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T-CELL ACTIVATION CONTROLLING EFFECTS OF ETHYL ACETATE FRACTION OF *KALANCHOE PINNATA* (LMK) PERS ON TMPD-TREATED LUPUS MICE

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Keywords:

T cell activation, Ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers, L-selectin, Leukocyte, Lupus

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ABSTRACT: T-cell activation, with the low expression of CD62L (L-selectin) marker in the spleen, will increase B cell activity to produce lupus antibodies. Therefore, lupus patients need drugs which could increase the L-selectin expression in the spleen to reduce the auto-reactive T cell. The tested drug to increase the L-selectin in this research was the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves (EF-KP), based on its immunosuppressant and anti-inflammatory activities. The research was performed by means of 2, 6, 10, 14 tetramethylpentadecane (TMPD) induced lupus-like mice. The experimental groups consisted of negative control (NC), EF-KP, and positive control (PC) groups. After 21 day treatment, the blood leukocyte was checked. Then, the spleen was prepared for the flow cytometry method to measure $CD4^+CD62L^+$ and $CD8^+CD62L^+$ T cells. The relative percentage of $CD4^+CD62L^+$ T cells in EF-KP groups increased insignificantly ($p>0.05$) (NC=7.91 ± 4.98%, EF-KP = 8.63 ± 3.07%, PC = 12.62 ± 8.09%). The relative percentage of $CD8^+CD62L^+$ T cells increased significantly ($p < 0.05$) (NC = 6.20 ± 4.91%, EF-KP = 15.94 ± 9.37%, PC = 24.32 ± 17.47%). Additionally, the total leukocytes reduced significantly ($p < 0.05$) (NC = 6.333 ± 1.078 cells/mm^3, EF-KP = 4.967 ± 635 cells/mm^3, PC = 4.367 ± 1.102 cells/mm^3). The results show that the active compounds in the EF-KP can mainly control the L-selectin regulatory function of $CD8^+$ T cells, and then reduce the inflammation process with the point view of reducing its total leukocytes. At last, the main compounds in the EF-KP could control the T-cell activation in lupus.

INTRODUCTION: T cell and B cell activation induces B cells to increase the production of lupus-specific antibodies [1, 2].

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T cell activation is regulated by L-selectin or CD62L as an activation marker. The L-selectin expressed on naïve CD4 T cells is needed for the recirculation between blood and lymph node. The L-selectin is an adhesion molecule that is able to reduce lymphocytes and bind the endothelial venule ligand. This regulation is important to control the traffic of T lymphocyte circulated to peripheral lymph node [3 - 8]. The naïve T cells in the spleen binds L-selectin, and then the L-selectin will

be shedded in the circulation become the memory T cells [9, 10]. The low number of L-selectin in the spleen will increase the lupus severity since the high number of T effector, some of them are auto-reactive, cannot be handled results in inflammation.

This severity can be inhibited by using lupus standard treatment. Lupus treatment by rheumatologists is sufficient to increase the quality of life, but it is not in an optimal outcome [11, 12]. Most of the drugs used are off-label nonselective immunosuppressive drugs, such as cyclophosphamide, methotrexate, azathioprine, and mycophenolate mofetil that are very toxic in a long term use [13 - 16]. Many efforts are performed to obtain new lupus drugs which are effective, safe, and acceptable. Many biotechnology manipulations are performed to find the appropriate treatment [14 - 17], but it needs high cost. The other effort is to find new lupus drugs from potential medicinal plants based on its immunosuppressive effect. However, medicinal plants have multiple effects which can beneficially use to reduce lupus manifestations. In this research, we try to exploit the multiple effects of *Kalanchoe pinnata* (Lmk) Pers leaves to increase the L-selectin in the spleen of in TMPD-treated lupus mice.

According to our previous study, the aqueous extract of *Kalanchoe pinnata* (Lmk) Pers leaves can reduce the kidney structural damage of TMPD-treated lupus mice. The main flavonoid compounds (mainly found in the ethyl acetate fraction) of this plant are active as immunosuppressant, anti-inflammatory, and antioxidant [18 –21]. The flavonoids of *Kalanchoe pinnata* (Lmk) Pers also inhibit T cell mitogenesis [22]. Besides, it inhibits IL-2 and IL-4 production without cell toxicity. *Kalanchoe pinnata* (Lmk) Pers is not toxic [23] and also safe for maternity since it reduces the uterine contractility [24]. This "miracle plant" is widely used as traditional in Africa and Bangladesh for many purposes [25, 26].

Based on many benefits of *Kalanchoe pinnata* (Lmk) Pers leaves for lupus, this research provide the data of the effects of flavonoids in the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) leaves in maintaining the L-selectin number in the spleen of lupus mice. The high number of L-selectin in spleen will control the excessive T cell activation in lupus. It could prevent the spread of circulating L-selectin, and then inhibit the systemic inflammation. The high level of leukocyte as an inflammatory outcome found in lupus model would be reduced if the L-selectin regulation was maintained well. Therefore, this research focused on the effect of flavonoids in the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) on L-selectin number in the spleen of TMPD-treated lupus mice.

MATERIALS AND METHODS:

Materials: The *Kalanchoe pinnata* (Lmk) Pers fresh leaves were obtained from a cultivation farm at Trenggalek, East Java. The botany identity of this plant was performed by the Conservation Unit of Indonesian Institute of Science, Purwodadi with the identification number of 0284/IPH.06/ HM/II/2015.

The female Balb/c mice (80 mice) aged 4 weeks were received from LPPT Gadjah Mada University, Indonesia. These mice were pathogen free species with the certificate number of 352/LP3HP/ 29/VII/2015. They were housed, randomized, and handled using standard maintenance on the Guide of the Care and Use of Laboratory Animal 8th edition, published by National Research Council 2011 [27].

TMPD >98% purity (Pristane) with the code number of Sigma-P2870 was obtained from Sigma-Aldrich supplier in Singapore. Anti-CD4, anti-CD8, and anti-L-selectin antibodies were obtained from Biogenesis, USA. Turk reagent, PBS, and aquadest were obtained from the laboratory of Faculty of Pharmacy, Universitas Airlangga. The chemicals used in the fractionation, *i.e.* ethyl acetate pro analysis, were obtained from Merck through PT. Dianum as Indonesian supplier. The chemicals used in the profiling were obtained from Biotechnology Laboratory of PUSPIPTEK Serpong, Indonesia.

Methods: The EF-KP was obtained by using a method used on the previous study. The ethyl acetate fraction quality control test was performed by quantitation of one active marker, rutin (a flavonoid compound), by means of LC-MS/MS system. The female Balb/c mice used were 7 weeks old when treated using 0.5mL TMPD. The administration was intraperitoneal injection.

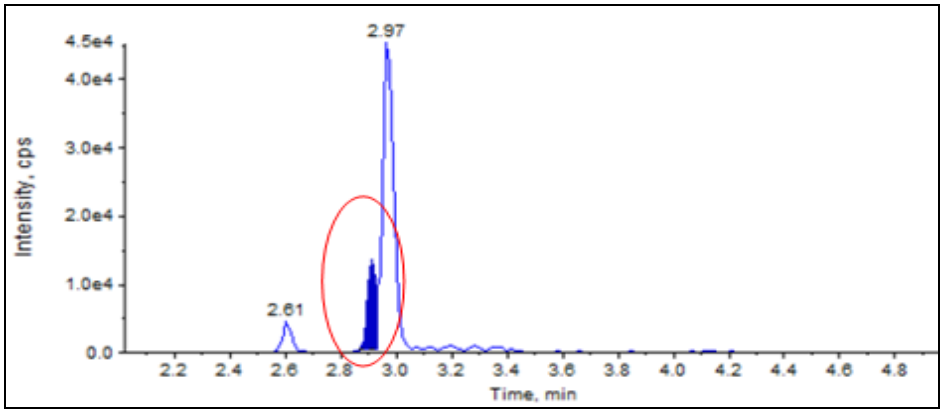
The induction time was lasted for 6 months [28 - 31]. At the end of the induction time, the proteinuria semi quantitative data showed a severe lupus manifestation. Then, the 30 mice were divided into 3 experimental groups, *i.e.* the negative control group that consists of lupus mice that received placebo, the EF-KP group that received the EF-KP at a dose which comparable to 400 mg/kgBB of crude extract, and the positive control group that received cyclophosphamide at a dose of 1 mg/kgBW. The compounds tested were orally administered on the daily treatment until 21 days.

Finally, the mice were sacrificed. The blood was prepared as a sample for total leukocyte count (TLC) assay by means of hemocytometer Neubauer-impruved and inverted microscope Olympus CKX41. The fresh spleen of each mouse was prepared to be spleen cells that were ready for flow cytometry assay. The T regulatory cells (T regs) $CD4^+$L-selectin$^+$ and $CD8^+$L-selectin$^+$ were measured by using flow cytometer BD FACS Calibur. Then, the data was analyzed by using BD Cell Quest program.

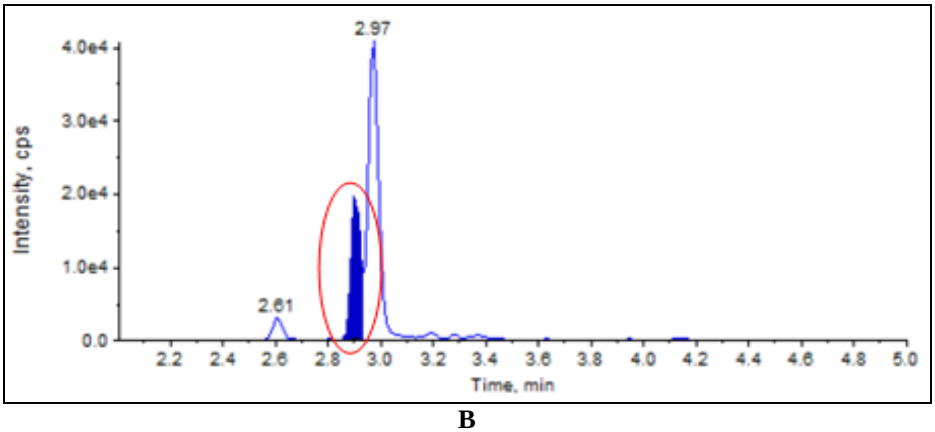
All of the data was analyzed by facilitating of One way ANOVA by means of SPSS Statistics (version 22). The ethical clearance of this research was approved by local ICUC of Veterinary Medicine Faculty of Universitas Airlangga at January 12, 2016, with the certificate number of 526-KE, then the research performed until April, 2017.

RESULTS:

The EF-KP Quality Control: The aqueous extract of *Kalanchoe pinnata* (Lmk) Pers was made from the fresh leaves. The extract was lyophilized and then fractionated by using liquid-liquid extraction. The EF-KP profile was measured by using LC/MS /MS tandem system. The result is shown in **Fig. 1**. According to the profile, the rutin is in the retention time of 2.90. The linearity of the rutin standard used for the rutin calculation in the EF-KP sample is shown in **Fig. 2**. Based on the standard, the rutin concentration in the EF-KP is in **Table 1**. Then, the EF-KP was prepared as solution form to be administered in each experimental mouse.



A



B

FIG. 1: THE EF-KP CHROMATOGRAM AND THE POSITION OF RUTIN MARKER IN EF-KP (A) AND RUTIN STANDARD (B)

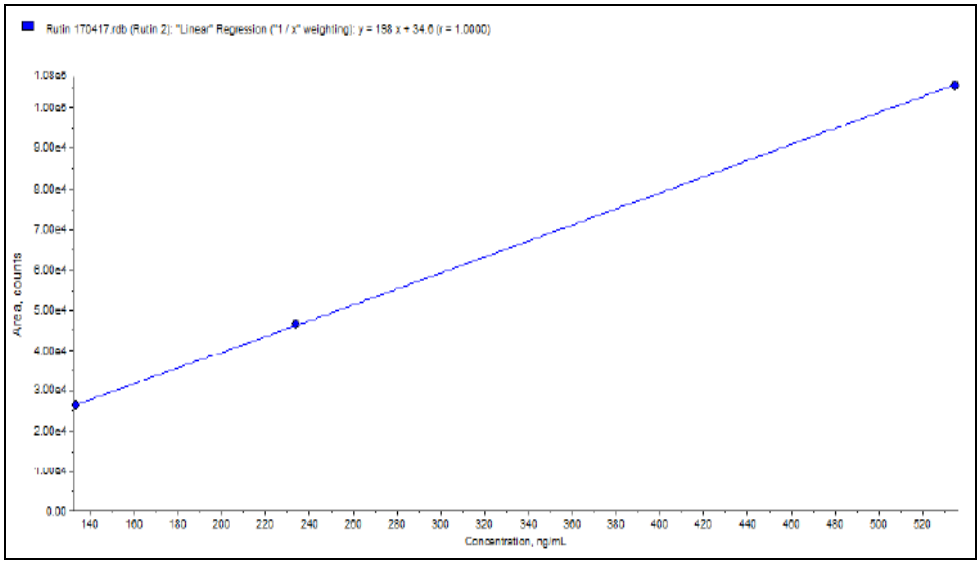


FIG. 2: THE STANDARD CURVE OF RUTIN STANDARD

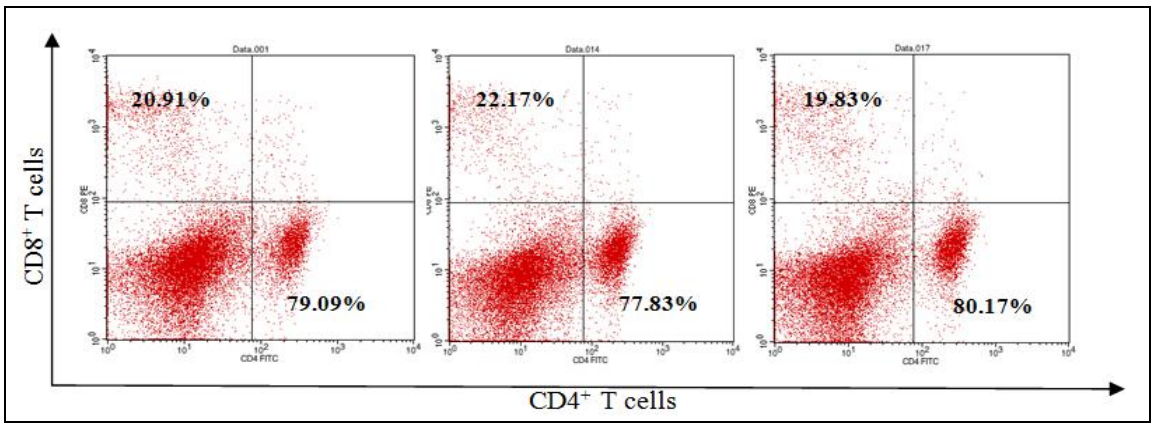
TABLE 1: CALCULATION OF RUTIN MARKER IN THE EF-KP SAMPLE

Description sample	Internal Code	Calculated concentration (ug/L)	Dilution Factor	Calculated concentration (ug/kg)	Average calc conc (ug/kg)	Result (mg/kg)
EF-KP	1	133	100	13,300	13,400	13.4
	2	135		13,500		

The Analysis of L-selectin and the Related Markers: CD62L or L-selectin is a T regulatory marker. The flow cytometry analysis was performed simultaneously on CD4, CD8, and CD62L markers. The relative percentages of $CD4^+$L-$selectin^+$ and $CD8^+$ L-$selectin^+$ T cells were calculated. The assessment of CD4:CD8 ratio was done by using the flow cytometry assay. The results are shown in **Table 2** and **Fig. 3 - 5**.

TABLE 2: THE RATIO OF $CD4^+$: $CD8^+$ T CELLS

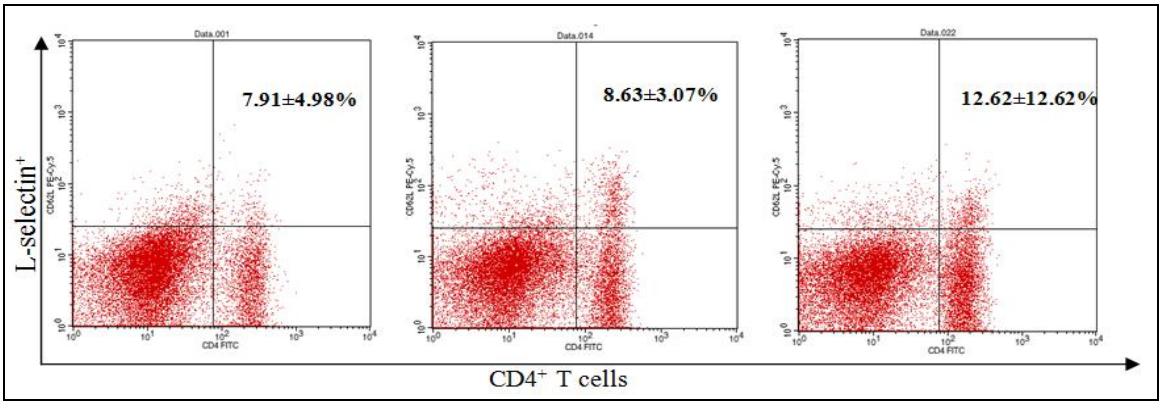
Groups	CD4:CD8 Ratio
Negative control	3.78 : 1
EF-KP	3.51 : 1
Positive control	4.04 :1



(a) (b) (c)

FIG. 3: THE RELATIVE PERCENTAGE OF $CD4^+CD8^+$ T CELLS AS A RESULT OF FLOW CYTOMETRY ANALYSIS OF THE FRESH SPLEEN OF THE THREE EXPERIMENTAL GROUPS
(a), EF-KP group (b), and positive control group (c) (n = 10 mice per group)

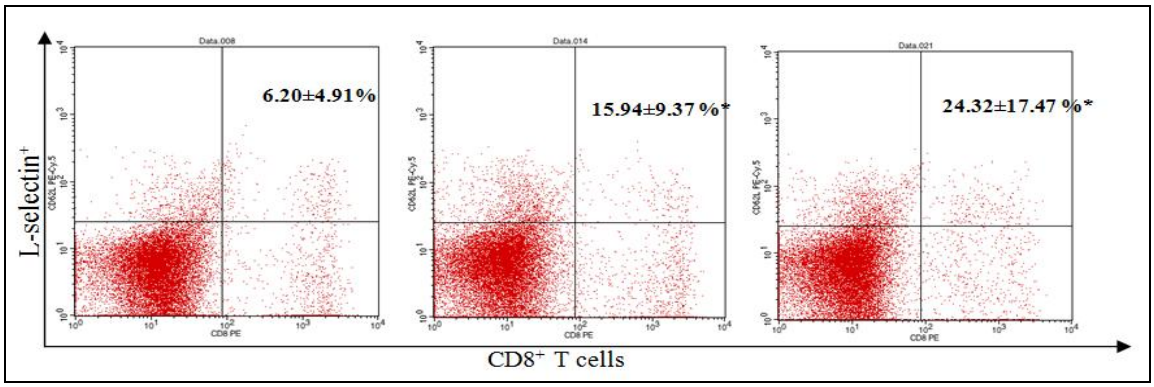
Then, the calculation result of the relative percentage of $CD4^+$ L-$selectin^+$ T cells on each group is shown in **Fig. 4**. This result shows the increase of the percentage of $CD4^+$L-$selectin^+$ after 21 day administration of the EF-KP. This increase is not significant, but it indicates an inhibition of naïve T-cell differentiation do not become the T cells effector. The flow cytometry analysis was continued to $CD8^+$L-$selectin^+$ T-cell parameter. The profile is shown in **Fig. 5**.



(a) (b) (c)

FIG. 4: THE RELATIVE PERCENTAGE PROFILE OF CD4$^+$ L-SELECTIN $^+$ T CELLS AS A RESULT OF FLOW CYTOMETRY ANALYSIS OF THE FRESH SPLEEN OF NEGATIVE CONTROL GROUP

(a) EF-KP group (b) and positive control group (c) (n = 10 mice per group)



(a) (b) (c)

FIG. 5: THE RELATIVE PERCENTAGE OF CD8$^+$L-SELECTIN$^+$ T CELLS AS A RESULT OF FLOW CYTOMETRY ANALYSIS IN THE SPLEEN CELLS IN CONTROL NEGATIVE GROUP

(a) EF-KP group (b), and positive control group (c) (n = 10 mice per group) *significantly different ($p < 0.05$) to negative and positive control groups

The CD8$^+$L-selectin$^+$ T cells are activation markers of CD8$^+$ T cells (T cytotoxic). The significant elevation of CD8$^+$L-selectin$^+$ in spleen shows a reduction of circulated T cell cytotoxic. It prevents attachments of auto-antigens and non-self recognition.

The Total Leukocyte Count (TLC) Result: The TLC assay was performed after 2 week treatment. The blood was taken from the tail vein. The mice were kept alive during this procedure for the next treatment. The results are shown in **Fig. 6**.

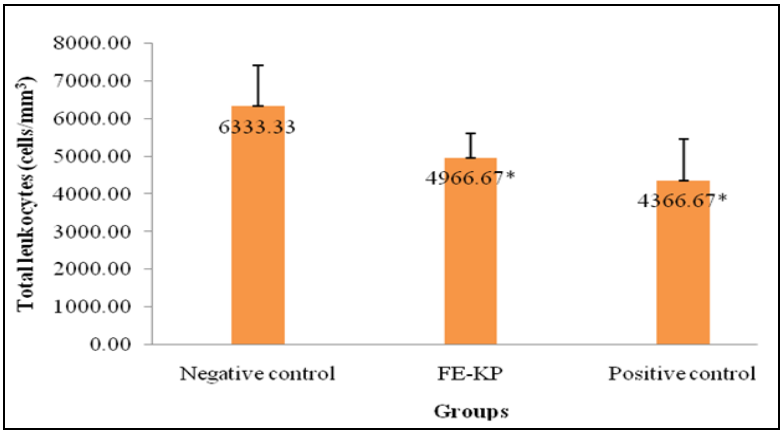


FIG. 6: THE TOTAL LEUKOCYTES OF THREE EXPERIMENTAL GROUPS. (n=3 MICE PER GROUP)

* Significantly different to the negative control group

This result shows a significant leukocytes reduction after the treatment. It indicates an anti-inflammatory effect and it is caused by the L-selectin regulation.

DISCUSSION: The EF-KP contains multiple compounds which are active as anti-inflammatory, immunosuppressant, and antioxidant. One of the active compounds quantitated for the quality control in this research was rutin, a flavonoid compound. According to the result on **Fig. 1**, the rutin compound was found in the retention time of 2.90. Based on a series concentration of rutin standard, the rutin measured in the EF-KP sample is 13.4mg/kg (**Table 1**). The limitation of the tandem system used was the precision of the molecular weight in the low retention time. It was resulted from the instrument validation which used a standard with the molecular weight of 500 Da. However, the limitation could be handled by comparing the spectrum with other previous publications about the active compounds of *Kalanchoe pinnata* (Lmk) Pers. Rutin, as one compound of flavonoids in this plant has also identified in other research [18, 32, 33].

After the treatment finished, the spleen of each mouse was isolated for the L-selectin$^+$ T cells assay. CD62L or L-selectin is one of the T regulatory markers. Ermann [34] states that T regs $CD4^+CD25^+$ with the co-transfer of L-selectin$^+$ is able to prevent tissue damage under the rejection of the immune system. This biomarker could be observed simultaneously by using flow cytometry method on the specific marker of $CD4^+CD8^+$L-selectin$^+$. Normally, the mice have the ratio of CD4+ T cells two times higher than CD8 T cells.

In this research, the ratio of CD4:CD8 is shown in **Table 2**. The results show a mild reduction of the ratio in EF-KP group. Although it did not achieve the normal condition, this reduction was a good sign of the effectiveness of the active compounds in EF-KP as a complementary drug for lupus patients. The effective drugs that could balance the ratio of CD4:CD8 have not been found [34 - 36]. The balance of the ratio could reduce the auto-reactive cells and then keep the tissue and organ normal. The positive control group showed an opposite effect. The ratio increased higher than the negative control group. It was assumed that the cyclophosphamide used in the positive control group had a toxic effect. It could be a consideration in the cyclophosphamide treatment. For further investigation, we observed the L-selectin$^+$ marker.

L-selectin$^+$ of T cells, a type 1 membrane protein, regulates lymphocyte homing into peripheral lymph node and accumulates leukocytes in inflammation area [3, 8, 37]. This molecule is expressed by naïve T cells $CD4^+$ L-selectin$^+$ and $CD8^+$ L-selectin$^+$. The naïve T cells circulate in blood, spleen, and peripheral lymph node and then it is attached by antigen. As a result, the naïve T cells are activated when L-selectin$^+$ is shedded. The L-selectin$^+$ T cells are expressed more than 80% in the normal condition. On the unhealthy condition, the expression decreases and then the T cells are activated [37, 38].

This research results in the relative percentages of $CD4^+$L-selectin$^+$ which are shown in **Table 2**. The EF-KP treatment increases the relative percentage of $CD4^+CD25^+$. The value is about 1% but the statistic calculation results a non-significant value. It suggests that EF-KP is able to inhibit the differentiation of naïve T cells to be the T-cell effector. The role of L-selectin$^+$ is as T-cell activation marker. $CD4^+$ L-selectin$^+$ T cells would be activated as CD4 T cells. The T-cell activation is avoided in autoimmune condition [39, 40]. The auto-reactive T cells can attach the normal cells and tissues because of a wrong recognition of self-autoantigen. The reduction of the $CD4^+$ T-cell activation reduces the immune complex formation. Auto-antigen that could not be eliminated by CD4 T cells will be attached by CD8 T cells.

The flow cytometry analysis results in the increase of the relative percentage of $CD8^+$L-selectin$^+$ T cells (**Table 3**). The relative percentage of $CD8^+$L-selectin$^+$ in the EF-KP group was 15.91%. It was more than two times higher than the negative control group. This increase is statistically significant. The high percentage of the EF-KP group and the positive control group shows the reduction of $CD8^+$L-selectin$^+$ T-cell differentiation. Thus, the CD8 T cell effector was reduced. This reduction is beneficial since the low number of CD8 has a direct impact on the reduction of CD8 T-cell reactivity [37, 41].

The low reactivity of CD8 T cell reduces the number of damage normal cells that are recognized as a non-self.

Total Leukocyte Count (TLC): The TLC assay was performed to prove the regulation of L-selectin$^+$ that binds the naïve T cells that stayed in the lymphoid organs. After 2 week treatment, we took the mice blood from the tail veins. The results in **Table 4** show a significant reduction of leukocytes in the group that received EF-KP and cyclophosphamide. The reduction of the total leukocyte is associated with the reduction of an inflammation. It is consistent with anti-inflammatory effect of EF-KP reported by Ferreira [19]. This effect is benefit for lupus condition. The adhesion molecule L-selectin$^+$ might play a role in this effect. However, further research is necessary to prove this suggestion.

CONCLUSION: The active compounds of EF-KP increase the L-selectin$^+$ T cells mainly CD8$^+$L-selectin$^+$ T cells in the spleen cause the inhibition of T-cell activation in the lupus model.

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CONFLICT OF INTEREST: We declare no conflict of interest.

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