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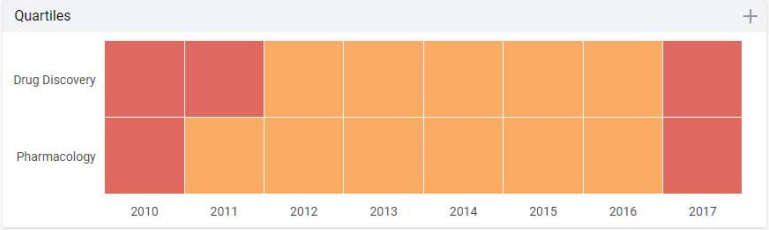

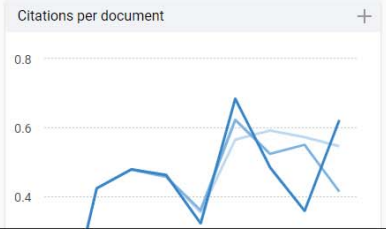

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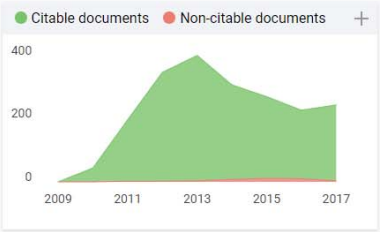

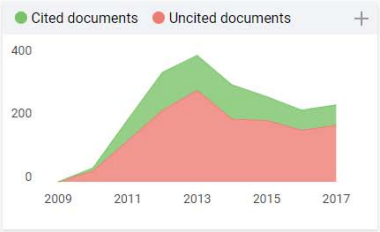

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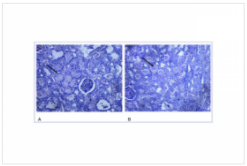

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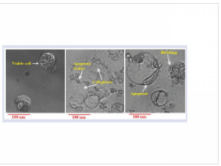

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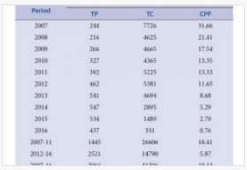

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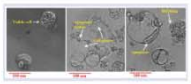

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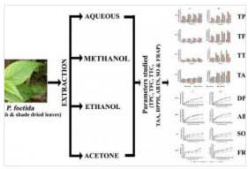
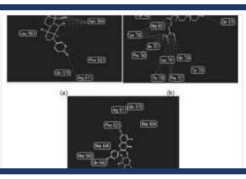


Original Article

Repairing Effects of Aqueous Extract of Kalanchoe pinnata (Lmk) Pers. on Lupus Nephritis Mice

Niken Indriyanti,Afrillia Nuryanti Garmana,Finna Setiawan



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Original Article

Repairing Effects of Aqueous Extract of *Kalanchoe pinnata* (Lmk) Pers. on Lupus Nephritis Mice

Niken Indriyanti[1*], Afrillia Nuryanti Garmana[2], Finna Setiawan[3]

ABSTRACT

Kalanchoe pinnata (Lmk) Pers (KP) has an immunosuppressive effect on delayed-type hypersensitivity test. Based on it, this research aimed to determine the repairing effects of aqueous extract of KP on lupus nephritis mice and identified its active compound. The KP extract profile was determined using UPLC-QTOF-MS/MS instrument. We examined six mice groups consisting of three curative treatment groups, one standard group receiving prednisone, one preventive group receiving KP extract, and one healthy (healthy and untreated) group. At the end of the experiment, we measured the proteinuria and renal histology parameters. To recognize the active compound in the KP profile, we performed *in silico* assays for the flavonoid compounds to bind to the glucocorticoid receptor. We played *in silico* tests for the flavonoid compounds to identify the active compound in the KP profile. We found the repairing effect of KP was detected in the kidney, demonstrated by its low proteinuria level and its better tissue structure. In the curative group, the urine protein level and its glomerular inflammation decreased. In the preventive group, the aqueous extract of KP could prevent lupus nephritis manifestations in the kidney. Bryophyllin A is the most active compound of the KP. However, further research is needed to understand the mechanism involved. We conclude, the aqueous extract, especially its bryophyllin A, have beneficial effects in repairing the function and tissue structure of lupus manifestations in mice kidney.

Key words: Lupus, Glomerulonephritis, Inflammation, Proteinuria, Docking.

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INTRODUCTION

Natural treatment for lupus nephritis is a very potent and challenging on drug development. A large number of medicinal plants have beneficial effects which can be used in lupus nephritis treatment, such as immunosuppressant, antioxidant, anti-inflammatory, and antidepressant. These plants have not been explored clearly on lupus condition.[1,2,3] Some Chinese Herbal Medicine, Ayurveda, and Traditional Kampo treatment which were used for treating lupus have been studied for its efficacy. However, some of the traditional medicines have side effects on liver enzyme abnormality.[4] Therefore, we investigated a lupus candidate drug from a medicinal plant which has a hepatoprotective activity[5,6] and also known as a nontoxic material, *Kalanchoe pinnata* (Lmk) Pers leaves (KP).

Polar compounds in aqueous extract of KP has beneficial effects for lupus condition. The activities include anticonvulsant,[2] anti-nociceptive and anti-inflammatory,[7,8] immunosuppressant,[9,10] and T cell suppression.[11] It also has a relaxant uterus effect so that it is safe for maternity.[12,13] fenoterol, in human myometrium. KP is also a safe material proved by using an accurate acute and subchronic toxicity test.[14] Compounds found KP are quercetin glycosyl conjugates, rutin, stigmasterol, 3,8-dimethoxy-4,5,7-trihydroxyflavone, friedelin, epigallocatechin-3-o-syringate, luteolin, kaempferol, quercetin,[15,16] quercetin-3L-rhamnosido-L-arabino furanoside, quercetin-3-O-diarabinoside, kaempferol-3 glucoside, bryophollone, bryophyllin A, Bryophyllin C, bersaldegenin-3-acetate, bersaldegenin-1,3,5-orthoacetate, daigremontianin.[17,18]

KP has many beneficial effects and safety profiles, so it is appropriate to be developed as a new drug candidate for lupus nephritis. Therefore, this research determined the efficacy of the KP extract used in a lupus model. A well-known lupus model, Pristane-induced lupus, was used based on its broad severe lupus nephritis manifestations.[3,19,20] The efficacy of the KP comes from multiple compounds, but we can predict the active compounds on lupus responsible receptors, such as glucocorticoid. Moreover, this research focused on the effect of aqueous extract of KP on repairing the lupus nephritis manifestations and the potential active compounds involved.

MATERIALS AND METHODS

MATERIALS

The *Kalanchoe pinnata* (Lmk) Pers fresh leaves (KP) were collected from Manoko, Bandung. All of the leaves below the fourth top leaves were used. The plant identification was confirmed at the Herbarium Bandungense, Bandung Institute of Technology.

Cite this article: Indriyanti N, Garmana AN, Setiawan F. Repairing Effects of Aqueous Extract of *Kalanchoe pinnata* (Lmk) Pers. on Lupus Nephritis Mice. Pharmacog J. 2018;10(3):548-52.

Pristane® which contains 2,6,10,14 tetramethylpentadecane (TMPD) ≥98% was purchased from Sigma-Aldrich.

Female Balb/c mice aged seven weeks were purchased from LPPT UGM. Mice were housed under specific conditions. They were fed by using standard diet and water ad-libitum. We also use reagent strips for urine analysis (Verify®) to check the level total protein in the urine of each mouse tested.

METHODS

Preparation of the extract

Aqueous extract of KP leaves was processed from pressed fresh leaves. The extract was dried by using freeze dryer equipment (Eyela). The KP extract profile was measured by using UPLC-QTOF-MS/MS tandem instrument (Waters) by using Acquity UPLC column BEH C18 1.7µm 2.1×50 mm, flow rate 0.3 ml/minute. The injected volume was 5µl (the concentration of 5000 µg/L). The temperature used was 40°C. Also, the eluent used was A: H2O+formic acid 0.1%, B: acetonitrile + formic acid 0.1%.

Experimental grouping

An animal model for lupus used was Pristane-treated mice. Female Balb/c mice were injected by using 0.5 mL Pristane intraperitoneally, and then the booster injection was done at the day 90th.[10] Proteinuria level was measured at 5th month after the induction and then lasted until the severe sign detected at month 8th. All of the induced mice experienced proteinuria at the level of ++ (100 mg/dL). The mice were grouped into some treatment groups (n=8 per group). Three treatment groups received the aqueous extract of KP at the doses of 200, 400, and 600 mg/kg body weight. There also positive control mice which received prednisone at a dose of 25 mg/kg body weight. The other groups were a preventive group that received extract at doses 200 mg/kg body weight and the standard group that did not induce by using Pristane.

Treatment

The KP extract was given orally to the treatment groups for 21 days. Protein level in the urine of all groups was measured every seven days. At the day 21st, mice were sacrificed then the renal histology observation was done by using Haematoxylin-Eosin (HE) staining. The data was analyzed by using Oneway ANOVA. This protocol was approved by the local ICUC of Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, with the number of 512-KE.

Docking analysis to predict the potentially active compounds

The *in silico* study was performed by using glucocorticoid receptor (GeneBank) and the chemical structures of some flavonoid compounds in KP, and also the prednisone chemical structure (PubChem). The structures were stabilized by using Chem3D Program, and then the docking was performed by using Molegro Virtual Docker. All of the programs used were licensed programs of Universitas Airlangga.

RESULTS

Characteristics of the aqueous extract of *Kalanchoe pinnata* (Lmk) Pers (KP)

The yield of the extract was 1.95%w/w. Based on the organoleptic observation, the extract was dark yellow hygroscopic crystals resembling light materials. It was kept in the freezer until it was used. The identity profile of aqueous extract used was provided as a chromatogram profile (**Figure 1**) obtained using UPLC-QTOF-MS/MS tandem system.

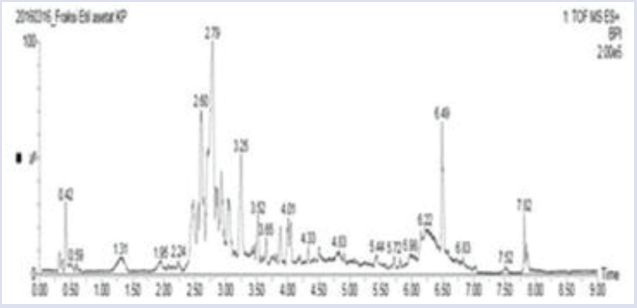

Figure 1: The profile of chemical compounds in aqueous extract of *Kalanchoe pinnata* (Lmk) Pers. Leaves.

The main compound was at the retention time of 2.79 and was predicted as flavonoid compounds. A lot of literature mention the name of specific flavonoid compounds found in this plant. This data supported by a phytochemical screening data that show the presence of flavonoids, saponin, tannin, and steroid/triterpenoid. Besides, the physical characteristics of the KP extract including water content 9.00% v/w, compounds soluble in water 54.34% w/w, and compounds soluble in ethanol 3.57% w/w. The characteristics exhibit beneficial and stable features of a solid material.

Three compounds in aqueous extract of KP has been widely studied. A lot of chemical compounds were found, such as steroids, terpenoids, flavonoids, phenolic, tannin, alkaloids, glycosides, carbohydrates, and proteins. The aqueous extract contains carbohydrates, proteins, flavonoids, phenolic, tannins, and glycosides. The leaves contain astragalin, 3,8-dimethoxy-4,5,7-trihydroxyflavone, friedelin, epigallocatechin-3-o-syringate, luteolin, rutin, kaempferol, quercetin, quercetin-3L-rhamnosido-L-arabino furanoside, quercetin-3-O-diarabinoside, and kaempferol-3-glucoside.[16,21] These multiple compounds might have the synergic effects that reduce lupus nephritis manifestations.

The effects of aqueous extract of *Kalanchoe pinnata* (Lmk) Pers leaves on lupus nephritis mice

The mice received a daily dose of aqueous extract of KP during 21 days. The protein level of the urine of the treated mice groups was decreased (**Table 1**). Proteinuria is a parameter for severity of nephritis.[22,23] (-/- Moreover, there was no severe proteinuria obtained on the mice until the 6th month after the induction. The mice only had mild proteinuria + (30 mg/dL) which revealed the low disease progress. This result was biased because the normal mice could reach proteinuria at the same level.

Then, the induction time was continued with a strict observation of proteinuria every seven days. As a result, the mice had proteinuria ++ (100 mg/dL) on the month 8th. Thus, they were ready for experimental grouping.

After the treatment period, the trend of proteinuria level decreased from level ++ (100 mg/dL) to level + (30 mg/dL) except the negative control groups. On the treatment groups, the dose of 200 mg/kgBW was the one that more active to decrease proteinuria level. The result was not as stable as the prednisone effect. On the preventive group, the proteinuria did not increase although they were injected twice using Pristane. Their physical condition showed a healthy behavior.

Proteinuria level was not the primary parameter to measure the severity of nephritis. Therefore, the observation was conducted in renal histology observation. Haematoxylin-Eosin (HE) staining showed the differences of each treatment effect on renal tissue structure. The results were in **Figure 2**.

The normal renal tissue of normal mice (**Figure 2a)** indicated by the mesangial area (long black arrow) seems thin with a regular structure,

Table 1: Proteinuria Level Before And After Kp Extract Treatment.

Group	Protein level in the urine (mg/dL)	
	Before treatment	After treatment
Treatment group at a dose of 200 mg/kg BW KP	200.00±115.47	65.00±40.41*
Treatment group at a dose of 400 mg/kg BW KP	65.00±40.41	22.50±8.66*
Treatment group at a dose of 600 mg/kg BW KP	112.00±110.77	55.00±41.53*
Prednisone at a dose of 25 mg/kgBW	200.00±115.47	30.00±0.00*
Negative control that received placebo	82.50± 35.00	132.50±116.44
Preventive group (treatment during induction time)	15.00±0.00	18.75±7.50*

* Different significantly compared to the negative control group with $p<0.05$. n = 8 mice per group using t-test Statistics

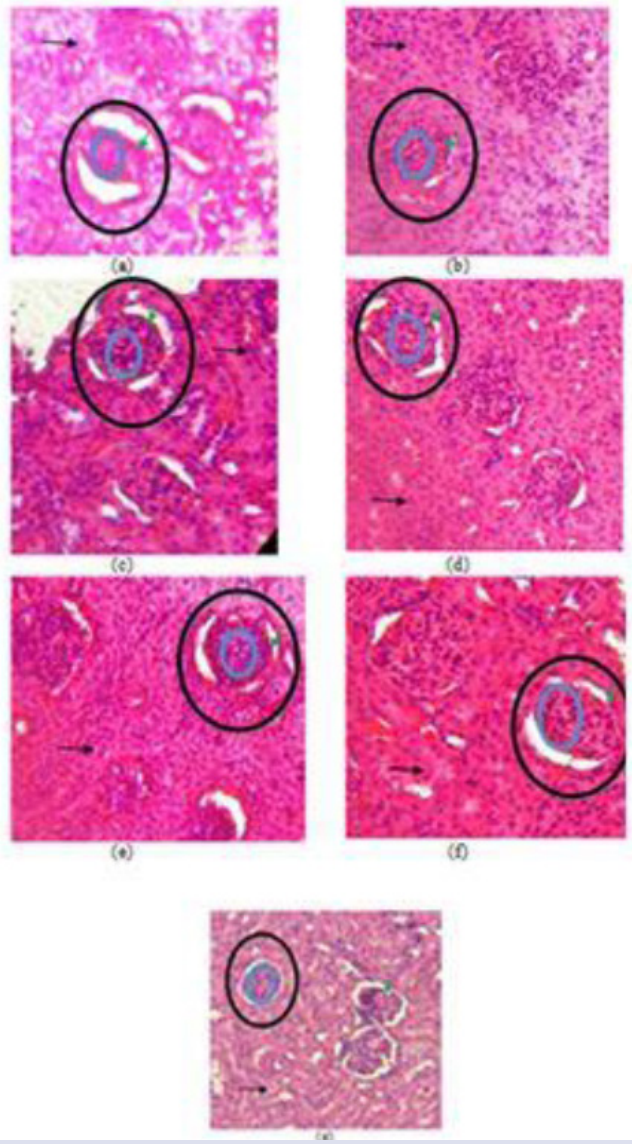

Figure 2 : Morphology of renal tissue at the magnitude of 400x by using light micrographs of a renal glomerulus of all groups Where; normal mouse of (a), negative control (b), treatment at the dose of 200 mg/kg BW (c), treatment at the dose of 400 mg/kg BW (d), treatment at the dose of 600 mg/kg BW (e), prednisone at the dose of 25 mg/kg BW (f), and preventive group (g). n= 8 mice per group.

and then the glomerulus (big circle) present between urinary spaces of Bowman's capsule. The glomerulus is a plaited mat of the capillary that has a filtration function. There is no proliferation of erythrocytes and inflammation cells inside the glomerulus (little circle). The area marked by green arrow is a position of glomerular basement membrane (GBM) that is thin in a healthy condition.

Furthermore, the positive control which only receiving placebo (**Figure 2b)** had many structural changes, such as the GBM is thicker than usual with a proliferation of glomerulus results in inflammation. This condition has been reported before.[10] Then, the glomerulus attaches the Bowman's capsule area, so the spaces are closed. In this situation, the filtration function performed difficultly. Besides, the mesangial area is thickened, are irregularly structured, and the cytoplasm cells are accumulated.

In the treatment group at the dose of 200 mg/kgBW (**Figure 2c**), the inflamed glomerulus was decreased. But, the GBM still looked thick. The cells accumulation is slightly reduced in the glomerulus. The mesangial area was shown the same structure with the **Figure 2b**. On the other groups received the extract at the dose of 400 and 600 mg/kgBW (**Figure 2 d and e**), the anti-inflammatory effect still present. The urinary spaces in Bowman's capsule looked wider although not achieve the healthy appearance. The mesangial area looked more regularly structured by the increase of the dose. The GBM thickness seemed similar to the low treatment dose, but it needs further measurement.

On the standard group received prednisone (**Figure 2f**), the accumulation of cells in the glomerulus and the glomerular inflammation were decreased although the GBM is still dense. The mesangial area looked thick, and not all of the area showed the repairing of the structure. At the last group, the preventive group, the histology structure is similar to the healthy mice.

The histology observation results show the repairing effect of aqueous extract of KP on many parts, such as tubules, glomerulus, GBM, and the Bowman's Capsule space. The treatment groups showed the alteration that it reduces the inflammation. The modification is not as good as the preventive group, but the effect is very potential to maintain the lupus patient stability.

Autoantigen-antibody complexes caused inflammation that occurred in glomerulus.[20,24] One of the autoantigens was nucleosome. Nucleosomes and anti-nucleosome antibodies have been shown to bind to the glomerular basal membrane (GBM). It is assumed that nucleosomes bind to the GBM via their histone proteins. The glomerulonephritis was developed by the formation of nucleosome-containing immune complexes. Also, there may be cross-reactivity of nucleosome-specific antibodies (e.g., with heparan sulfate or a so-far-unknown surface antigen) whereby these autoantibodies reach the glomeruli and cause inflammation.[25,26]

This result showed a capability of aqueous extract of KP in reducing the inflammation of glomerulus in the lupus nephritis mice model. It also prevents the mice from Pristane induction effect, so the mice keep normal although they received the second injection. The result showed a curative and preventive potency. But, further investigation needed to ensure this effect. These data indicate that aqueous extract of KP was useful to maintain immune system in lupus nephritis condition. This efficacy data is supported by the toxicity data reported by Ozolua[14] stating that there was no toxicity occurred in acute and subchronic toxicity test. Because of the efficacy and the safety, aqueous extract of KP could be a good candidate for a natural treatment for lupus nephritis.

The docking of some compounds in KP in glucocorticoid receptor

The docking result did not show a correlation with cell culture test, the effect on the animal model or others. Docking did not calculate the bioavailability, toxicity, and other factors in the body. The main reason for the use of docking is to predict the compound which binds to a specific receptor (protein) well. We also could see how is the three dimensions geometric structure of a compound bound to the active site of the protein.

In this research, we focused on three compounds found in KP, i.e., bryophyllin A, beta-sitosterol, and kaempferol-3-glucoside. The receptor was glucocorticoid receptor. This receptor has a very crucial role in immune homeostasis. The receptor is shown as a free water receptor. The ligand would be shed before the tested compound was inserted into the ligand space.

The compounds tested were prepared as 3D structures with an optimum geometric stability, using the Chem3D program. Then, the docking results are shown in **Figure 3.**

The ligand binding of bryophyllin A to glucocorticoid receptor presents through a hydrogen bond with amino acid residue Arg 611 (**Figure 3**). There is also a hydrophobic bonding with Gln 570, Leu 563, Met 560, Ile 747, Thr 739, Phe 749, Glu 748, Thr 561, Asn 564, and Phe 623. **Figure 3b** shows beta-sitosterol binds the receptor with hydrogen bonds to Arg 611 and Gln 570, and also hydrophobic bonds with Gln 738, Try 735, Phe 737, Leu 741, Thr 739. Ile 757, Phe 740, Cys 736, Met 601, Phe 749, Leu 753, Trp 600, Asn 564 and Leu 563. Fig. 5Sc showed that kaempferol-3-glucoside binds into glucocorticoid receptor using hydrogen bonds with Arg 611 and Asn 564, and hydrophobic bonds with Tyr 735, Leu 732, Cys 736, Phe 749, Met 639, Gln 642, Met 560, Met 646, Phe 623, Gln 570, and Met 604.

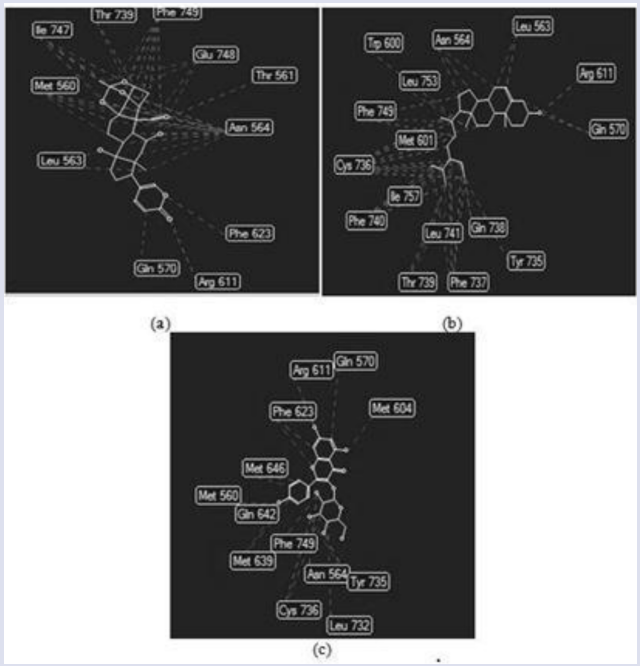

Figure 3: The Docking Result of Bryophyllin A (a), Beta-Sitosterol (b) and Kaempferol-3-glucoside (c) in the Glucocorticoid Receptor Tested Using Molegro Virtual Docker Program.

According to the scoring calculation, the free binding energy of bryophylin A compound is -66.6975 kcal/mol, beta sitosterol is -106.1340 kcal/mol, and the kaempferol-3-glucoside is -105.5710 kcal/mol. The results show the lowest free binding energy was on bryophyllin A compound. This little power makes it easy to bind to the glucocorticoid receptor, so the anti-inflammatory effect occurs selectively. At last, the *in silico* prediction of the most active compound results in the bryophyllin A as the compound which has the lowest free binding energy in the glucocorticoid receptor. The experiment needs to be conducted using other receptors responsible for the lupus pathogenesis to support this result.

DISCUSSION

Aqueous extract of KP is sufficient to reduce inflammatory signs on kidney parameters of lupus nephritis mice. This anti-inflammatory effect reduces the high pro-inflammatory cytokines released when the glomerular inflamed due to the increasing number of immune complexes deposited inside. Flavonoid compounds in the KP have a high anti-inflammatory effect and also anti-oxidative results[7,27,28,29] which support the regulation of immune responses. Lupus marked with irregular immune reactions which released suddenly if the triggering factors come.[30,31] The KP treatment in all doses gives many beneficial outcomes in the kidney to reduce and inhibit the glomerular swelling. It followed by the lower proteinuria level. Both results show that the glomerulus moves to its normal condition.

The glomerulus disorder in lupus is different with the kidney failure signs, which cannot be repaired. Glomerulonephritis in lupus occurred as a result of complex immune deposit.[32,33] It is possible for the immune complex to split and then eliminated from glomerulus, so the glomerulus function repaired.

In this research, all doses tested result in good outcomes to repair the kidney structure and function (**Figure 2 and Table 1**). It leads a further question regarding the active compound which responsible for the effect. According to the profile of the aqueous extract of KP, the primary compounds are flavonoid compounds. Therefore, we challenged some active compounds which potentially have an anti-inflammatory effect, i.e., bryophyllin A, beta-sitosterol, and kaempferol-3-glucoside. The compounds have good affinity to bind the glucocorticoid receptor, a receptor involved in the inflammatory process. The glucocorticoid receptor is a specific target for anti-inflammatory drugs. The compound which has the lowest binding energy to the glucocorticoid receptor was bryophyllin A (**Figure 3**). It means that bryophyllin A can efficiently bind the target site in glucocorticoid and results in the highest anti-inflammatory effect. However, the further research to calculate the concentration of bryophyllin A in the KP is necessary.

CONCLUSION

The aqueous extract of *Kalanchoe pinnata* (Lmk) Pers repairs on the kidney damage of Pristane-induced lupus nephritis mice. The potential flavonoid active compound which could be its active anti-inflammatory marker in this result is bryophyllin A.

ABBREVIATIONS USED

KP: *Kalanchoe pinnata;* **TMPD:** 2, 6, 10, 14-tetramethylpentadecane; **GBM:** glomerular basement membrane.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUMMARY

Drug development for advance safe lupus therapy is highly needed. The aqueous extract of *Kalanchoe pinnata* (Lmk) Pers (KP) fresh leaves has the activity to reduce lupus manifestation in the kidney of lupus mice. It reduces the glomerular inflammation, so the level of protein present in the urine can be decreased. Furthermore, the aqueous extract of KP has multiple compounds. The most potent compound of this material which can bind the glucocorticoid receptor efficiently is the structure of Bryoplyllin A, based on the *in silico* measurement.

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Repairing Effects of Aqueous Extract of Kalanchoe pinnata (Lmk) Pers. on Lupus Nephritis Mice

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Original Article

Repairing Effects of Aqueous Extract of *Kalanchoe pinnata* (Lmk) Pers. on Lupus Nephritis Mice

Niken Indriyanti[1*], Afrillia Nuryanti Garmana[2], Finna Setiawan[3]

ABSTRACT

Kalanchoe pinnata (Lmk) Pers (KP) has an immunosuppressive effect on delayed-type hypersensitivity test. Based on it, this research aimed to determine the repairing effects of aqueous extract of KP on lupus nephritis mice and identified its active compound. The KP extract profile was determined using UPLC-QTOF-MS/MS instrument. We examined six mice groups consisting of three curative treatment groups, one standard group receiving prednisone, one preventive group receiving KP extract, and one healthy (healthy and untreated) group. At the end of the experiment, we measured the proteinuria and renal histology parameters. To recognize the active compound in the KP profile, we performed *in silico* assays for the flavonoid compounds to bind to the glucocorticoid receptor. We played *in silico* tests for the flavonoid compounds to identify the active compound in the KP profile. We found the repairing effect of KP was detected in the kidney, demonstrated by its low proteinuria level and its better tissue structure. In the curative group, the urine protein level and its glomerular inflammation decreased. In the preventive group, the aqueous extract of KP could prevent lupus nephritis manifestations in the kidney. Bryophyllin A is the most active compound of the KP. However, further research is needed to understand the mechanism involved. We conclude, the aqueous extract, especially its bryophyllin A, have beneficial effects in repairing the function and tissue structure of lupus manifestations in mice kidney.

Key words: Lupus, Glomerulonephritis, Inflammation, Proteinuria, Docking.

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INTRODUCTION

Natural treatment for lupus nephritis is a very potent and challenging on drug development. A large number of medicinal plants have beneficial effects which can be used in lupus nephritis treatment, such as immunosuppressant, antioxidant, anti-inflammatory, and antidepressant. These plants have not been explored clearly on lupus condition.[1,2,3] Some Chinese Herbal Medicine, Ayurveda, and Traditional Kampo treatment which were used for treating lupus have been studied for its efficacy. However, some of the traditional medicines have side effects on liver enzyme abnormality.[4] Therefore, we investigated a lupus candidate drug from a medicinal plant which has a hepatoprotective activity[5,6] and also known as a nontoxic material, *Kalanchoe pinnata* (Lmk) Pers leaves (KP).

Polar compounds in aqueous extract of KP has beneficial effects for lupus condition. The activities include anticonvulsant,[2] anti-nociceptive and anti-inflammatory,[7,8] immunosuppressant,[9,10] and T cell suppression.[11] It also has a relaxant uterus effect so that it is safe for maternity.[12,13] fenoterol, in human myometrium. KP is also a safe material proved by using an accurate acute and subchronic toxicity test.[14] Compounds found KP are quercetin glycosyl conjugates, rutin, stigmasterol, 3,8-dimethoxy-4,5,7-trihydroxyflavone, friedelin, epigallocatechin-3-o-syringate, luteolin, kaempferol, quercetin,[15,16] quercetin-3L-rhamnosido-L-arabino furanoside, quercetin-3-O-diarabinoside, kaempferol-3 glucoside, bryophollone, bryophyllin A, Bryophyllin C, bersaldegenin-3-acetate, bersaldegenin-1,3,5-orthoacetate, daigremontianin.[17,18]

KP has many beneficial effects and safety profiles, so it is appropriate to be developed as a new drug candidate for lupus nephritis. Therefore, this research determined the efficacy of the KP extract used in a lupus model. A well-known lupus model, Pristane-induced lupus, was used based on its broad severe lupus nephritis manifestations.[3,19,20] The efficacy of the KP comes from multiple compounds, but we can predict the active compounds on lupus responsible receptors, such as glucocorticoid. Moreover, this research focused on the effect of aqueous extract of KP on repairing the lupus nephritis manifestations and the potential active compounds involved.

MATERIALS AND METHODS

MATERIALS

The *Kalanchoe pinnata* (Lmk) Pers fresh leaves (KP) were collected from Manoko, Bandung. All of the leaves below the fourth top leaves were used. The plant identification was confirmed at the Herbarium Bandungense, Bandung Institute of Technology.

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Pristane® which contains 2,6,10,14 tetramethylpentadecane (TMPD) ≥98% was purchased from Sigma-Aldrich.

Female Balb/c mice aged seven weeks were purchased from LPPT UGM. Mice were housed under specific conditions. They were fed by using standard diet and water ad-libitum. We also use reagent strips for urine analysis (Verify®) to check the level total protein in the urine of each mouse tested.

METHODS

Preparation of the extract

Aqueous extract of KP leaves was processed from pressed fresh leaves. The extract was dried by using freeze dryer equipment (Eyela). The KP extract profile was measured by using UPLC-QTOF-MS/MS tandem instrument (Waters) by using Acquity UPLC column BEH C18 1.7µm 2.1×50 mm, flow rate 0.3 ml/minute. The injected volume was 5µl (the concentration of 5000 µg/L). The temperature used was 40°C. Also, the eluent used was A: H2O+formic acid 0.1%, B: acetonitrile + formic acid 0.1%.

Experimental grouping

An animal model for lupus used was Pristane-treated mice. Female Balb/c mice were injected by using 0.5 mL Pristane intraperitoneally, and then the booster injection was done at the day 90th.[10] Proteinuria level was measured at 5th month after the induction and then lasted until the severe sign detected at month 8th. All of the induced mice experienced proteinuria at the level of ++ (100 mg/dL). The mice were grouped into some treatment groups (n=8 per group). Three treatment groups received the aqueous extract of KP at the doses of 200, 400, and 600 mg/kg body weight. There also positive control mice which received prednisone at a dose of 25 mg/kg body weight. The other groups were a preventive group that received extract at doses 200 mg/kg body weight and the standard group that did not induce by using Pristane.

Treatment

The KP extract was given orally to the treatment groups for 21 days. Protein level in the urine of all groups was measured every seven days. At the day 21st, mice were sacrificed then the renal histology observation was done by using Haematoxylin-Eosin (HE) staining. The data was analyzed by using Oneway ANOVA. This protocol was approved by the local ICUC of Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, with the number of 512-KE.

Docking analysis to predict the potentially active compounds

The *in silico* study was performed by using glucocorticoid receptor (GeneBank) and the chemical structures of some flavonoid compounds in KP, and also the prednisone chemical structure (PubChem). The structures were stabilized by using Chem3D Program, and then the docking was performed by using Molegro Virtual Docker. All of the programs used were licensed programs of Universitas Airlangga.

RESULTS

Characteristics of the aqueous extract of *Kalanchoe pinnata* (Lmk) Pers (KP)

The yield of the extract was 1.95%w/w. Based on the organoleptic observation, the extract was dark yellow hygroscopic crystals resembling light materials. It was kept in the freezer until it was used. The identity profile of aqueous extract used was provided as a chromatogram profile (**Figure 1**) obtained using UPLC-QTOF-MS/MS tandem system.

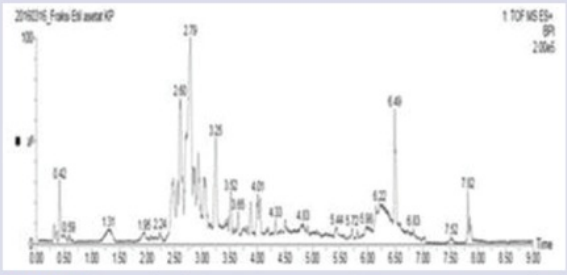

Figure 1: The profile of chemical compounds in aqueous extract of *Kalanchoe pinnata* (Lmk) Pers. Leaves.

The main compound was at the retention time of 2.79 and was predicted as flavonoid compounds. A lot of literature mention the name of specific flavonoid compounds found in this plant. This data supported by a phytochemical screening data that show the presence of flavonoids, saponin, tannin, and steroid/triterpenoid. Besides, the physical characteristics of the KP extract including water content 9.00% v/w, compounds soluble in water 54.34% w/w, and compounds soluble in ethanol 3.57% w/w. The characteristics exhibit beneficial and stable features of a solid material.

Three compounds in aqueous extract of KP has been widely studied. A lot of chemical compounds were found, such as steroids, terpenoids, flavonoids, phenolic, tannin, alkaloids, glycosides, carbohydrates, and proteins. The aqueous extract contains carbohydrates, proteins, flavonoids, phenolic, tannins, and glycosides. The leaves contain astragalin, 3,8-dimethoxy-4,5,7-trihydroxyflavone, friedelin, epigallocatechin-3-o-syringate, luteolin, rutin, kaempferol, quercetin, quercetin-3L-rhamnosido-L-arabino furanoside, quercetin-3-O-diarabinoside, and kaempferol-3-glucoside.[16,21] These multiple compounds might have the synergic effects that reduce lupus nephritis manifestations.

The effects of aqueous extract of *Kalanchoe pinnata* (Lmk) Pers leaves on lupus nephritis mice

The mice received a daily dose of aqueous extract of KP during 21 days. The protein level of the urine of the treated mice groups was decreased (**Table 1**). Proteinuria is a parameter for severity of nephritis.[22,23] (-/- Moreover, there was no severe proteinuria obtained on the mice until the 6th month after the induction. The mice only had mild proteinuria + (30 mg/dL) which revealed the low disease progress. This result was biased because the normal mice could reach proteinuria at the same level.

Then, the induction time was continued with a strict observation of proteinuria every seven days. As a result, the mice had proteinuria ++ (100 mg/dL) on the month 8th. Thus, they were ready for experimental grouping.

After the treatment period, the trend of proteinuria level decreased from level ++ (100 mg/dL) to level + (30 mg/dL) except the negative control groups. On the treatment groups, the dose of 200 mg/kgBW was the one that more active to decrease proteinuria level. The result was not as stable as the prednisone effect. On the preventive group, the proteinuria did not increase although they were injected twice using Pristane. Their physical condition showed a healthy behavior.

Proteinuria level was not the primary parameter to measure the severity of nephritis. Therefore, the observation was conducted in renal histology observation. Haematoxylin-Eosin (HE) staining showed the differences of each treatment effect on renal tissue structure. The results were in **Figure 2**.

The normal renal tissue of normal mice (**Figure 2a**) indicated by the mesangial area (long black arrow) seems thin with a regular structure,

Table 1: Proteinuria Level Before And After Kp Extract Treatment.

Group	Protein level in the urine (mg/dL)	
	Before treatment	After treatment
Treatment group at a dose of 200 mg/kg BW KP	200.00±115.47	65.00±40.41*
Treatment group at a dose of 400 mg/kg BW KP	65.00±40.41	22.50±8.66*
Treatment group at a dose of 600 mg/kg BW KP	112.00±110.77	55.00±41.53*
Prednisone at a dose of 25 mg/kgBW	200.00±115.47	30.00±0.00*
Negative control that received placebo	82.50± 35.00	132.50±116.44
Preventive group (treatment during induction time)	15.00±0.00	18.75±7.50*

* Different significantly compared to the negative control group with $p<0.05$. n = 8 mice per group using t-test Statistics

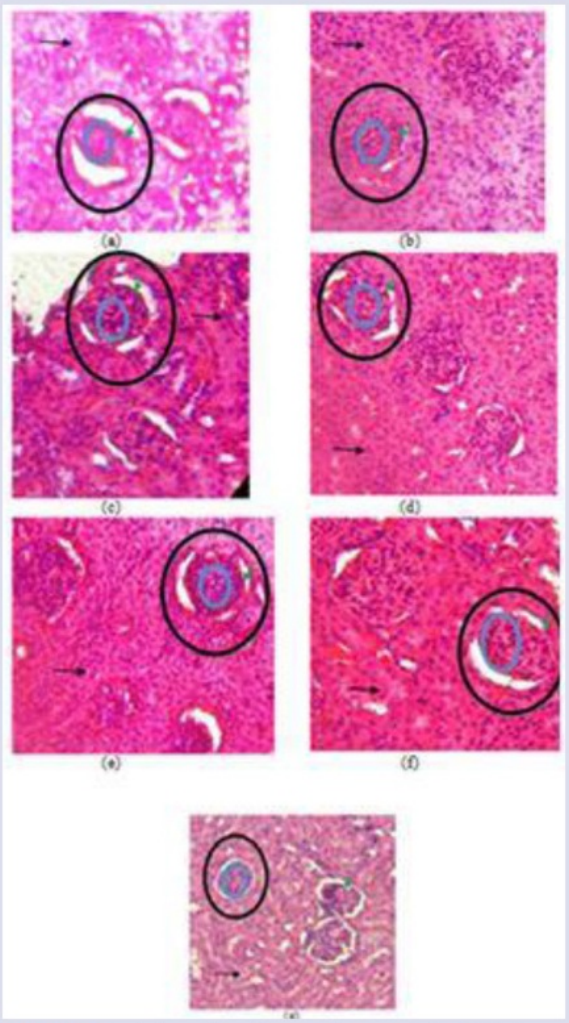

Figure 2 : Morphology of renal tissue at the magnitude of 400x by using light micrographs of a renal glomerulus of all groups Where; normal mouse of (a), negative control (b), treatment at the dose of 200 mg/kg BW (c), treatment at the dose of 400 mg/kg BW (d), treatment at the dose of 600 mg/kg BW (e), prednisone at the dose of 25 mg/kg BW (f), and preventive group (g). n= 8 mice per group.

and then the glomerulus (big circle) present between urinary spaces of Bowman's capsule. The glomerulus is a plaited mat of the capillary that has a filtration function. There is no proliferation of erythrocytes and inflammation cells inside the glomerulus (little circle). The area marked by green arrow is a position of glomerular basement membrane (GBM) that is thin in a healthy condition.

Furthermore, the positive control which only receiving placebo (**Figure 2b**) had many structural changes, such as the GBM is thicker than usual with a proliferation of glomerulus results in inflammation. This condition has been reported before.[10] Then, the glomerulus attaches the Bowman's capsule area, so the spaces are closed. In this situation, the filtration function performed difficultly. Besides, the mesangial area is thickened, are irregularly structured, and the cytoplasm cells are accumulated.

In the treatment group at the dose of 200 mg/kgBW (**Figure 2c**), the inflamed glomerulus was decreased. But, the GBM still looked thick. The cells accumulation is slightly reduced in the glomerulus. The mesangial area was shown the same structure with the **Figure 2b**. On the other groups received the extract at the dose of 400 and 600 mg/kgBW (**Figure 2 d and e**), the anti-inflammatory effect still present. The urinary spaces in Bowman's capsule looked wider although not achieve the healthy appearance. The mesangial area looked more regularly structured by the increase of the dose. The GBM thickness seemed similar to the low treatment dose, but it needs further measurement.

On the standard group received prednisone (**Figure 2f**), the accumulation of cells in the glomerulus and the glomerular inflammation were decreased although the GBM is still dense. The mesangial area looked thick, and not all of the area showed the repairing of the structure. At the last group, the preventive group, the histology structure is similar to the healthy mice.

The histology observation results show the repairing effect of aqueous extract of KP on many parts, such as tubules, glomerulus, GBM, and the Bowman's Capsule space. The treatment groups showed the alteration that it reduces the inflammation. The modification is not as good as the preventive group, but the effect is very potential to maintain the lupus patient stability.

Autoantigen-antibody complexes caused inflammation that occurred in glomerulus.[20,24] One of the autoantigens was nucleosome. Nucleosomes and anti-nucleosome antibodies have been shown to bind to the glomerular basal membrane (GBM). It is assumed that nucleosomes bind to the GBM via their histone proteins. The glomerulonephritis was developed by the formation of nucleosome-containing immune complexes. Also, there may be cross-reactivity of nucleosome-specific antibodies (e.g., with heparan sulfate or a so-far-unknown surface antigen) whereby these autoantibodies reach the glomeruli and cause inflammation.[25,26]

This result showed a capability of aqueous extract of KP in reducing the inflammation of glomerulus in the lupus nephritis mice model. It also prevents the mice from Pristane induction effect, so the mice keep normal although they received the second injection. The result showed a curative and preventive potency. But, further investigation needed to ensure this effect. These data indicate that aqueous extract of KP was useful to maintain immune system in lupus nephritis condition. This efficacy data is supported by the toxicity data reported by Ozolua[14] stating that there was no toxicity occurred in acute and subchronic toxicity test. Because of the efficacy and the safety, aqueous extract of KP could be a good candidate for a natural treatment for lupus nephritis.

The docking of some compounds in KP in glucocorticoid receptor

The docking result did not show a correlation with cell culture test, the effect on the animal model or others. Docking did not calculate the bioavailability, toxicity, and other factors in the body. The main reason for the use of docking is to predict the compound which binds to a specific receptor (protein) well. We also could see how is the three dimensions geometric structure of a compound bound to the active site of the protein.

In this research, we focused on three compounds found in KP, i.e., bryophyllin A, beta-sitosterol, and kaempferol-3-glucoside. The receptor was glucocorticoid receptor. This receptor has a very crucial role in immune homeostasis. The receptor is shown as a free water receptor. The ligand would be shed before the tested compound was inserted into the ligand space.

The compounds tested were prepared as 3D structures with an optimum geometric stability, using the Chem3D program. Then, the docking results are shown in **Figure 3.**

The ligand binding of bryophyllin A to glucocorticoid receptor presents through a hydrogen bond with amino acid residue Arg 611 (**Figure 3**). There is also a hydrophobic bonding with Gln 570, Leu 563, Met 560, Ile 747, Thr 739, Phe 749, Glu 748, Thr 561, Asn 564, and Phe 623. **Figure 3b** shows beta-sitosterol binds the receptor with hydrogen bonds to Arg 611 and Gln 570, and also hydrophobic bonds with Gln 738, Try 735, Phe 737, Leu 741, Thr 739. Ile 757, Phe 740, Cys 736, Met 601, Phe 749, Leu 753, Trp 600, Asn 564 and Leu 563. Fig. 5Sc showed that kaempferol-3-glucoside binds into glucocorticoid receptor using hydrogen bonds with Arg 611 and Asn 564, and hydrophobic bonds with Tyr 735, Leu 732, Cys 736, Phe 749, Met 639, Gln 642, Met 560, Met 646, Phe 623, Gln 570, and Met 604.

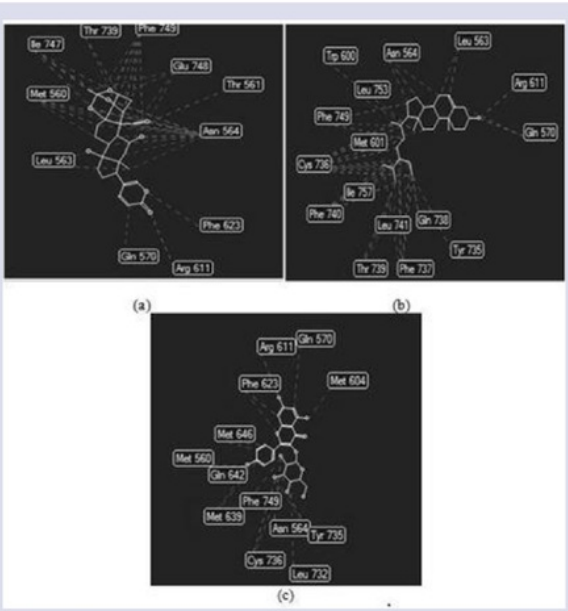

Figure 3: The Docking Result of Bryophyllin A (a), Beta-Sitosterol (b) and Kaempferol-3-glucoside (c) in the Glucocorticoid Receptor Tested Using Molegro Virtual Docker Program.

According to the scoring calculation, the free binding energy of bryophylin A compound is -66.6975 kcal/mol, beta sitosterol is -106.1340 kcal/mol, and the kaempferol-3-glucoside is -105.5710 kcal/mol. The results show the lowest free binding energy was on bryophyllin A compound. This little power makes it easy to bind to the glucocorticoid receptor, so the anti-inflammatory effect occurs selectively. At last, the *in silico* prediction of the most active compound results in the bryophyllin A as the compound which has the lowest free binding energy in the glucocorticoid receptor. The experiment needs to be conducted using other receptors responsible for the lupus pathogenesis to support this result.

DISCUSSION

Aqueous extract of KP is sufficient to reduce inflammatory signs on kidney parameters of lupus nephritis mice. This anti-inflammatory effect reduces the high pro-inflammatory cytokines released when the glomerular inflamed due to the increasing number of immune complexes deposited inside. Flavonoid compounds in the KP have a high anti-inflammatory effect and also anti-oxidative results[7,27,28,29] which support the regulation of immune responses. Lupus marked with irregular immune reactions which released suddenly if the triggering factors come.[30,31] The KP treatment in all doses gives many beneficial outcomes in the kidney to reduce and inhibit the glomerular swelling. It followed by the lower proteinuria level. Both results show that the glomerulus moves to its normal condition.

The glomerulus disorder in lupus is different with the kidney failure signs, which cannot be repaired. Glomerulonephritis in lupus occurred as a result of complex immune deposit.[32,33] It is possible for the immune complex to split and then eliminated from glomerulus, so the glomerulus function repaired.

In this research, all doses tested result in good outcomes to repair the kidney structure and function (**Figure 2 and Table 1**). It leads a further question regarding the active compound which responsible for the effect. According to the profile of the aqueous extract of KP, the primary compounds are flavonoid compounds. Therefore, we challenged some active compounds which potentially have an anti-inflammatory effect, i.e., bryophyllin A, beta-sitosterol, and kaempferol-3-glucoside. The compounds have good affinity to bind the glucocorticoid receptor, a receptor involved in the inflammatory process. The glucocorticoid receptor is a specific target for anti-inflammatory drugs. The compound which has the lowest binding energy to the glucocorticoid receptor was bryophyllin A (**Figure 3**). It means that bryophyllin A can efficiently bind the target site in glucocorticoid and results in the highest anti-inflammatory effect. However, the further research to calculate the concentration of bryophyllin A in the KP is necessary.

CONCLUSION

The aqueous extract of *Kalanchoe pinnata* (Lmk) Pers repairs on the kidney damage of Pristane-induced lupus nephritis mice. The potential flavonoid active compound which could be its active anti-inflammatory marker in this result is bryophyllin A.

ABBREVIATIONS USED

KP: *Kalanchoe pinnata;* **TMPD:** 2, 6, 10, 14-tetramethylpentadecane; **GBM:** glomerular basement membrane.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUMMARY

Drug development for advance safe lupus therapy is highly needed. The aqueous extract of *Kalanchoe pinnata* (Lmk) Pers (KP) fresh leaves has the activity to reduce lupus manifestation in the kidney of lupus mice. It reduces the glomerular inflammation, so the level of protein present in the urine can be decreased. Furthermore, the aqueous extract of KP has multiple compounds. The most potent compound of this material which can bind the glucocorticoid receptor efficiently is the structure of Bryoplyllin A, based on the *in silico* measurement.

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