash with aqua dest, observed under a microscope.

Ethical test

In this research using the mice, the subject of study, so it required an ethical test that qualifies to mitigate the treatment gave an adverse impact on a human subject in this research. The ethical test conducted by the Commission of Ethics Faculty of Medicine Universitas Andalas, Padang, Sumatera Barat.

RESULTS AND DISCUSSION

The extract of *Etlingera elatior* (Jack) R.M.Sm. was semi-solid extract with characterisation black-brown colour, characteristic odour, and sour taste. Its yield of 14.11%, and according to Indonesian Pharmacopoeia Herbal, the yield percentage of kincung extract is not less than 9.86%. Shrinkage of dried kincung flower extract is 6.65%, and according to Indonesian Pharmacopoeia Herbal, it does not more than 10%. The total ash content of kincung flower extract was 4.57%, and according to Indonesian Herbal Pharmacopoeia it does not more than 7.5%, while the ash content of kincung flower extract was insoluble in acid 0.02% and according to Indonesian Pharmacopoeia Herbal, it does not more than 0.1%.[24]

Determinating of total flavonoid used a routine as a standard that tested in UV-vis Spectro that obtain wavelength was 418 nm. The linear regression of the routine comparison calibration curve for the calculation of total flavonoid levels was y = 0.0046x - 0.0562 with R2 = 0.998 so that the total flavonoid concentration of kincung flower extract (*Etlingera elatior* (Jack) RMSm.) obtained was 1.564% and according to Indonesian Pharmacopoeia Herbal, the total flavonoid content of kincung extract (*Etlingera elatior* (Jack) M. Sm.) not less than 0.58% which is calculated as routine[24] Total flavonoid extract rate calculated at 418 nm wavelength. The spectrum of UV showed in Figure 1.

According to Indonesian Pharmacopoeia Herbal, the TLC test of *Etlingera elatior* (Jack) RM.Sm. extract using ethyl acetate P: formic acid P: water (100:15:17) and silica gel 60 F254 and also using a rutin comparison. Results Rf value extract obtained as can be seen in Figure 2.

This study was conducted with the sensitisation of experimental animals with 20% albumen as much as 0.2 mL/20 g bw intraperitoneally. Purebred albumen are used as antigens because these albumen immunogenic properties are quite high[25], the protein content is around 12.[26] The *in vitro* test in this study used sensitised mast cell. The purpose of this sensitisation is to generate a primary immune response where the

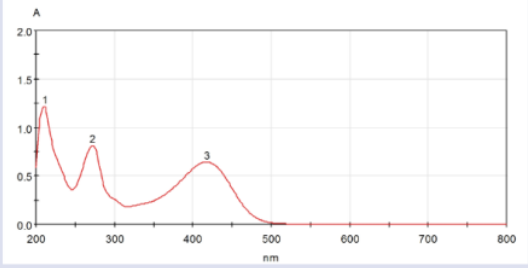

Figure 1: Ultraviolet-visible spectrum comparison of rutin-aluminum chloride.

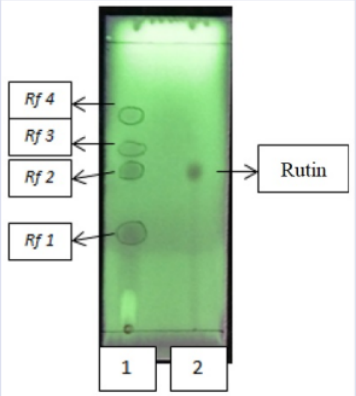

Figure 2: TLC kincung flower extract with eluent ethyl acetate P: formic acid P: water (100:15-17) and silica gel 60 F254. Description: 1. TLC kincung flower extract 2. routine comparison. There are four marks, and the routine showed in Rf2 with value of 0.446. This extract has suitable for Indonesian Pharmacopoeia Herbal.

first antigen injection is performed intraperitoneally so that the process of antigen recognition is faster by lymphocyte cells. This recognition process is carried out by macrophages, where macrophages are one of the presenting cell antigens and widely found in the abdominal cavity. On the seventh day, a subcutaneous second injection of antigens was carried out to increase the formation of IgE antibodies, so that allergic reactions get worse. In a preliminary study of *Etlingera elatior* (Jack) R.M.Sm.) was tested *in vitro* to inhibit desensitised mast cell in white male mice.[7] Desensitised mast cell-induced with albumen and allergic mice will be shown redness to red spots or bumps around the body and injection site. This reaction occurs when antigens bind to IgE antibodies that are on the surface of mast cells and basophils. This bond in a matter of minutes can cause mastocyte degranulation which results in the release of mediators, especially histamine.[27] Allergy mice will then used for further treatment, except for the negative control group used normal mice. The formula for calculating the number of leukocytes per µm is: cells counted x 20 (1:20) x 10 (0.1mm): 4 (number of boxes in µm²) or number of cells counted in boxes multiplied by 5.[28]

Total leukocyte counts of male mice type 1 hypersensitivity mice showed in Table 1, and the relationship between total leukocyte counts by giving dosage variations showed in Figure 3. One-way Anova analysis of total leukocyte cells after ethanol extract of kincung flowers at doses of 100, 300, and 1000 mg/kg in type I hypersensitivity mice showed a significant increase in the number of mouse leukocyte cells ($p<0.05$). However, this increase is still in the normal range where normal mice cell leukocytes range from 2000-10000 cell/µL.[29-31] The result of an increase in the total number of leukocyte cells showed in Table 2. An increase in the total number of leukocytes represents a humoral and cellular response against pathogenic agents or indicates an increase in the body's defence capability,[32] where the function of leukocytes is to protect the body from pathogens by producing antibodies and phagocytic processes. Increased leukocytes are thought to contain flavonoids in kincung flowers. Flavonoids can enhance the immunomodulatory system by increasing the effectiveness of lymphokine proliferation produced by T cells so that it will stimulate phagocytic cells to respond to phagocytosis.[33] Higher doses of flavonoids make leukocyte cells (phagocytes) more active against phagocytic bacterial cells, and more bacteria can be damaged and digested with leukocyte cells.[34]

The percentage of leukocytes, performed by Romanowsky staining method because this staining can give satisfactory results on peripheral blood smears. Giemsa and wright colouring is included in Romanowsky colouring .wright-stain to see basophil cells and neutrophil, lymphocytes, monocytes, eosinophils using Giemsa. Leukocyte count done by cross-sectioned or leukocyte count, which starts from the edge of the blood sample by snaking until 100 leukocyte cells are obtained then expressed in percentage.[35-37] The types of leukocytes that can be seen using Giemsa colouring showed in Figure 4. For the percentage of leukocytes, it showed in Table 2, and the relationship of percentage leukocytes with administering dose variations showed in Figure 5.

After one-way Anova analysis on the percentage of leukocytes after administration of kincung flower ethanol extract at doses of 100, 300, and 1000 mg/kg bw in type I hypersensitivity mice (Figure 4) showed that there is a significant decrease in lymphocytes ($p<0.05$). The percentage of normal lymphocytes in mice is 70-80% of the total differential leukocytes.[29,31,38] Decreased lymphocytes can be triggered by several things such as stress during the treatment process[29,39], or with increasing age and when the number of neutrophils increases.[38,39]

The results of the One-way ANOVA analysis showed a significant increase in neutrophils ($p<0.05$). The standard percentage of neutrophils in mice is 20-30% of the total differential leukocytes.[29,31] An increase in neutrophils or so-called neutrophilia is associated with responses to stress or excitement[38] and usually increases in cases of bacterial infection and acute inflammation.[40-42]

The results of the one-way ANOVA analysis showed that monocytes were not significantly increased or decreased ($p>0.05$). Monocytes are

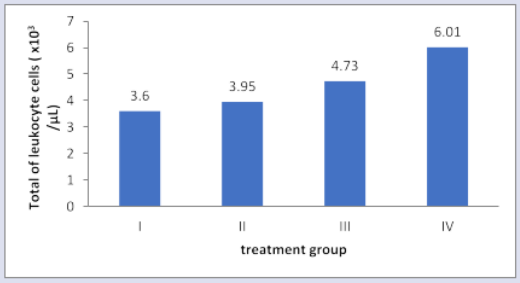

Figure 3: Relationship between dose variance with total leukocyte white male mice hypersensitivity.

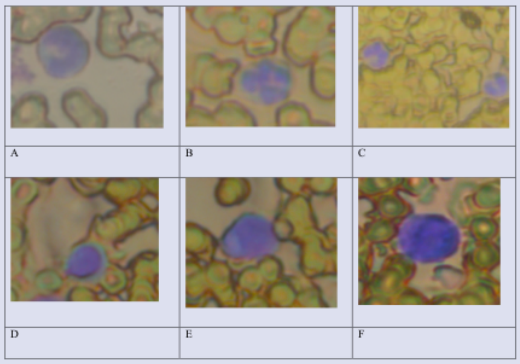

Figure 4: Differential of leukocyte cells. Descrption: A.stem neutrophils B.segment neutrophils C.eosinophils D.lymphocytes E.monocytes F. basophils.

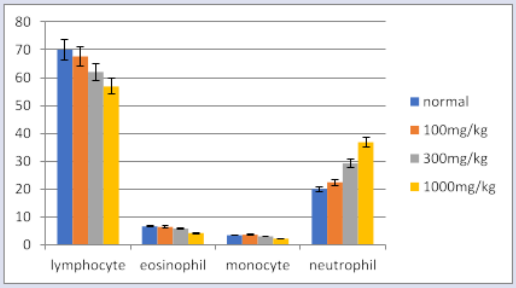

Figure 5: Relationship between dose variance with percentage of leukocytes in white male hypersensitivity mice.

Table 1. Total of leukocyte cells in white male mice's blood after kincung flower (*Etlingera elatior* (Jack) R.M.Sm.) extract given for six days.

Group	Total of leukocytes (x10³/µL)					Mean±SD
	I	II	III	IV	V	
I. Control group	4.05	3.15	3.8	3.4	3.6	3.6± 0.35
II. 100 mg/kg	3.65	4.3	3.95	4.1	3.75	3.95± 0.26
III. 300 mg/kg	4.3	4.9	4.55	5.3	4.6	4.73± 0.38
IV. 1000 mg/kg	6.05	5.9	5.75	5.25	7.1	6.01± 0.68

*Note: total leukocytes showed a significant increase (p <0.05)

Table 2. Percentage of leukocyte cells in white male mice's blood after kincung flower (*Etlingera elatior* (Jack) R.M.Sm.) extract given for six days.

Group	No	Percentage of Leukocyte Cells (%)			
		Lymphocytes	eosinophils	monocytes	neutrophils
	1	70	7	4	19
	2	71	5	2	22
Control group	3	69	7	3	21
	4	67	9	4	20
	5	73	5	4	18
Mean ± SD		70.0±2.23	6.6±1.67	3.4±0.89	20.0±1.58
	1	71	6	4	19
	2	65	8	5	22
Doses 100 mg/kg bw	3	69	7	3	21
	4	68	6	3	23
	5	65	5	3	27
Mean ± SD		67.6 ±2.60	6.4± 1.14	3.6±1.07	22.4±3.39
	1	63	5	2	30
	2	64	6	3	27
Doses 300 mg/kg bw	3	62	5	4	29
	4	60	6	3	31
	5	61	7	3	29
Mean ± SD		62.0 ±1.58	5.8±0.83	3.0±1.03	29.2±1.48
	1	51	4	3	42
	2	58	3	2	37
Doses 1000 mg/kg bw	3	68	4	2	26
	4	51	4	3	42
	5	56	6	1	37
Mean ± SD		56.8±6.97	4.2±1.09	2.2±1.07	36.8±6.53

Table 3. Percentage of basophils in white male mice's blood after kincung flower (*Etlingera elatior* (Jack) R.M.Sm.) extract given for six days.

Group treatment	Mice					%Mean±SD
	I	II	III	IV	V	
I .Control group	2	1	2	3	2	2.0± 0.70
II. 100 mg/kg bw	1	3	2	1	2	1.8±0.83
III. 300 mg/kg bw	1	2	2	1	1	1.4±0.57
IV. 1000 mg/kg bw	1	0	1	1	1	0.8±0.44

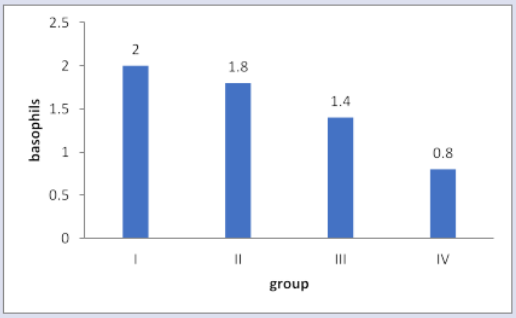

Figure 6: Relationship between dose variance with percentage of basophils in white male hypersensitivity mice.

the largest leukocytes, and the standard percentage of monocytes in mice ranges between 2-6% of the circulating cell population and their numbers increase in response to infection.[43] Decrease in the number of monocytes at doses of 300 and 1000 mg/kg is estimated because monocytes migrate to tissue or to the location of damage or infection where they then mature into macrophages. Monocytes, along with macrophages and tissue neutrophils are the primary cells involved in first-line defence against pathogenic organisms or foreign cells.[44]

Percentage of basophils in male white hypersensitivity mice showed in Table 3, and the relationship of the percentage of leukocytes administering dose variations showed in Figure 6.

The results of the one-way ANOVA analysis showed a significant decrease in eosinophils and basophils ($p<0.05$). The number of basophil cells in the blood is deficient, the percentage of healthy basophil cells <2%[45] and eosinophil 0-7% of the total differential leukocytes.[29,31] Decreasing basophils and eosinophils can used as an indicator of anti-hypersensitivity. Basophils or eosinophils will increase during the response to antigens, parasites, and allergies.[46,47] Basophils are one of the granulocytes involved in the thought process where the antigen enters a second time, and then the antigen is immediately bound by the IgE pair that is on the surface of the basophil cell.[45,47] Cells will change the degranulation process and release mediators such as histamine, serotonin, prostaglandins, et cetera. The mediator is responsible for the onset of reactions such as itching, redness, oedema, and tissue dysfunction.[47,48]

In previous studies, it stated that flavonoids could inhibit IL-4 and IL-13 by activation of basophil cells.[49] Routine also stated have an antiallergic activity that affect to mast cell activity mediated by IgE.[50,51]

CONCLUSION

The results of this study concluded that kincung flowers could increase the number of leukocytes significantly ($p<0.05$), decrease lymphocytes, eosinophils, basophils significantly ($p<0.05$), increase neutrophils significantly ($p<0.05$) and does not increase monocytes significantly ($p>0.05$). Increased total leukocytes after gave kincung flower extract can be used as an immunomodulator, and a decrease in the percentage of basophil cells and eosinophils can used as an allergic drug.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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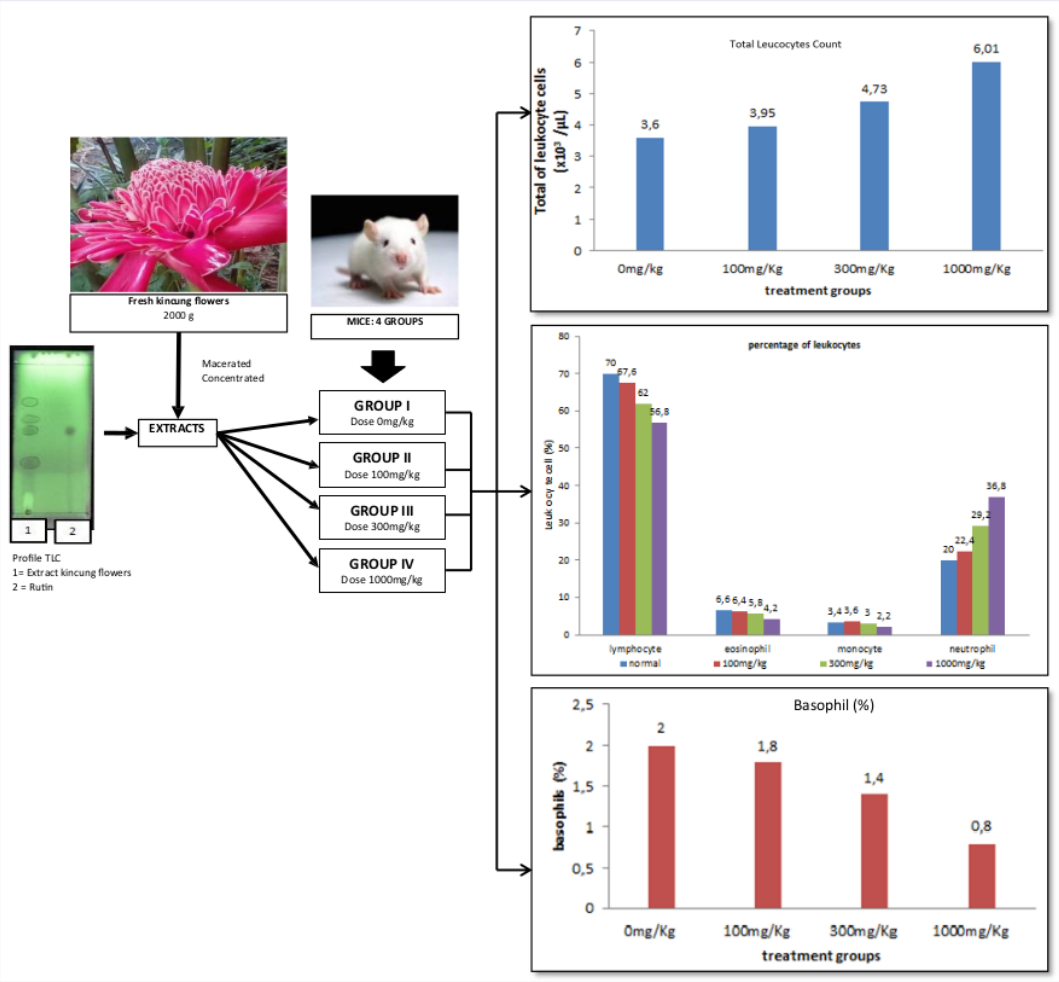
GRAPHICAL ABSTRACT



SUMMARY

Research on the activity of kincung flower extract (*Etlingera elatior* (Jack) R.M.Sm.) on the total number of leukocytes and the percentage of leukocytes was carried out. Kincung flower (*Etlingera elatior* (Jack) R.M.Sm.) tested in the form of thick extract that had standardised according to Indonesian herbal pharmacopoeia. Kincung flower extract (*Etlingera elatior* (Jack) R.M.Sm.) was given to male white mice for seven days at doses of 100, 300, 1000 mg/kg body weight. Kincung flower extract (*Etlingera elatior* (Jack) R.M.Sm.) can increase the total number of leukocytes and neutrophils significantly, decrease lymphocytes, eosinophils, and basophils significantly, and decrease monocytes insignificantly.

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