

Immunomodulation Effects of *Bryophyllum Pinnatum* on Pregnant Pristane-Induced Lupus Mice Model

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ABSTRACT

Determining the effect of *Bryophyllum pinnatum* treatment in modulating immune response and the pregnancy outcomes of pregnant pristane-induced lupus mice model. Sixteen Balb/c mice were intraperitoneally injected with single 0.5 cc pristane to induce lupus manifestations. After 12 weeks of injection, mice were mated and considered as gestational day 0 (GDO). Mice were divided into 4 groups based on the dosages of *Bryophyllum pinnatum*: control (no treatment), B1 (10.5 mg/kg), B2 (21 mg/kg), and B3 (42 mg/kg). The treatment was given orally every day started from GD9 until 9 days. At the end of the study, blood pressure and fetal size were measured. Serum anti-dsDNA and urine albumin levels were measured by ELISA. Spleen T helper (Th) and mature B cells percentages were measured by flow cytometry. Administration of *Bryophyllum pinnatum* reduced the percentages of Th1 ($p=0.006$), Th2 ($p=0.005$), Th17 ($p=0.000$), and mature B cells ($p=0.007$) in dose-dependent manner. B1 and B2 had significantly lower of systolic blood pressure

compared to control ($p=0.026$ and $p=0.022$ respectively). Significantly lower of anti-dsDNA levels were found in B1 group compared to control ($p=0.014$). However, no significantly different of urine albumin levels were found between groups. *Bryophyllum pinnatum* also significantly increased the fetus body weight in dose-dependent manner ($p=0.000$). Treatment of *Bryophyllum pinnatum* could improve the pregnancy outcome and modulate the immune response in pregnant pristane-induced lupus mice. Therefore, *Bryophyllum pinnatum* is a potential herb, which can be developed as an immunosuppressive agent in the future.

Keywords: Systemic lupus erythematosus, *Bryophyllum pinnatum*, pregnancy, T helper, mature B cells

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multiple organ involvements that primarily affect women of childbearing ages. Pregnancy in women with SLE is associated with an increased risk of adverse maternal

and fetal outcomes. Recent studies reveal that lupus patients were associated with a 20-fold increase in maternal mortality and maternal morbidity, including a higher risk of lupus flares, preeclampsia, hypertension, higher rates of preterm delivery, and the presence of anti-phospholipid antibodies. Fetal mortality and morbidity in SLE during pregnancy are also documented in various studies; premature birth, intrauterine growth retardation (IUGR), and neonatal lupus syndrome are the most complications found in the fetus from SLE mother.

Improving the pregnancy outcomes in SLE patients is the main concern of physicians in the world until today. In spite of that, the treatment of SLE is still being a big problem in some developing countries, including Indonesia. Glucocorticoid and immunosuppressant are the first line therapy in managing SLE patients in Indonesia, however, these drugs have various side effects and some are reported to have teratogenic effects. The use of biologic agents has shown some promising results against SLE during pregnancy by reducing mortality and morbidity while preserving the safety in pregnancy. However, biologic agents are still not widely available in some developing countries and limited only in major health facilities. Therefore, there is a need to develop a new drug for SLE treatment especially during pregnancy with good efficacy and maintain the safety in pregnancy.

A recent approach to developing a new immunosuppressive drug is by using herbal medicines as an adjunctive treatment. One of the herbs which have immunosuppressive effects is *Bryophyllum pinnatum*. *Bryophyllum pinnatum* belong to the plant family Crassulaceae. It is a perennial herb growing widely and used in folkloric medicine in tropical countries, such

as Indonesia, India, and tropical Africa. In traditional medicine, the leaves of this plant have been used for antimicrobial, antiulcer, anti-inflammatory, antihypertensive, and potent anti-allergic activity. As an immunosuppressive agent, the first study was done by Rossi-Bergmann et al. shows the aqueous extract of *Bryophyllum pinnatum* leaves cause significant inhibition of cell-mediated and humoral immune response in mice. Almeida et al. also finds that leaves extracts of *Bryophyllum pinnatum* inhibit in vivo lymphocyte proliferation. Similar to other studies, our previous research shows the efficacy of ethanolic leaves extracts of *Bryophyllum pinnatum* in modulating in vitro plasma cell differentiation and survival in B cell cultures from pristane-induced lupus mice model (unpublished data). However, the use of *Bryophyllum pinnatum* in pregnancy with SLE is never been reported.

Therefore, our present study was aimed to determine the efficacy of *Bryophyllum pinnatum* in modulating the immune response in pregnant pristane-induced lupus mice model, including the autoantibody production and the differentiation of T helper (Th) and B cells. We also aimed to monitor the effects of *Bryophyllum pinnatum* treatment on the pregnancy outcome of pristane-induced lupus mice model, including the outcomes of the mother and fetuses.

METHODS

Mice and Pristane Induction.

This study was approved by Ethical Committee for Animal Experimentation of Faculty of Medicine University of Brawijaya. A total of 16 female Balb/c mice, 9-10 weeks old were obtained from Veterinarian Center (Surabaya, Indonesia). All mice were housed at Pharmacology Department,

Faculty of Medicine University of Brawijaya under pathogen-free condition. Food and water were given ad libitum. To develop the mice model of SLE, all mice were induced by a single intraperitoneal injection of 0.5 cc pristane. After 12 weeks of pristane injection, mice were mated. To standardize the pregnancy age, males were kept only for 1 night in the breeding cages. Detection of the vaginal plug was defined as gestation day (GD) 0.

Preparation and Treatment of *Bryophyllum Pinnatum*.

Bryophyllum pinnatum leaves were obtained from Agricultural Center in Batu, Indonesia. Extraction of *Bryophyllum pinnatum* was done according to the previous study. Briefly, dried and powdered leaves of *Bryophyllum pinnatum* (800 gr) were extracted with ethanol (2.5 L) by static maceration at room temperature every 48 h for 20 times. The ethanol extract was filtered and evaporated in a rotary vacuum evaporator at controlled temperature (50-60°C). Mice were divided into 4 groups based on the dosages of the *Bryophyllum pinnatum*: control group (no *Bryophyllum pinnatum* given), B1 group (10.5 mg/kg body weight (BW) of *Bryophyllum pinnatum*), B2 group (21 mg/kgBW of *Bryophyllum pinnatum*), and B3 group (42 mg/kgBW of *Bryophyllum pinnatum*). Ethanolic extract of *Bryophyllum pinnatum* was given orally and daily started from GD9 up to 9 days.

Tissue and Specimen Collection.

At GD18, mice were euthanized by chloroform inhalation. All specimens including serum, spleen, and fetuses were collected. The number, weight, and length of the fetuses were measured. Prior to euthanasia, blood pressure from all mice was measured by CODA (Kent Scientific Cooperation, USA) and then mice were

collected into a collecting cage to take the 24 h urine sample.

Flow Cytometry Assay.

Flow cytometry assay was done to measure the percentages of mature B cells and T helper (Th) cells, including Th1, Th2, and Th17. Single cell suspensions were prepared from spleen using 100 µm-cell strainers (Falcon, BD Biosciences, USA). This suspension then stained extracellularly with FITC-conjugated anti-mouse CD19 antibody (Biolegend, USA) and PE-conjugated anti-mouse CD22 antibody (Biolegend, USA) to measure the percentages of mature B cells. On the other hand, detection of Th cells was done by extracellular staining with FITC-conjugated anti-mouse CD4 antibody (Biolegend, USA) and subsequent intracellular staining, including PE-conjugated anti-mouse IFN γ (Biolegend, USA), PerCP-conjugated anti-mouse IL-4 (Biolegend, USA), and PE-conjugated anti-mouse IL-17A (Biolegend, USA). Prior to intracellular staining, cells were stimulated and fixed-permeabilized for 5 h with phorbol myristate acetate (PMA) (Sigma-Aldrich, USA), ionomycin and Brefeldin-A (Sigma-Aldrich, USA). Mature B cells were considered as cells which expressed CD19+ CD22+ while Th1 expressed CD4+ IFN γ +, Th2 expressed CD4+ IL-4+, and Th17 expressed CD4+ IL-17A+. Quantification was done in 105 cells with an FACS Calibur (BD Biosciences, USA).

Enzyme-Linked Immunosorbent Assay (ELISA).

ELISA was performed to measure the serum anti-dsDNA levels and urine albumin levels. Mouse anti-dsDNA ELISA kit (MyBioSource, USA) was used to measure the serum anti-dsDNA level while mouse albumin ELISA kit (Elabscience, USA) was used to measure the urine albumin level. All ELISA

procedures were done according to the manufacture’s protocols.

Statistical Analysis.

Comparison between groups was done by ANOVA and followed by posthoc multi-comparison analysis. Data was shown as the mean ±standard deviation (SD). Comparison between groups was considered significant if p <0.05. Correlation analysis between clinical manifestation with Th and B cell percentages was done by Pearson correlation analysis. Correlation between groups was considered significant if p <0.05. All statistical analysis and table generation were done by SPSS version 16.0 for Windows.

RESULT AND DISCUSSION

Effect of *Bryophyllum Pinnatum* Treatment on Th and B Cells Percentages of Pregnant Pristane-Induced Lupus Mice.

T helper and mature B cells percentages were measured by flow cytometry assay. Figure 1 showed the representative dot plot analysis from the flow cytometer for the measurement of Th and mature B cells percentages. Th1 cells which expressed CD4⁺ IFN γ ⁺ were significantly lower in all groups treated with *Bryophyllum pinnatum* compared to control group (p=0.022, p=0.018, and p=0.020 in B1, B2, and B3

respectively as shown in table 1). On the other hand, Th2 cells which expressed CD4⁺ IL4⁺ were also significantly lower in B2 and B3 groups compared to control group (p=0.019 and p=0.036, respectively) but not with B1 group. Similarly, significantly lower of Th17 cells percentages which expressed CD4⁺ IL17A⁺ were also significantly lower in B2 and B3 groups compared to control as seen in table 1 (p=0.019 and p=0.036). Lastly, mature B cells percentages which expressed CD19⁺ CD22⁺ were significantly lower only in B3 group compared to control (p=0.036).

Effect of *Bryophyllum pinnatum* treatment on clinical manifestations of pregnant pristane-induced lupus mice.

Clinical characteristics of pregnant pristane-induced lupus mice from each group were shown in table 2. Serum anti-dsDNA levels in B1 group were significantly lower compared to control group (p=0.014). However, serum anti-dsDNA levels in B2 and B3 groups were not significantly different compared to control group (p=0.118 and p=0.998 respectively, as shown clinical hallmarks of pregnant pristane-induced lupus mice were elevated blood pressure and the presence of albuminuria, thus we measured the blood pressure and also the urine albumin levels from all group.

Table 1. Percentages of T Helper and Mature B Cells on Each Group.

Percentage of cells	Control (n=4)	B1 (n=4)	p*	B2 (n=4)	p*	B3 (n=4)	p*
Th1 cells CD4 ⁺ IFN γ ⁺ (%)	9.58±2.07	3.62±1.38	0.022	3.43±2.61	0.018	3.54±1.60	0.020
Th2 cells CD4 ⁺ IL4 ⁺ (%)	10.20±4.29	8.64±0.28	0.934	2.59±0.43	0.012	2.95±0.22	0.017
Th17 cells CD4 ⁺ IL17A ⁺ (%)	5.66±1.03	7.56±0.81	0.063	2.79±1.48	0.019	3.08±0.15	0.036
Mature B cells CD19 ⁺ CD22 ⁺ (%)	57.35±8.35	60.75±0.13	0.914	41.11±9.75	0.053	39.78±3.65	0.036

*p value was considered as comparison with control group, considered significant if p < 0.05.

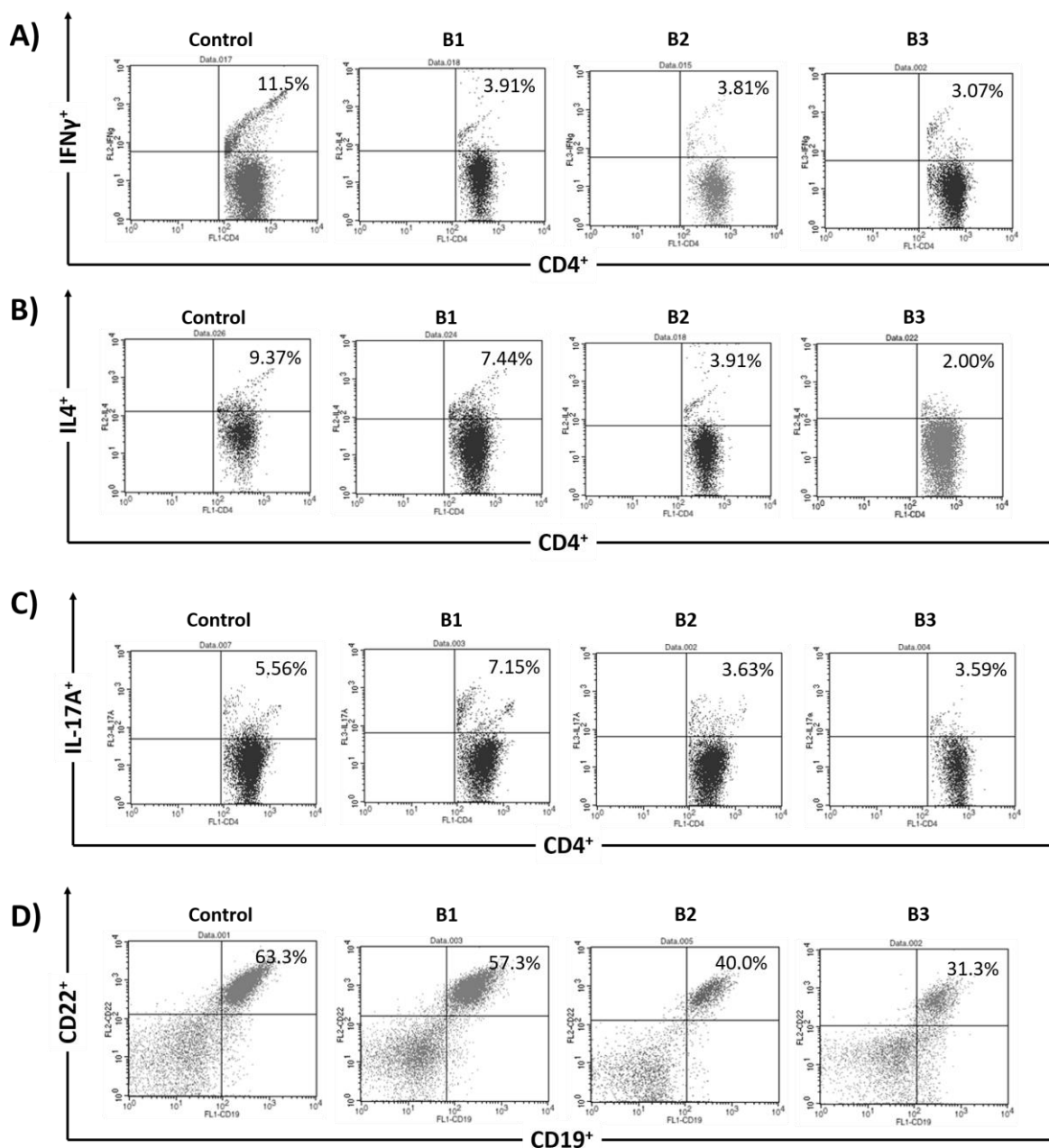


Figure 1. Representative of dot plot analysis from flow cytometer; A) Th1 cells which expressed CD4⁺ IFN γ ⁺, B) Th2 cells which expressed CD4⁺ IL-4⁺, C) Th17 cells which expressed CD4⁺ IL-17A⁺, and D) Mature B cells which expressed CD19⁺ CD22⁺.

As shown in table 2, systolic blood pressures were significantly lower in B1 and B2 groups compared to control ($p=0.026$ and $p=0.022$), but the systolic blood pressure was not significantly different in B3 group ($p=0.058$). On the other hand, diastolic blood pressures were not significantly different in all group compared to control (ANOVA, $p=0.304$). In addition, even though the urine albumin in levels in all groups treated with *Bryophyllum pinnatum* were slightly lower compared to control group, they were not statistically different (ANOVA, $p=0.494$).

Table 2. Clinical Characteristics of Pregnant Pristane-Induced Lupus Mice on Each Group.

Characteristics	Control (n=4)	B1 (n=4)	p*	B2 (n=4)	p*	B3 (n=4)	p*
Serum anti-dsDNA levels (OD)	0.746±0.08	0.591±0.01	0.014	0.648±0.02	0.118	0.739±0.03	0.998
Blood pressure							
Systole (mmHg)	146.0±22.1	101.0±14.2	0.026	99.3±16.2	0.022	107.7±6.4	0.058
Diastole (mmHg)	89.0±17.1	75.7±12.7	0.635	73.0±14.7	0.500	78.3±10.9	0.770
Urine albumin (ng/ml)	1,649±296.3	1,064±567.5	0.123	1446±118.6	0.823	1,377±87.0	0.665
Fetus profiles							
Number of fetuses	8.3±2.3	12.3±2.3	0.104	13.4±1.5	0.040	7.7±0.6	0.968
Fetus weight (gr)	0.68±0.14	0.85±0.06	0.128	0.98±0.05	0.009	1.31±0.04	0.000
Fetus length (cm)	2.28±0.09	2.19±0.29	0.889	1.95±0.11	0.061	2.06±0.02	0.292

*p value was a comparison with control group, considered significant if p < 0.05.

Effect of *Bryophyllum Pinnatum* Treatment on the Fetus Profiles of Pregnant Pristane-Induced Lupus Mice Mother.

Intrauterine growth retardation (IUGR) was one of the main characteristics of the fetuses from SLE mother. Thus, we measured the fetus profiles, including the number, body weight, and length of the fetuses. We found that the number of fetuses was significantly higher in B2 group compared to control group (p=0.040) while the number of fetuses from B1 and B3 did not statistically different compared to control (p=0.104 and p=0.968, respectively). Significantly higher of fetus weight was found in B2 and B3 groups compared to control (p=0.009 and p=0.000). On the other hand, there was no statistically different of fetus length in all groups treated with *Bryophyllum pinnatum* compared to control group (ANOVA, p=0.066), as seen in table 2.

Correlation between Clinical Manifestations with Th and B Cell Percentages.

To determine which factors were involved in the improvement of clinical manifestations after treatment of *Bryophyllum pinnatum*, we correlated the

clinical data which improved significantly after treatment of *Bryophyllum pinnatum* with Th and B cells percentages. Although albuminuria levels did not significantly higher in the group treated by *Bryophyllum pinnatum*, we still included it in this analysis. As shown in table 3, anti-dsDNA and systolic blood pressure only correlated significantly with Th1 percentages (p=0.024, r=0.582 and p=0.018, r=0.583 respectively). Albuminuria had significant positive correlations with Th1 and Th2 percentages (p=0.024, r=0.589 and p=0.043, r=0.474 respectively). The fetal body weight had significant negative correlations with all Th and B cell percentages (p=0.020, r=-0.599; p=0.001, r=-0.796; p=0.019, r=-0.603; and p=0.033, r=-0.547 for Th1, Th2, Th17, and mature B cells respectively) as shown in table 3.

Interestingly, this fetal body weight only had a significant positive correlation with albuminuria (p=0.035 and r=-0.540) but not with other clinical data, including anti-dsDNA and systolic or diastolic blood pressure.

Table 3. Correlation Analyses Between Clinical Manifestation with Th and B Cells Percentages.

Variable	% of Th1	% of Th2	% of Th17	% of mature B cell
Anti-dsDNA level	p = 0.024* r = 0.582	p = 0.291 r = 0.177	p = 0.222 r = -0.245	p = 0.078 r = -0.437
Systolic blood pressure	p = 0.018* r = 0.583	p = 0.150 r = 0.312	p = 0.457 r = 0.034	p = 0.195 r = 0.274
Albuminuria	p = 0.024* r = 0.589	p = 0.043* r = 0.474	p = 0.407 r = 0.070	p = 0.330 r = 0.135
Fetal body weight	p = 0.013* r = -0.599	p = 0.001* r = -0.796	p = 0.019* r = -0.603	p = 0.033* r = -0.547

*correlated significantly with $p < 0.05$

RESULT & DISCUSSION

In this current study, we found that mice treated with *Bryophyllum pinnatum* had significantly lower serum anti-dsDNA levels, lower mature B cell percentages, and lower Th cell percentages including Th1, Th2, and Th17 in a dose-dependent manner. The use of *Bryophyllum pinnatum* as an immunosuppressive agent was first described by Rossi-Bergmann et al. they determined that aqueous extract of *Bryophyllum pinnatum* leaves caused significant inhibition of cell-mediated and humoral immune response in mice. The spleen cells of animal treated with this extract showed a decreased ability to proliferate in response to both mitogens and to antigen in vitro. Almeida et al. also showed that ethanolic extract of *Bryophyllum pinnatum* had a potent suppressive activity against lymphocyte proliferations. Similar results also found in other studies; they determined that *Bryophyllum pinnatum* inhibited Th2-driven diseases such as asthma and anaphylactic shock by inhibiting mast cell activation, reduce the production of IgE antibodies, reduce eosinophilia, and reduce some pro-inflammatory cytokines production. Our findings added by the previous studies

strengthen the evidence that *Bryophyllum pinnatum* is a potent immunosuppressive agent.

Similar with the other reports, the clinical manifestations of SLE during pregnancy found in our present study were the increase of blood pressure, albuminuria, and the decrease of fetal body weight and lengths which manifested as IUGR. Our present study found that daily oral treatment of ethanolic extract of 10.5 and 21 mg/kgBW *Bryophyllum pinnatum* could decrease the systolic blood pressure significantly in pristane-induced lupus mice model. Although the diastolic blood pressure in *Bryophyllum pinnatum* treated groups were lower than control, it was not statistically significant. The antihypertensive properties of *Bryophyllum pinnatum* had been determined before by Ojewole et al. that showed the leaf extracts of *Bryophyllum pinnatum* could decrease the arterial blood pressure and heart rate in hypertensive rats which were resistant to physiological doses and concentration of standard antagonist drugs. Moreover, the leaves extracts also produced dose dependent, significant decreases in the rate and force of contractions of guinea-pig isolated atria, and inhibited electrical field stimulation (ES)-

provoked, as well as potassium and receptor-mediated agonist drugs-induced contractions of the rat isolated thoracic aortic strips in a non-specific manner. In addition, cardio depression and vasodilation effects would appear to contribute significantly to the antihypertensive effect of the herb. This result indicate that *Bryophyllum pinnatum* is a potential herb that can be developed as a hypotensive agents in some cases, including SLE during pregnancy.

Our analysis obtained that the clinical manifestation of pregnant pristane-induced lupus mice, such as increased of systolic blood pressure and serum anti-dsDNA levels were correlated with the increase of Th1 percentages. Although the albuminuria was not significantly lower in the group treated by *Bryophyllum pinnatum*, we did find correlations between the increasing of urine albumin levels with Th1 and Th2 percentages. This result indicates the importance of Th1/Th2 balance in the pathogenesis of SLE during pregnancy. Although SLE is often considered as Th2-driven disease, it had been studied before that there was a shift in the Th1/Th2 balance in SLE during pregnancy. In normal pregnancy, Th2 frequency and activity will be more dominant than Th1. However, Munoz-Valle et al. found that there was an increase of Th1 cytokines production at the second and third semester in pregnant SLE patients while the Th2 cytokines are decreased at the same time. The increase of Th1 activity has been postulated as one of the cause for the pathologic conditions in pregnancy.

In this current study we found that the mice treated with *Bryophyllum pinnatum* had significantly higher body weight in dose dependent manner even though the fetal body lengths were not statistically different.

Adverse fetal outcomes in SLE during pregnancy are associated with variety maternal factors, such as hypertension, proteinuria, and presence of anti-dsDNA and anti-phospholipid antibody which lead to placental insufficiency. However, we found that only urine albumin levels had significant negative correlation with the fetal body weight. Our result is similar with a systematic review done by Smyth et al. that there was a correlation between active nephritis with the fetal outcomes in SLE patients. However, it is still not clear what factors may contribute to the fetal outcomes in SLE during pregnancy. Our findings also showed there were inverse correlations between the fetal weight with Th and mature B cell percentages indicating the importance of immunologic involvement in the pathogenesis of SLE during pregnancy. Previous studies determined that the increase of Th1 subsets in pregnancy is associated with apoptosis activation which leads to placental insufficiency, fetal growth retardation, and spontaneous abortion. This result indicates that the fetal size in pregnant pristane-induced lupus mice is associated with various factors, including immunological or other clinical factors.

In conclusion, treatment of *Bryophyllum pinnatum* can improve the pregnancy outcomes in pregnant pristane-induced lupus mice, including lowering anti-dsDNA levels, lowering systolic blood pressure, and preventing the fetal growth retardations. *Bryophyllum pinnatum* also has immunosuppressive ability to suppress the Th and mature B cells percentages. Thus, *Bryophyllum pinnatum* is a potential agent that can be developed as an adjunctive immunosuppressive treatment against autoimmune disease, such as SLE during pregnancy. However, further studies are necessary to monitor the safety of this herb

during pregnancy. In addition, studies in a bigger group of population also required because at this point this study is only limited in a small group of animal model.

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