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Positive Impact of Ethyl Acetate Fraction of *Kalanchoe pinnata* on Anti-Smith Antibody and T Reg in Lupus Mice

(Dampak Positif Fraksi Etil Asetat dari *Kalanchoe pinnata* terhadap Antibodi Anti-Smith dan Sel T Reg pada Mencit Lupus)

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Abstract: *Kalanchoe pinnata* (Lmk) Pers is a medicinal plant that has many activities, such as antioxidant, anti-inflammation and immunosuppressant activities. Based on our previous study, aqueous extract of this plant leaves has a repairing effect on lupus nephritis model. The expected active compounds are flavonoids. Those compounds are separated in the ethyl acetate fraction. Therefore, this study observed the activity of the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers on reducing the level of Anti-Smith autoantibody and the regulation function of $CD4^+CD25^+$ T reg in lupus mice. The experimental groups were a negative control group that received placebo, ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) group and a positive control group that received cyclophosphamide. The anti-Sm level was measured by using indirect ELISA. The spleen cells were prepared for $CD4^+CD25^+$ T reg assay using flow cytometry. The result of this experiment was a reduction of anti-Sm antibody level. The decrease was sufficient to stabilize lupus condition. The flow cytometry assay results showed the increase in the relative percentage of $CD4^+CD25^+$ T reg. This function can inhibit the reactivity of autoantigens, so it prevents tissue and organ damage from lupus. However, further research is needed to observe the complete mechanism.

Keywords: *Kalanchoe pinnata* (Lmk) Pers, lupus, anti-Sm, $CD4^+CD25^+$ T reg, immunosuppressant.

Abstrak: *Kalanchoe pinnata* (Lmk) Pers memiliki banyak aktivitas, seperti antioksidan, antiinflamasi, dan imunosupresan. Berdasarkan penelitian sebelumnya, ekstrak air daun *Kalanchoe pinnata* (Lmk) Pers memiliki efek memperbaiki struktur dan fungsi ginjal pada model lupus nefritis. Senyawa aktif yang diduga adalah senyawa-senyawa golongan flavonoid yang masuk ke dalam fraksi etil asetat sesuai metode fraksinasi Cruz(1). Tujuan penelitian ini adalah membuktikan aktivitas fraksi etil asetat *Kalanchoe pinnata* (Lmk) Pers (EF-KP) untuk menurunkan kadar antibodi Anti-Smith dan mengatur fungsi regulasi sel T regulator $CD4^+CD25^+$. Kelompok uji terdiri dari kontrol negatif (placebo), FE-KP, dan kontrol positif (siklofosfamid). Kadar anti-Sm diukur menggunakan *indirect* ELISA. Selanjutnya sel-sel limpa diisolasi untuk pengukuran sel T reg $CD4^+CD25^+$ dengan metode *flow cytometry*. Hasil penelitian menunjukkkan bahwa ada penurunan rata-rata kadar anti-Sm yang cukup untuk menstabilkan kondisi model lupus. Hasil pengukuran T reg menunjukkan peningkatan jumlah *marker* sel T reg $CD4^+CD25^+$. Peningkatan fungsi regulasi ini mampu menghambat reaktivitas autoantigen, sehingga mencegah kerusakan jaringan dan organ pada penyakit lupus. Namun, penelitian selanjutnya perlu dilakukan untuk mengamati mekanisme kerja FE-KP pada *biomarker* lupus yang lain.

Kata kunci: *Kalanchoe pinnata* (Lmk) Pers, lupus, Anti-Sm, sel T $CD4^+CD25^+$, immunosupresan.

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INTRODUCTION

THE MEDICINAL plant is a promising topic for lupus drug development. One of the potential herbal medicines is *Kalanchoe pinnata* (Lmk) Pers. According to previously reported research, flavonoid compounds that expected to be separated in ethyl acetate fraction have immunosuppressant, antiinflammation and antioxidant activity[2-6]. Compounds found on *Kalanchoe pinnata* (Lmk) Pers are quercetin glycosyl conjugates, rutin, stigmasterol, 3,8-dimethoxy-4,5,7-trihydroxyflavone, friedelin, epigallocatechin-3-o-syringate, luteolin, kaempferol and quercetin[1].

The ethyl acetate fraction activity needs to be tested using in vivo model. Lupus has many manifestations. One of them is the presence of specific antibody for lupus, i.e., Anti-Smith antibody, anti-ribosomal P and anti-dsDNA[8]. These autoantibodies attach autoantigens from self. Then, this immune disorder develops as systemic inflammation. Autoantigens attached by Anti-Sm are U1, U2, U4-6 snRNP (protein B', D, E, F , G)[8]. This antibody present in Pristane-treated mice[8,9]. Besides antibody, T regulation function also plays a critical role. Depletion of the T cell regulatory (T reg) $CD4^+CD25^+$ could be a trigger that makes a cell population induce autoimmune diseases, in immunocompromised patients. High CD25 expression ($CD4^+CD25^+$) on T reg cells shows that there is an immunosuppressive activity[10].

This research observed how the active compounds in ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers impacted on the Anti-Sm antibody level in the blood. In addition, the spleen cells were isolated to measure the percentage of $CD4^+CD25^+$ T reg in lupus mice.

MATERIALS AND METHODS

MATERIALS. *Kalanchoe pinnata* (Lmk) Pers fresh leaves were taken from a cultivation farm in Trenggalek, East Java. It was identified botanically by Conservation Unit of Indonesian Institute of Science, Purwodadi with the identification number of 0284/IPH.06/HM/II/2015.

Female Balb/c mice aged four weeks were received from LPPT UGM. These mice were species pathogen free with the certificate number of 352/LP3HP/29/VII/2015. TMPD (Pristane) (Cat. Number P2870 SIGMA, >98% purity) was purchased from Sigma-Aldrich, Singapore. The Mouse anti-Smith antibody ELISA Kit (Code: CSB-E15976) was purchased from Cusabio via CV. Kristallindo Biolab as an Indonesian supplier. The anti-CD4 and anti-CD25 were obtained from Biogenesis, USA and purchased by Laboratory of Molecular Biology, Biology Department of Brawijaya University. The PBS and aqua bidestillata (Ikapharmindo) were obtained from LDB Laboratory. The Verify reagent strips for urinalysis were purchased from CV. Rachmandjaya Surabaya. The ethyl acetate (pro analysis grade) was obtained from Merck via PT Dianum Surabaya.

METHODS. The Flavonoid-Rich Compound in Aqueous Extract of *Kalanchoe pinnata* (Lmk) Pers. Leaves were Separated Using Liquid-Liquid Extraction (LLE). The solvent was aquadest and ethyl acetate. The profiling was done by using a UPLC-QTOF-MS/MS system (Waters). Then, the rutin profile was analyzed by using densitometry method with Camag 3 densitometer.

The experimental groups were a negative control group that received placebo, ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) group with a dose that similar to 400 mg/kg BW of crude extract and a positive control group that received cyclophosphamide with a dose of 1 mg/kg BW. The treatment lasted for 21 days. The proteinuria measurement was done every 7 days. At the end of the experiment, mice were sacrificed. The blood plasma was used for measuring the Anti-Sm level by using indirect ELISA. Then, the fresh spleen cells immediately prepared to be measured by using flow cytometry method. This $CD4^+CD25^+$ T reg measurement would be analyzed using BD CellQuest program. Ethical clearance of this research was approved by ICUC of Veterinary Faculty on January 12, 2016, with number 526-KE.

RESULTS AND DISCUSSIONS

RESULTS. Profiling of Ethyl Acetate Fraction of *Kalanchoe pinnata* (Lmk) Pers Leaves. The ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves contained a less amount of compounds than the crude extract. It was separated well using LLE methods. The profile was obtained using UPLC-QTOF-MS/MS system. The chromatogram of this fraction is shown in Figure 1.

Based on the previous reports[4,11], the compounds which responsible for the anti-inflammation and antioxidant activity are quercetin glycosyl conjugates. At the MS/MS system, the glycosides are fragmented become quercetin and its glycosides. The peak at the retention time of 2.74 had monomolecular weight 302 Da, identified as quercetin. The mass spectrum is in Figure 2.

The quercetin found in the EF-KP was not a pure quercetin, but it was a glycoside form. For the

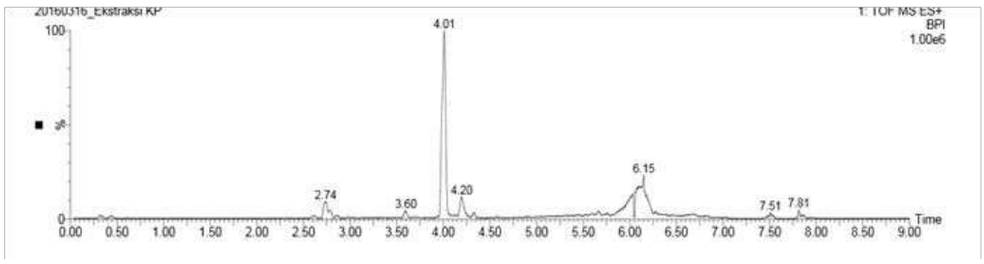

Figure 1. Profile of ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves

profiling purpose by using thin layer chromatography, we decided to use rutin as the standard (Figure 3). The spot which similar to rutin spot was analyzed by using a densitometer. The result is shown in Figure 4. The chromatogram shows the presence of rutin in EF-KP sample. It can be concluded that rutin and quercetin are compounds found in the EF-K

Impacts on Anti-Sm Antibody Level. Pristane is a hydrocarbon that could induce anti-nRNP, anti-Sm, anti-Su, anti-dsDNA, glomerulonephritis, and arthritis autoantibodies in mice[12]. The presence of these autoantibodies was triggered by a breakdown of immune tolerance. Genetic, hormonal and immunology factor together with environmental factor would enhance the disease onset and severity. Antibody modulation in Pristane-treated mice regulated by Neutrophil Gelatinase-Associated Lipocalin (NGAL). After the injection of Pristane, NGAL expression present in serum and spleen. Mice that lack in NGAL expression easily induced to be autoimmune mice[13].

The mice used in this experiment were the same strain, sex, and age. The expectation was the similar expression of NGAL between mice. The anti-Sm autoantibody level measured using indirect ELISA. The result is in Table 1. This result shows that the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves could slightly inhibit the production of Anti-Sm. The inhibition is not as high as positive control activity, neither statistically significant. However, the inhibition of this autoantibody production predicted to stabilize lupus patient condition.

The measurement of Anti-Sm antibody level was done previously on lupus model research[8,14]. No other published article says about the level of Anti-Sm after treatment using a drug candidate. However, the autoantibody level in human decreases after 6-month treatment.

In this research, the cyclophosphamide was used as a standard drug. Its off-label activity as immunosuppressant can decrease lupus manifestation. In this observation, the decreasing effect on Anti-Sm by means of cyclophosphamide administration was not drastic. This result was enough because there was not found other materials that had an extreme decreasing on the Anti-Sm level.

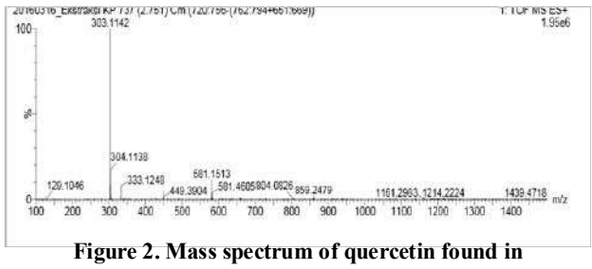

Figure 2. Mass spectrum of quercetin found in ethyl acetate fraction.

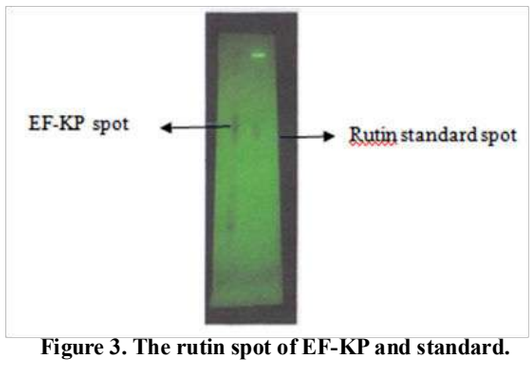

Figure 3. The rutin spot of EF-KP and standard.

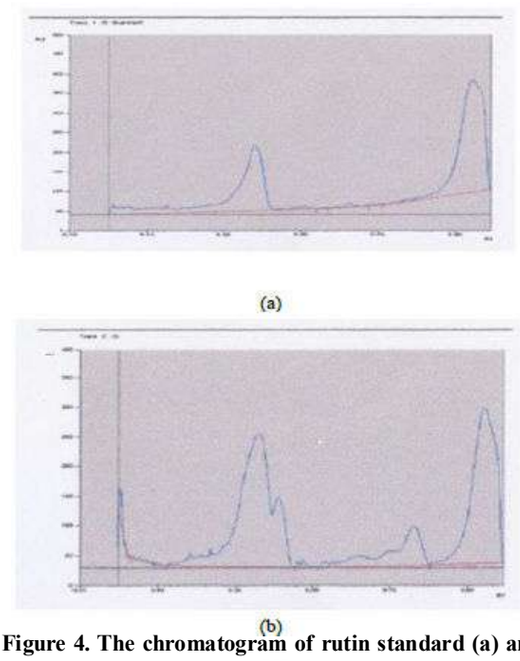

Figure 4. The chromatogram of rutin standard (a) and EF-KP (b)

Table 1. Anti-Sm level in each experimental group.

Group	Mean of Anti-Sm level (pg/mL) ± SD
Negative control	111.15 ± 12.08
EF-KP	107.22 ± 16.82
Positive control	100.74 ± 4.99

Impacts on $CD4^+CD25^+$ T reg. T regulatory (T reg) plays a crucial role in immune tolerance regulation. $CD4^+CD25^+$ is one of the T regs that not only expressed by T cells but also by dendritic cells and others. T reg cells prevent a person from autoimmune diseases by preventing the development of autoreactive cells[15]. The relative percentage of $CD4^+CD25^+$ T reg cells in this research was measured on cells which isolated from spleen, by using flow cytometry method. The result is in Table 2 and Figure 5.

The data shows that the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers can increase $CD4^+CD25^+$ T reg cells. The increase of $CD4^+CD25^+$ T reg cells is also reported in another publication which using *Andrographis paniculata* on rheumatoid arthritis model[16]. This effect leads to an expectation that there is a balance on immune regulation so that it prevents any autoimmune inflammation in mice[15].

DISCUSSIONS. The Ethyl Acetate Fraction of *Kalanchoe pinnata* (Lmk) Pers Profile. This research using ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) in line with an anti inflammation mechanism that previously used by Fereira (2014)[3]. The EF-KP profile was detected by using UPLC-QTOF-MS/MS tandem instrument. This pattern is important as an identity of the multiple compounds found in EF-KP. The chromatogram peaks represent the number of compounds in the material. The peaks not only single peak but also accumulated peak depends on how many compounds found at each retention time. The area of the peak determines the level of a chemical compound semiquantitatively.

The method chosen in the next profiling was densitometry. The standard used is quercetin as previously reported by Muzitano (2011)[17] says

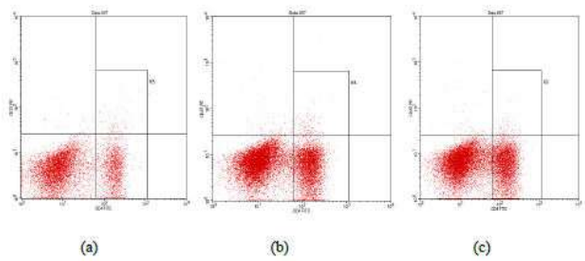

Figure 5. Profile of the relative percentage of CD4+CD25+ T reg cells in the spleen of control negative group (a), EF-KP group (b), and positive control group (c).

Table 2. Profile of the relative percentage of CD4+CD25+ T reg cell.

Group	$CD4^+CD25^+$ T reg cells (%) ± SD
Negative control	3.01 ± 3.43
EF-KP	5.05 ± 7.07
Positive control	3.26 ± 4.92

that the quercetin qlycosides in the crude extract of *Kalanchoe pinnata* (Lmk) Pers is a significant compound. Unfortunately, the result of the previous experiment did not show a good value. Therefore, we used another standard, rutin. The result is in Figure 3-4. The chromatogram indicates the presence of rutin.

Impacts on Anti-Sm antibody. Lupus-specific antibody productions increase as the increasing on lupus triggering factor. It would increase the severity of targeted organs [9,18]. On the Pristane-treated mice, antibody occurred on the 2nd month is anti-nuclear antibody (ANA). Then, the manifestation continued to the production of lupus-specific antibodies until the 6th month[8]. A new antibody occurred after the 6th month is not reported in any publications.

In this research, the anti-Sm antibody is decreased, but the decrease is not significant (Table 1). The reduction of antibody level is a challenging effort as long as the presence of the triggering factors and the feedback-loop mechanisms. The fact that antibody level did not decrease after treatment is not only a problem in herbal medicine but also is a limitation on the conventional therapy. The non-specific immunosuppressant could not decrease antibody level drastically.

Based on the previous publication, the antibody level on the 2nd month is still low so that it could not make any changes on the organ structure and function. This fact could be explained by the immune homeostatic mechanism that could inhibit the immune complex formation. The level and the kind of antibodies develop by time as a result of feedback-loop mechanism as reported by Rottmann and Willis (2005) [9]. At a point after the 5th month, the homeostatic system is unable to make any immune balance. The result is the formation of immune complexes at targeted organs[18]. The pristane targeted organs is kidney, joint, and lung[9,14,19,20]. Modulating the factors that could increase the activity of B cells will reduce the immune complexes. The B cells activities are mostly triggered by T cells. The T cells are regulated by regulatory T cells (T regs). A large number of T regs would control the T cells reactivity[10].

Impacts on T Regs. T regs are biomarkers that present in a low expression in autoimmune diseases. The T regs have an optimal regulation function on the normal state to reduce the auto-reactive T cells[10]. The

low T regs functions impact on the unregulated T cells activity for triggering B cells activity.

In this research, marker used was $CD4^+CD25^+$ T reg. This T reg plays a central role in the autoimmune disease as reported by Scheniecker (2010)[10]. The result (Table 2 and Figure 5) shows an increase on the relative percentage of $CD4^+CD25^+$ T reg of EF-KP group.

It indicated that the active compounds in EF-KP could increase the T cells regulation function. This effect will give a benefit for lupus patient because a large number of $CD4^+CD25^+$ T reg will make the reactive T effector cells decrease, then it maintains the self-tolerance. There is some evidence that $CD4^+CD25^+$ regulatory T cells have an ability to prevent the development of auto-reactive cells [21-27].

CONCLUSIONS

The research concluded that the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers has a capability to slightly reduce the Anti-Sm antibody level, and increase the percentage of $CD4^+CD25^+$ T reg. The activities would maintain the immune system stability. However, further research to observe the effect on other biomarkers needs to be conducted.

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CONFLICT OF INTEREST

Declared none

REFERENCES

1. Cruz EA, Reutera S, Martina H, Dehzada N, Muzitano M, Costa SS, *et al. Kalanchoe pinnata* inhibits mast cell activation and prevents allergic airway disease. Phytomedicine. 2012.19:115– 21.
2. Gupta R, Lohani M, Arora S. Anti-inflammatory activity of the leaf extracts/fractions of *Bryophyllum pinnatum*. Int J Pharm Sci Rev & Res. 2010.3(1):16-8.
3. Ferreira RT, Coutinho MAS, Carmo Malvar D, Costa EA, Florentino IF, Costa SS, *et al*. Mechanisms underlying the antinociceptive, antiedematogenic, and anti-inflammatory activity of the main flavonoid from *Kalanchoe pinnata*. Evid Base Compl Alternative Med. 2014. Article ID 429256, 8 pages.
4. Cruz EA, Da-Silva SAG, Muzitano MF, Silva PMR, Costa SS, Rossi-Bergmann B. Immunomodulatory pretreatment with *Kalanchoe pinnata* extract and its quercitrin flavonoid effectively protects mice against anaphylactic shock. Int Immunopharmacol. 8:1616-21.
5. Bergmann R, Costa SS, Borges MBS, Silva SA, Noleto GR, Souza MLM. Immunosuppressive effect of the aqueous extract of *Kalanchoe pinnata* in mice. Phytotherapy Res. 1994.8:399-402.
6. Coutinho MA, Muzitano MF, Cruz EA, Bergonzi MC, Kaiser CR, Tinoco LW, Bilia AR, Vincieric FF, Rossi-Bergmann B, Costa SS. Flowers from *Kalanchoe pinnata* are a rich source of T cell-suppressive flavonoids. Nat Product Commun. 2012.7(2):175-8.
7. Yu C, Gershwin ME, Chang C. Diagnostic criteria for systemic lupus erythematosus: A critical review. J of Autoimmun. 2014.5:48-59.
8. Reeves HW, Lee PY, Weinstein JS, Satoh M, Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. Trends in Immunol. 2009.30(9):455–64.
9. Rottman JB, Willis CR. Mouse models of systemic lupus erythematosus reveal a complex pathogenesis. Vet Pathol. 2010.47(4):664-76.
10. Scheinecker C, Bonelli M, Smolen JS. Pathogenetic aspects of systemic lupus erythematosus with an emphasis on regulatory T cells. J Autoimmun. 2010.35:269-75.
11. Nascimento LB, Leal-Costa MV, Coutinho MA, Moreira Ndos S, Lage CL, Barbi Ndos S, *et al*. Increased antioxidant activity and changes in phenolic profile of *Kalanchoe pinnata* (Lamarck) Persoon (*Crassulaceae*) specimens grown under supplemental blue light. Photochem and Photobiol. 2013.89(2): 391-9.
12. Satoh M, Kuroda Y, Yoshida H, Behney KM, Mizutani A, Akaogi J, *et al*. Induction of lupus autoantibodies by adjuvants. J Autoimmun. 2003.2:1–9.
13. Pawar RD, Goilav B, Xia Y, Zhuang H, Herlitz L, Reeves WH, Putterman C. Serum autoantibodies in pristane-induced lupus are regulated by neutrophil gelatinase-associated lipocalin. Clin Immunol. 2014.154:49–65.
14. Calvani N, Satoh M, Croker BP, Reeves WH, Richards HB. Nephritogenic autoantibodies but absence of nephritis in Il-12p35–deficient mice with pristane-induced lupus. Kidney Int. 2003.64:897–905.
15. Rifa'i M. Protective effect of $CD4^+CD25^+$ regulatory T cells on mice model of rheumatoid arthritis. Annales Bogorienses. 2011.15(1):48-57.
16. Qarni UA, Rifa'i M. Uji aktifitas biologis fraksi etanol daun sambiloto (*Andrographis paniculata*) terhadap perubahan kuantitatif sel T regulator pada mencit BALB/c (*Mus musculus*). Jurnal Biotropika. 2013.1(5):15-21.
17. Muzitano MF, Bergonzi MC, Melo GOD, Lage CLS, Bilia AR, *et al*. Influence of conditions, season of collection and extraction method on the content of antileishmanial flavonoids from *Kalanchoe pinnata*. J Ethnopharmacol. 2011.133:132-7.
18. Wallace DC and Hahn BH. Dubois's lupus erythematosus and related syndromes. New York: Elsevier Saunder; 2013:237-51.
19. Satoh M, Reeves WH, Induction of lupus-associated

autoantibodies in BALB/c mice by intraperitoneal injection of pristane. J Exp Med. 1994.180(6):23-41.
20. Leiss H, Niederreiter B, Bandur T, Schwarzecker B, Steiner G, *et al*. Pristane-induced lupus as a model of human lupus arthritis: evolvement of autoantibodies, internal organ and joint inflammation. Lupus. 2013.22:778–92.
21. Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. $CD4^{+}CD25^{+}$ regulatory T cells in rheumatoid arthritis. Differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheumatol. 2004.50:2775-85.
22. Bleesing MR, Brown SE, Straus JK, Siegel MD, Johnson M. Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. Blood. 2001.98(8):2466-73.
23. Chatenoud LB, Salomon, Bluestone JA. Suppressor T cells-they're back and critical for regulation of autoimmunity. Immunol Rev. 2001.182:149-63.
24. Chen W, Jin N, Hardegen KJ, Lei I, Marinos. Conversion of peripheral $CD4^{+}CD25^{+}$ naive T cells to $CD4^{+}CD25^{+}$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 2003.198:1875-86.
25. Malek TR, Bayer AR. Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol. 2004. 4:665-74.
26. Seddon B, Mason D. The third function of the thymus. Immunol. 2001.21:95-9.
27. Wan Y, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl. Acad Sci. 2005.102:5126–31.