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Research Article

THE BENEFITS OF ACTIVE COMPOUNDS IN *KALANCHOE PINNATA* (LMK) PERS ETHYL ACETATE FRACTION ON LUPUS ARTHRITIS MICE

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ABSTRACT

Objective: The aim of this research was to measure the effects of the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) on lupus arthritis mice.

Methods: The research was performed by testing of $CD123^+$ interferon-α ($IFN-\alpha^+$) dendritic cells and $CD68^+$ interleukin 6 ($IL-6^+$) macrophages as the main biomarkers using flow cytometry method, and then, the outcomes were directly observed in the joint's tissue structure. The results of the research were analyzed using statistics.

Results: The EF-KP reduced the relative percentages of $CD123^+IFN-\alpha^+$ dendritic cells significantly ($p<0.05$) with the percentage of 32.95±8.25% (negative control); 23.28±9.31%* (EF-KP); and 22.98±10.39%* (positive control). It also reduces the relative percentages of $CD68^+IL-6^+$ macrophages but not significantly. Finally, the outcome to the grade of joint damage was scored using Pritzker method. The treated groups have one grade lower, and the joint spaces were narrower than the untreated group.

Conclusion: The results show the ability of the active compounds in EF-KP, which are comparable to 0.042 mg/kg of quercetin, to inhibit the progress of lupus arthritis pathogenesis in mice. It might reveals the effectiveness of the EF-KP in human with lupus arthritis. However, the further clinical research is necessary.

Keywords: *Kalanchoe pinnata* (Lmk) Pers, Flavonoids, Lupus arthritis, $CD123^+$ interferon-α dendritic cells, $CD68^+$ interleukin 6 macrophages.

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INTRODUCTION

Lupus arthritis, as a kind of lupus manifestation mainly attacks to joints, develops fast by the regulation of feedback loop mechanism to produce lupus autoantibodies. The most important biomarker in this mechanism is interferon-α (IFN-α) [1-3]. The high release of IFN-α impacts the activation of T-helper to induce B-cell activity and also directly increases the B-cells activity to produce lupus-specific antibodies [4]. The excessive amount of IFN-α will make the manifestation of systemic lupus inflammation appears. The pro-inflammatory cytokine which has the main role in lupus arthritis is interleukin 6 (IL-6) [5,6]. The high level of IL-6 will be comparable to the severity grade of the joint damage.

The severe lupus arthritis is a life-threatening disease without any possibilities to be cured. Its treatments are focused on increasing the quality of life, but the long-term treatments lead to many harmful side effects. A new breakthrough to find appropriate therapeutical outcomes for lupus patients is based on the promising effects of herbal medicines [7-9]. In a very long history, the Chinese Herbal Medicine (CHM), Ayurveda (India), and Kampo (Japan) treatments develop herbal prescriptions for lupus based on their experiences. Most of them are used without any sufficient scientific data but have good outcomes [8]. Despite the efficacy, some herbal medicines are known to induce liver enzyme abnormalities [10]. Moreover, one widely known plant, *Kalanchoe pinnata* (Lmk) Pers extract inhibits lupus manifestations without hepatotoxicity in our previous study.

Flavonoid compounds in ethyl acetate fraction of an aqueous extract of *K. pinnata* (Lmk) Pers leaves (EF-KP) have many useful effects for lupus treatment, especially immunosuppressive [11,12] and anti-inflammatory effects [13-15]. The crude extract repairs the kidney tissue structure of lupus mice induced using 2, 6, 10, 14-tetramethylpentadecane (TMPD) in the previous study [16]. This material is also not toxic, not affects to the hematology and biochemistry properties of blood [17], and safe for maternity [18], so it can be a better choice for lupus since the current treatment has too many serious side effects [19-22]. *K. pinnata* (Lmk) Pers also has the hepatoprotective effect [23,24], so this plant is highly potent for lupus drug development. Therefore, this research purpose was to determine the activity of the active multiple compounds in the EF-KP on severe lupus arthritis mice.

In this study, the active compounds in the EF-KP potentially effective to inhibit the IFN-α production and also reduce the IL-6 in lupus arthritis mice. The EF-KP quality control was performed using total quercetin as an active marker. The test of IFN-α was focused on the IFN-α produced by dendritic cells, with the marker of $CD123^+IFN-\alpha^+$ dendritic cells. In addition, the IL-6 measurement was focused on IL-6 produced by macrophages ($CD68^+IL6^+$ macrophages). Then, the outcome was observed in the joint tissue structure of all experimental mice using Pritzker method [25]. The data will be useful to find an effective and safe drug for lupus arthritis which is ready to be continued to a clinical study.

MATERIALS AND METHODS

Materials

K. pinnata (Lmk) Pers fresh leaves were collected from our cultivation farm in Trenggalek, East Java, Indonesia. The female BALB/c mice were obtained from LPPT Gadjah Mada University, and the induction of the

mice was performed using TMPD in the trade name of Pristane (Sigma-Aldrich) with the code of Sigma-P2870 and the purity of >98%. The materials used in the flow cytometry assay were obtained from Biology Molecular Laboratory of Brawijaya University. The anti-CD68, anti-IL-6, anti-CD123, and anti-IFN-α were obtained from Biogenesis, USA. The cyclophosphamide with the purity >98% was obtained from PT. Kimia Farma, Indonesia. In addition, the materials used in the hematoxylin-eosin staining were obtained from the laboratory of Veterinary Medicine of Universitas Airlangga.

Methods

The preparation of EF-KP as the tested material

The plant determination was performed in UPT BKT Kebun Raya Purwodadi, Indonesia, with the identification number of 0284/IPH.06/HM/II/2015. *K. pinnata* (Lmk) Pers fresh leaves at the harvest time of 10 months were obtained from our cultivation farm in Trenggalek, East Java, Indonesia. The aqueous extract of the leaves was taken by a pressing method, and then, the yellow extract was dried by facilitating of freeze dryer. About 20 g of the dried extract was fractionated using liquid-liquid extraction method to obtain the EF-KP. Then, the EF-KP was prepared for liquid chromatography-mass spectrometry (LC-MS)/MS assay to measure its total quercetin.

A series concentration of quercetin standard (Sigma), with the purity of >95%, was calculated to make a standard curve. Nearly 0.5 g of the EF-KP was added with 5 ml of methanol 0.5%. This mixture was vortexed to obtain a solution. About 0.1 ml of the solution was taken, and then, 0.9 ml of methanol 50% was added. The mixture was vortexed. The dilution factor of 100,000 was used for the quercetin quantitation. Finally, the solution was injected in a particular volume to the LC-MS/MS instrument (AB Sciex 4000). Then, the chromatogram data were translated into the peak area data, so the concentration of quercetin in the EF-KP could be calculated by the instrument program Analyst Instrument Control and Data Processing Software AB Sciex.

The lupus mice preparation

The mice used in this research were female Balb/c mice which were pathogen-free species. The mice aged 7 weeks when induced using TMPD. The positive lupus mice were obtained after 6 months [26,27]. The lupus mice were divided into 3 experimental groups (10 mice per group). The negative control group consisted of lupus mice which received placebo. The EF-KP group consisted of lupus mice which received EF-KP with a dose which comparable to 400 mg/kg BW of the crude extract [16]. In addition, the positive control group consisted of lupus mice which received a dose which comparable to 1 mg/kg BW cyclophosphamide as a standard treatment. The treatment lasted for 21 days. After the treatment, mice were sacrificed, and then, the femur bones, spleens, and joints were isolated as the sample for the parameters observation. This procedure was approved by local ICUC of the Faculty of Veterinary Medicine, Universitas Airlangga, with the certificate number of 526-KE, in January 2016.

The measurement of tested parameters

Isolation of dendritic cells from the mice femur bones and macrophages from the spleen

Each spleen was washed in phosphate-buffered saline (PBS) solution and then isolated in a small chamber contained PBS using an apparatus to separate the cells from its connective tissues. The isolated cells of each spleen were placed in a flask, and the volume was homogenized into 15 ml/flask.

Each femur was isolated from other parts of the mice hip. The bone marrow was flushed along both points of the femur until the bone seemed white. The flushing result was collected in a flask, and the volume was added until the final volume of 15 ml using PBS.

Staining process

Each flask was centrifuged at the speed of 2500 rpm and 10°C for 5 minutes. The supernatants were thrown away to obtain the pellets. The cell pellets were added with 1 ml PBS and then divided into some microtube based on the need of staining combination. The microtubes contained PBS before the pellets were added. In addition, the suspense in the microtubes was separated from the cells using centrifugation at the speed of 2,500 rpm, 10°C in 5 minutes. The supernatant was thrown away, so the pellets were ready for the staining process.

The $CD123^+IFN\alpha^+$ dendritic cells and $CD68^+IL\text{-}6^+$ macrophages were stained by performing extracellular antibody phycoerythrin staining. In the $CD123^+IFN\alpha^+$ dendritic cells staining, each bone marrow pellet in each microtube was added with the volume of 50 µl of specific antibodies anti-CD123 and then anti-IFN-α before incubated. Besides, in the $CD68^+IL\text{-}6^+$ macrophages staining, the spleen pellet in each microtube was added with anti-CD68 and anti-IL-6 antibody. All of the microtubes were incubated for 20 minutes at the temperature of 4°C, in the dark place. Finally, in each microtube, PBS with the volume of 400 µl was added, and then, the sample was moved into a cuvette for flow cytometry analysis. The program used to interpret the result was BD Cell Quest.

The joint observation

The structural tissue of knee joint of each mice was observed under an Optilab microscope. The mice knees were isolated from each mice tested. Then, the fixation process began using formalin 10%, and then, the decalcification was performed using nitric acid 5%. The dehydration processes were step by step using ethanol 70%, 80%, and 90%, one concentration in a day. The clearing and impregnating processes were done, and then the blocking step of the sample preparation was also done well to provide the joint tissues to be observed. The sectioning was done with the thickness of 3-5 µm, so the staining using hematoxylin and eosin can be done well. The observation materials were fixed in object glass for the microscope observation. The statistics used in the one-way ANOVA test of the data, with $p<0.05^*$ and $p<0.001^{**}$.

RESULTS

Quercetin as the active marker in the EF-KP

The *K. pinnata* (Lmk) Pers plants were successfully cultivated in one cultivation place. The sunlight during the day and enough water [28] made them grow about 1 m in 10 months and be predicted to have the homogeny active compounds. After the fresh leaves were extracted and then fractionated as the EF-KP, its quercetin was analyzed for the quality control. The quercetin was chosen since most of the flavonoid content of *K. pinnata* (Lmk) Pers leaves was quercetin in the form of glycoside [29]. The fragmentation process in the instrument separated them into its aglycone, so the quantitated marker was total quercetin.

The quercetin concentration in the EF-KP was calculated, and the result is shown in Table 1. Total quercetin found in a high concentration of 253 mg/kg. It resembles other research [28] which counted the level of each quercetin glycoside in *K. pinnata* (Lmk) Pers leaves. Since quercetin has anti-inflammatory [30], antinociceptive, antioxidant [31], and immunosuppressive [32] effects, quercetin can be an active marker compound in this research.

The effects of the EF-KP on IFN-α, IL-6, and tissue structure of the joint

The EF-KP was tested in experimental lupus mice for 21 days, with placebo and cyclophosphamide as the negative and positive control. The parameters that reveal the inhibition of the active compounds of the EF-KP on the lupus arthritis pathogenesis were IFN-α and IL-6. Finally, the outcome was observed by observing the tissue structure of the joint.

The first tested biomarker was IFN-α, expressed by dendritic cells as $CD123^+IFN\text{-}\alpha^+$ dendritic cells.

Table 1: Calculation of the quercetin concentration in the EF-KP sample obtained using analyst instrument control and data processing software AB Sciex (y=2.41e+0.04x+(−2.02e)+0.04 and R value=0.9993)

Sample	Replication	Quercetin concentration calculated (µg/g)	Dilution factor	Concentration calculated (µg/g)	Mean (µg/kg)±SD	Result (mg/kg)
EF-KP	1	2.53	100,000	253,000	252,500±707	253±0.707
	2	2.52		252,000		

EF: Ethyl acetate fraction, KP: *Kalanchoe pinnata*

CD123+IFN-α dendritic cells

IFN-α has the central role in lupus development because it leads the dendritic cells to increase the activity of CD4+T-helper 1, so the T helper increases the cell-cell communication to the B cells to increase the production of autoantibodies [4]. Besides, IFN-α acts directly to the B cells to induce the high level of lupus-specific antibodies. IFN-α is mainly secreted by dendritic cells [33]. Surface markers of dendritic cells are CD11c and CD123. Therefore, the parameter used in this case was the $CD123^+IFN\text{-}\alpha^+$ dendritic cell. The results are shown in Fig. 1.

This result shows that the EF-KP reduces the relative percentage of $CD123^+IFN\text{-}\alpha^+$ dendritic cells. The decrease is comparable to the cyclophosphamide effect. The low expression of IFN-α inhibits the feedback loop mechanism in producing the excessive amount of lupus autoantibodies by B cells. It will reduce the frequency and severity of the lupus manifestations. It is also predicted to inhibit the other biomarkers which have the main role in lupus inflammation process.

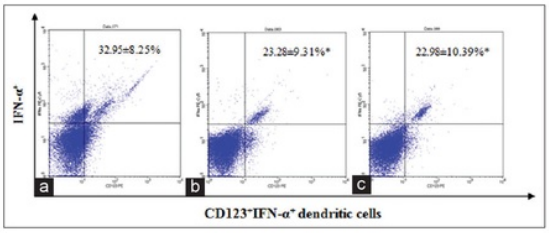

Fig. 1: The mean ± standard deviation of the relative percentage of $CD123^+$ interferon-α dendritic cells analyzed flow cytometer from the bone-marrow sample of the control negative group (n=10) (a), ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers group (n=9) (b), and positive control group (n=10) (c). *Significantly different (p<0.05) to the negative control group and analyzed by one-way ANOVA.

$CD68^+IL\text{-}6^+$ macrophages

The pro-inflammatory cytokine that has the high correlation to lupus arthritis is IL-6 [4]. In this experiment, the inflammatory parameter chosen was the surface marker of $CD68^+IL\text{-}6^+$ macrophages in all mice used. The results are shown in Fig. 2.

The mean of the relative percentage of $CD68^+IL\text{-}6^+$ macrophages of the EF-KP group seems lower than the negative control, but in fact, the decrease is not significantly ($p>0.05$) under statistic calculation. It can be explained by performing further IL-6 calculation using the IL-6 secreted by T-cells. It has been known that macrophages mainly used in the innate immune response, whether the T-cells act in adaptive immune response [34]. The macrophage autophagy in lupus is one cause that might be considered as the leading caution of the insignificant IL-6 decrease [27,35]. It seems logical since the passive lupus manifestation still reveals the organ disorder, without severe inflammations occur.

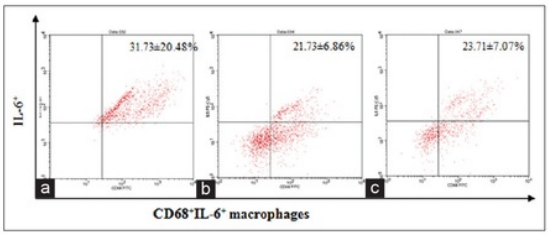

Fig. 2: The mean ± standard deviation of the relative percentage of $CD68^+$ interleukin 6 macrophages analyzed using flow cytometer from the spleen sample of the negative control group (n=10) (a), ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers group (n=9) (b), and positive control group (n=10) (c). The results are not significantly different (p>0.05) to the negative control group and analyzed by one-way ANOVA

The EF-KP effects on the structural changes of joint tissue

The results of the HE staining were shown in Fig. 3. The results show that the three experimental groups have serious erosions on its hyaline layer. The erosions mainly occur in the middle of the fibrous layer and chondrogenic layer. This area is a part of ligament cruciatum posterius. Besides, the synovial membrane was thickened as a sign of inflammation process. Nucleus pulposus seemed more than normal mice. All of the abnormalities are not reach the compacta bone, so the joint can freely move as usual.

Fig. 3 shows that there is no significant difference of the joints in three experimental groups. The erosions still present and not being repaired after the EF-KP administration and also after cyclophosphamide administration. The number of its nucleus pulposus did not seem to decrease too. Moreover, if joint space narrowing (JSN) can be considered as a representative parameter, the JSN in both EF-KP and positive control cyclophosphamide groups is narrower than the negative control as a sign of the inhibition of inflammation processes in the joint.

The grade of joint damage severity was calculated semi-quantitatively according to Pritzker method [25]. The results are shown in Table 2.

The grade of joint damage in the EF-KP group and the positive control group is decreased. It means that the active compounds of EF-KP could inhibit the progress of the joint erosion or repair the structure of hyaline layer. The further research to know more about this effect is necessary. The data are homogeny according to the Levene test ($p>0.05$). However, the decrease of the grade of joint damage is not statistically significant ($p>0.05$). Then, the JSN measurement was performed to assess the joint disorder. The JSN measurement results can support the hyaline layer damage grade data and represent the anti-inflammatory effect. The EF-KP cannot cure the severe damage in the joint of lupus mice, but it can be a drug candidate to inhibit lupus arthritis manifestation.

DISCUSSION

K. pinnata (Lmk) Pers has major flavonoid compounds which have anti-inflammatory, antinociceptive [13], immunosuppressive [12], antioxidant, and anti-depressive effects [36,37]. It is needed in the lupus condition. The aqueous extract of *K. pinnata* (Lmk) Pers leaves has a strong effect in lupus nephritis model induced by TMPD in our previous study. The flavonoids can be separated into ethyl acetate fraction (unpublished data).

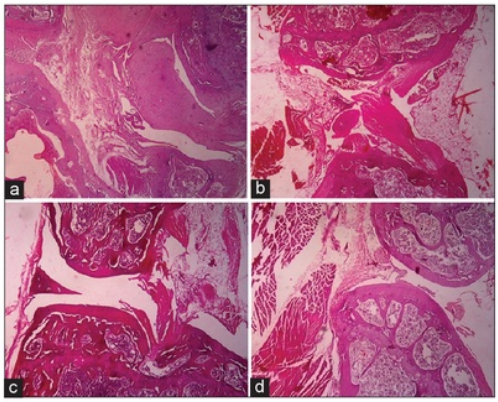

Fig. 3: The histopathology observation of tissue structure of the knee of each mouse in the experiment, which consists of the negative control group (n=10) (a), ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers group (n=9) (b), positive control (n=10) (c), and the normal mouse (d) under Optilab microscope with the magnitude of ×40

Table 2: The grade of damage of joint tissue in the three experimental groups

Group	The mean±SD of ASS	The mean±SD of JSN (μm)
Negative control	3.70±1.06	672.34±454.97
EF-KP	2.89±1.27	548.32±266.66
Positive control	2.90±1.97	415.31±274.62

Negative control group n=10; EF-KP group n=9; Positive control group n=10.
ASS: Arthritis severity score, JSN: Joint space narrowing, SD: Standard deviation

In this research, based on Table 1, the EF-KP dose was equal to 0.042 mg of quercetin as its active marker. The EF-KP reduces the relative percentage of the $CD123^+IFN\text{-}\alpha^+$ dendritic cell. The dendritic cells lead to an excessive amount of IFN-α which has the central role in lupus pathogenesis [38,39]. Its decrease (Fig. 1) leads to the low lupus arthritis manifestation. IL-6 is also the most important pro-inflammatory cytokine in lupus arthritis [40,41]. The pro-inflammatory cytokines IL-6 expressed by macrophages ($CD68^+IL\text{-}6^+$ macrophages) are lower than the negative control group (Fig. 2) and reveal the inhibition of inflammation in the joint so that the lowering sign of arthritis inflammation occurs (Fig. 3) and can be observed in the JSN parameter (Table 2). The EF-KP antioxidant and anti-inflammatory [12,42,43] effects could inhibit but not repair the damage of hyaline layer erosion as well since the severity of the joints was at the severe level when the mice were treated.

Lupus treatments using the medicinal plants are traditionally used in CHM, Ayurveda, and Kampo in particular herbal prescriptions based on generation to generation experiences [8,9]. Most of them are applied without sufficient scientific data but giving satisfying outcomes in lupus patients [8]. Some of the herbal treatments for lupus which has studied are Huo-luo-Xiao-Ling Dan herbal formula, *Celastrus aculeatus* Merr., and green tea. The active compounds regulate a lot of cytokines in lupus model and result in anti-arthritic activity [7]. There are no data that state the risk of those three herbal medicines when used in a long lupus treatment. Many individual lupus manifestations can be diagnosed well in traditional Chinese medicine using latent class model, so the individual CHM treatment can be done. The efficacy and toxicity data of herbal prescription in many CHM lupus treatments are also incomplete [8]. Moreover, taking traditional medicine in a long-term treatment might lead to liver enzyme abnormalities [10].

In other cases, dietary factors also regulate cytokines in lupus and have the beneficial impacts on reducing lupus manifestations. Calorie restriction, vitamin A, vitamin D, vitamin E, phytoestrogens, and herbal medicine are used to maintain the cytokine normal in lupus patients. It can be a beneficial material to maintenance the immune regulation stability in lupus [44-46].

To find the safe and effective herbal medicine for lupus, *K. pinnata* (Lmk) Pers in the form of its EF-KP has beneficial effects as mentioned before. It also has hepatoprotective compounds which can minimize the side effects of the liver injury. This data supported by another study by Ozolua [17] which states no toxic effect of *K. pinnata* (Lmk) Pers in a subchronic toxicity test. The advantages support the efficacy of the EF-KP and then increase its value to be a safe lupus drug candidate which is ready for the next step of clinical study.

CONCLUSION

The active compounds of the EF-KP with the dose that comparable to 400 mg/kg BW extract and contains 0.042 mg of quercetin reduce the IFN-α produced by dendritic cells and the IL-6 produced by macrophages, so the arthritis manifestations in the joints are decreased. Therefore, the active compounds in the EF-KP inhibit the severity of lupus arthritis progressions and manifestations.

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