

RESEARCH ARTICLE

The Protective Effect of *Andrographis paniculata* Against Lipopolysaccharide-induced Sepsis in Lung Tissues of a Rat Model Through the Decrease of ICAM-1 and E-selectin Expression

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Abstract

BACKGROUND: *Andrographis paniculata* has anti-inflammatory, anti-cancer, and immunomodulatory effects. Intercellular adhesion molecule-1 (ICAM-1) and E-selectin are released in inflammatory conditions due to sepsis. In sepsis-associated fatalities, E-selectin is strongly expressed on pulmonary microvasculature and ICAM-1 is strongly expressed on pulmonary microvasculature endothelial cells, pulmonary macrophages, and lymphocytes. Therefore, this study was conducted to analyze the protective effect of *A. paniculata* leaf extract against lung injury in lipopolysaccharide (LPS)-induced sepsis mouse model.

METHODS: Thirty male Wistar rats were divided into 1 control group and 4 sepsis groups. The control group was fed *ad libitum* for 14 days, followed by an intraperitoneal injection of 5 mg/kgBW 0.9% NaCl on day-21. While the sepsis groups were fed *ad libitum* and orally administered 0, 200, 400, and 500 mg/kgBW of *A.*

paniculata leaf extract, respectively, for 14 days. All four sepsis groups were intraperitoneally injected with 5 mg/kgBW LPS on day-21. Mice termination and measurement of ICAM-1 and E-selectin were performed on day-25. The immunohistochemical examination of ICAM-1 expression was performed using ICAM-1/CD54 antibody, meanwhile E-selectin expression was examined using primary antibody E-Selectin (D-7) rabbit IgG antibody.

RESULTS: All groups given *A. paniculata* leaf extract showed lower expression of ICAM-1 ($p < 0.001$) and E-selectin ($p < 0.001$) than the group without extract. *A. paniculata* leaf extract 500 mg/kgBW gives the best effectiveness compared to other doses.

CONCLUSION: *A. paniculata* leaf extract has a protective effect against lung injury by lowering the expression of ICAM-1 and E-selectin in lung tissue.

KEYWORDS: ICAM-1, E-selectin, sepsis, *Andrographis paniculata*, andrographolide

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Introduction

Sepsis is a life-threatening condition of organ failure or dysfunction caused by impaired regulation of the host's immune response to an infection. The causes of dysregulation

in sepsis are multifactorial, including pathophysiological, pathobiological, and immunologic responses.(1,2) Sepsis activates the inflammatory response and homeostatic pathways in the form of a coagulation cascade response simultaneously.(3) The stress reaction in sepsis also triggers a series of inflammatory reactions, leukocyte binding to

vascular endothelial cells, and induction of the body's immune response through the action of cytokines, selectins (E-selectin), and other complements such as intercellular adhesion molecules-1 (ICAM-1).(4-6)

ICAM-1 is a glycoprotein found on the cell surface and adhesion receptor, which functions as a regulator of leukocytes' movement from the circulation to the inflammation site. Its expression occurs in vascular endothelial, epithelial, and immune cells in response to inflammation. Furthermore, ICAM-1 expression is induced by various inflammatory cytokines, hence, it is recognized as a major regulator of various cellular functions under pathological conditions.(7-10) Investigations on animal models as well as in the serum of patients with chronic obstructive pulmonary disease (COPD), asthma, sepsis, atherosclerosis, coronary heart disease, and cancer, found elevated serum levels of ICAM-1. Meanwhile, this is associated with inflammatory conditions, classification of infectious or non-infectious cases, Systemic Inflammatory Response Syndrome (SIRS), inflammatory disorders, and in some clinical studies used to monitor response to therapy especially in cancer patients.(7,11,12)

E-selectin is an adhesion molecule expressed on endothelial cells activated by cytokines.(13-15) During inflammation or tissue damage, it permits adhesion between leukocytes and platelets in contact with the vascular endothelium.(6,8) Moreover, E-selectin upregulation is based on the binding of NF- κ B to the regulatory domain at its promoter.(16,17) Various studies have shown that it can predict acute lung injury in pneumonia patients, and is an independent predictive factor for mortality from acute respiratory diseases, including interstitial pneumonia and infectious pneumonia.(13,16,18,19)

The prevention and control of sepsis is a challenge that is always being investigated, including with the use of traditional or herbal medicines. According to WHO data, 65% and 80% of the population in developed and developing countries respectively use herbs as a form of traditional medicine.(20-22) One of the widely used medicinal plants is *Andrographis paniculata*, which leaves are widely used as herbal medicine in Asia. Several studies reported that this plant has potential as an antimicrobial, regulates the balance of pro-inflammatory and anti-inflammatory immune responses, maintains host cells to fight infection, reduces drug side effects (8,23), and as an alternative therapy to boost the immune system (23-25).

Only a few investigations have been conducted on *A. paniculata* and sepsis. A previous study performed

using andrographolide, the main biologically active ingredient of *A. paniculata*, with a concentration of 1-10 mg/kgBW, showed improvement in acute lung damage due to lipopolysaccharide (LPS) in rats. Its mechanism of action is through the suppression of nuclear factor kappa B (NF- κ B) activation, decreased expression of cell adhesion molecule E-selectin, prevention of E-selectin-mediated leukocyte adhesion, and downregulation of tumor necrosis factor (TNF)- α -induced ICAM-1 expression, leading to the inhibition of endothelial-monocyte adhesion.(12,23-25) Therefore, it is assumed that andrographolide can inhibit the activation of endothelial cell adhesion molecules. (11) Aside from suppressing E-selectin expression, andrographolide also downregulates TNF-induced ICAM-1 expression, leading to inhibition of endothelial-monocyte adhesion.(12)

Based on the various phytochemical and pharmacological properties of *A. paniculata* leaf extract, it is a potential candidate for complementary anti-inflammatory treatment in lung tissue damage due to sepsis. However, the information regarding the direct relationship between *A. paniculata* administration and the expression of ICAM-1 and E-selectin in sepsis analyzed with immunohistochemical examination is limited. Therefore, this study was conducted to analyze the protective effect of *A. paniculata* leaf extract as a pre-treatment against lung injury in LPS-induced sepsis mouse model.

Methods

Preparation of *A. paniculata* Leaf Extract

The *A. paniculata* plant was purchased from local suppliers. For the extraction process of *A. paniculata* leaf extract, the leaves were separated from contaminants, cleaned, and dried in the oven. Clean ingredients were formulated and grounded into coarse pieces/powder. Maceration was done by extracting the ingredients for 24 hours using 90% alcohol. Percolation was done with liquid for 2 hours at a temperature of 60°C. The filtrate was filtered and thickened by evaporation using a vacuum evaporator until a thick extract was obtained. *A. paniculata* leaf extract then was obtained in the form of standardized powder and dissolved in a 1% Na-CMC solution.

Animal Model

Thirty male Wistar rats (*Rattus norvegicus*) aged 2-3 months with a body weight of 150-200 grams were obtained

from the Inter University Center (IUC) Universitas Gadjah Mada animal cage laboratory. The protocol of this study had been approved by the Health Research Ethics Commission, Faculty of Medicine, Universitas Diponegoro, Semarang (No. 87/EC/H/FK-UNDIP/VIII/2022). The handling and treatment of experimental animals were conducted in accordance with the European Union on Animal Care (CEE Council 86/609) used by the Health Research Ethics Commission, Faculty of Medicine, Universitas Diponegoro, Semarang.

Animal Treatment

Thirty rats were acclimatized for 7 days and kept at room temperature of $28.0 \pm 2.0^\circ\text{C}$, before randomized and separated into 1 control group and 4 sepsis groups (Table 1). The non-sepsis group were only given food and drink *ad libitum* for 14 days. Meanwhile, the 4 sepsis groups were given food and drink *ad libitum*, as well as orally administered 0, 200, 400, and 500 mg/kgBW of *A. paniculata* leaf extract, respectively, for 14 days. The non-sepsis group received an intraperitoneal injection of 5mg/kgBW 0.9% NaCl on day-21, while the sepsis groups received intraperitoneal LPS injection of *Escherichia coli* O111:BA at a dose of 5 mg/kgBW (which was dissolved in 0.9% NaCl solution at a dose of 3 mL/200 grams) on day-21 (7 days after the extract administration). The sepsis induction was performed according to previously reported procedures.(26,27) The lung was harvested for 3 days after the induction of sepsis, and then on day-25, the rats were euthanized using chloroform. Figure 1 showed the schematic illustration of the animal treatment and timeline.

Immunohistochemistry (IHC) Analysis

Three days after the induction of sepsis, the rats were terminated, then the lungs were taken, fixated with formalin 10%, trimmed into pieces, and made into paraffin block.

The block then sectioned into 4–5 μm thick slices, and a microscope slide was placed under the selected tissue section, and the slide was dried with oven at 37°C .

The IHC examination of ICAM-1 expression was performed using ICAM-1/CD54 antibody (Cat No. A11110; Boster Biological Technology, Pleasanton, CA, USA) and E-selectin expression was performed using primary antibody E-selectin (D-7) rabbit IgG antibody (Cat No. sc-137054; Santa Cruz Biotechnology, Dallas, TX, USA). ICAM-1 and E-selectin staining was done by Steptavidine-Biotin method. Deparaffinization was done with xylol for 4x5 minutes; then rehydrated with absolute ethanol, 96% ethanol, and 70% ethanol; washed with distilled water, running water, another distilled water, and phosphate buffered saline (PBS).

Antigen retrieval was carried out in a microwave oven with Tris EDTA pH 9 at 90°C for 3 minutes then continued at low temperature for 10 minutes. After cooling, the slide was washed with PBS, endogenous 3% H_2O_2 methanol peroxidase was added, then washed with running water, before the addition of blocking serum. A primary antibody (ICAM-1/CD54 antibody or E-selectin rabbit IgG antibody) with a dilution of 1: 200 was added, then the slides were incubated at 4°C for 18 hours, washed with PBS, dripped with biotin, washed with PBS, dripped with streptavidin, washed with PBS, given peroxidase enzyme substrate 3,3' Diaminobenzidine (DAB), washed with running water, dripped with hematoxylin, and finally washed with running water. The final stage of the preparation was the drying process at room temperature, then mounted using entellan, and covered with a glass cover.

Immunoreactive cells were counted under a light microscope with a magnification of 400x, and the mean of cells stained at 5 randomly-selected spots was counted. ICAM-1 and E-selectin expressions in the form of broad fractions were calculated using ImageJ software (National Institutes of Health, Bethesda, MY, USA).

Table 1. Interventions given to the rat models.

Group	Treatment	
	Oral Pretreatment	Sepsis Induction
Group 1	-	NaCL
Group 2	-	LPS
Group 3	200 mg/kgBW	LPS
Group 4	400 mg/kgBW	LPS
Group 5	500 mg/kgBW	LPS

Oral pretreatment of *A. paniculata* leaf extract in various concentrations was performed for 14 days. Sepsis induction was performed 7 days after the oral pretreatment with intraperitoneal 5 mg/kgBW NaCL or 5 mg/kgBW LPS.

Results

ICAM-1 Expression

The lowest ICAM-1 expression was found in Group 1, with a value of $1.29 \pm 0.35\%$, followed by Group 5, with of $2.86 \pm 0.74\%$. Meanwhile, the highest ICAM-1 expression was found in Group 2, with a value of $23.08 \pm 0.90\%$ (Figure 2).

Based on the One-Way ANOVA test results, there was a significant difference in ICAM-1 expression levels

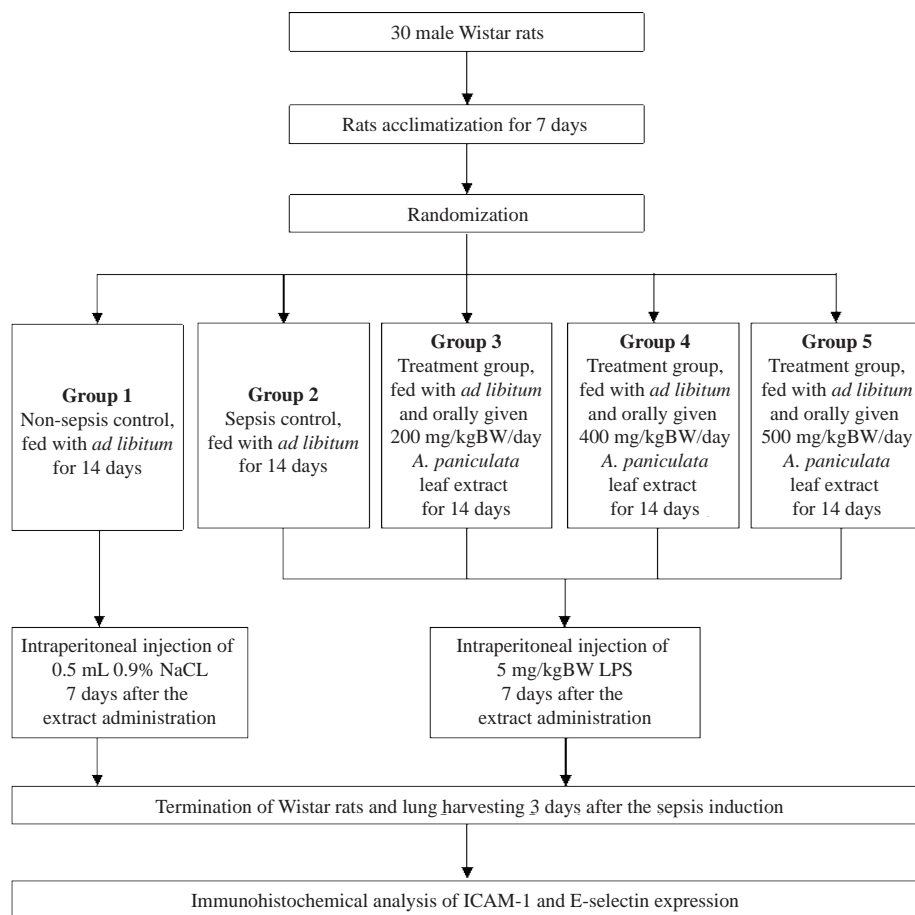


Figure 1. Schematic diagram of the study design and timeline.

between the non-sepsis control, sepsis control, and treatment groups ($p < 0.001$). After the post-hoc analysis, it was known that the administration of *A. paniculata* leaf extract for 14 days at a dose of 500 mg/kgBW before sepsis-induction was significantly reduced the ICAM-1 expression compared to the group that received 200 and 400 mg/kgBW *A. paniculata* leaf extract ($p < 0.001$) (Figure 3).

E-selectin Expression

E-selectin expression obtained had similar results with the ICAM-1 expression, where the lowest value was found in Group 1 with an area fraction value of $0.40 \pm 0.32\%$, followed by Group 5 ($4.62 \pm 0.35\%$). The highest expression of E-selectin was found in Group 3, with $16.92 \pm 0.32\%$ (Figure 4).

There were significant differences in E-selectin expression levels between all groups based on the One-Way ANOVA test results ($p < 0.001$). The results of the post-hoc analysis showed that the administration of *A. paniculata* leaf extract pre-treatment at a dose of 500 mg/kgBW significantly reduced E-selectin expression compared to the group that received 200 and 400 mg/kgBW ($p < 0.001$) (Figure 5).

Discussion

ICAM-1 expression in the lungs of Wistar rats that were not given *A. paniculata* leaf extract was significantly higher than the healthy control group with $p < 0.001$. ICAM-1, or commonly referred to as Cluster of Difference 54 (CD54), is a protein that plays a role in mediating bonds within cells and with the extracellular matrix. ICAM-1 levels increase with vascular endothelial cell damage in patients with sepsis.(28)

Based on the results, ICAM-1 expressions in the group that received *A. paniculata* leaf extract at a dose of 200, 400, and 500 mg/kg BW were lower than the sepsis control group ($p < 0.001$). There were significant differences between the *A. paniculata* leaf extract treated groups that received a dose of 200, 400, and 500 mg/kgBW. These results are consistent with previous study, which reported that the administration of Andrographolide, which is one of the active substances in *A. paniculata*, reduced ICAM-1 and TNF- α expression levels.(28) Administration of ethanol extract from *A. paniculata* also shown to significantly decreased the expression of ICAM-1 and TNF- α levels in

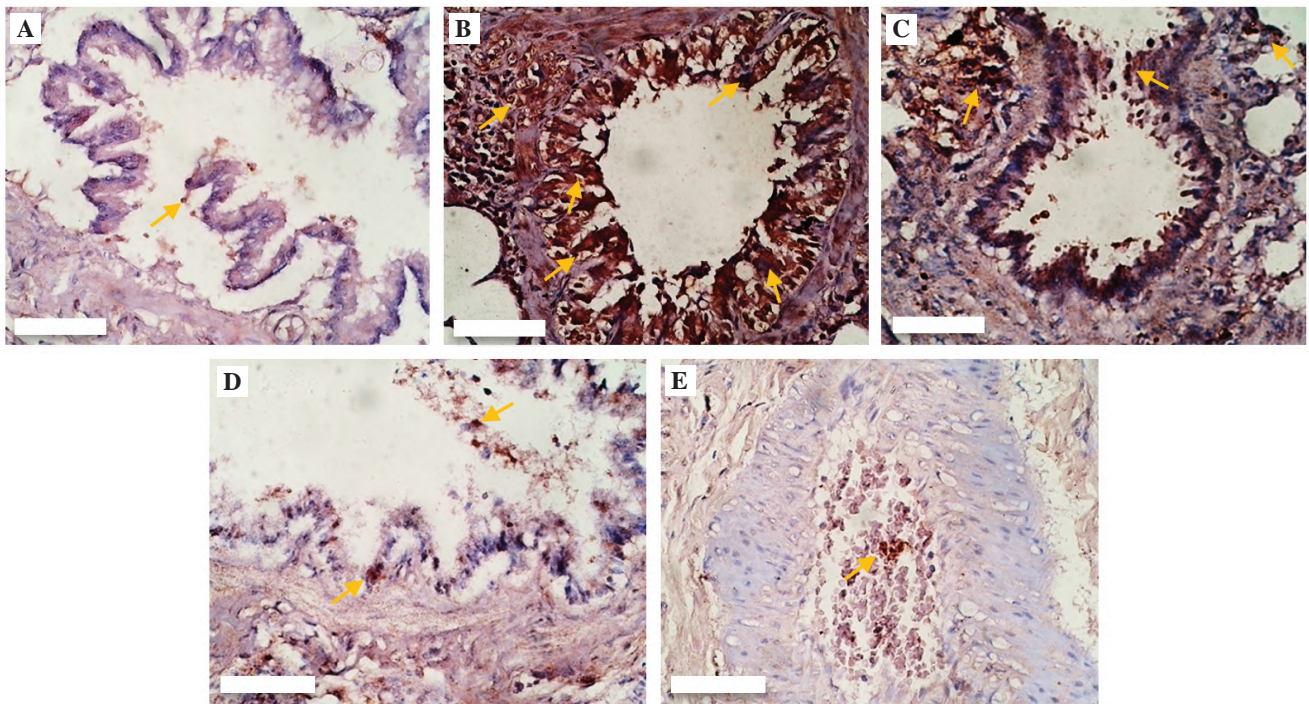


Figure 2. IHC staining results of ICAM-1 antibody on lung tissue of Wistar rats. ICAM-1 expression area fraction was calculated using ImageJ software. A: non-sepsis control (Group 1); B: sepsis control (Group 2); C: 200 mg/kgBW *A. paniculata* leaf extract (Group 3); D: 400 mg/kgBW *A. paniculata* leaf extract (Group 4); E: 500 mg/kgBW *A. paniculata* leaf extract (Group 5). White bar: 50 µm. Yellow arrow: the area of ICAM-1 expression.

EA.hy926 cells through inhibition of the IKK/I κ B/ NF- κ B activation pathway.(29) Administration of Andrographolide to cells at risk of endothelial damage led to the activation of the PI3K/Akt and endothelial nitric oxide synthase (eNOS) pathways culminating in the production of Nitric Oxide (NO) and cyclic guanosine-3',5'-monophosphate (cGMP), and decreased expression of NF- κ B, and expression of ICAM-1 levels.(30-32) Administration of andrographolide with a

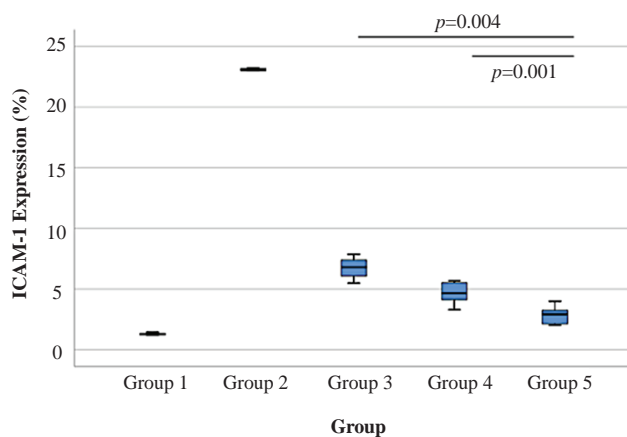


Figure 3. Boxplot graph of ICAM-1 expression in different groups. The p -value for all group comparison other than Group 3 vs. Group 5 and Group 4 vs. Group 5 are $p < 0.001$, based on Benferonni post-hoc analysis.

dose of 1 mg/kgBW for 7 days successfully downregulates specific antibody expression in response to LPS in rats. (33) *A. paniculata* stem extract with a dose of 200 mg/kgBW were comparable with 10 mg/kgBW Ibuprofen (as a standardized anti-inflammatory drug), hence, proved another anti-inflammatory effect of this extract.(34)

ICAM-1 functions in mediating the relationship between cells and the extracellular matrix. It is also released in a series of processes (cascade) caused by damage to the endothelium in septic conditions in response to LPS which triggers the release of several pro-inflammatory cytokines and increases ICAM-1 expression. Various studies mentioned that the expression of ICAM-1 increased in patients with sepsis. The expression of ICAM-1 has high sensitivity and specificity in detecting sepsis, hence, it is considered a good biomarker and effective in the diagnosis of sepsis. Moreover, ICAM-1 expression is increased with the rising severity of sepsis. High mortality rates were found in septic patients with high ICAM-1 expression. This is due to the inactivation of the endogenous fibrinolytic system which is a consequence of elevated plasminogen activator inhibitor-1 (PAI-1) levels. In the pathogenesis of sepsis, PAI-1 plays an important role in the biological process of organ dysfunction.(28)

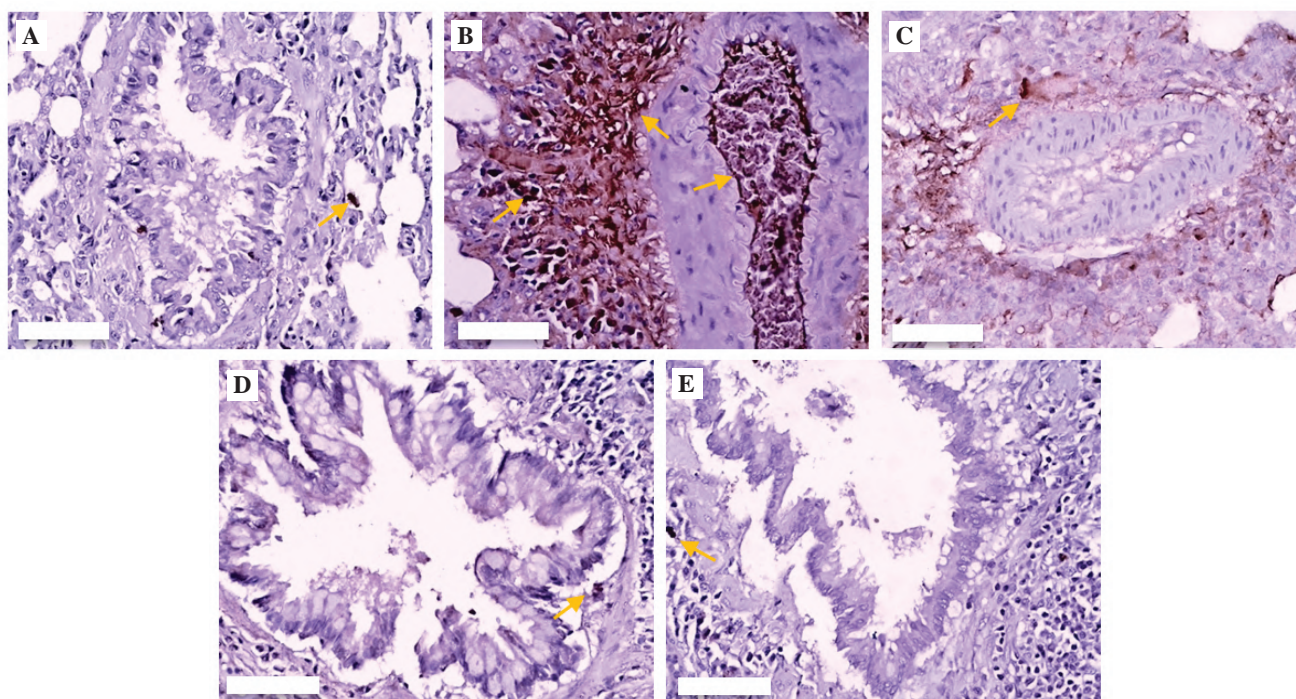


Figure 4. IHC staining results of E-selectin antibody on lung tissue of Wistar rats. E-selectin expression area fraction was calculated using ImageJ software. A: non-sepsis control (Group 1); B: sepsis control (Group 2); C: 200 mg/kgBW *A. paniculata* leaf extract (Group 3); D: 400 mg/kgBW *A. paniculata* leaf extract (Group 4); E: 500 mg/kgBW *A. paniculata* leaf extract (Group 5). White bar: 50 μm. Yellow arrow: the area of E-selectin expression.

The administration of *A. paniculata* leaf extract for 14 days at a dose of 200, 400, and 500 mg/kgBW before LPS-induced sepsis significantly reduced ICAM-1 expression. This is because andrographolide compounds found in *A. paniculata* leaf extract increase cellular antioxidant activity, have anti-inflammatory and chemopreventive properties. (28) Furthermore, the lowest ICAM-1 expression was found in the group that received the leaf extract dose of 500 mg/kgBW. This condition indicates that *A. paniculata* leaf extract acts dose-dependently on ICAM-1 expression and

the most common dose widely consumed by the general public is 500 mg/kgBW.

E-selectin expression in sepsis control was higher than the non-sepsis control group. It was known that increased levels of E-selectin expression were found as part of a heterogeneous response to the administration of LPS in rats. An increase in the levels of endothelial cell adhesion molecules, such as E-selectin and ICAM-1 as mediators of extravasation from leukocytes during the inflammatory process was reported.(35,36)

Sepsis causes a systemic inflammatory response which occurs in response to the presence of LPS, such as during microvascular inflammation and secondary endothelial dysfunction. E-selectin expression is closely related to the severity of sepsis measured by mortality and morbidity rate. The higher the expression of E-selectin the more severe the sepsis. This condition indicates the occurrence of endothelial cell activation in sepsis.(37) Shedding of E-selectin is believed to decrease the number of leukocyte-endothelial interactions by reducing the cell surface density of endothelial cells. This shedding process also produces a substance that competes with leukocytes in the bloodstream, ultimately reducing harm to the host.(38) Furthermore, E-selectin plays a role in regulating leukocyte turnover in

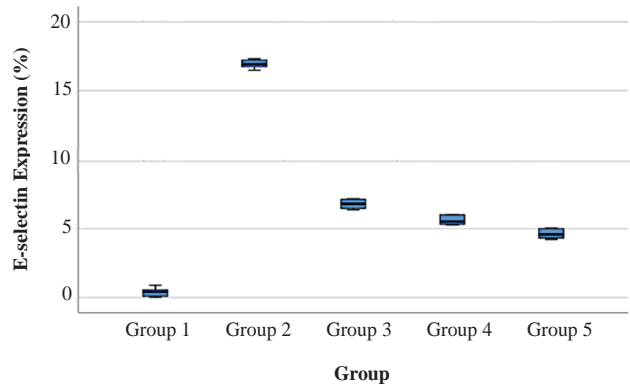


Figure 5. Boxplot graph of E-selectin expression different groups. The *p*-value for all group comparison *p*<0.001.

the endothelium, while ICAM-1 functions in leukocyte attachment. The inflammation in septic conditions causes the production and increase in the number of pro-inflammatory cytokines and elevated levels of E-selectin and ICAM-1 molecule that plays an important role in rolling, adhesion, and displacement of polymorphonuclear leukocytes (PMN).(36) The leukocyte count, as well as E-selectin, and ICAM-1 expression levels are all increased in septic conditions.(4,39)

Moreover, andrographolide can reduce expression levels of tissue factors, such as E-selectin and ICAM-1, and also reduce the risk of vascular damage and adhesion by lowering the expression level of E-selectin. Another study found that the administration of andrographolide at a dose of 200 mg/kgBW for 2 days significantly reduced E-selectin expression levels and decreased matrix metalloproteinase (MMP)-9 expression levels.(11,35,40)

Results of this study showed that the administration of *A. paniculata* leaf extract for 14 days at a dose of 200, 400, and 500 mg/kgBW before LPS-induced sepsis significantly reduced E-selectin expression. This was due to the presence of andrographolide that suppressed the production of NF- κ B and TNF- α in endothelial cells under inflammatory conditions. The lowest E-selectin expression was found in the group that received *A. paniculata* leaf extract at a dose of 500 mg/kgBW compared to 200 and 400 mg/kgBW. This indicates that the *A. paniculata* leaf extract acts in a dose-dependent manner on the expression of E-selectin.

Based on the results of post-test only measurement of ICAM-1 and E-selectin, it is suggested that the administration of *A. paniculata* leaf extract before sepsis might protect lung tissue against damage. However, a further study assessing the relationship of immunohistochemistry analysis and other serum sepsis markers after *A. paniculata* leaf extract administration in sepsis model might be necessary to provide a clearer knowledge of sepsis marker expressions.

Conclusion

The administrations of *A. paniculata* leaf extract for 14 days at a dose of 200, 400, and 500 mg/kgBW before induction of sepsis decrease the ICAM-1 and E-selectin expression in the lung tissue, with the most effective dose of 500 mg/kgBW. The administration of *A. paniculata* leaf extract before sepsis can protect lung tissue against damage by modulating the expression of proinflammatory mediators in lung tissues.

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Authors Contribution

MK, NSW, SB, YWP, and NS were involved in concepting and planning the research. NSW and NS performed the data acquisition/collection. SB and YWP calculated the experimental data. MK performed the analysis, drafted the manuscript, designed the figures, and aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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