Biomedical & Pharmacology Journal, March 2025. Vol. **18**(1), p. 671-680

The Effects of Andrographis paniculata Leaf Extract on NF-κB and iNOS Expression in the Heart of Lipopolysaccharide-Induced Septic Rats

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https://dx.doi.org/10.13005/bpj/3118

(Received: 06 September 2024; accepted: 19 March 2025)

Sepsis is a severe medical condition characterized by an excessive inflammatory response to infection, often leading to organ failure, particularly in the heart. Cardiac dysfunction in sepsis results from multiple mechanisms and significantly worsens patient prognosis. Given the limited therapeutic options for sepsis, there is an urgent need to explore new substances that can help modulate the inflammatory response. This study aimed to evaluate the effects of Andrographis paniculata on transcription factors and proinflammatory cytokines, specifically NF-?B and iNOS. This study used a 100% pure, standardized Andrographis paniculata leaf extract with andrographolide content of 5.68%. The extract was diluted in 1.0% Na-CMC to a total volume of 100 mL and administered orally. A negative control group received 100 mL of 1.0% Na-CMC. Lipopolysaccharide (LPS) was injected intraperitoneally to induce inflammation. Thirty male Wistar rats were randomly divided into five groups (n=6 per group): K1 (positive control), P1 (negative control), P2 (200 mg extract), P3 (400 mg extract), and P4 (500 mg extract). The treatment lasted 21 days, after which histopathological samples were analyzed, and NF-?B and iNOS levels were measured using immunohistochemical staining. All three dosages of Andrographis paniculata extract significantly reduced NF-?B and iNOS expression. The highest dosage group (500 mg) showed the most substantial decrease in expression compared to the other treatment groups. Andrographis paniculata leaf extract effectively reduced NF-?B and iNOS expression in a sepsis-induced animal model, highlighting its potential as an anti-inflammatory agent in sepsis management

Keywords: Andrographis paniculata; Andrographolide; iNOS; NF-kB; Sepsis.

Sepsis is a severe medical condition characterized by a strong inflammatory response to infection. Sepsis can cause cardiac dysfunction through multiple pathophysiological mechanisms, such as inflammatory pathways, endothelial dysfunction, oxidative stress, mitochondrial

dysfunction, and immune paralysis. The inflammatory pathway releases inflammatory mediators that can disrupt the contractility of cardiac muscle cells and calcium signaling. Endothelial dysfunction exacerbates the condition through increased vascular permeability, impaired vasodilation, and activation of the pro-coagulant pathway. Sepsis can also induce the production of reactive oxygen species (ROS), leading to oxidative stress in the heart. Additionally, mitochondrial dysfunction and immune paralysis contribute to the development of cardiac dysfunction, which is associated with a poorer prognosis.[1–3] The incidence of sepsis increases by approximately 9.0% annually, with 600,000 cases in 2000 rising to over 1,000,000 hospitalizations in 2008. Healthcare expenditures followed suit as one of the most expensive medical conditions in 2009, comprising 5.0% of total hospital expenses in the United States. Advancements in sepsis management introduced by the Surviving Sepsis Campaign have contributed to a decrease in mortality rates from 16.50% in 2009 to 13.80% in 2012. However, severe cases still lead to fatal outcomes in 25.0% of patients and 50.0% of patients with septic shock. This rate is influenced by the speed of treatment, demographic factors including age, ethnicity, and gender, as well as pre-existing health conditions and organ dysfunction. Inpatient mortality is predominantly predicted by the degree and extent of organ damage, especially respiratory, cardiovascular, liver, and neurological organ.[4] In the United States, cohort studies show over 2 million cases of sepsis occur each year, with approximately 30.0% receiving sepsis diagnosis, while septic shock occurs in around 19 per 1000 inpatient visits. This number was reported to have increased by nearly 50.0% over the past decade according to ICD-9.[4]

Sepsis initiates when pathogens trigger an inflammatory response, primarily recognized by macrophages and surface receptors on polymorphonuclear leukocytes (PMNs). Pathogen-associated compounds like lipopolysaccharides and exotoxins can stimulate this response by inducing the production of enzymes such as inducible nitric oxide synthase (iNOS) and the nuclear transcription factor NF-êB.[5,6] iNOS is an enzyme possessing the ability to catalyze the production of nitric oxide (NO) from arginine in response to various stimuli, including endotoxins and bacterial cytokines. NO is a potent vasodilator and plays a crucial role in regulating blood vessel tone and blood flow. Excessive NO production by iNOS is implicated in the pathogenesis of sepsis, potentially leading to vasodilation, hypotension, and tissue damage. Additionally, NO can interact with superoxide to form peroxynitrite, a highly reactive molecule that contributes to oxidative damage of biomolecules.[7,8] NF-êB is a transcription factor that regulates the expression of various genes in inflammation, immunity, and cell survival by processes such as translocating into the nucleus, where it binds to specific DNA sequences. During sepsis, NF-êB plays a key role in immune response by regulating the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. However, excessive activation can lead to prolonged inflammation, tissue damage, and organ dysfunction.[9]

The roles of iNOS and NF-êB in sepsis have been extensively investigated through experimental and clinical studies, providing insights into their mechanisms of action and therapeutic potential. While iNOS inhibition has shown potential in reducing mortality and improving hemodynamic parameters in animal models, clinical trials have not yielded successful results. Targeting NF-êB signaling appears promising, as several agents inhibiting NF-êB activation or downstream pathways have improved survival rates and reduced inflammation in animal models of sepsis.[10] In a previous study conducted on rats, the use of liquid *Andrographis paniculata* extract indicates its ability to reduce the regulation of p38 MAPK, STAT3, and NF-êB in the circulatory system rate. NF-êB triggers the inflammatory response by activating IL-1â, which in turn stimulates iNOS production and promotes inflammation.[11] iNOS and NF-êB levels may serve as valuable markers to evaluate the effects of *Andrographis paniculata* leaf extract in sepsis management.

Andrographis paniculata is a widely used medicinal plant, classified as 'jamu' in Indonesian traditional herbal medicine and traditionally known as 'sambiloto'. [12–16] It contains andrographolide compounds, which are believed to modulate inflammatory responses and mitigate the side effects of chemical treatments.[17–22] This extract in pill or powder form is widely available and sold

over the counter and in traditional market stalls and supermarkets.[12–16] Research has investigated the anti-inflammatory and immunomodulatory effects of *Andrographis paniculata* and its bioactive compounds, particularly their influence on NF-êB signaling and adhesion molecule expression. Studies have shown that a semisynthetic diterpenoid lactone inhibits NF-êB signaling, reducing inflammation and airway hyperresponsiveness in a mouse asthma model, indicating its potential as an anti-inflammatory agent. Furthermore, studies reported that andrographolide, a major bioactive compound of *Andrographis paniculata*, inhibits the adhesion of gastric cancer cells to endothelial cells by blocking E-selectin expression, indicating a role in preventing tumor metastasis. Additionally, examined the expression of microRNA-23b in septic patients and found its regulatory effects on leukocyte activity, E-selectin, and ICAM-1 expression, demonstrating its involvement in modulating immune responses during sepsis. These findings highlight the potential of *Andrographis paniculata* in regulating NF-êB and inflammation-related pathways, which may be relevant in understanding its effects on NF-êB and iNOS expression in the heart of lipopolysaccharide-induced septic rats.

MATERIALS AND METHODS

Study Design

Andrographis paniculata plant identification was conducted by Medanense Herbarium (MEDA), affiliated with the Faculty of Mathematics and Natural Sciences, University of North Sumatra, Indonesia. The Andrographis paniculata plant is categorized as follows: Kingdom: Plantae Division Class Order Family Genus Species: Spermatophyta: Dicotyledonae: Lamiales: Acanthaceae: Andrographis: Andrographis paniculata (Burm. fil.) Ness. Local Name: Sambiloto. The target population for this study consisted of male Wistar rats aged 2-4 months, with a weight range of 150 to 200 grams. These rats were obtained from the Central Inter-University Experimental Animal Laboratory at the Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. The inclusion criteria were male Wistar rats, aged 2-3 months, weighing 150-200 grams, and showing good health, activity, and normal behavior. Rats that were sick during the study or had anatomical abnormalities were excluded. Sample size calculations followed WHO guidelines and the Institutional Animal Care and Use Committee Guidebook. The sample consists of 5 groups of 5 rats each and an additional 20.0% (1 rat) as a backup per group. Consequently, the total number of male Wistar rats across all test groups was 30, with 5 groups of 6 rats each. The *Andrographis paniculata* leaf extract was a pure 100.0% standardized powdered leaf extract with andrographolide content of 5.68% obtained from PT Jamu Sido Muncul, a reputable herbal extract company. *Andrographis paniculata* leaf extract was diluted into a solution of 100ml 1.0% Na-CMC in water.

Rats Model of Sepsis

Male Wistar rats were selected due to their cost-effectiveness, small size, physiological similarity to humans, and stability against the influence of menstrual cycles and pregnancy. Before the study commenced, facilities for housing the experimental animals were provided, including cages, wood shavings, food and water containers, as well as rodent feed. The rats were acclimatized for 7 days in a room with a controlled temperature of 28.0 ± 2.0°C, a 12-hour light cycle from 09:00 to 21:00, and provided with ad libitum food and water. After 7 days of acclimatization, *Andrographis paniculata* leaf extract is diluted in a solution of 1.0%Na-CMC in 100mL of water and administered orally once every day for 14 days to ensure even intake and minimize the effects of external factors over time.[23] The same solution *Andrographis paniculata* leaf extract is administered as a placebo for the control groups. The rats were then administered a lipopolysaccharide injection intraperitoneally at 5 mg per kilogram of body weight on day 22. During the injection, the position of the rats was adjusted so that their heads were lower than their abdomens, and the needle was inserted at an angle of approximately 100 degrees from the abdomen. The injection site is located slightly off-center from the midline to prevent bladder and liver exposure. The side effect of this injection is short-lived discomfort as the injected lipopolysaccharide is quickly absorbed into the circulatory system of the rats.

Time and Location of The Study

The sample collection is carried out over

25 days, including the acclimatization of the rats in a laboratory environment and tissue sample collection. Sepsis induction and the extraction of myocardium were conducted at the Central University Experimental Animal Laboratory located at the Faculty of Medicine, Gadjah Mada University, Yogyakarta. Histopathological preparations in the form of paraffin blocks of myocardium tissue samples were collected at the Stem Cell and Cancer Research Laboratory, Semarang, Central Java. The scope of the study is limited to the histological analysis of the extent of tissue stress induced by increased levels of iNOs and NF-êB in the sample. This study does not observe other physiological or behavioral effects on the study sample or tissue damage outside of the heart.

Study Variables

The independent variable of this study was *Andrographis paniculata* leaf extract, and the dependent variables were iNOS and NF-kB transcription factor expression.

Treatment Procedure

This study had five groups of rats, each receiving different treatments supported by previous study of safety evaluation of AP-Bio®, a similar widely available andrographolide product with median lethal dose (LD_{50}) to be more than 5000 mg/kg rat body weight[24] and median effective dose (ED50) of aqueous andrographolide in heart tissue to be 8.6 mg/kg.[25] The healthy control group (K) was orally administered a standard diet for 14 days, with 1.0%Na-CMC in 100mL of water as a placebo once a day and then induced with 0.5 mL of NaCl intraperitoneally on day 22. The negative control group (K-) was also fed a standard diet for 14 days, administered with 100mL of 1.0%Na-CNC in water as a placebo. The treatment groups 1 (P1), 2 (P2), and 3 (P3) received a standard diet and *Andrographis paniculata* leaf extract dilution in 100mL of 1.0%Na-CNC in water for 14 days. The dose is adjusted so that P1 received a dose of 200 mg/kgBW, P2 received 400 mg/kgBW, and P3 received a dose of 500 mg/kgBW. On day 22, the negative control group and all three treatment groups were injected with lipopolysaccharide intraperitoneally at a dose of 5 mg/kgBW. The collection of myocardium tissue samples, rich in blood vessels, was carried out on day 25 of the study. Euthanasia was performed on Wistar rats using chloroform prior to the sample collection, and this method was chosen due to the availability and reliability of chloroform in euthanizing rats.[26] Thoracotomy is then performed to retrieve the heart tissue. The tissue was rinsed with sterile NaCl to remove any remaining blood and placed in a container/pot containing a 10.0% formalin solution, followed by histopathological preparations in the form of paraffin blocks. Meanwhile, blocks that were previously cut using a microtome had a thickness of 4 ìm and were placed on glass slides that had undergone a coating process. The coating process was carried out by placing glass slides that had been coated with poly-L-lysine on top of the paraffin blocks. Subsequently, the blocks were incubated at a temperature of 37°C overnight. After the coating process was completed, the blocks on the glass slides passed through staining using the immunohistochemistry method with the streptavidin-biotin method. This method was chosen to increase the retention of cardiac cells in the glass slide preparate. The next step was deparaffinization by immersing the blocks in xylene four times for 5 minutes each. Rehydration was carried out with absolute ethanol, 96.0% ethanol, and 70.0% ethanol, each for 5 minutes, followed by washing with distilled water for five minutes and then rinsing with running water. Further washing with distilled water for five minutes was performed, followed by washing with phosphate-buffered saline (PBS) solution twice for five minutes. Antigen retrieval was carried out using a microwave oven with Tris EDTA pH 9 at a temperature of 90°C for 3 minutes, followed by a low temperature for 10 minutes. After reaching a low temperature, the blocks were cooled and washed with PBS twice for 5 minutes each. Endogenous peroxidase methanol H2O2 3.0% was added for 20 minutes, and washed with running water. Blocking serum was added for 10 minutes, and the block was drained. Finally, the NF-êB p65 Polyclonal Antibody from ThermoFisher-Invitrogen and Anti-iNOS antibody (EPR 16635) ab178945 from Abcam, were applied. The results of the study were evaluated based on the percentage of staining area fraction (%) read at 400x magnification under a light microscope using ImageJ software.

Data Analysis

After data collection, cleaning, coding,

and tabulation of data were carried out. Analyzing data included testing hypotheses and descriptive analysis. Data on the iNOS and NF-kB expression were reported in terms of mean and standard deviation. The Shapiro-Wilk test was used to perform the normality test, and the Levene test was employed to carry out the homogeneity test. Since the data obtained were normally distributed and homogeneous, a comparative analysis between groups was performed using the parametric ANOVA test and the Bonferroni post hoc test. All data analyses were performed using SPSS version 26.0 for Windows.

RESULTS

The expression of iNOS and NF-kB was assessed using Immunohistochemistry (IHC). Normality tests using the Shapiro-Wilk method indicated that the levels of iNOS and NF-kB followed a normal distribution. Consequently, further comparative analysis was conducted using

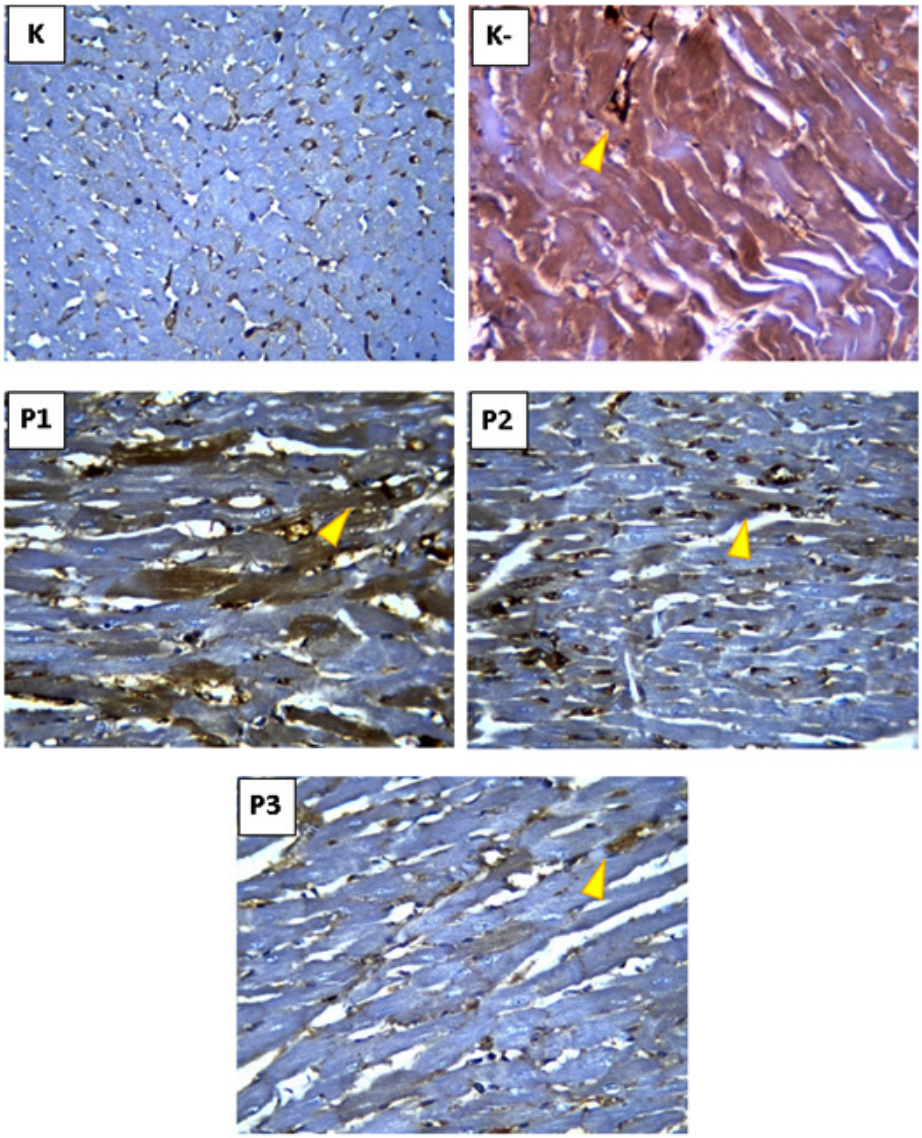

Fig. 1. Results of immunohistochemical staining for iNOS expression (yellow arrow) in the heart of Rattus norvegicus rats in the sepsis model

one-way ANOVA as demonstrated in table 1 and table 2. The results of one-way ANOVA showed that the levels of iNOS and NF-kB had a significant difference with a p-value of <0.001, both below the 0.05 significance level. The Bonferroni post-hoc test for iNOS expression indicated significant differences between all groups. The results showed significant differences in iNOS and NF-kB expression among the treatment groups. This indicated the potential impact of the administered treatments on the inflammatory markers.

DISCUSSION

Inducible Nitric Oxide Synthase (iNOS) is an enzyme that produces nitric oxide (NO) in response to various types of cells, including immune and non-immune cells, as well as stimuli such as inflammation and infection. While the production of nitric oxide (NO) is essential for maintaining the normal structure and function of blood vessels, produced by both parenchymal (functional) and hematopoietic (blood-forming)

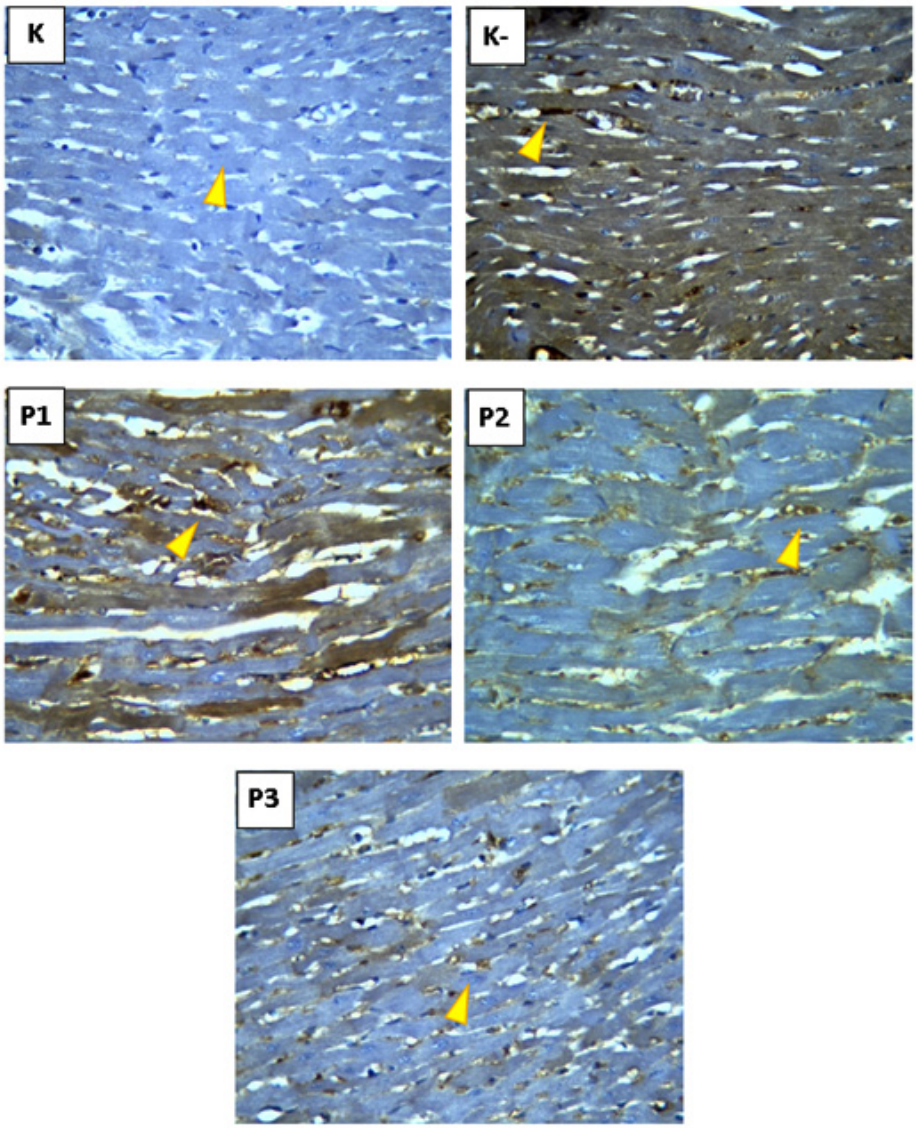

Fig. 2. Results of immunohistochemical staining for NF-kB expression (yellow arrow) in the heart of Rattus norvegicus rats in the sepsis model

Table 1. Descriptive Analysis with One-Way ANOVA test for iNOS and NF-kB

Groups	P value iNOS	P value NF-kB
K	0.574*	0.906*
K-	0.751*	0.332*
P1	0.961*	0.305*
P2	0.516*	0.286*
P3	0.420*	0.362*

Table 2. Analysis of One-Way ANOVA test for iNOS and NF-kB

Groups	Mean ± SD NF-kB	Mean ± SD iNOS
K	11.42 ± 1.17	4.00 ± 0.96
K-	44.54 ± 3.38	38.32 ± 2.43
P1	34.32 ± 2.84	28.79 ± 1.13
P2	25.08 ± 0.91	24.76 ± 0.69
P3	16.10 ± 1.79	12.46 ± 2.22

cells, several inflammatory molecules might trigger and activate the nitric oxide synthase isoform–iNOS, which may function beyond its intended physiological role. Furthermore, iNOS can also generate reactive nitrogen species (RNS) under certain conditions, potentially causing further damage due to its dual role as an immunotoxin and a signaling molecule in the cardiovascular process.[27,28]

The expression of iNOS in the hearts of Wistar rats fed ad libitum and injected with 5 mg/kg BW lipopolysaccharide (LPS) intraperitoneally was significantly higher compared to groups that were intraperitoneally injected with a 0.9% NaCl solution and the healthy control group (K(S)). This difference was highly statistically significant ($p < 0.001$). At the same time, the expression of iNOS in the groups that received *Andrographis paniculata* leaf extract (P1, P2, P3) were significantly lower than in the negative control group (K(-)), with a statistical significance of $p<0.001$). Among the administered groups, a statistically significant difference was observed between (P3), (P1), and (P2), with p-values of $p<0.001$ and $p = 0.001$, respectively. This result is consistent with other research observing the effects of andrographolide compounds in other conditions that elevate iNOS levels. A study examining the use of andrographolide to inhibit induced iNOS levels in patients with cervical cancer, which showed a decrease in iNOS levels with the administration of andrographolide compounds.[29] Dehydroandrographolide (DA), one of the major components of *Andrographis paniculata*, was found to induce autophagy and caused cell death in human oral cancer cells by inhibiting iNOS.[30] According to another study the administration of Andrographolide to endothelial risk cells activated the PI3K/Akt pathway and iNOS, leading to the production of *Nitric Oxide* (NO) and cGMP, while reducing NF-kB expression and ICAM-1 expression levels.[31]

NF-êB (*Nuclear Factor-Kappa B*) is a family of transcription factors, which regulate gene expression within cells. The primary function of NF-êB is to regulate the expression of genes in various biological processes, including the immune response and inflammation, particularly in the context of sepsis. Furthermore, it plays a crucial role in maintaining the balance and proper immune response of the immune system to infections and tissue damage.[30] The role of NF-kB in sepsis has been well-documented in previous studies.

The expression of NF-kB in the hearts of Wistar rats fed ad libitum and injected with 5 mg/kg BW lipopolysaccharide (LPS) intraperitoneally was significantly higher than in the groups of rats that received ad libitum feeding and were intraperitoneally injected with a 0.9% NaCl solution or the healthy control group (K(S)). This difference was highly statistically significant ($p < 0.001$). These findings align with previous studies regarding the role of the NF-kB system in response to various medical conditions. study on bladder cancer patients found that andrographolide suppressed the activation of the NF-kB system.[32] Andrographolide also showed the ability to inhibit TNF-alpha production by inhibiting the NF-kB system.[32]

Administration of emodin for Acute Lung Injury (ALI) in sepsis patients has demonstrated that NF-êB inhibition reduces proinflammatory cytokine production, alleviating sepsis.[33] Another study reported that the suppression of NF-kB expression was associated with an improvement in the sepsis condition in experimental animals.[34] NF-êB is responsible for regulating the transcription of a large number of genes, and the products of these

genes play a crucial role in the pathophysiology of sepsis. Another study with rats lacking NF-êB-dependent genes demonstrated resistance to septic shock and mortality. The inhibition of the NF-êB pathway improved sepsis-related abnormalities, thereby restoring normal blood pressure, improving heart function, and stabilizing blood vessels during sepsis. Furthermore, it reduced proinflammatory gene expression, blood clotting, neutrophil infiltration, and vascular leakage. The inhibition of NF-êB activation also prevented damage to multiple organs and improved survival in septic shock animal models.[35]

CONCLUSION

In conclusion, this study demonstrated the beneficial effects of *Andrographis paniculata* leaf extract as a pre-treatment in a sepsis-induced Wistar rat model. The results showed that the administration of *Andrographis paniculata* leaf extract for 14 days significantly reduced the expression of *inducible Nitric Oxide Synthase* (iNOS) and *Nuclear Factor-Kappa B* (NF-kB) in the heart tissues of the sepsis model rats compared to the negative control group. This reduction suggested that *Andrographis paniculata* leaf extract pre-treatment may mitigate sepsis severity, leading to a decreased mortality rate associated with sepsis. Additionally, the higher dosage of *Andrographis paniculata* leaf extract (500 mg/kgBW) yielded more pronounced effects in lowering iNOS and NF-kB expression, confirming its potential as a therapeutic intervention to combat sepsis-related complications.

These results provided insight into the pharmacological properties of *Andrographis paniculata* leaf extract and its promising role in modulating the inflammatory and immune response pathways in sepsis. Moreover, further investigations and clinical studies are recommended to explore the translational potential of *Andrographis paniculata* extract in sepsis management and to elucidate the underlying molecular mechanisms contributing to its therapeutic effects.

The limitations of this study included the omission of an in-depth investigation into the molecular mechanisms underlying the impact of *Andrographis paniculata* leaf extract (Sambiloto) on sepsis, particularly regarding iNOS and NF-kB, as well as the omission of the probable, wider physiological and behavioral effects on the rats. The experiment was conducted in vivo on Wistar rats, thus requiring further studies on its application to human subjects. Additionally, more study is required to examine other pro-inflammatory cytokines such as iNOS and NF-kB levels, to substantiate the influence of *Andrographis paniculata* leaf extract (Sambiloto) on the heart tissues of sepsis model animals.

ACKNOWLEDGMENT

The authors would like to thank Faculty of Medicine Diponegoro University for granting this postgraduate research work. We also extend our gratitude to the Biomedical Science Postgraduate Program, Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia, for providing the academic environment and resources that facilitated this research.

Funding Disclosure

This research was conducted as a part of work supported by Universitas Diponegoro research grant number 1664/UN7.5.4.2/PP/2021.

Conflict Of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This study received ethical approval from the Experimental Animal Laboratory at the Faculty of Medicine, Diponegoro University with Ethical Clearance No.113/EC-H/KEPK/FK-UNDIP/IX/2023.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

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Not Applicable.

Author Contributions

Johan Eko Saputro: Data Collection,Writing, Analysis, Funding Acquisition;

Selamat Budijitno: Supervision, Funding Acquisition; Nani Maharani: Supervision, Project Management; Erlangga Pradipta Harianto: Analysis, Writing, Review and Editing; Neni Susilaningsih: Conceptualization, Methodology, Writing; Nyoman Suci Widyastiti: Corresponding Author, Conceptualization, Methodology, supervision, Writing.

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