

Basic Medical Research

***Bryophyllum pinnatum* leaves ethanol extract inhibit maturation and promote apoptosis of systemic lupus erythematosus BALB/c mice B cells**

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ABSTRAK

Latar belakang: Sel B memegang peranan penting pada lupus eritematosus sistemik (LES). Menargetkan sel B sebagai terapi LES merupakan pendekatan yang paling memungkinkan. *Bryophyllum pinnatum* dengan kandungan metabolit sekundernya memiliki agen imunomodulator potensial. Studi ini menyelidiki efek potensial dari ekstrak etanol daun *B. pinnatum* dalam menurunkan persentase maturasi, meningkatkan persentase apoptosis, dan menurunkan ekspresi NF- κ B p65 sel B mencit BALB/c dengan LES.

Metode: Studi *in vitro* dilakukan dengan melakukan kultur sel B dari limpa mencit BALB/c dengan LES yang diinduksi pristan. Sel B distimulasi oleh BAFF, LPS, IL-4, dan anti-CD40 menghasilkan CD19+ >80%. Sel B dikultur dengan menambahkan stimulan tersebut dengan dan tanpa ekstrak etanol daun *B. pinnatum* (dosis 0; 0,02; 0,1; atau 0,5 μ g/ml) selama 72 jam pada suhu 37°C. Persentase maturasi (CD19+CD38+) dan apoptosis (Annexin V+PI+) sel B diukur menggunakan Flow cytometry. Analisis lebih lanjut untuk mengetahui ekspresi faktor transkripsi dari maturasi dan apoptosis sel B, NF- κ B p65, diukur menggunakan imunositokimia. Data yang terkumpul dianalisa menggunakan perangkat lunak SPSS versi 22.

Hasil: Uji Flow cytometry menunjukkan adanya penurunan signifikan terhadap persentase maturasi sel B pada semua dosis pemberian dan peningkatan signifikan terhadap persentase apoptosis sel B pada dosis 0,5 μ g/ml. Hasil pemeriksaan imunositokimia menunjukkan penurunan signifikan terhadap ekspresi NF- κ B p65 pada semua dosis. Persentase maturasi, apoptosis, dan ekspresi NF- κ B p65 dari sel B memiliki korelasi satu sama lain.

Kesimpulan: Penelitian *in vitro* ini menunjukkan bahwa ekstrak etanol daun *B. pinnatum* menurunkan persentase maturasi, meningkatkan persentase apoptosis, dan menurunkan ekspresi NF- κ B p65 sel B mencit BALB/c dengan LES secara signifikan.

Keywords: B cells, *Bryophyllum pinnatum*, pristane, SLE

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ABSTRACT

Background: B cells play a key role in systemic lupus erythematosus (SLE). Targeting B cells as SLE therapy is a plausible approach. This study investigated the potential effects of *Bryophyllum pinnatum* leaves with ethanol extract in decreasing percentages of maturation, increasing percentages of apoptosis, and decreasing NF- κ B p65 expressions of SLE BALB/c mice B cells.

Methods: Culturing B cells from pristane induced SLE BALB/c mice's spleen will result in this *in vitro* study. B cells were activated by BAFF, LPS, IL-4, and anti-CD40 yielding CD19+ >80%. B cells were cultured by adding those stimulants with and without *B. pinnatum* leaves (0, 0.02, 0.1, or 0.5 μ g/ml) for 72 hours at 37°C. Flow cytometry was performed to determine the Percentages of maturation (CD19+CD38+) and apoptosis (Annexin V+PI+) of B cells. Further analysis to determine the expressions of transcription factor of maturation and apoptosis of B cells, NF- κ B p65, were performed using immunocytochemistry. Data were analyzed using SPSS version 22.

Results: Flow cytometry assay showed significant decrease in percentages of maturation of B cells in all doses and significant increase in percentage of apoptosis of B cells in dose 0.5 μ g/ml. Immunocytochemistry results showed significant decrease expressions of NF- κ B p65 in all doses. Percentages of maturation, apoptosis, and expressions of NF- κ B p65 of B cells were significantly correlated.

Conclusion: This *in vitro* study revealed that *B. pinnatum* leaves with ethanol extract decreased the percentages of maturation, increased the percentage of apoptosis, and decreased NF- κ B p65 expressions of SLE BALB/c mice B cells significantly.

Among many autoimmune disease, SLE is one of the complex ones. It display heterogeneity in clinical manifestation followed by several abnormalities such as: autoantibodies formation, immune complex deposition, and damages to the organs. B cells play a key role in SLE such as secreting autoantibodies by plasma cells, providing antigens as co-stimulant to T cells, inducing dendritic cell (DC) immunogenicity, producing proinflammatory cytokines and chemokines, and influencing the regulation of immunity and limphogeneity in SLE.¹ The plausible approach in therapy of SLE includes supression of autoantibodies production due to B cells maturation and proliferation, or B cell function modulation.²

To investigate the mechanisms of the disease, we need to understand how important are several SLE animal models to the mechanisms itself. One model, characterized by the development of several autoantibodies and clinical manifestation, is called pristane induced lupus model.³ Intraperitoneal injection of pristane in BALB/c mice causes a SLE with high levels of immunoglobulin G (IgG), anti-double stranded DNA (anti-dsDNA), anti-Smith (anti-Sm), anti-RNP, anti-Su, anti-ribosomal P, and other lupus-related autoantibodies starting 12 weeks after injection.⁴ This model may cause abnormalities in interferon production followed by defective clearance of apoptotic cells and over-active B-cell signalling. This is a clear impact of pristine-induced SLE BALB/c mice model.⁵

Due to earlier diagnosis and better treatment options of SLE, the prognosis has markedly improved in the last decades.⁶ In the past decade, we have learn to fully understand the process behind pathogenesis of SLE, which led to a more effective therapeutic approach⁷. One of the examples of recent therapeutic approach is biologic agent for SLE. Biologic agent has generated substantial research interest for developing therapy of several autoimmune diseases.⁸

In 2011, biologic agent belimumab, a monoclonal antibody targeting human B cell activating factor (BAFF), was shown in randomized clinical trials to be efficacious in SLE and has now become the first approved targeted therapy for SLE.⁸ Belimumab treatment is quite expensive. The total cost for the first year of treatment is around \$28,000.⁹

The study of BAFF and its clinical inhibition are gaining notable interests over the year. It was mainly due to the test of similar biologic agent in clinical trials.¹⁰ Therefore, more effective drugs with an affordable monetary cost are urgently needed. In nature, there are many natural compounds which can be developed to treat SLE because their effects in modulate immunity.

B. pinnatum is a wild perennial succulent herb that is usually used as a traditional medicinal plant in tropical countries like Africa, Indonesia, and India. *B. pinnatum* is known by numerous vernacular names, such as life plant, love plant, and miracle leaf.¹¹ It has been used in anthroposophic medicine to treat various disorders caused by hyperactive conditions. Various secondary metabolites of *B. pinnatum*, especially flavonoid and bufadienolid, have been reported by several pharmacological studies as broad spectrum therapeutic potential such as immunomodulatory, cytotoxic and antitumor promoting activity, antiallergic, anti-inflammatory, antioxidant, analgesic, and antihypertensive. Other constituents of *B. pinnatum* include steroids, triterpenes, phenanthrenes, and some ubiquitous compounds.¹² Despite having broad spectrum therapeutic potential, *B. pinnatum*'s immunomodulatory activity in general and B cell apoptosis inducing property in particular have not been explored as yet. Therefore, this study evaluated whether *B. pinnatum* could decrease the percentages of maturation, increase the percentages of apoptosis, and decrease NF- κ B p65 expressions of B cells furthermore prevent the development of pristane-induced lupus in SLE BALB/c mice B cells.

METHODS

Animals

This experimental and randomized post-test only controlled group design study was conducted in Malang, East Java, Indonesia between April and December 2016. Female BALB/c mice aged 6–8 weeks (25–35g) were purchased and certified from Pusat Veteriner Farma (Surabaya, East Java, Indonesia). All the BALB/c mice were housed at Pharmacology Laboratory of Universitas Brawijaya and acclimatized in the laboratory for 1 week prior to the experiments. The housing conditions were controlled, with a

room temperature and a diurnal 12-hour light/dark cycle. All experimental protocols described in this study were approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya (Ethical clearance approval number: No. 328/EC/KEPK/05/2015).

Induction and treatment of pristane-induced SLE in mice

Female BALB/c mice were treated with single injection of pristane (Santa Cruz, California, USA) 0.5 ml intraperitoneally per mice. Spleen and serum were collected 16 weeks after the pristane injection. Clinical manifestations were also observed after 16 weeks of pristane injection. Consistent with our previous findings, treatment with single injection of pristane 0.5 ml intraperitoneally increased antinuclear antibodies (ANA) serum titers compared with control group as early 16 weeks (data not shown).

Plant material

B. pinnatum leaves were collected from Batu, East Java, Indonesia in August 2016. Leaves were identified and authenticated at Unit Pelaksana Teknis (UPT) Balai Materia Medica, Batu, East Java, Indonesia. They were air dried and grounded into fine powder. A sample specimen was deposited at the herbarium.

Extract preparation

Dried and powdered leaves (100 g) were exhaustively extracted with ethanol (900 ml) by static maceration at room temperature every 24 hours for 3 times. The ethanol extract was filtered and evaporated under a rotary vacuum evaporator at controlled temperature (70-75°C). This extract was filtered through whatman paper no. 42 (125 mm) and every 1 mg of stock solution was prepared in 1 ml (0.01%) of dimethyl sulfoxide (DMSO). The DMSO concentration was ignored because it was lower than 1%.

Cell preparation and culture

Mice were sacrificed by cervical dislocation 16 weeks after receiving the single pristane injection, and spleens were collected for analysis. Cell suspensions were prepared by homogenization in a tissue grinder. B cell enriched suspensions were obtained by stimulating suspensions with 100 ng/ml BAFF

(Biolegend), 10 µg/ml anti-CD40 antibody (Biolegend), 20 µg/ml LPS from *Escherichia coli* (Sigma-Aldrich), and 50 ng/ml IL-4 (Biolegend). Our preliminary experiments demonstrated that this procedure yielded an enriched B-cell population >80% CD19+ cells as determined by flow cytometry analysis (data not shown). B cells enriched suspensions were cultured at a concentration of 5×10^5 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS and 100 U/ml penicillin-streptomycin (Sigma-Aldrich, USA), those stimulating factors mentioned above, with or without ethanol extract of *B. pinnatum* leaves (0, 0.02, 0.1, or 0.5 µg/ml) for 72 hours at 37°C without DMSO control.

CD19 and CD38

To determine the immunomodulatory activity of *B. pinnatum* leaves with ethanol extract against B cells maturation process *in vitro*, expressions of typical markers (CD19 and CD38) associated with the maturation status of B cells were measured. After 72 hours, cells were harvested for flow cytometry analysis of the percentages of CD19+CD38+ B cells. Cells were labeled with fluorescein isothiocyanate (FITC)-anti mouse CD19 (Biolegend) and phycoerythrin (PE) anti-mouse CD38 (Biolegend) following the protocol indicated by the manufacturer.

Apoptosis assay

Apoptosis of B cells were measured using flow cytometry staining with the FITC annexin V detection kit with propidium iodide (PI) (Biolegend). In detail, cells were stained for 20 minutes at room temperature in the dark with an annexin V. Afterwards, PI was added to the wells, and cells were further incubated for 5 minutes. After staining, cells were analyzed by flow cytometry to determine the percentages of apoptotic cells (Annexin V+PI+).

Detection NF-κB p65 expressions by immunocytochemistry

To understand the molecular mechanisms of B cells depletion, the expressions of protein controller of maturation and apoptosis of B cells, NF-κB p65, were examined using immunocytochemistry labeled with NF-κB p65. Immunocytochemistry was performed by using the streptavidin-biotin/indirect immunoperoxidase method.

Thin smears were fixed in poly-L-lysine coated slides by methanol for 30 minutes. They were washed in FBS 3 times for 5 minutes, and endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 20 minutes at room temperature. Smears were washed in FBS 3 times for 5 minutes. Unspecific proteins were blocked using blocking buffer (3% FBS and 0.25% Triton X-100) for 6 minutes at room temperature. The primary antibody (NF- κ B p65, Santa Cruz) was applied at the desired dilution, and slides were incubated overnight at 4°C in a moist chamber. They were washed 3 times with FBS and further incubated with the secondary antibody (Biotin conjugate) for 60 minutes, washed in FBS, and incubated with horseradish peroxidase for 40 minutes. By washing the slides again with FBS three times, and rinsing it with distilling water, can result in the development of the color 3–3' diaminobenzidine and hydrogen peroxide. Hematoxylin can be used afterwards to counterstain the smears. The final output, the brown reaction product (NF- κ B p65), then would be mounted and observed under the light microscope. Total numbers of apoptotic cells were determined by calculating the number of brown color cells in 10 fields of view then divided by 10.

Statistical Analysis

The percentages of CD19+CD38+, Annexin V+PI+, and NF- κ B p65 expressions of B cells

were tested for normality using the Kolmogorov Smirnov test and for homogeneity variances prior to further statistical analysis. The data were normally distributed and were expressed as means \pm standard error of mean (SEM). Significant differences among groups were analyzed by one way ANOVA followed by Tukey's post-test for multiple comparisons using SPSS software, version 22 (IBM Corp., Armonk, NY, USA). Correlations between the percentages of CD19+CD38+, Annexin V+PI+, and NF- κ B p65 expressions of B cells were examined by Pearson's correlation coefficient. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Pristane-induced SLE BALB/c mice model

Clinical evidence of inflammation of joints, gait changes, alopecia, and ascites occurred 16 weeks after pristane injection in BALB/c mice (Figure 1). The first sign of joint inflammation was seen at 12 weeks after pristane injection (Figure 1A).

B. pinnatum leaves with ethanol extract inhibited maturation of pristane-induced SLE BALB/c mice B cells

This study showed that treatment with 0, 0.02, 0.1, or 0.5 μ g/ml *B. pinnatum* leaves with

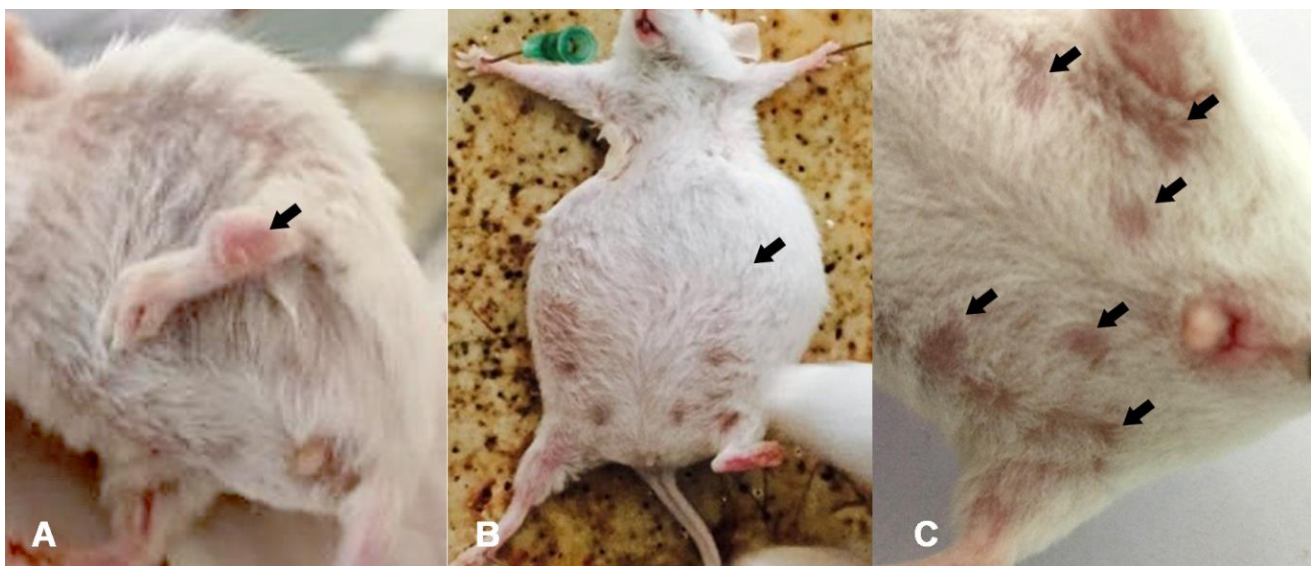


Figure 1. SLE clinical manifestations after 16 weeks of pristane injection. A) Joint inflammation was seen at 12 weeks after pristane injection (black arrow); B) Production of abdominal fluid or ascites (black arrow); C) The most frequent signs were alopecia (black arrows)

ethanol extract inhibited maturation rates of $6.64 \pm 0.14\%$, $5.90 \pm 0.46\%$, $5.54 \pm 0.31\%$, and $4.89 \pm 0.37\%$ (Figure 2), respectively. CD19⁺CD38⁺ B cells percentages were significantly decreased compared to the untreated group ($p=0.006$, $p=0$, and $p=0$, respectively) (Figure 3A).

***B. pinnatum* leaves with ethanol extract promoted apoptosis of pristane-induced SLE BALB/c mice B cells**

This study showed that treatment with 0, 0.02, 0.1, or 0.5 $\mu\text{g/ml}$ *B. pinnatum* leaves with ethanol

extract promoted apoptotic rates of $35.80 \pm 1.54\%$, $38.41 \pm 5.19\%$, $40.28 \pm 2.51\%$, and $44.01 \pm 2.60\%$ (Figure 2), respectively. B cells treated with 0.5 $\mu\text{g/ml}$ *B. pinnatum* leaves with ethanol extract significantly increased the percentage of apoptosis compared to the untreated group ($p=0.002$). However, lower dose treatment of 0.02 and 0.1 $\mu\text{g/ml}$ *B. pinnatum* leaves with ethanol extract showed insignificant effect on increasing the percentage of apoptosis compared to the untreated group ($p=0.520$ and $p=0.114$, respectively) (Figure 3B).

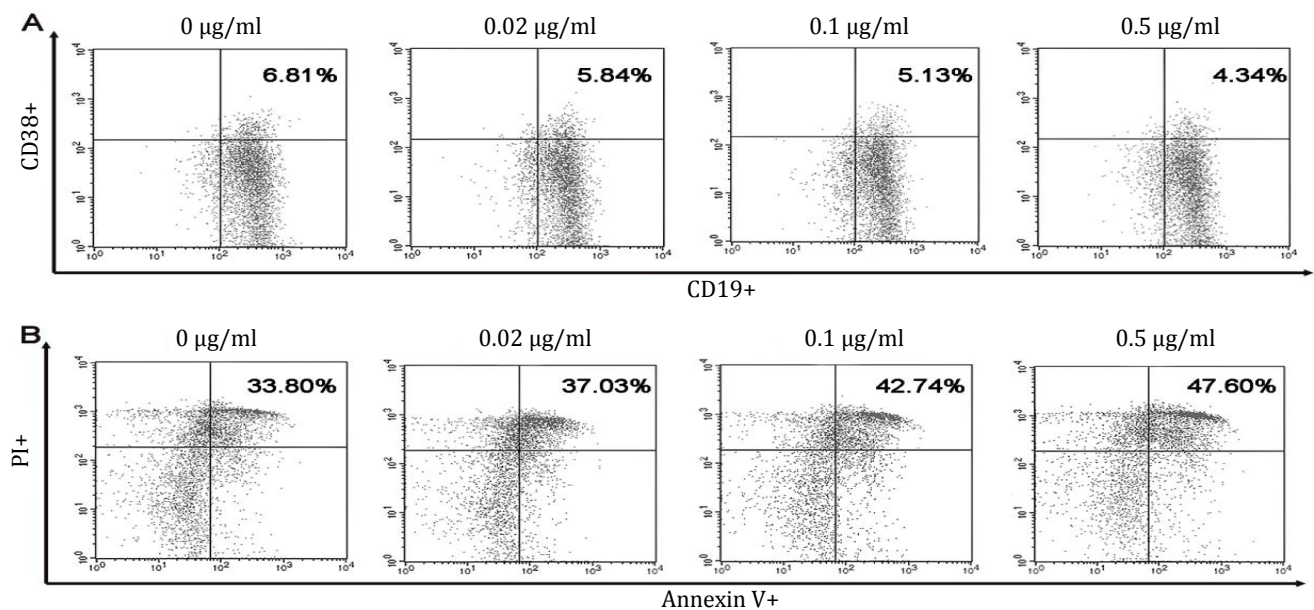


Figure 2. Dot plots of B cells maturation and apoptosis in vitro. Spleen of pristane-induced SLE BALB/c mice model cultured with BAFF, anti-CD40, LPS, IL-4 and without or with different doses of *B. pinnatum* leaves with ethanol extract (0, 0.02, 0.1, or 0.5 $\mu\text{g/ml}$). Cells were harvested and measured percentages of maturation (CD19⁺CD38⁺) (A) and apoptosis (Annexin V⁺PI⁺) (B) of B cells using flow cytometry

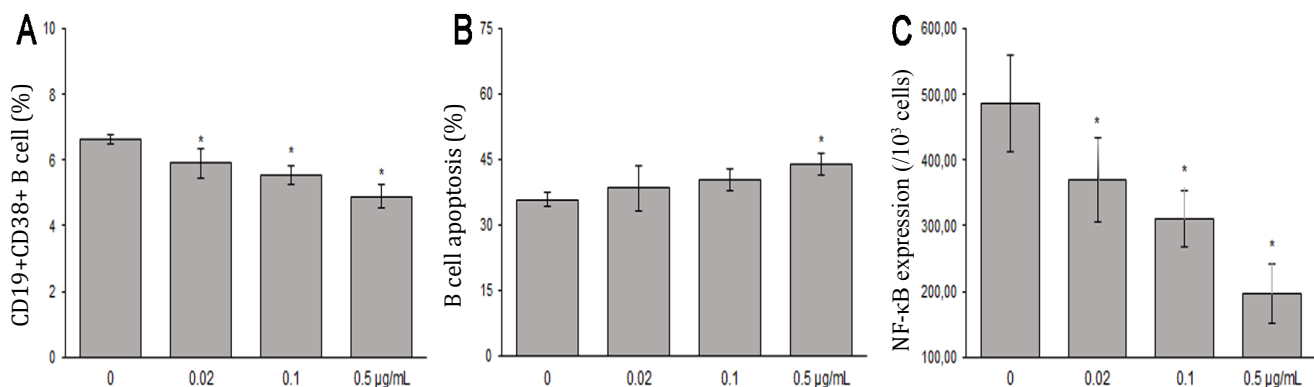


Figure 3. The effects of *B. pinnatum* leaves with ethanol extract on B cells in vitro. A) *B. pinnatum* leaves with ethanol extract inhibit maturation (CD19⁺CD38⁺) of B cells; B) *B. pinnatum* leaves extract promote apoptosis (Annexin V⁺PI⁺) of B cells; C) *B. pinnatum* leaves with extract decrease expressions of NF- κ B p65 (mean \pm SD; n = 6, * $p < 0.05$)

***B. pinnatum* leaves with ethanol extract decreased NF-κB p65 in pristane-induced SLE BALB/c mice B cells**

B. pinnatum leaves with ethanol extract group had significant lower expressions of NF-κB p65 compared to the untreated group ($p=0.013$,

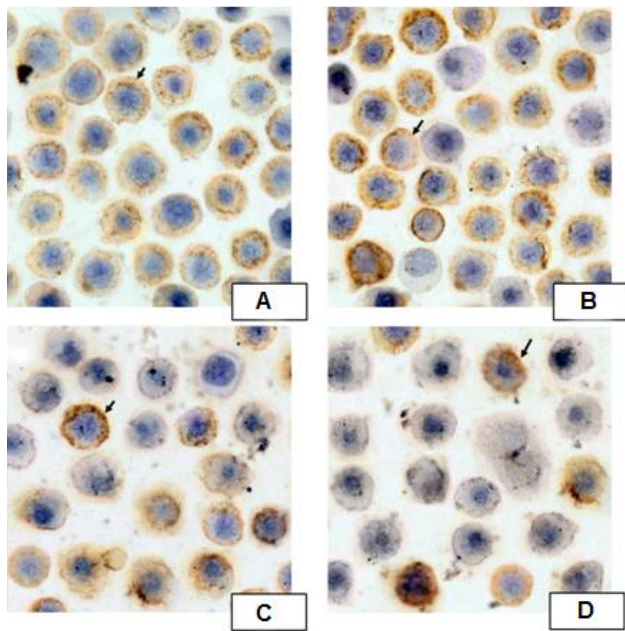


Figure 4. Expressions of NF-κB p65 by Immunocytochemistry. Spleen of pristane-induced SLE BALB/c mice model cultured with BAFF, anti-CD40, LPS, IL-4 and treated without or with different doses of *B. pinnatum* leaves with ethanol extract (0, 0.02, 0.1, or 0.5 μg/ml) for 72 hours. Cells were processed for immunocytochemistry using NF-κB p65 antibody as described in Materials and Methods

$p<0.01$, and $p<0.01$, respectively). *B. pinnatum* leaves with ethanol extract decreased the expressions of NF-κB p65 (Figure 4).

Correlations between the percentages of maturation, apoptosis, and expressions of NF-κB p65 of B cells

The percentages of maturation of B cells had moderate and significant correlation with apoptosis of B cells ($r=-0.512$, $p<0.05$). Significant and strong correlation was observed in the percentages of maturation and NF-κB p65 expressions of B cells ($r=0.849$, $p<0.001$). Significant and moderate correlation was observed in the percentage of apoptosis and NF-κB p65 expressions of B cells ($n=6$, $r=-0.692$, $p<0.001$) (Figure 5).

DISCUSSION

B. pinnatum is a wild plant that has been proven to have benefits as anti-inflammatory, antitumor, and immunomodulatory agents.¹¹ Phytochemistry studies showed that *B. pinnatum* contains elements such as alkaloids, phenol, flavonoids, tannins, anthocyanin, glycosides, bufadienolide, saponins, coumarin, sitosterol, quinine, carotenoids, tocopherol, mucilago, lignin, and lectin.¹³⁻¹⁵ The therapeutic effects of *B. pinnatum* in pristane induced SLE BALB/c mice B cells explored in this study. *B. pinnatum* leaves with ethanol extract decreased percentages of

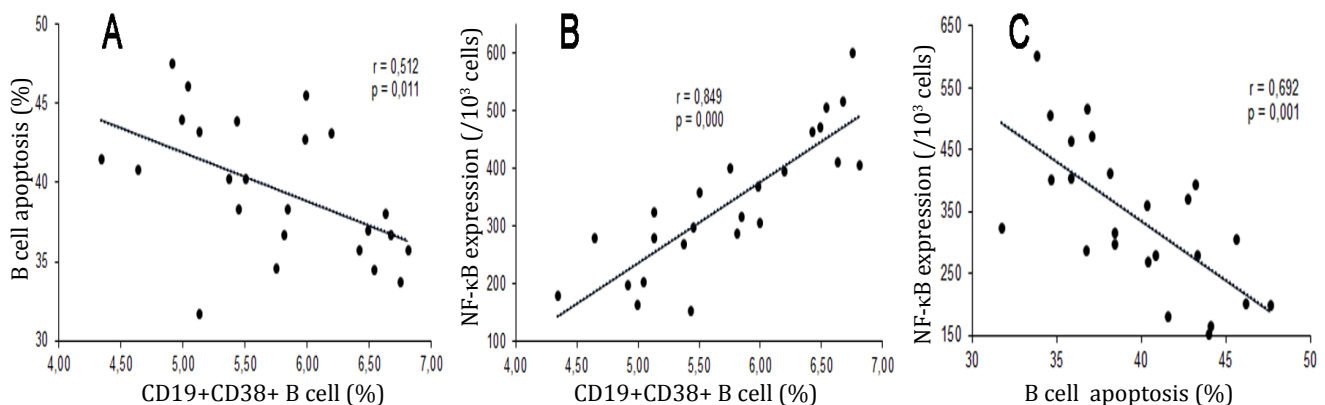


Figure 5. Correlations between percentages of maturation, apoptosis, and NF-κB p65 expressions of B cells. Diagrams showed correlations among the percentages of maturation (CD19+CD38+), apoptosis (Annexin V+PI+), and expressions of NF-κB p65 of B Cells. A) Significant and moderate correlation was observed in percentages of maturation and apoptosis of B cells ($r=-0.512$, $p=0,011$); B) Significant and strong correlation was observed in the percentages of maturation and expressions NF-κB p65 of B cells ($r=0.849$, $p=0,000$); C) Significant and moderate correlation was observed in the percentage of apoptosis and expressions NF-κB p65 of B cells ($n=6$, $r=-0.692$, $p<0,001$)

maturation (CD19+CD38+) of B cells, increased the percentage of apoptosis (Annexin V+PI+), and decreased NF- κ B p65 expressions in pristane-induced SLE BALB/c mice B cells *in vitro*.

The parenteral use of *B. pinnatum* has been granted permission by the German and Swissmedic E commission.¹⁶ *B. pinnatum* preparations are available in tablets, powders, and parenterals (*Bryophyllum* 5%), produced by Weleda AG, and used as a tocolytic agent in premature pregnancy and various other medical conditions as well.¹¹ Previous study compared various parenteral agents and showed that administering of *Bryophyllum* 5% did not have significant effects to apoptosis and necrosis of lymphocytes and DC *in vitro*. However, this study showed different findings compared to Gründemann et al.¹⁷ The administration of *B. pinnatum* leaves with ethanol extract increased percentages of B cells apoptosis.

Several strategies have been developed to suppress the production of autoantibodies by depletion of B cells, inhibition of B cells proliferation, and modulation of B cells. Recent studies were still focused on the mechanism of BAFF in depleting B cells.¹⁸ This study performed B cells activation models by stimulating it with BAFF, IL-4, LPS, and anti-CD40 antibody. This study suggested that *B. pinnatum* leaves with ethanol extract could decrease B cells maturation, NF- κ B expressions, and increase apoptosis of B cells. The limitation of this study was that it did not compare the effects of BAFF, IL-4, LPS, and anti-CD40 antibody stimulation respectively. Further research may require to understand the mechanisms of *B. pinnatum* metabolites in disrupting the ligand-receptor binding of B cells.

Bufadienolide is one of the main active metabolites in *B. pinnatum*. About 40.5–52 mg bufadienolide contained in every 100 grams of *B. pinnatum*.¹² Our unpublished *in silico* study was conducted to understand the affinity of bufadienolide active compounds (Bryophyllin A, Bryophyllin B, and Bryotoxin B) in *B. pinnatum* against BAFF and its receptors: BAFF-R, TACI, and BCMA. Docking results suggested that those *B. pinnatum* compounds interacted with BAFF-R, TACI, BCMA and BAFF through hydrogen bonds and hydrophobic interactions. These results showed that bufadienolide active compounds disrupted BAFF interactions with its receptors.

These study results demonstrated that single dose of pristane injection intraperitoneally in BALB/c mice induced arthritis, alopecia, and ascites after 16 weeks of injection. These results supported our previous study that manifestations of SLE such as arthritis, alopecia, and ascites appear in BALB/c mice model after single dose of 0.5 mg pristane injection intraperitoneally.¹⁹ Based on the kinetics of autoantibody development following exposure to pristane, 16 weeks of the first clinical manifestation period was chosen.²⁰ The results showed that pristane induced immunity dysregulation and induced autoreactive of B cells shown by producing autoantibodies like ANA.^{19,21} ANA serum titers are increased in the week 16.

The clinical impact of this study is expected to find a new complementary therapy derived from the original leaves of Indonesia, *B. Pinnatum*, to improve the success of therapy in patients with SLE in Indonesia. However, this study still can not explain which specific secondary metabolite content has a role to the maturation and induction of B cell apoptosis. Besides, we also have not compared the effect of *B. pinnatum* leaves with ethanol extract between each stimulant to know specifically the role of secondary metabolite contained to the activation pathway of B cells development. The study would have yielded different results if a negative or normal control group had been included. Thus, further research should address this limitation in order to reveal more important findings.

In conclusion, *B. pinnatum* leaves with ethanol extract decreased the percentages of maturation (CD19+CD38+) of B cells, increased the percentage of apoptosis (Annexin V+PI+), and decreased NF- κ B p65 expressions in pristane-induced SLE BALB/c mice B cells *in vitro*. Further studies in other lupus models and good-designed clinical trials are required to confirm these studies and identify its therapeutic effects, especially in humans.

Conflicts of interest

The authors affirm no conflict of interests in this study.

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