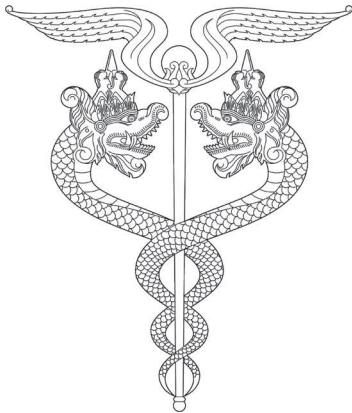


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RESEARCH ARTICLE

***Andrographis paniculata* Leaves Extract Inhibit TNF- α and Caspase-3 Expression of Septic Rats' Intestinal Tissues**

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Abstract

BACKGROUND: Microcirculation and cellular disturbances caused by sepsis might trigger significant intestinal damage. *Andrographis paniculata* extract decreases inflammatory intestinal epithelial cells with its role as an antiparasitic and anti-inflammatory agent. However, *A. paniculata* extract's effect on sepsis have not been commonly studied, especially in the intestinal tissues. Therefore, this study was conducted to determine *A. paniculata* leaves extract (APLE) effect in sepsis-induced intestinal tissues of rats by examining the expression of inflammatory cytokines involved in sepsis, namely tumor necrosis factor (TNF)- α and Caspase-3.

METHODS: Rats were divided into five groups; two groups received no pretreatment and the other three groups received 200, 400, and 500 mg/kg BW/day APLE, respectively. Three pretreated groups and one group with no pretreatment were then injected with 1 mg/200 g BW lipopolysaccharides (LPS) intraperitoneally to create septic rat models. Three days after the LPS-induction, rats were euthanized and the expression of TNF- α and Caspase-3

were assessed based on the immunohistochemical staining of rats' intestinal tissues.

RESULTS: Compared with NaCl (sham), LPS significantly ($p < 0.001$) induced TNF- α expression from 6.60 ± 1.36 to 25.37 ± 1.74 . Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced TNF- α expression (18.82 ± 1.36 , 11.45 ± 1.18 , and 6.89 ± 1.90 , respectively). Similar with TNF- α , compared with NaCl (sham), LPS significantly ($p < 0.001$) induced Caspase-3 expression from 6.92 ± 1.66 to 23.59 ± 2.25 . Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced Caspase-3 expression (17.47 ± 1.68 , 12.99 ± 1.51 , and 5.59 ± 1.51 , respectively).

CONCLUSION: The pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent.

KEYWORDS: *Andrographis paniculata*, sepsis, TNF- α , Caspase-3, lipopolysaccharide

Indones Biomed J. 2024; 16(1): 66-71

Introduction

Sepsis has been associated with substantial morbidity and mortality. This condition has become one of the growing global burdens with its complexity. The World Health Organization (WHO) has reported a significant increase of sepsis-related deaths throughout the years, with the alarming incidence rate made sepsis as a global health priority.(1) Although significant improvement and advancement have been made in sepsis management, the mortality risk remains high and many survivors never recovered fully, which led them to long-term morbidities.(2) These problems highlight the need for novel alternative treatment that could augment or enhance current strategies.

Sepsis is a condition of dysregulated host response to infection (3), which is predominantly caused by Gram-positive bacteria. The most frequently isolated bacteria are *Staphylococcus aureus* and *Streptococcus pneumoniae*. (4) Considering the complexity of sepsis, a multifactorial mechanism has been thought to elicit the condition, which involves a variety of pro- and anti-inflammatory mediators. Microcirculation, cellular, and coagulation impairment cause tissue hypoperfusion that leads to tissue damage.(5) Previous studies showed that sepsis causes reduced blood flow to the digestive organs, inflicting a rapidly occurred ischemia and leads to acute intestinal damage, especially in the colon. Many inflammatory cytokines are involved in sepsis, mainly tumor necrosis factor (TNF)- α and Caspase-3. (6) Studies showed that Caspase-3 inhibition leads to reduced TNF- α production in various cell types, making it a potential therapeutic target for inflammatory diseases.(7,8) These findings highlight the promising potential and benefits of TNF- α and Caspase-3 as a new therapeutic approach in reducing sepsis-associated morbidity and mortality.(9)

Traditional medicines made from botanical substances or herbal have been extensively utilized for their many potencies (10-14), particularly in developing nations. WHO reported an approximately 65% of individuals in developing nations incorporated herbal medicines in their healthcare practices.(15) *Andrographis paniculata*, in Indonesia known as Sambiloto, is a type of herbal plant often used in traditional medicine due to its anti-inflammatory, antioxidant, and immunomodulatory properties.(16,17) *A. paniculata* has been found to enhance bacterial clearance in an intra-abdominal sepsis that alleviated pathological organ injury induced by sepsis.(18) Administration of *A. paniculata* extract could decrease inflammatory intestinal epithelial cells, highlighting its role as an antiparasitic and

anti-inflammatory agent. Administration of *A. paniculata* could also modulate the expression of apoptotic genes such as Caspase-9 and Caspase-3.(19,20) In our knowledge, *A. paniculata* extract's effect on sepsis have not been studied, especially in the intestinal tissues. Thus, this study was conducted to determine the effect of *A. paniculata* extract in the sepsis-induced intestinal tissues of rats, by focusing on the expression of TNF- α and Caspase-3.

Methods

A. paniculata Leaves Extract (APLE) Production

APLE production was performed at PT Sido Muncul, a herbal and pharmaceutical company located in Semarang, Indonesia. Briefly, after macroscopical and microscopical ingredient and quality control checks, the simplicia (100 g *A. paniculata* leaves) was cleansed, dried, minced, macerated with 90% ethanol for 24 h and percolated with digital shaker at a speed of 50 rpm for 2 h at 60°C. Resulted filtrate was filtered and evaporated for 2 h to obtain brownish green APLE with 8% yield (8 g).

Study Design, Ethical Approval and Animal Acclimatization

An animal experimental, randomized, post-test-only with control group study was designed. The study protocol was reviewed and approved by the Medical Research and Ethics Committee Universitas Diponegoro (No. 112/EC-H/KEPK/FK-UNDIP/IX/2023). Thirty healthy male rats (*Rattus norvegicus*), aged 2-3 months, weighted 150-200 g, were obtained from Animal Laboratory of Universitas Gadjah Mada, Yogyakarta. The rats were individually caged, acclimated with standard diet of COMFEED AD II (Japfa, Jakarta, Indonesia) and drink for 7 days.

APLE Pretreatment and Lipopolysaccharide (LPS) Induction

After the 7 day-acclimatization, the rats were randomly divided into 5 groups (n=6). No pretreatment was performed for Group 1 and 2. For Group 3, 4 and 5, the rats were pretreated with 200 mg/kg BW/day, 400 mg/kg BW/day and 500 mg/kg BW/day APLE, respectively. The administration was performed orally with feeding tube for 14 consecutive days, in conjunction with standard diet. On the next day (day 22), septic induction was performed by an intraperitoneal injection of 1 mg/200 g BW LPS (Merck, St. Louis, MO, USA) for Group 2, 3, 4 and 5. For Group 1, injection was performed as well with 0.5 mL NaCl. The rats' heads were

positioned lower than the abdomen when injecting the needle at approximately 100 degrees from the surface. The injection was slightly away from the midline to avoid hitting the bladder and slightly lower to avoid the liver.

TNF- α and Caspase-3 Immunohistochemical Staining

Three days after LPS induction (day 25), the rats were euthanized using chloroform, and then a laparotomy was performed to collect intestinal tissue. The tissue samples were fixed in 10% formalin, dehydrated, and embedded in paraffin. The paraffin block was sliced into 5 μ m thick sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated with 0.5% H₂O₂ and blocked with 5% bovine serum albumin. For primary antibody, 1:100

Rabbit Polyclonal TNF- α (A0277) (ABclonal, Woburn, MA, USA) or 1:100 Mouse Monoclonal Caspase 3 (74T2) (ThermoFisher Scientific, Waltham, MA, USA) Antibody was used. For secondary antibody and streptavidin-biotin immunoenzymatic antigen detection system, Mouse and Rabbit Specific HRP (ABC) Detection IHC Kit (Abcam, Cambridge, UK) was used. For the chromogen, Diamino benzidine (DAB) Substrate Kit (Abcam) was used. Counterstaining was performed with Meyer's hematoxylin. Two pathologists, who were blinded to the study design, performed the histopathological examinations. An intraclass correlation coefficient analysis was conducted to evaluate the results. For each stained intestinal tissue section, 5 different areas were selected and documented at 100x magnification under upright light microscope. Each documented image was measured using ImageJ (US National Institutes of Health, Bethesda, MD, USA). Color deconvolution and threshold feature were set to measure protein expression.

Results

Two pathologists, who were blinded to the study design, had intraclass correlation coefficient values of $\kappa = 0.999$ for TNF- α and $\kappa = 0.998$ for Caspase-3 immunohistochemical expression assessments. This indicated a very good agreement between the assessments of the pathologists on TNF- α and Caspase-3 immunohistochemical expression.

APLE Inhibited LPS-induced TNF- α Expression of Rats' Intestinal Tissues

Under 1 mg/200 g BW LPS induction, TNF- α expression of rats' intestinal tissues was significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure

1). This indicated that the LPS could induce inflammatory rats' intestinal tissues. However, under pretreatment of APLE, the TNF- α expression of rats' intestinal tissues could be inhibited. Significant APLE inhibition on LPS-induced TNF- α expression of rats' intestinal tissues was started in concentration of 200 mg/kg BW/day (Group 3). This APLE inhibition was in concentration manner. Therefore, the TNF- α expression of rats' intestinal tissues with pretreatment of 400 mg/kg BW/day APLE (Group 4) was lower significantly than the one of 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the group that was pretreated with 500 mg/kg BW/day (Group 5) had the lowest TNF- α expression of rats' intestinal tissues. The TNF- α expression of rats' intestinal tissues in this Group 5 was similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest that the 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced TNF- α expression of rats' intestinal tissues.

APLE Inhibited LPS-induced Caspase-3 Expression of Rats' Intestinal Tissues

Under 1 mg/200 g BW LPS induction, Caspase-3 expression of rats' intestinal tissues was significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure 1). This indicated that the LPS could induce apoptotic rats' intestinal tissues. However, under pretreatment of APLE, the Caspase-3 expression of rats' intestinal tissues could be inhibited. Significant APLE inhibition on LPS-induced Caspase-3 expression of rats' intestinal tissues was started in concentration of 200 mg/kg BW/day (Group 3). This APLE inhibition was in concentration manner. Therefore, the Caspase-3 expression of rats' intestinal tissues with pretreatment of 400 mg/kg BW/day APLE (Group 4) was significantly lower than the 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the group that was pretreated with 500 mg/kg BW/day (Group 5) had the lowest Caspase-3 expression of rats' intestinal tissues. The Caspase-3 expression of rats' intestinal tissues in this Group 5 was similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest that the 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced Caspase-3 expression of rats' intestinal tissues.

Discussion

Current study showed that the induction using LPS injection could resemble sepsis condition, which increased the expression of TNF- α and Caspase-3. A similar result has

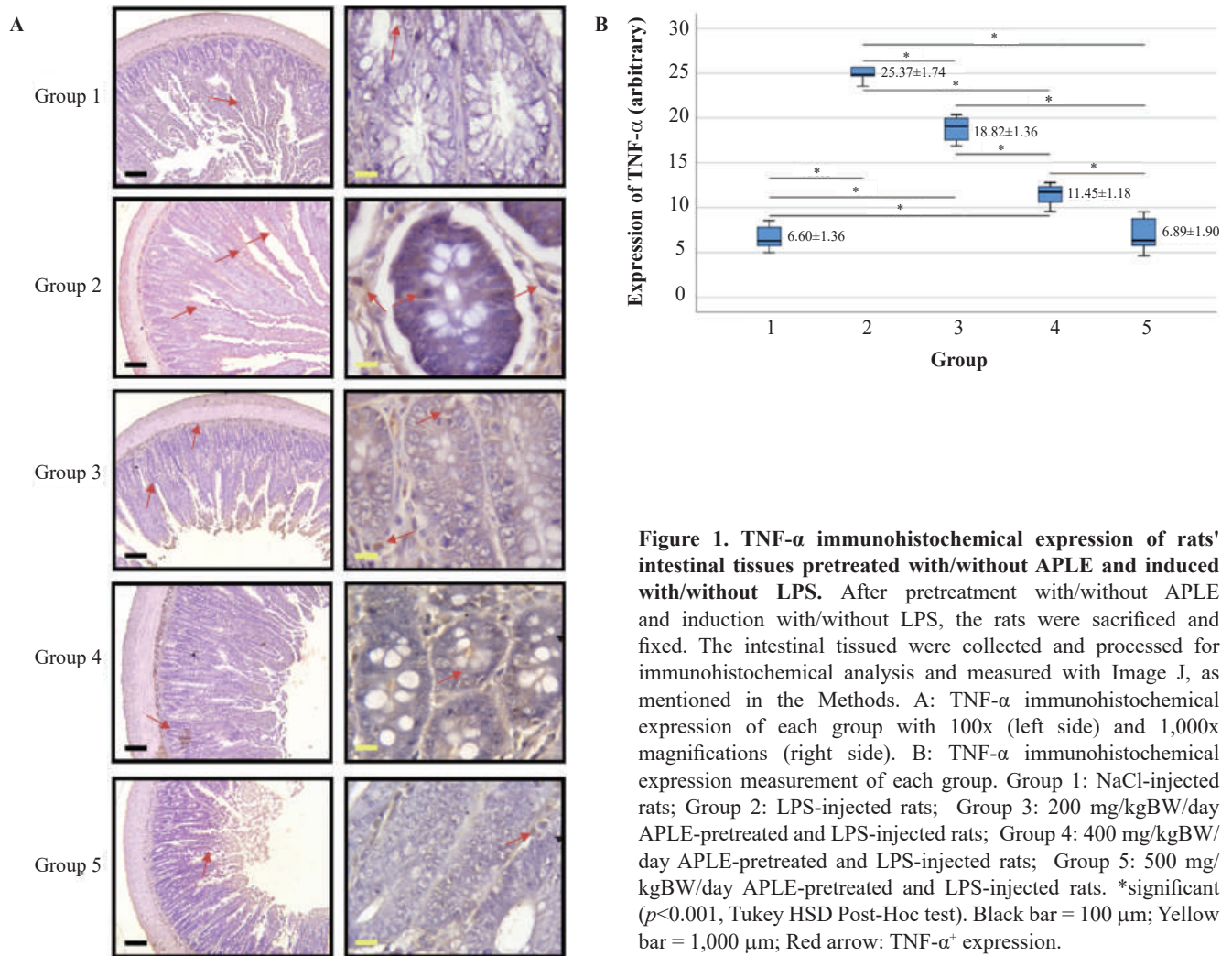


Figure 1. TNF- α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS. After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were collected and processed for immunohistochemical analysis and measured with Image J, as mentioned in the Methods. A: TNF- α immunohistochemical expression of each group with 100x (left side) and 1,000x magnifications (right side). B: TNF- α immunohistochemical expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α^+ expression.

been reported with the use of Septimed, a herbal medicine with an anti-inflammatory properties. Compared to a control group that were administered with standard sepsis treatment, Septimed was found to significantly decrease the severity of sepsis based on the reduction of the inflammatory markers.(21) Another study also reports a successful sepsis induction in BALB/c mice using sub-lethal dose of LPS, proven by the increase of TNF- α , nuclear factor kappa B (NF- κ B), and nitrate concentrations that were measured using real-time polymerase chain reaction.(22) Induction with intraperitoneal LPS injection has been found to cause apoptosis of intestinal epithelial cells.(23) The apoptosis of intestinal epithelial cells was induced by a cascade that leads to the production of proinflammatory cytokines and interferons, including TNF- α .(24) Release of TNF- α into the blood circulation will trigger the activation of Caspase-3 which then cleave various structural, cell cycle, and DNase proteins causing apoptosis.(25) In current

study, pretreatment of APLE could inhibit the expression of TNF- α and Caspase-3 in a concentration dependent manner. The 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced TNF- α and Caspase-3 expression of rats' intestinal tissues. Inhibition of TNF- α , interleukin (IL)-6, and nitric oxide (NO) production by APLE in a dose-dependent pattern was reported as well.(26)

Andrographolide, an active metabolite of *A. paniculata*, is an effective anti-inflammatory agent, which has been found to significantly decrease TNF- α level. Moreover, Andrographolide can form a covalent that inhibits the production of pro-inflammatory cytokines, thus could alleviate or prevent inflammation.(27) *A. paniculata* extract contains some non-standardized constituents that belongs to the flavonoid and phenylcarboxylic acid class. Both of this compounds are known and commonly used in scavenging free radicals and its antioxidant properties, which in this case, oxidative stress is a substantial component in

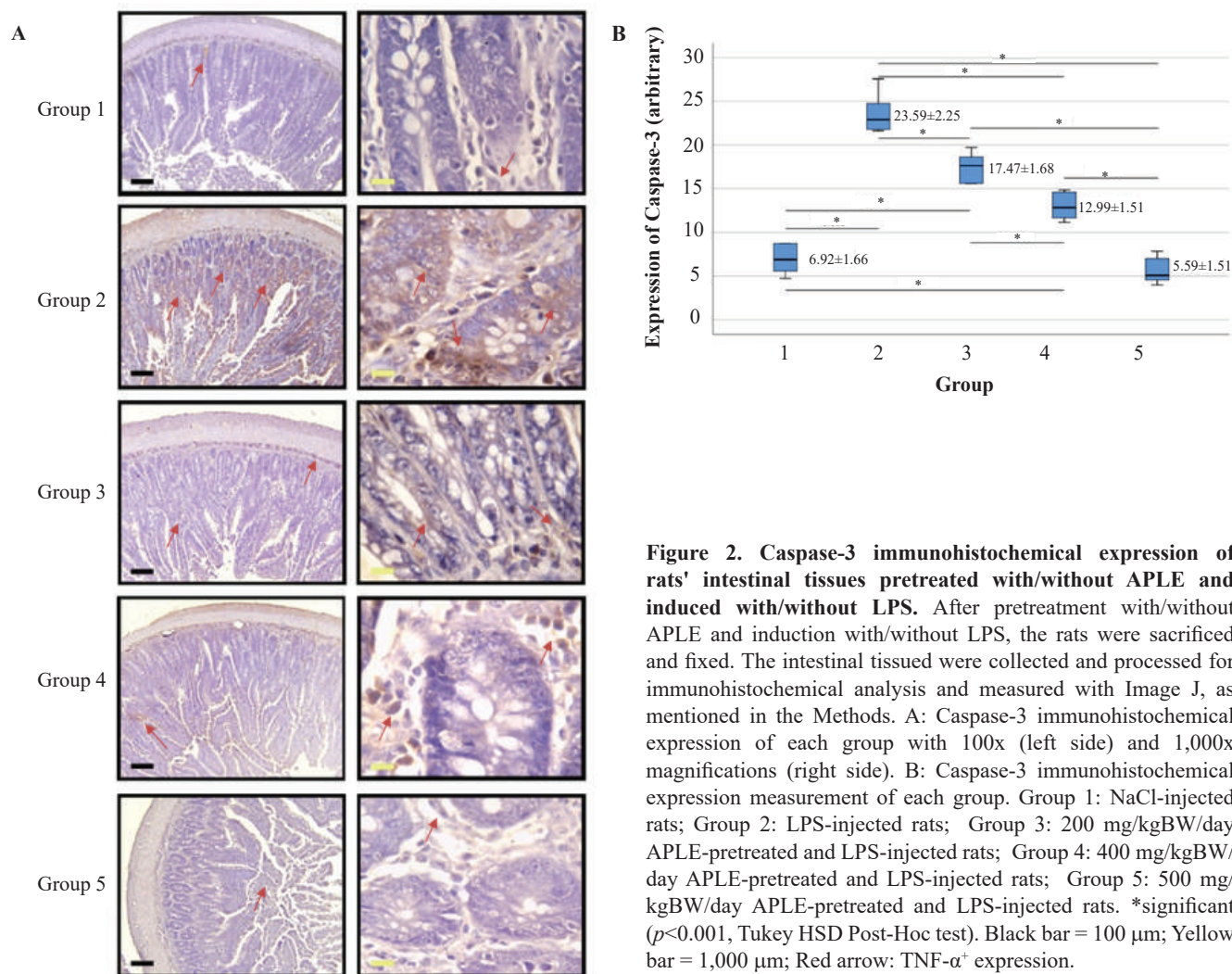


Figure 2. Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS. After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were collected and processed for immunohistochemical analysis and measured with Image J, as mentioned in the Methods. A: Caspase-3 immunohistochemical expression of each group with 100x (left side) and 1,000x magnifications (right side). B: Caspase-3 immunohistochemical expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α expression.

inflammatory tissue damage and cytokine signaling.(28) Overall, APLE could reduce cytokine production mediated by LPS through negative regulation, so that inflammation can be controlled and resolved quickly with minimal acute organ damage. However, our study did not analyze the bioactive compounds contained in APLE, therefore further research is needed.

Conclusion

LPS could induce septic rats' intestinal tissues by increasing the expression of TNF- α and Caspase-3. And the pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent. However, the compound and mechanism of APLE should be further investigated.

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Authors Contribution

RGA, PB, NSW was involved in the concepting and planning of the study and collected the data samples. RGA and FS performed the analysis of the data, designed the figures, as well as drafted and revised the manuscript. PB, NSW, NM, NS, and FS critically revised the draft for important intellectual content. All authors have read and approved the final manuscript.

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RESEARCH ARTICLE

Andrographis paniculata Leaves Extract Inhibit TNF- α and Caspase-3 Expression of Septic Rats' Intestinal Tissues

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Abstract

BACKGROUND: Microcirculation and cellular disturbances caused by sepsis might trigger significant intestinal damage. *Andrographis paniculata* extract decreases inflammatory intestinal epithelial cells with its role as an antiparasitic and anti-inflammatory agent. However, *A. paniculata* extract's effect on sepsis have not been commonly studied, especially in the intestinal tissues. Therefore, this study was conducted to determine *A. paniculata* leaves extract (APLE) effect in sepsis-induced intestinal tissues of rats by examining the expression of inflammatory cytokines involved in sepsis, namely tumor necrosis factor (TNF)- α and Caspase-3.

METHODS: Rats were divided into five groups, two groups received no pretreatment and the other three groups received 200, 400, and 500 mg/kg BW/day APLE, respectively. Three pretreated groups and one group with no pretreatment were then injected with 1 mg/200 g BW lipopolysaccharides (LPS) intraperitoneally to create septic rat models. Three days after the LPS-induction, rats were euthanized and the expression of TNF- α and Caspase-3

were assessed based on the immunohistochemical staining of rats' intestinal tissues.

RESULTS: Compared with NaCl (sham), LPS significantly ($p < 0.001$) induced TNF- α expression from 6.60 \pm 1.36 to 23.37 \pm 1.74. Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced TNF- α expression (18.82 \pm 1.36, 11.45 \pm 1.18, and 6.89 \pm 1.90, respectively). Similar with TNF- α , compared with NaCl (sham), LPS significantly ($p < 0.001$) induced Caspase-3 expression from 6.92 \pm 1.66 to 23.59 \pm 2.25. Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced Caspase-3 expression (17.47 \pm 1.68, 12.99 \pm 1.51, and 5.59 \pm 1.51, respectively).

CONCLUSION: The pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent.

KEYWORDS: *Andrographis paniculata*, sepsis, TNF- α , Caspase-3, lipopolysaccharide

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RESEARCH ARTICLE

***Andrographis paniculata* Leaves Extract Inhibit TNF- α and Caspase-3 Expression of Septic Rats' Intestinal Tissues**Ryco Giftyan Ardika¹, Bernardus Parish Budiono², Nyoman Suci Widiastiti³, Nani Maharani⁴, Neni Susilaningsih⁵, Ferry Sandra^{6,*}¹Biomedical Science Postgraduate Program, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia²Digestive Surgery Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia³Clinical Pathology Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia⁴Pharmacology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia⁵Histology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia⁶Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

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METHODS: Rats were divided into five groups; two groups received no pretreatment and the other three groups received 200, 400, and 500 mg/kg BW/day APLE, respectively. Three pretreated groups and one group with no pretreatment were then injected with 1 mg/200 g BW lipopolysaccharides (LPS) intraperitoneally to create septic rat models. Three days after the LPS-induction, rats were euthanized and the expression of TNF- α and Caspase-3

were assessed based on the immunohistochemical staining of rats' intestinal tissues.

RESULTS: Compared with NaCl (sham), LPS significantly ($p < 0.001$) induced TNF- α expression from 6.60 ± 1.36 to 25.37 ± 1.74 . Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced TNF- α expression (18.82 ± 1.36 , 11.45 ± 1.18 , and 6.89 ± 1.90 , respectively). Similar with TNF- α , compared with NaCl (sham), LPS significantly ($p < 0.001$) induced Caspase-3 expression from 6.92 ± 1.66 to 23.59 ± 2.25 . Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit the LPS-induced Caspase-3 expression (17.47 ± 1.68 , 12.99 ± 1.51 , and 5.59 ± 1.51 , respectively).

CONCLUSION: The pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent.

KEYWORDS: *Andrographis paniculata*, sepsis, TNF- α , Caspase-3, lipopolysaccharide

Indones Biomed J. 2024; 16(1): 66-71

Introduction

Sepsis has been associated with substantial morbidity and mortality. This condition has become one of the growing global burdens with its complexity. The World Health Organization (WHO) has reported a significant increase of sepsis-related deaths throughout the years, with the alarming incidence rate made sepsis as a global health priority.(1) Although significant improvement and advancement have been made in sepsis management, the mortality risk remains high and many survivors never recovered fully, which led them to long-term morbidities.(2) These problems highlight the need for novel alternative treatment that could augment or enhance current strategies.

Sepsis is a condition of dysregulated host response to infection (3), which is predominantly caused by Gram-positive bacteria. The most frequently isolated bacteria are *Staphylococcus aureus* and *Streptococcus pneumoniae*. (4) Considering the complexity of sepsis, a multifactorial mechanism has been thought to elicit the condition, which involves a variety of pro- and anti-inflammatory mediators. Microcirculation, cellular, and coagulation impairment cause tissue hypoperfusion that leads to tissue damage.(5) Previous studies showed that sepsis causes reduced blood flow to the digestive organs, inflicting a rapidly occurred ischemia and leads to acute intestinal damage, especially in the colon. Many inflammatory cytokines are involved in sepsis, mainly tumor necrosis factor (TNF)- α and Caspase-3. (6) Studies showed that Caspase-3 inhibition leads to reduced TNF- α production in various cell types, making it a potential therapeutic target for inflammatory diseases.(7,8) These findings highlight the promising potential and benefits of TNF- α and Caspase-3 as a new therapeutic approach in reducing sepsis-associated morbidity and mortality.(9)

Traditional medicines made from botanical substances or herbal have been extensively utilized for their many potencies (10-14), particularly in developing nations. WHO reported an approximately 65% of individuals in developing nations incorporated herbal medicines in their healthcare practices.(15) *Andrographis paniculata*, in Indonesia known as Sambiloto, is a type of herbal plant often used in traditional medicine due to its anti-inflammatory, antioxidant, and immunomodulatory properties.(16,17) *A. paniculata* has been found to enhance bacterial clearance in an intra-abdominal sepsis that alleviated pathological organ injury induced by sepsis.(18) Administration of *A. paniculata* extract could decrease inflammatory intestinal epithelial cells, highlighting its role as an antiparasitic and

anti-inflammatory agent. Administration of *A. paniculata* could also modulate the expression of apoptotic genes such as Caspase-9 and Caspase-3.(19,20) In our knowledge, *A. paniculata* extract's effect on sepsis have not been studied, especially in the intestinal tissues. Thus, this study was conducted to determine the effect of *A. paniculata* extract in the sepsis-induced intestinal tissues of rats, by focusing on the expression of TNF- α and Caspase-3.

Methods

A. paniculata Leaves Extract (APLE) Production

APLE production was performed at PT Sido Muncul, a herbal and pharmaceutical company located in Semarang, Indonesia. Briefly, after macroscopical and microscopical ingredient and quality control checks, the simplicia (100 g *A. paniculata* leaves) was cleansed, dried, minced, macerated with 90% ethanol for 24 h and perlocated with digital shaker at a speed of 50 rpm for 2 h at 60°C. Resulted filtrate was filtered and evaporated for 2 h to obtain brownish green APLE with 8% yield (8 g).

Study Design, Ethical Approval and Animal Acclimatization

An animal experimental, randomized, post-test-only with control group study was designed. The study protocol was reviewed and approved by the Medical Research and Ethics Committee Universitas Diponegoro (No. 112/EC-H/KEPK/FK-UNDIP/IX/2023). Thirty healthy male rats (*Rattus norvegicus*), aged 2-3 months, weighted 150-200 g, were obtained from Animal Laboratory of Universitas Gadjah Mada, Yogyakarta. The rats were individually caged, acclimated with standard diet of COMFEED AD II (Japfa, Jakarta, Indonesia) and drink for 7 days.

APLE Pretreatment and Lipopolysaccharide (LPS) Induction

After the 7 day-acclimatization, the rats were randomly divided into 5 groups (n=6). No pretreatment was performed for Group 1 and 2. For Group 3, 4 and 5, the rats were pretreated with 200 mg/kg BW/day, 400 mg/kg BW/day and 500 mg/kg BW/day APLE, respectively. The administration was performed orally with feeding tube for 14 consecutive days, in conjunction with standard diet. On the next day (day 22), septic induction was performed by an intraperitoneal injection of 1 mg/200 g BW LPS (Merck, St. Louis, MO, USA) for Group 2, 3, 4 and 5. For Group 1, injection was performed as well with 0.5 mL NaCl. The rats' heads were

positioned lower than the abdomen when injecting the needle at approximately 100 degrees from the surface. The injection was slightly away from the midline to avoid hitting the bladder and slightly lower to avoid the liver.

TNF- α and Caspase-3 Immunohistochemical Staining

Three days after LPS induction (day 25), the rats were euthanized using chloroform, and then a laparotomy was performed to collect intestinal tissue. The tissue samples were fixed in 10% formalin, dehydrated, and embedded in paraffin. The paraffin block was sliced into 5 μ m thick sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated with 0.5% H₂O₂ and blocked with 5% bovine serum albumin. For primary antibody, 1:100

Rabbit Polyclonal TNF- α (A0277) (ABclonal, Woburn, MA, USA) or 1:100 Mouse Monoclonal Caspase 3 (74T2) (ThermoFisher Scientific, Waltham, MA, USA) Antibody was used. For secondary antibody and streptavidin-biotin immunoenzymatic antigen detection system, Mouse and Rabbit Specific HRP (ABC) Detection IHC Kit (Abcam, Cambridge, UK) was used. For the chromogen, Diamino benzidine (DAB) Substrate Kit (Abcam) was used. Counterstaining was performed with Meyer's hematoxylin. Two pathologists, who were blinded to the study design, performed the histopathological examinations. An intraclass correlation coefficient analysis was conducted to evaluate the results. For each stained intestinal tissue section, 5 different areas were selected and documented at 100x magnification under upright light microscope. Each documented image was measured using ImageJ (US National Institutes of Health, Bethesda, MD, USA). Color deconvolution and threshold feature were set to measure protein expression.

Results

Two pathologists, who were blinded to the study design, had intraclass correlation coefficient values of $\kappa = 0.999$ for TNF- α and $\kappa = 0.998$ for Caspase-3 immunohistochemical expression assessments. This indicated a very good agreement between the assessments of the pathologists on TNF- α and Caspase-3 immunohistochemical expression.

APLE Inhibited LPS-induced TNF- α Expression of Rats' Intestinal Tissues

Under 1 mg/200 g BW LPS induction, TNF- α expression of rats' intestinal tissues was significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure

1). This indicated that the LPS could induce inflammatory rats' intestinal tissues. However, under pretreatment of APLE, the TNF- α expression of rats' intestinal tissues could be inhibited. Significant APLE inhibition on LPS-induced TNF- α expression of rats' intestinal tissues was started in concentration of 200 mg/kg BW/day (Group 3). This APLE inhibition was in concentration manner. Therefore, the TNF- α expression of rats' intestinal tissues with pretreatment of 400 mg/kg BW/day APLE (Group 4) was lower significantly than the one of 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the group that was pretreated with 500 mg/kg BW/day (Group 5) had the lowest TNF- α expression of rats' intestinal tissues. The TNF- α expression of rats' intestinal tissues in this Group 5 was similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest that the 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced TNF- α expression of rats' intestinal tissues.

APLE Inhibited LPS-induced Caspase-3 Expression of Rats' Intestinal Tissues

Under 1 mg/200 g BW LPS induction, Caspase-3 expression of rats' intestinal tissues was significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure 1). This indicated that the LPS could induce apoptotic rats' intestinal tissues. However, under pretreatment of APLE, the Caspase-3 expression of rats' intestinal tissues could be inhibited. Significant APLE inhibition on LPS-induced Caspase-3 expression of rats' intestinal tissues was started in concentration of 200 mg/kg BW/day (Group 3). This APLE inhibition was in concentration manner. Therefore, the Caspase-3 expression of rats' intestinal tissues with pretreatment of 400 mg/kg BW/day APLE (Group 4) was significantly lower than the 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the group that was pretreated with 500 mg/kg BW/day (Group 5) had the lowest Caspase-3 expression of rats' intestinal tissues. The Caspase-3 expression of rats' intestinal tissues in this Group 5 was similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest that the 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced Caspase-3 expression of rats' intestinal tissues.

Discussion

Current study showed that the induction using LPS injection could resemble sepsis condition, which increased the expression of TNF- α and Caspase-3. A similar result has

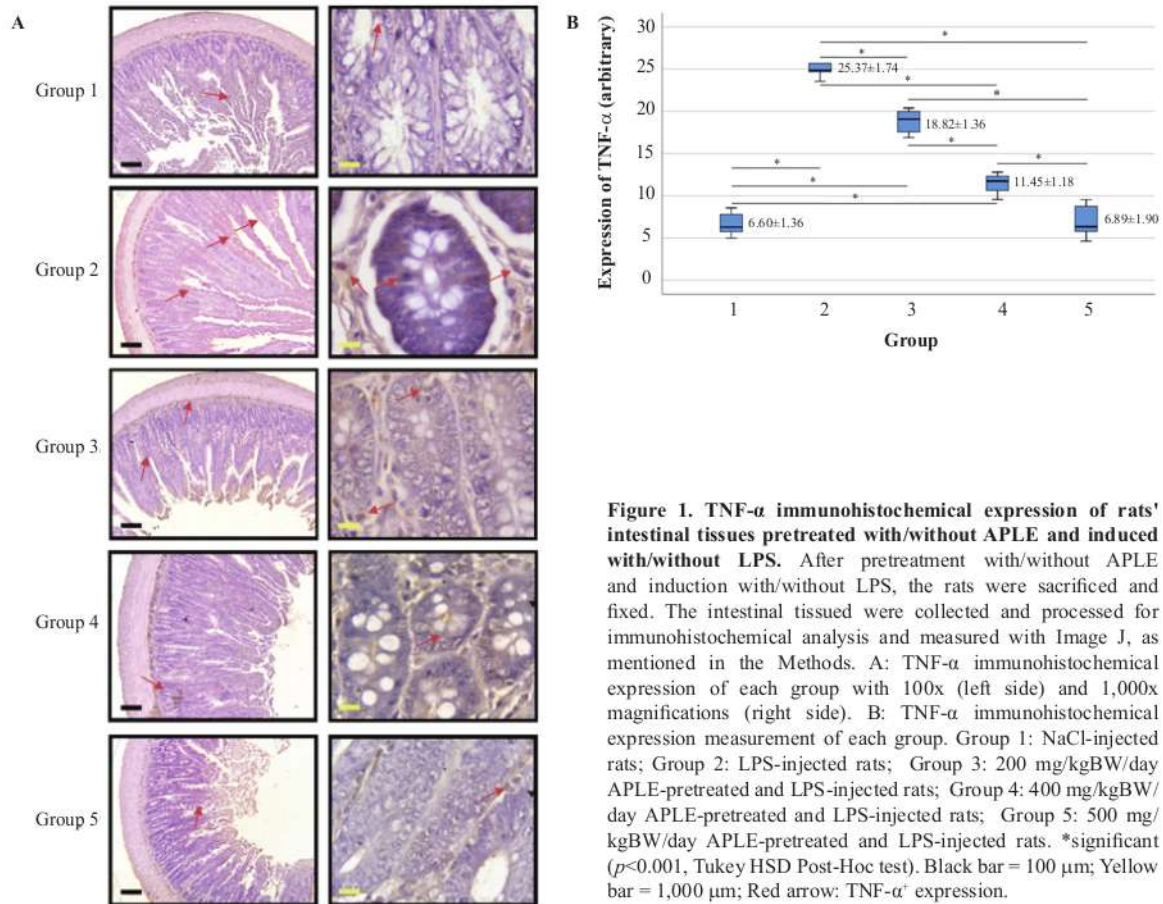


Figure 1. TNF- α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS. After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were collected and processed for immunohistochemical analysis and measured with Image J, as mentioned in the Methods. A: TNF- α immunohistochemical expression of each group with 100x (left side) and 1,000x magnifications (right side). B: TNF- α immunohistochemical expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α expression.

been reported with the use of Septimed, a herbal medicine with an anti-inflammatory properties. Compared to a control group that were administered with standard sepsis treatment, Septimed was found to significantly decrease the severity of sepsis based on the reduction of the inflammatory markers.(21) Another study also reports a successful sepsis induction in BALB/c mice using sub-lethal dose of LPS, proven by the increase of TNF- α , nuclear factor kappa B (NF- κ B), and nitrate concentrations that were measured using real-time polymerase chain reaction.(22) Induction with intraperitoneal LPS injection has been found to cause apoptosis of intestinal epithelial cells.(23) The apoptosis of intestinal epithelial cells was induced by a cascade that leads to the production of proinflammatory cytokines and interferons, including TNF- α .(24) Release of TNF- α into the blood circulation will trigger the activation of Caspase-3 which then cleave various structural, cell cycle, and DNase proteins causing apoptosis.(25) In current

study, pretreatment of APLE could inhibit the expression of TNF- α and Caspase-3 in a concentration dependent manner. The 500 mg/kg BW/day APLE could almost totally inhibit the LPS-induced TNF- α and Caspase-3 expression of rats' intestinal tissues. Inhibition of TNF- α , interleukin (IL)-6, and nitric oxide (NO) production by APLE in a dose-dependent pattern was reported as well.(26)

Andrographolide, an active metabolite of *A. paniculata*, is an effective anti-inflammatory agent, which has been found to significantly decrease TNF- α level. Moreover, Andrographolide can form a covalent that inhibits the production of pro-inflammatory cytokines, thus could alleviate or prevent inflammation.(27) *A. paniculata* extract contains some non-standardized constituents that belongs to the flavonoid and phenylcarboxylic acid class. Both of this compounds are known and commonly used in scavenging free radicals and its antioxidant properties, which in this case, oxidative stress is a substantial component in

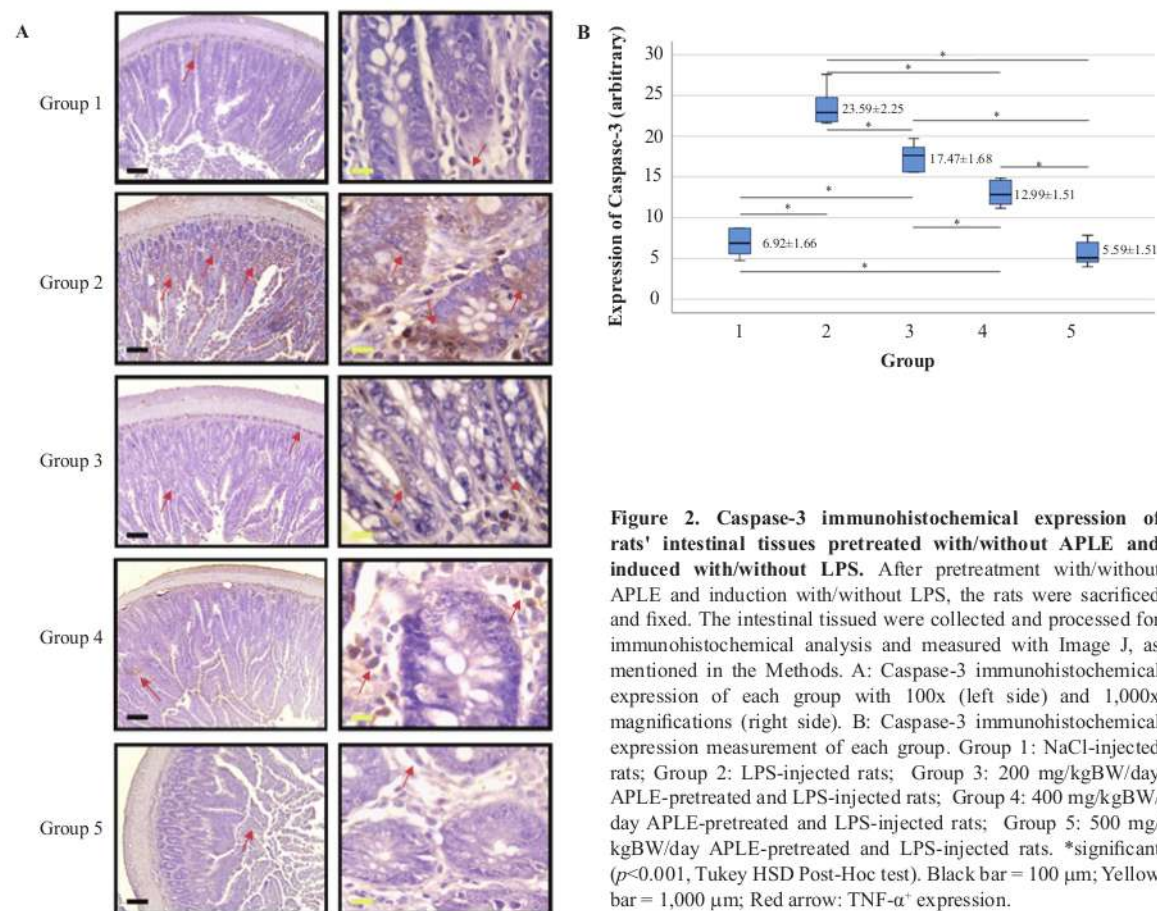


Figure 2. Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS. After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were collected and processed for immunohistochemical analysis and measured with Image J, as mentioned in the Methods. A: Caspase-3 immunohistochemical expression of each group with 100x (left side) and 1,000x magnifications (right side). B: Caspase-3 immunohistochemical expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α expression.

inflammatory tissue damage and cytokine signaling.(28) Overall, APLE could reduce cytokine production mediated by LPS through negative regulation, so that inflammation can be controlled and resolved quickly with minimal acute organ damage. However, our study did not analyze the bioactive compounds contained in APLE, therefore further research is needed.

Conclusion

LPS could induce septic rats' intestinal tissues by increasing the expression of TNF- α and Caspase-3. And the pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent. However, the compound and mechanism of APLE should be further investigated.

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Authors Contribution

RGA, PB, NSW was involved in the concepting and planning of the study and collected the data samples. RGA and FS performed the analysis of the data, designed the figures, as well as drafted and revised the manuscript. PB, NSW, NM, NS, and FS critically revised the draft for important intellectual content. All authors have read and approved the final manuscript.

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Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2023322 Editor Decision - Resubmit for Review

Secretariat of InaBJ <secretariat@inabj@gmail.com>
To: ferry@trisakti.ac.id

Tue, Nov 28, 2023 at 11:26 AM

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, manuscript M2023322 entitled "**Sambiloto Leave (*Andrographis paniculata*) Extract Decrease TNF- α And Caspase-3 Expression in the Colon Epithelial Cell of Sepsis Induced Mice**".

Our decision is: **Resubmit for Review.**

Find the files attached to see detailed comments from reviewers. Please make sure you read all the comments and revise the manuscript based on the suggestions given. Revise this manuscript thoroughly before **December 12, 2023**.

When you are done, you can upload it in: <https://inabj.org/index.php/ibj/author/submissionReview/2727>, or simply send us an email of your revised manuscript and response letter.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--

Secretariat of The Indonesian Biomedical Journal

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Manuscript Review Form

Reviewer	: Reviewer 1
Manuscript #	: M202333
Manuscript Title	: Sambiloto Leave (<i>Andrographis paniculata</i>) Extract Decrease TNF- α And Caspase-3 Expression in the Colon Epithelial Cell of Sepsis Induced Mice

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published? Notes: A new idea is the effect of Sambiloto extract on TNF-alpha and Caspase 3	√	
2.	Are the title and abstract of the manuscript appropriate? Notes: Appropriate	√	
3	Do the title and abstract reflect the study result/content? Notes: Appropriate	√	
4.	Is the significance of the study well explained at the Background? Notes: Appropriate	√	
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists? Notes:	√	
6.	Are the results, ideas, and data presented in this manuscript important	√	



	enough for publication?		
	Notes:		
7.	Are all figures and tables necessarily presented?	√	
	Notes: Appropriate		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?		√
	Notes: This research does not explain in detail what compounds contained in the Sambiloto extract affect the role of TNF-alpha and Caspase 3. Please add literature that explains what specific compounds are found in sambiloto, maybe you can look for bioactive compounds		
9.	Are the conclusions and interpretations valid and supported by the data?	√	
	Notes: It is necessary to clarify in conclusion the factors that cause a decrease in the expression of pro-inflammatory cytokines TNF-alpha and caspase-3		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?		√
	Notes: consistency of word writing e.g bitter - sambiloto		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

Simple research, perhaps it can be applied to society to deal with the problem of sepsis. Writers are advised to provide opinions or suggestions on how to make Sambiloto extract so that it can be easily used for the benefit of society.



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Reviewer's Recommendation (Please tick only one option)	<input checked="" type="checkbox"/>
Accept Submission (No significant alterations suggested)	<input checked="" type="checkbox"/>
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Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	<input type="checkbox"/>

Further Reviewer's Comments Regarding Disposition of the Manuscript:

Manuscript is acceptable with some minor corrections.

Date and Sign:

November 27, 2023

Reviewer 1



Manuscript Review Form

Reviewer	: Reviewer 2
Manuscript #	: M2023322
Manuscript Title	: Sambiloto Leave (<i>Andrographis paniculata</i>) Extract Decrease TNF- α And Caspase-3 Expression in the Colon Epithelial Cell of Sepsis Induced Mice

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published? Notes: A lot of research has been done on bitter leaves, especially those related to infection and inflammation		V
2.	Are the title and abstract of the manuscript appropriate? Notes: The abstract already describes the title but needs to be sharpened further regarding the reasons for the urgency of this research		V
3	Do the title and abstract reflect the study result/content? Notes: The conclusions drawn were too early because the dose given was too large and did not explain the toxic effects		V
4.	Is the significance of the study well explained at the Background? Notes:	V	
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists? Notes:	V	



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6.	Are the results, ideas, and data presented in this manuscript important enough for publication?		V
	Notes: Add to the background the urgency of this research being carried out		
7.	Are all figures and tables necessarily presented?	V	
	Notes:		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	V	
	Notes:		
9.	Are the conclusions and interpretations valid and supported by the data?	V	
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?		V
	Notes: some grammatical sentences need to be corrected		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

Overall, this manuscript is suitable for publication, but there are several important notes that must be corrected and added according to the comments above



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Reviewer's Recommendation (Please tick only one option)	
Accept Submission (No significant alterations suggested)	√
Revisions Required (Suggest changes to the manuscript as specified in this review)	V
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

Further Reviewer's Comments Regarding Disposition of the Manuscript:

Overall, this manuscript is suitable for publication, but there are several important notes that must be corrected and added according to the comments above

Date and Sign:

November 22, 2023

Reviewer 2

1 **Sambiloto Leave (*Andrographis paniculata*) Extract Decrease TNF- α And Caspase-3**
2 **Expression in the Colon Epithelial Cell of Sepsis Induced Mice**

3
4 **Abstract**

5 **Background:** Sepsis is a condition that has been correlated with significant morbidity and
6 mortality. Inflammatory, in response to the cause of microcirculation and cellular disturbances,
7 can trigger acute intestinal damage especially in colon. Pro-inflammatory cytokines such as
8 TNF- α and caspase-3 were increased in sepsis and triggering an inflammatory response and
9 cell apoptosis. Traditional treatment with Sambiloto leaves (*Andrographis paniculata*) extract
10 has been widely used because of its beneficial effects. Thus, this study aims to determine the
11 effect of Sambiloto extract on the expression of TNF- α and caspase-3 in a mouse model of
12 sepsis.

13 **Methods:** Thirty male *Rattus norvegicus* mice were divided randomly into five groups: healthy
14 control, negative control, and three treatment groups administered with graded doses
15 of Sambiloto extract orally. The treatment was carried out for 14 days, and on the 22nd day,
16 sepsis was induced by intraperitoneal injection of 5 mg/kgBW lipopolysaccharide. The
17 expression of TNF- α and caspase-3 was assessed based on microscopic examination of
18 immunohistochemical staining of colon tissue samples taken on day 25.

19 **Results:** Sambiloto extract significantly reduced the expression of TNF- α and caspase-3 in
20 mice experiencing sepsis compared to negative controls ($p < 0.05$). Administration of Sambiloto
21 extract at a dose of 500 mg/kgBW resulted in the lowest expression of TNF- α and caspase-3
22 and was similar in reaching levels comparable to those observed in the healthy control group.

23 **Conclusion:** Administration of Sambiloto extract at a dose of 500 mg/kgBW can prevent the
24 condition of sepsis.

25 **Keywords:** *Andrographis paniculata*, sepsis, TNF- α , caspase-3

26

Commented [H11]: Explain on the background of what specific bacteria that cause sepsis are intervened in mice and does the effect of this leaf extract apply generally to all infections?

27 **Introduction**

28 Sepsis is a condition that has been associated with substantial morbidity and mortality. This
29 condition is triggered by the host's dysregulated inflammatory response to pathogens
30 recognized by immune cells, especially macrophages and polymorphonuclear leukocyte
31 surface receptors (PMN).¹ Microcirculation, cellular, and coagulation impairment cause tissue
32 hypoperfusion which leads to tissue damage.² Previous studies showed that sepsis causes to
33 reduce blood flow to the digestive organs, so ischemia can occur acutely and cause acute
34 intestinal damage, especially in the colon. Several cytokines that are mainly involved in sepsis
35 are TNF- α and caspase-3.³ TNF- α is produced by immune cells in response to microbial
36 infections or other inflammatory stimuli and has been shown to increase caspase-3, an essential
37 enzyme involved in cell apoptosis. Moreover, the inhibition of caspase-3 has been shown to
38 reduce TNF- α production in various cell types, making it a potential therapeutic target for
39 inflammatory diseases, including sepsis.^{4,5}

40 Furthermore, traditional medicines made from botanical substances have been
41 extensively utilized, particularly in developing nations. According to the World Health
42 Organization (WHO), a significant proportion of individuals in developed nations,
43 approximately 65%, and in developing countries, approximately 80%, include herbal
44 medicines in their healthcare practices as a component of traditional medicine.⁶ In connection,
45 *Andrographis paniculata*, or Sambiloto, is a type of herbal plant often used in traditional
46 medicine because it has anti-inflammatory, antioxidant, and immunomodulator properties.⁷
47 *Andrographis paniculata* has been found to enhance bacterial clearance in an intra-abdominal
48 sepsis, shown by the decrease of TNF- α and IL-6, which alleviated pathological organ injury
49 induced by sepsis.⁸ Administration of *Andrographis paniculata* could decrease the number of
50 inflammatory in colon epithelial cells, highlighting its role as an antiparasitic and anti-
51 inflammatory agent.⁹ The extract could also modulates the expression of apoptotic genes such

Commented [H12]: Add some types of bacteria that cause sepsis

52 as caspase-9 and caspase-3.¹⁰ Thus, this study aims to determine the effect of *Andrographis*
53 *paniculata* (Sambiloto) leave extract on the expression of TNF- α and caspase-3 in the colon
54 tissues of *Rattus norvegicus* mice in the sepsis model.

55

56

57 **Methods**

58 *Animal Study*

59 For the purpose of this investigation, an experimental, randomized, post-test-only study with a
60 control group design was adapted and given permission to be carried by the Medical Research
61 and Ethics Committee Diponegoro University (112/EC-H/KEPK/FK-UNDIP/IX/2023). Thirty
62 healthy male *Rattus norvegicus* mice aged two up to three (2-3) months, weighted 150-200
63 grams, were obtained from Gadjah Mada University Animal Laboratory, Yogyakarta. The mice
64 were individually caged, acclimated with standard diet and drink for seven (7) days, and
65 randomly assigned into five (5) groups consisted of 6 mice each; (Group 1) Sham; (Group 2)
66 sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day;
67 (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis
68 induced and Sambiloto leave extract 500 mg/kgBW/day. The entire course of treatment was
69 administered orally for a duration of 14 consecutive days, in conjunction with typical food and
70 water consumption.

71 *Sambiloto Leave Extract Preparation*

72 The selected Sambiloto leaves were cleaned, drained, and dried to stop the enzymatic reaction.
73 The dried Sambiloto leaves were ground using a blender, and the powder was sieved with a 20-
74 mesh sieve. One hundred grams of Sambiloto leaf powder were dissolved in water and put into
75 a jar, stirred, and water was added as solvent until the powder was submerged. The solvent used
76 was at least 2x the weight of the powder or more. The jar was closed tightly for 24 hours,

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77 processed on a digital shaker at a speed of 50 rpm until it became homogeneous, and filtered
78 with a cloth filter. The liquid extract was re-macerated twice on the dregs by putting it back
79 into the jar and adding solvent until submerged. The first to last extracts were put together and
80 evaporated using a rotary evaporator for two (2) hours to obtain water and bitter leaf extract.
81 The obtained Sambiloto leaf extract was brownish green in color and is a 100% pure extract
82 preparation.

83 *Induction of Sepsis*

84 Sepsis was induced at the 22nd day after all the mice were given the assigned diet for 14 days.

85 Induction was done by injecting lipopolysaccharide (LPS) intraperitoneally with a dose of 1
86 mg/200 gBW. The rat's head were positioned lower than the abdomen when injecting the needle
87 at approximately 100 degrees from the surface. The injection was given slightly away from the
88 midline to avoid hitting the bladder and lower to avoid the liver. Sham-operated mice were
89 given intraperitoneal injection of 0,5 mL NaCl.

90 *Outcome Analysis*

91 Intestinal tissue sampling was carried out on the 25th day, in which all mice were euthanized
92 using chloroform, and then a laparotomy was performed to remove intestinal tissue. The tissue
93 samples were made into a histopathological preparation. Immunohistochemistry staining was
94 used to investigate TNF- α and caspase-3 expressions. Paraffin block pieces that have been
95 sliced with a microtome are placed on a glass object that has been coated with a protective
96 layer. This coating process involves placing a glass slide that has been coated with poly-L-
97 lysine on top of a layer of paraffin. Block sections were treated with TNF- α rabbit IgG and
98 caspase-3 rabbit antibody, then washed with PBS. Biotin was added for about 15 minutes,
99 followed by washing with PBS. Streptavidin was added for about 10 minutes and rewashed
100 with PBS. The DAB peroxidase enzyme substrate is given for about 3-5 minutes, which is then
101 washed with running water for about 10 minutes. The addition of hematoxylin was carried out

Commented [H13]: What bacteria or what is induced in mice to cause the mice to become septic? and what are your indicators that say the mice have sepsis?

102 for about 4 minutes, followed by washing with running water for about 10 minutes. The
103 preparation is dried and continued with microscopic reading. The process was conducted in
104 *Stem Cell and Cancer Research* (SCCR) laboratory, Semarang. A pathologist, who was blinded
105 to the study design, performed the histopathological examinations. The colon tissue sample
106 was measured at 400x magnification in 5 different areas in each samples from each tissue block
107 to reduce bias, using an objective tool from the ImageJ software.

108 ***Statistical Analysis***

109 Statistical analysis was performed using the IBM SPSS® Statistic software, version 23 (New
110 York, Manhattan). Results are expressed as means \pm SD. Statistical comparisons were
111 conducted using One Way ANOVA and Bonferroni Post Hoc test. The differences were
112 considered significant at $p < 0,05$ with 95% confidence interval.

113

114 **Results**

115 ***Sepsis Model***

116 Sepsis was induced using intraperitoneal LPS injection in the allocated groups. The success of
117 sepsis induction was assessed by the difference in TNF- α dan Caspase-3 expression between
118 the healthy control subjects (group 1) and the sepsis induced negative control subjects (group
119 2). Analysis on both of the markers showed a significant difference in TNF- α dan Caspase-3
120 expression between the two groups, indicating there is an increase of the inflammatory markers
121 with sepsis.

122 ***Sambiloto Leave Extract Decrease TNF- α Expression***

123 The TNF- α expression was significantly lower in the groups treated with Sambiloto leave
124 extract compared to the negative control group (Table 1). There was no difference between the
125 health control (Group 1) and group 5 revealed that administrating Sambiloto leave extract with

126 a dose of 500 mg/kgBW/day resulted in a comparable TNF- α expression level with the healthy
127 subjects (Figure 1, 2, and 5).

128 ***Sambiloto Leave Extract Decrease Caspase-3 Expression***

129 Caspase-3 expression was significantly lower in the groups treated with Sambiloto leave
130 extract compared to the negative control group (Table 2). There was no difference between the
131 health control group (Group 1) and group 5, showing that administrating Sambiloto leave
132 extract with a dose of 500 mg/kgBW/day resulted in a comparable caspase-3 expression level
133 with the healthy subjects (Figure 3, 4, and 6).

134

135 **Discussion**

136 This current study showed that the expression levels of TNF- α and caspase-3 from the healthy
137 control group were the lowest. It indicated that the induction using LPS injection was proven
138 to resemble sepsis conditions, which increased the expression of TNF- α and caspase-3. This
139 study does not use positive control group because the objective was to study the preventive
140 effect of Sambiloto leave extract on sepsis. A similar result has been reported with the use of
141 Septimed, a herbal medicine with an anti-inflammatory properties. Compared to a control
142 group that were administered with standard sepsis treatment, Septimed was found to
143 significantly decrease the severity of sepsis based on the reduction of the inflammatory markers
144 .¹¹ Another study also reports a successful sepsis induction in BALB/c mice using sub-lethal
145 dose of LPS, proven by the increase in TNF- α , Nf-kB, and nitrate concentrations that were
146 measured using real-time polymerase chain reaction.¹²

147 Induction using intraperitoneal LPS has been found to cause apoptosis of intestinal
148 epithelial cells.¹³ The apoptosis of intestinal epithelial cells is induced by a mechanism that is
149 dependent on TLR4. In this mechanism, mononuclear cells expressing TLR4 require the
150 adapter protein MD-2. MD-2 directly binds to and recognizes the lipophilic portion of the LPS,

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151 resulting in the formation of a heterodimer complex. This complex subsequently initiates a
152 cascade of protein interactions. This cascade leads to the production of proinflammatory
153 cytokines and interferons, increasing the expression of TNF- α .¹⁴ TNF- α released into the blood
154 circulation will bind to TNFR1 on intestinal epithelial cells and trigger activation of caspase-9
155 through the NF- κ B, activating caspase-3. Increased caspase-3 will cleave various structural,
156 cell cycle, and DNase proteins causing apoptosis.¹⁵

157 Analysis showed that the administration of Sambiloto leaf extract significantly reduced
158 the expression of TNF- α and caspase-3, with administration of the larger dose producing
159 expression levels similar to healthy conditions. The Sambiloto leaf extract could inhibit the
160 activation of NF- κ B in a dose-dependent pattern and inhibit the production of TNF- α , IL-6,
161 and NO. Activation of NF- κ B is central to activate various inflammatory mediators and
162 complex cytokines in the pathogenesis of septic shock and inflammation. Activation of NF- κ B,
163 which is inhibited by sambiloto leaf extract, will suppress the production of inflammatory
164 mediators TNF- α , IL-2, MIP-2 and NO.¹⁶ TNF- α level was also found to significantly decrease
165 through a dose-dependent effect when given andrographolide sulfonate, one of the active
166 components of Sambiloto leaf extract.

167 Moreover, Andrographolide can form a covalent that inhibits the binding of NF- κ B
168 oligonucleotides to nuclear proteins and inhibits the phosphorylation of NF- κ B and p38, along
169 with suppressing the activation of STAT3. This transcription factor plays a role in the
170 production of pro-inflammatory cytokines.¹⁷ Overall, the anti-inflammatory effect of bitter leaf
171 extract can reduce cytokine production mediated by LPS through negative regulation involving
172 the activation of p38 MAPK, STAT3, and NF- κ B so that inflammation can be controlled and
173 resolved quickly and minimize acute organ damage. In conclusion, administration of
174 *Andrographis paniculata* extract at a dose of 500 mg/kgBW can prevent the condition of sepsis.
175 In this case, it can be noticed by decreasing the expression of the pro-inflammatory cytokines

176 TNF- α and caspase-3 to levels in healthy subjects. Further studies need to explore the
177 difference between methods of *Andrographis paniculata* extract administration and a routine
178 timed assessment to explore the optimal dose and duration of administering *Andrographis*
179 *paniculata* extract.

180

181 **Conclusion**

182 Administration of *Andrographis paniculata* extract at a dose of 500 mg/kgBW can prevent the
183 condition of sepsis, which is characterized by decreasing the expression of the pro-
184 inflammatory cytokines TNF- α and caspase-3 to levels in healthy subjects.

185

186 **Acknowledgments**

187 We would like to thank Clara and Ardi Prasetyo from Stem Cell and Cancer Research Indonesia
188 for the support given during the study.

189

190 **Authors' Contributions**

191 RGA: Planned the study, collected the data, performed the analysis, and wrote the manuscript;
192 PB, NSW, NM, NS: critically revised the draft for important intellectual content and finally
193 approved the manuscript. All authors read and approved the final manuscript.

194

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- 249
- 250

251 **Tables**252 **Table 1.** TNF- α Expression Analysis.

Groups	Mean \pm SD (%)	Median (min – max)	p-value
1	6.60 \pm 1.36	6.28 (4.98 – 8.56)	0.688*
2	25.37 \pm 1.74	24.86 (23.55 – 28.65)	0.121*
3	18.82 \pm 1.36	19.06 (16.89 – 0.39)	0.701*
4	11.45 \pm 1.18	11.72 (9.56 – 12.80)	0.738*
5	6.89 \pm 1.90	6.33 (4.62 – 9.54)	0.549*

253 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
 254 Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave
 255 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500
 256 mg/kgBW/day. *Normal distribution ($p>0,05$)

257

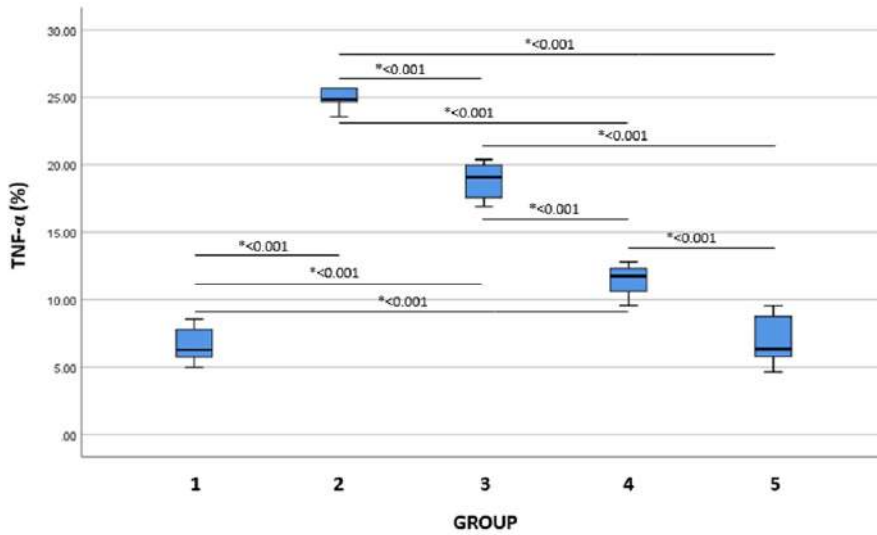
258 **Table 2.** Caspase-3 Expression Analysis.

Groups	Mean \pm SD (%)	Median (min – max)	p-value
1	6.92 \pm 1.66	6.88 (4.75 – 8.72)	0.445*
2	23.59 \pm 2.25	22.90 (21.62 – 27.59)	0.190*
3	17.47 \pm 1.68	17.64 (15.61 – 19.73)	0.481*
4	12.99 \pm 1.51	12.84 (11.16 – 14.85)	0.555*
5	5.59 \pm 1.51	5.08 (3.98 – 7.84)	0.433*

259 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
 260 Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave
 261 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500
 262 mg/kgBW/day. *Normal distribution ($p>0,05$)

263

264 **Figure 1.** Boxplot analysis of TNF- α expression.



265

266 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
267 Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave
268 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500
269 mg/kgBW/day. * Significant (p<0,05)

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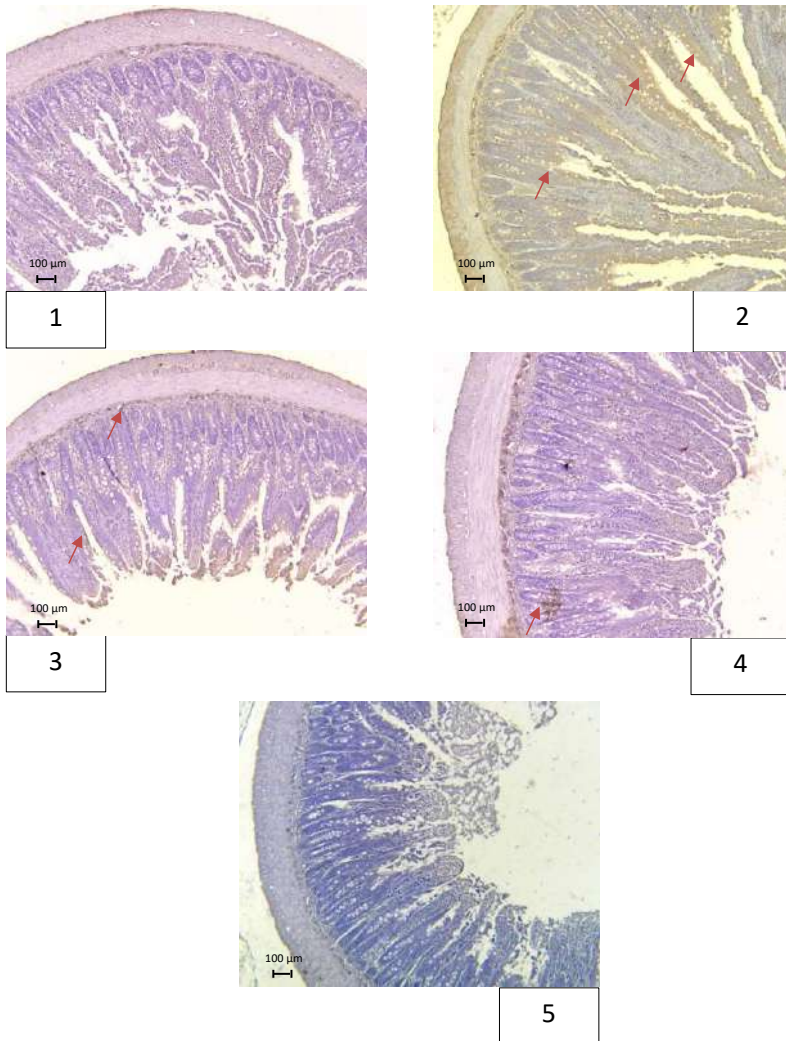
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278 **Figure 2.** Immunohistochemical staining of TNF- α expression at 100x magnification of
279 colon.

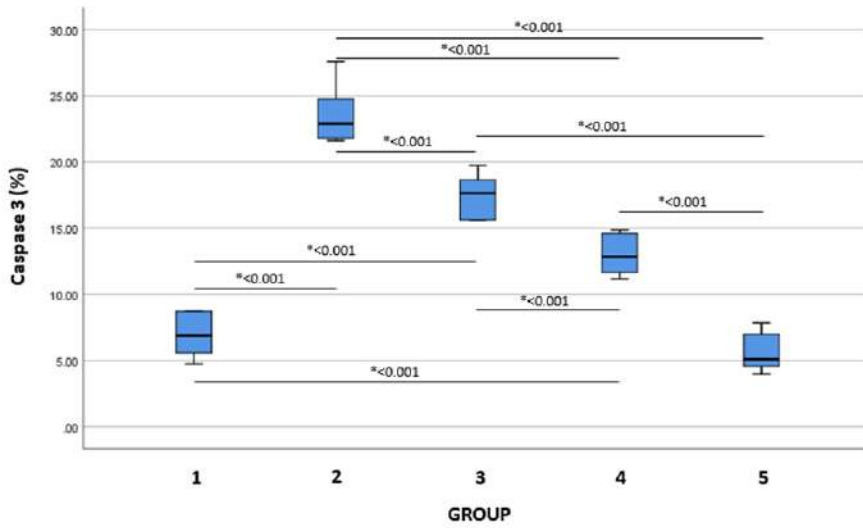


299 Description: (1) Sham; (2) sepsis induced; (3) sepsis induced and Sambiloto leave extract 200
300 mg/kgBW/day; (4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (5)
301 sepsis induced and Sambiloto leave extract 500 mg/kgBW/day.

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303

304 **Figure 3.** Boxplot analysis of Caspase-3 expression.

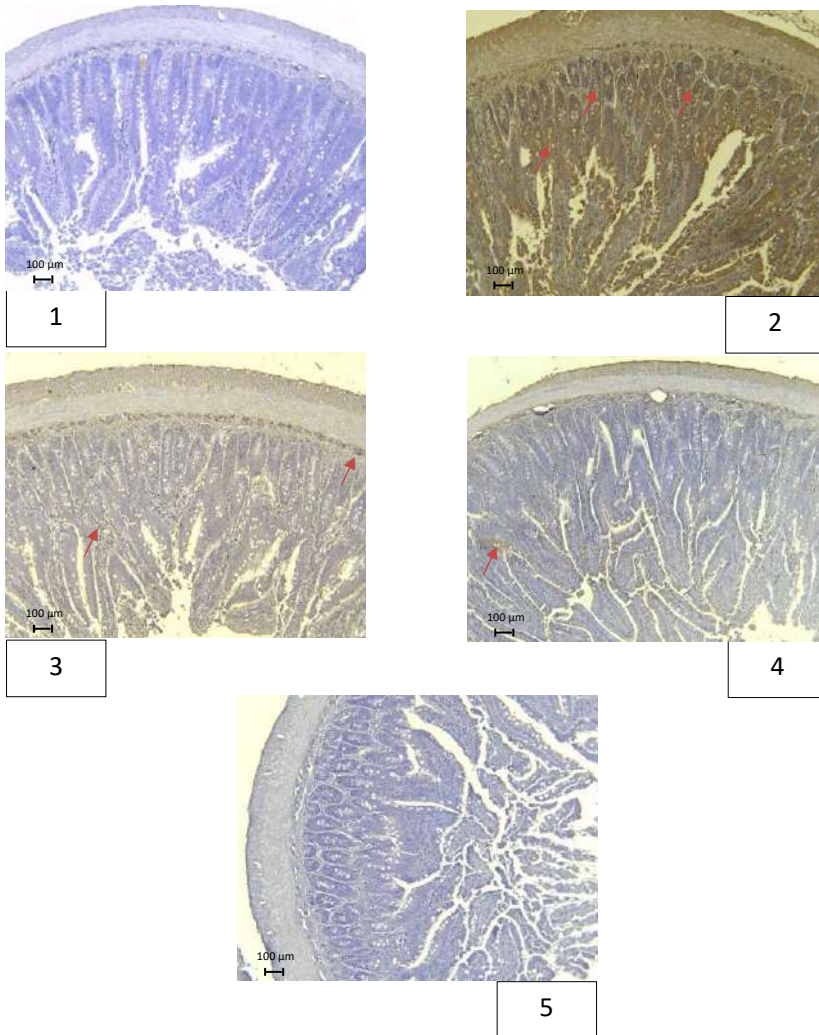


305

306 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
307 Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave
308 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500
309 mg/kgBW/day. * Significant ($p < 0,05$)

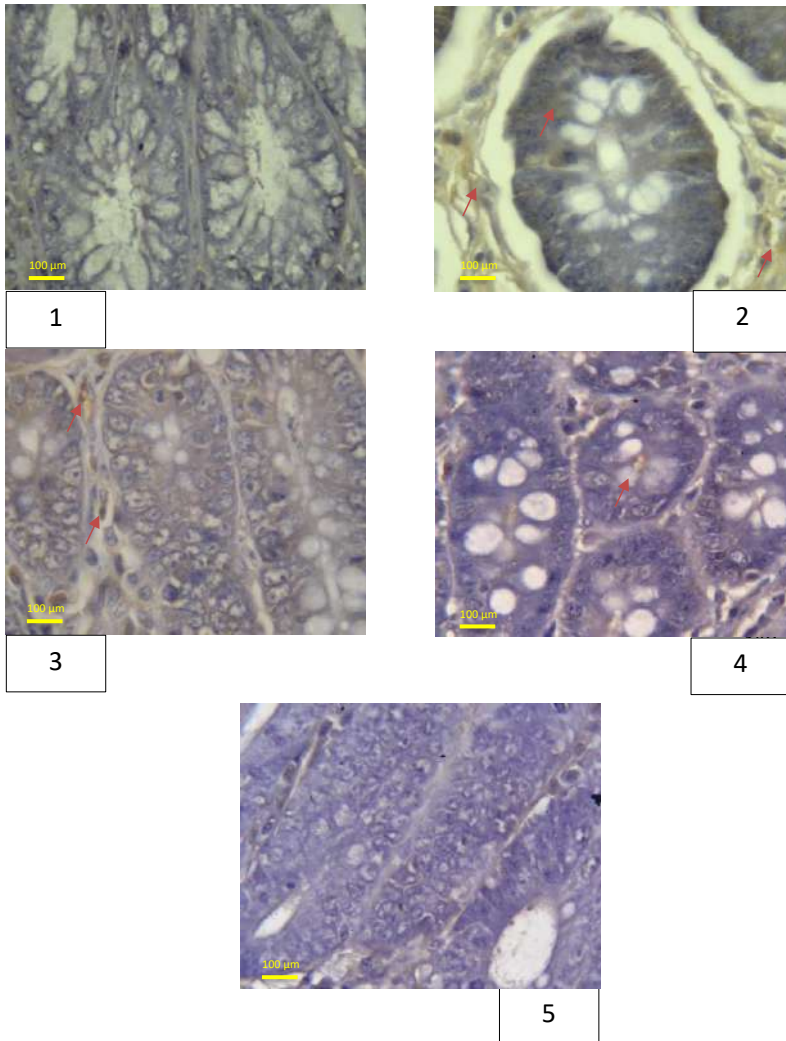
310

311 **Figure 4.** Immunohistochemical staining of Caspase-3 expression at 100x magnification of
312 colon.



332 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
333 Sambiloto leaf extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leaf
334 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leaf extract 500
335 mg/kgBW/day.

336 **Figure 5.** Immunohistochemical staining of TNF- α expression at 1000x magnification of
337 colon.



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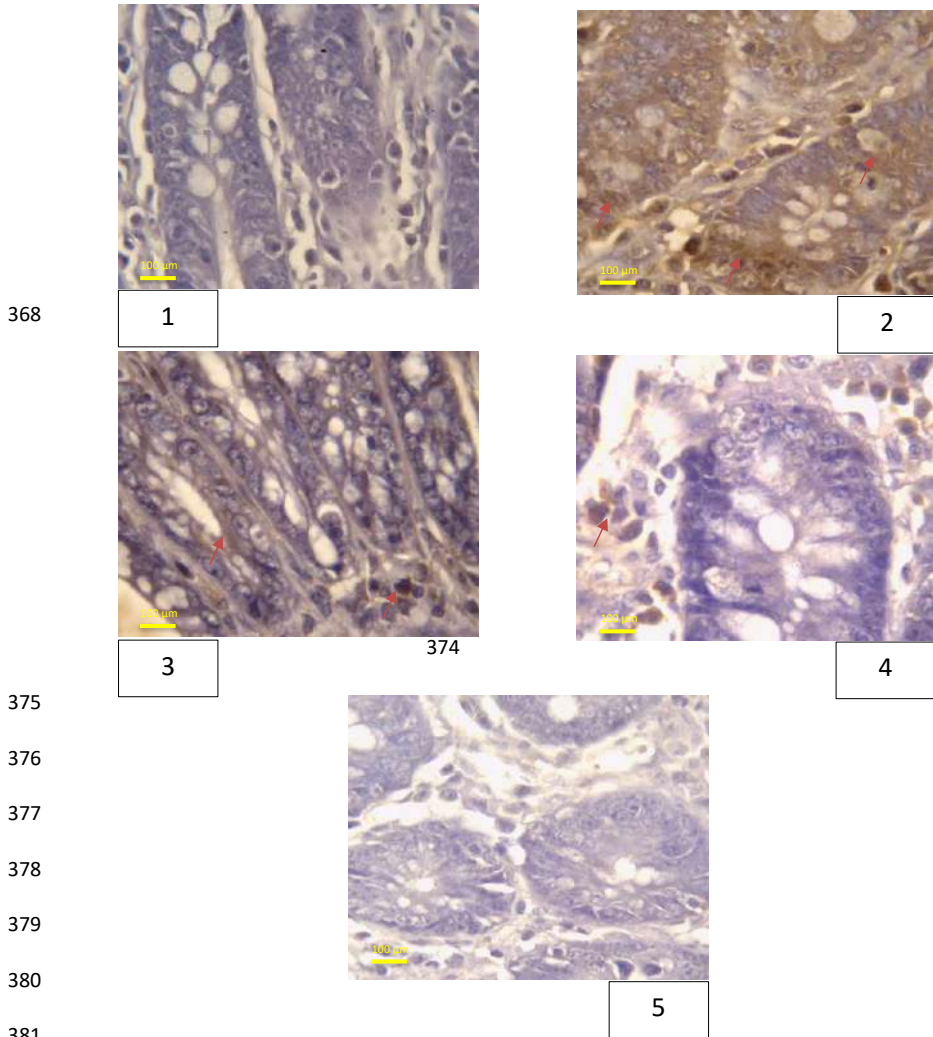
357 Description: (1) Sham; (2) sepsis induced; (3) sepsis induced and Sambiloto leave extract 200

358 mg/kgBW/day; (4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (5)

359 sepsis induced and Sambiloto leave extract 500 mg/kgBW/day.

360

361 **Figure 6.** Immunohistochemical staining of Caspase-3 expression at 1000x magnification of
362 colon.



382 Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and
383 Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave
384 extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500
385 mg/kgBW/day.



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[InaBJ] M2023322 Editor Decision - Resubmit for Review

Ferry Sandra <ferry@trisakti.ac.id>

Tue, Feb 13, 2024 at 8:53 AM

To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

Please find the revised version of the manuscript M2023322. I sincerely apologize for the delay, however I have made major corrections to the manuscript and all comments from the reviewers have been revised accordingly.

Thank you.

Regards,
Ferry Sandra

[Quoted text hidden]

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Head of Medical Research Center
Universitas Trisakti

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1 *Andrographis paniculata* Leaf Extract Inhibits TNF- α and Caspase-3 Expression of
2 Septic Rats' Intestinal Tissues

3
4 **Abstract**

5 **Background:** Microcirculation and cellular disturbances caused by sepsis might trigger
6 significant intestinal damage. *Andrographis paniculata* extract decreases inflammatory
7 intestinal epithelial cells with its role as an antiparasitic and anti-inflammatory agent. However,
8 *A. paniculate* extract's effect on sepsis have not been commonly studied, especially in intestinal
9 tissues. Therefore, this study was conducted to determine *A. paniculate* leave extract (APLE)
10 effect in sepsis-induced intestinal tissues of rats by examining the expression of inflammatory
11 cytokines involved in sepsis, namely TNF- α and Caspase-3.

12 **Methods:** Rats were divided into five groups; two groups received no pretreatment and the
13 other three groups received 200, 400, and 500 mg/kg BW/day APLE, respectively. Three
14 pretreated groups and one group with no pretreatment were then injected with 1 mg/200 g BW
15 lipopolysaccharides (LPS) intraperitoneally to create septic rat models. Three days after the
16 LPS-induction, rats were euthanized and the expression of TNF- α and Caspase-3 were assessed
17 based on the immunohistochemical staining of rats' intestinal tissues.

18 **Results:** Compared with NaCl (sham), LPS significantly ($p < 0.001$) induced TNF- α expression
19 from 6.60 ± 1.36 to 25.37 ± 1.74 . Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could
20 significantly ($p < 0.001$) inhibit the LPS-induced TNF- α expression (18.82 ± 1.36 , 11.45 ± 1.18 ,
21 and 6.89 ± 1.90 , respectively). Similar with TNF- α , compared with NaCl (sham), LPS
22 significantly ($p < 0.001$) induced Caspase-3 expression from 6.92 ± 1.66 to 23.59 ± 2.25 .
23 Pretreatment of 200, 400, and 500 mg/kg BW/day APLE could significantly ($p < 0.001$) inhibit
24 the LPS-induced Caspase-3 expression (17.47 ± 1.68 , 12.99 ± 1.51 , and 5.59 ± 1.51 , respectively).

25 **Conclusion:** The pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-3
26 expression, therefore APLE could be suggested as a potential sepsis-preventing agent.

27

28 **Keywords:** *Andrographis paniculata*, sepsis, TNF- α , Caspase-3, lipopolysaccharide

29

30 **Introduction**

31 Sepsis has been associated with substantial morbidity and mortality. This condition has become
32 one of the growing global burdens with its complexity. The World Health Organization (WHO)
33 has reported a significant increase of sepsis-related deaths throughout the years, with the
34 alarming incidence rate made sepsis as a global health priority.(1) Although significant
35 improvement and advancement have been made in sepsis management, the mortality risk
36 remains high and many survivors never recovered fully, which led them to long-term
37 morbidities.(2) These problems highlight the need for novel alternative treatment that could
38 augment or enhance current strategies.

39 Sepsis is a condition of dysregulated host response to infection (3), which is
40 predominantly caused by Gram-positive bacteria. The most frequently isolated bacteria are
41 *Staphylococcus aureus* and *Streptococcus pneumoniae*.(4) Considering the complexity of
42 sepsis, a multifactorial mechanism has been thought to elicit the condition, which involves a
43 variety of pro- and anti-inflammatory mediators. Microcirculation, cellular, and coagulation
44 impairment cause tissue hypoperfusion that leads to tissue damage.(5) Previous studies showed
45 that sepsis causes reduced blood flow to the digestive organs, inflicting a rapidly occurred
46 ischemia and leads to acute intestinal damage, especially in the colon. Many inflammatory
47 cytokines are involved in sepsis, mainly tumor necrosis factor (TNF)- α and Caspase-3.(6)
48 Studies showed that Caspase-3 inhibition leads to reduced TNF- α production in various cell
49 types, making it a potential therapeutic target for inflammatory diseases.(7,8) These findings

50 highlight the promising potential and benefits of TNF- α and Caspase-3 as a new therapeutic
51 approach in reducing sepsis-associated morbidity and mortality.(9)

52 Traditional medicines made from botanical substances or herbal have been extensively
53 utilized for their many potencies (10-14), particularly in developing nations. WHO reported an
54 approximately 65% of individuals in developing nations incorporated herbal medicines in their
55 healthcare practices.(15) *Andrographis paniculata*, in Indonesia known as Sambiloto, is a type
56 of herbal plant often used in traditional medicine due to its anti-inflammatory, antioxidant, and
57 immunomodulatory properties.(16,17) *A. paniculata* has been found to enhance bacterial
58 clearance in an intra-abdominal sepsis that alleviated pathological organ injury induced by
59 sepsis.(18) Administration of *A. paniculata* extract could decrease inflammatory intestinal
60 epithelial cells, highlighting its role as an antiparasitic and anti-inflammatory agent.
61 Administration of *A. paniculata* could also modulate the expression of apoptotic genes such as
62 Caspase-9 and Caspase-3.(19,20) In our knowledge, *A. paniculate* extract's effect on sepsis
63 have not been studied, especially in the intestinal tissues. Thus, this study was conducted to
64 determine the effect of *A. paniculata* extract in the sepsis-induced intestinal tissues of rats, by
65 focusing on the expression of TNF- α and Caspase-3.

66

67 **Methods**

68 ***A. paniculata* Leave Extract (APLE) Production**

69 APLE production was performed at PT Sido Muncul, a herbal and pharmaceutical company
70 located in Semarang, Indonesia. Briefly, after macroscopical and microscopical ingredient and
71 quality control checks, the simplicia (100 g *A. paniculata* leaves) was cleansed, dried, minced,
72 macerated with 90% ethanol for 24 h and perlocated with digital shaker at a speed of 50 rpm
73 for 2 h at 60°C. Resulted filtrate was filtered and evaporated for 2 h to obtain brownish green
74 APLE with 8% yield (8 g).

75 **Study Design, Ethical Approval and Animal Acclimatization**

76 An animal experimental, randomized, post-test-only with control group study was designed.
77 The study protocol was reviewed and approved by the Medical Research and Ethics Committee
78 Universitas Diponegoro (No. 112/EC-H/KEPK/FK-UNDIP/IX/2023). Thirty healthy male rats
79 (*Rattus norvegicus*), aged 2-3 months, weighted 150-200 grams, were obtained from Animal
80 Laboratory of Universitas Gadjah Mada, Yogyakarta. The rats were individually caged,
81 acclimated with standard diet of COMFEED AD II (Japfa, Jakarta, Indonesia) and drink for 7
82 days.

83 **APLE Pretreatment and Lipopolysaccharide (LPS) Induction**

84 After the 7 day-acclimatization, the rats were randomly divided into 5 groups (n=6). No
85 pretreatment was performed for Group 1 and 2. For Group 3, 4 and 5, the rats were pretreated
86 with 200 mg/kg BW/day, 400 mg/kg BW/day and 500 mg/kg BW/day APLE, respectively. The
87 administration was performed orally with feeding tube for 14 consecutive days, in conjunction
88 with standard diet. On the next day (day 22), septic induction was performed by an
89 intraperitoneal injection of 1 mg/200 g BW LPS (Merck, St. Louis, MO, USA) for Group 2, 3,
90 4 and 5. For Group 1, injection was performed as well with 0.5 mL NaCl. The rats' heads were
91 positioned lower than the abdomen when injecting the needle at approximately 100 degrees
92 from the surface. The injection was slightly away from the midline to avoid hitting the bladder
93 and slightly lower to avoid the liver.

94 **TNF- α and Caspase-3 Immunohistochemical Staining**

95 Three days after LPS induction (day 25), the rats were euthanized using chloroform, and then
96 a laparotomy was performed to collect intestinal tissue. The tissue samples were fixed in 10%
97 formalin, dehydrated, and embedded in paraffin. The paraffin block was sliced into 5 μ m thick
98 sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated
99 with 0.5% H₂O₂ and blocked with 5% bovine serum albumin. For primary antibody, 1:100

100 Rabbit Polyclonal TNF- α (A0277) (ABclonal, Woburn, MA, USA) or 1:100 Mouse
101 Monoclonal Caspase 3 (74T2) (ThermoFisher Scientific, Waltham, MA, USA) Antibody was
102 used. For secondary antibody and streptavidin-biotin immunoenzymatic antigen detection
103 system, Mouse and Rabbit Specific HRP (ABC) Detection IHC Kit (Abcam, Cambridge, UK)
104 was used. For the chromogen, Diamino benzidine (DAB) Substrate Kit (Abcam) was used.
105 Counterstaining was performed with Meyer's hematoxylin. Two pathologists, who were
106 blinded to the study design, performed the histopathological examinations. An intraclass
107 correlation coefficient analysis was conducted to evaluate the results. For each stained
108 intestinal tissue section, 5 different areas were selected and documented at 100x magnification
109 under upright light microscope. Each documented image was measured using ImageJ (US
110 National Institutes of Health, Bethesda, MD, USA). Color deconvolution and threshold feature
111 were set to measure protein expression.

112

113 **Results**

114 Two pathologists, who were blinded to the study design, had intraclass correlation coefficient
115 values of $\kappa = 0.999$ for TNF- α and $\kappa = 0.998$ for Caspase-3 immunohistochemical expression
116 assessments. This indicated a very good agreement between the assessments of the pathologists
117 on TNF- α and Caspase-3 immunohistochemical expression.

118 **APPLE Inhibited LPS-induced TNF- α Expression of Rats' Intestinal Tissues**

119 Under 1 mg/200 g BW LPS induction, TNF- α expression of rats' intestinal tissues was
120 significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure
121 1). This indicated that the LPS could induce inflammatory rats' intestinal tissues. However,
122 under pretreatment of APPLE, the TNF- α expression of rats' intestinal tissues could be inhibited.
123 Significant APPLE inhibition on LPS-induced TNF- α expression of rats' intestinal tissues was
124 started in concentration of 200 mg/kg BW/day (Group 3). This APPLE inhibition was in

125 concentration manner. Therefore, the TNF- α expression of rats' intestinal tissues with
126 pretreatment of 400 mg/kg BW/day APLE (Group 4) was lower significantly than the one of
127 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the
128 group that was pretreated with 500 mg/kgBW/day (Group 5) had the lowest TNF- α expression
129 of rats' intestinal tissues. The TNF- α expression of rats' intestinal tissues in this Group 5 was
130 similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest that the 500
131 mg/kg BW/day APLE could almost totally inhibit the LPS-induced TNF- α expression of rats'
132 intestinal tissues.

133 **APLE Inhibited LPS-induced Caspase-3 Expression of Rats' Intestinal Tissues**

134 Under 1 mg/200 g BW LPS induction, Caspase-3 expression of rats' intestinal tissues was
135 significantly increased in Group 2 than Group 1 ($p < 0.001$, Tukey HSD Post-Hoc test) (Figure
136 1). This indicated that the LPS could induce apoptotic rats' intestinal tissues. However, under
137 pretreatment of APLE, the Caspase-3 expression of rats' intestinal tissues could be inhibited.
138 Significant APLE inhibition on LPS-induced Caspase-3 expression of rats' intestinal tissues
139 was started in concentration of 200 mg/kg BW/day (Group 3). This APLE inhibition was in
140 concentration manner. Therefore, the Caspase-3 expression of rats' intestinal tissues with
141 pretreatment of 400 mg/kg BW/day APLE (Group 4) was lower significantly than the one of
142 200 mg/kg BW/day APLE (Group 3). Hence, among these 3 APLE-pretreated groups, the
143 group that was pretreated with 500 mg/kg BW/day (Group 5) had the lowest Caspase-3
144 expression of rats' intestinal tissues. The Caspase-3 expression of rats' intestinal tissues in this
145 Group 5 was similar to the one in Group 1 (sham group, NaCl-injected rats). This could suggest
146 that the 500 mg/kgBW/day APLE could almost totally inhibit the LPS-induced Caspase-3
147 expression of rats' intestinal tissues.

148

149 **Discussion**

150 Current study showed that the induction using LPS injection could resemble sepsis condition,
151 which increased the expression of TNF- α and Caspase-3. A similar result has been reported
152 with the use of Septimed, a herbal medicine with an anti-inflammatory properties. Compared
153 to a control group that were administered with standard sepsis treatment, Septimed was found
154 to significantly decrease the severity of sepsis based on the reduction of the inflammatory
155 markers.(21) Another study also reports a successful sepsis induction in BALB/c mice using
156 sub-lethal dose of LPS, proven by the increase of TNF- α , nuclear factor kappa B (NF- κ B), and
157 nitrate concentrations that were measured using real-time polymerase chain reaction.(22)
158 Induction with intraperitoneal LPS injection has been found to cause apoptosis of intestinal
159 epithelial cells.(23) The apoptosis of intestinal epithelial cells was induced by a cascade that
160 leads to the production of proinflammatory cytokines and interferons, including TNF- α .(24)
161 Release of TNF- α into the blood circulation will trigger the activation of Caspase-3 which then
162 cleave various structural, cell cycle, and DNase proteins causing apoptosis.(25) In current
163 study, pretreatment of APLE could inhibit the expression of TNF- α and Caspase-3 in a
164 concentration dependent manner. The 500 mg/kg BW/day APLE could almost totally inhibit
165 the LPS-induced TNF- α and Caspase-3 expression of rats' intestinal tissues. Inhibition of TNF-
166 α , interleukin (IL)-6, and nitric oxide (NO) production by APLE in a dose-dependent pattern
167 was reported as well.(26)

168 Andrographolide, an active metabolite of *A. paniculate*, is an effective anti-
169 inflammatory agent, which has been found to significantly decrease TNF- α level. Moreover,
170 Andrographolide can form a covalent that inhibits the production of pro-inflammatory
171 cytokines, thus could alleviate or prevent inflammation.(27) Another phytochemical study has
172 shown that *A. paniculate* extract contains some non-standardized constituents that belongs to
173 the flavonoid and phenylcarboxylic acid class. Both of this compounds are known and
174 commonly used in scavenging free radicals and its antioxidant properties, which in this case,

175 oxidative stress is a substantial component in inflammatory tissue damage and cytokine
176 signaling.(28) Overall, APLE could reduce cytokine production mediated by LPS through
177 negative regulation, so that inflammation can be controlled and resolved quickly with minimal
178 acute organ damage. However, our study did not analyze the bioactive compounds contained
179 in APLE, therefore further research is needed.

180

181 **Conclusion**

182 LPS could induce septic rats' intestinal tissues by increasing the expression of TNF- α and
183 Caspase-3. And the pretreatment of APLE could inhibit the LPS-induced TNF- α and Caspase-
184 3 expression, therefore APLE could be suggested as a potential sepsis-preventing agent.
185 However, the compound and mechanism of APLE should be further investigated.

186

187 **Acknowledgments**

188 We would like to thank the Prodia Education and Research Institute as well as Clara and Ardi
189 Prasetio from Stem Cell and Cancer Research Indonesia for the support in this study.

190

191 **Authors' Contributions**

192 RGA was involved in concepting and planning of the study and collected the data samples.
193 RGA and FS performed the analysis of the data, designed the figures, as well as drafted and
194 revised the manuscript. PB, NSW, NM, NS, and FS critically revised the draft for important
195 intellectual content. All authors have read and approved the final manuscript.

196

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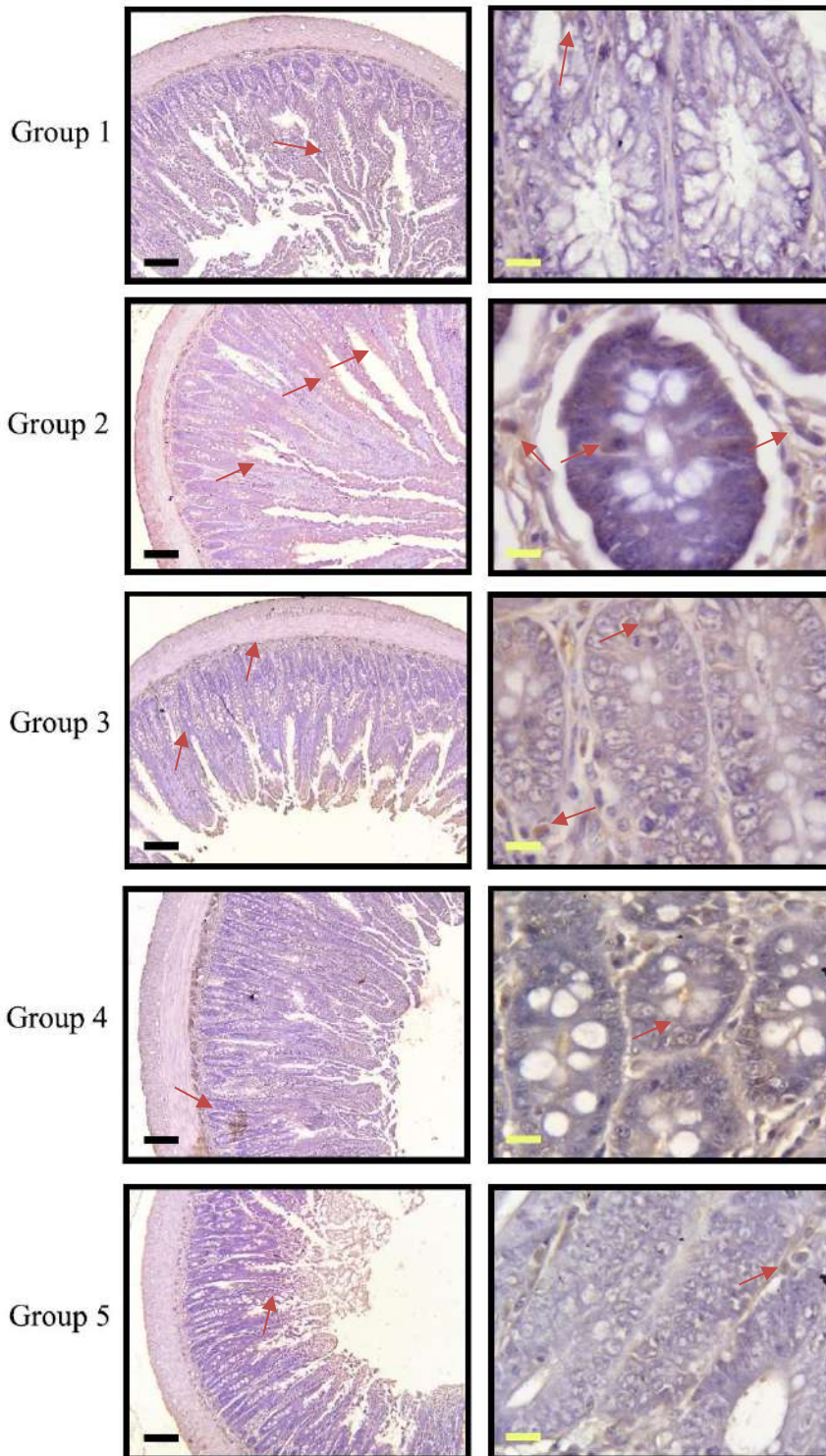
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287 **Figures**

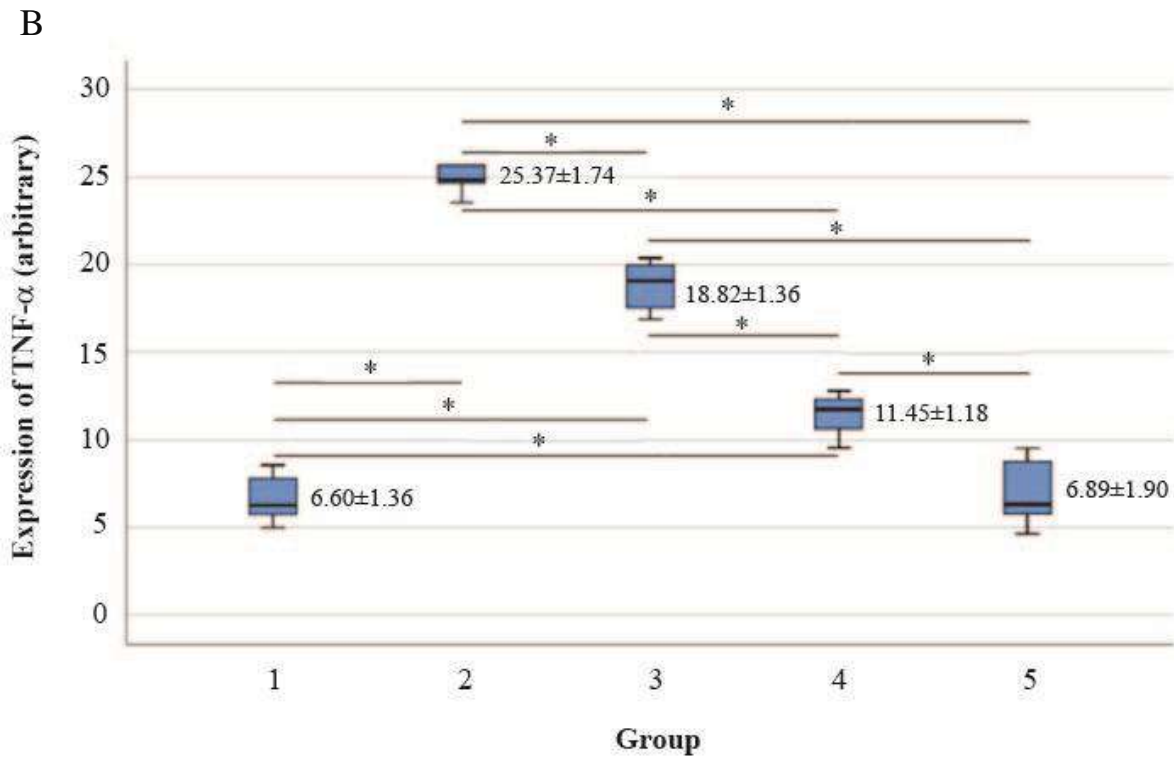
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292 **Figure 1.** TNF- α immunohistochemical expression of rats' intestinal tissues pretreated
 293 with/without APLE and induced with/without LPS. After pretreatment with/without APLE and
 294 induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were
 295 collected and processed for immunohistochemical analysis and measured with Image J, as
 296 mentioned in the Methods. A: TNF- α immunohistochemical expression of each group with
 297 100x (left side) and 1,000x magnifications (right side). B: TNF- α immunohistochemical
 298 expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected
 299 rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400
 300 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-
 301 pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar
 302 = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α^+ expression.

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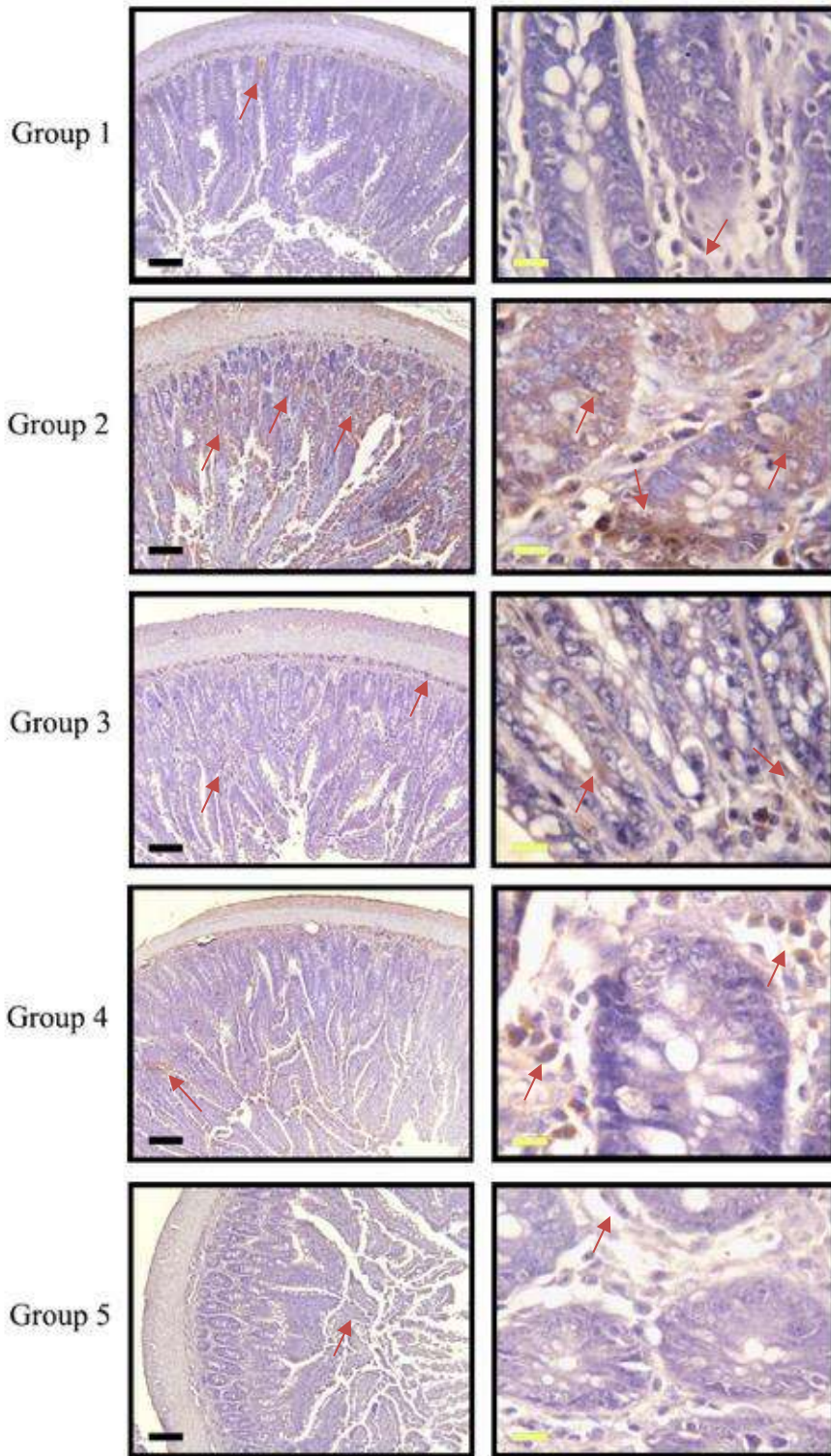
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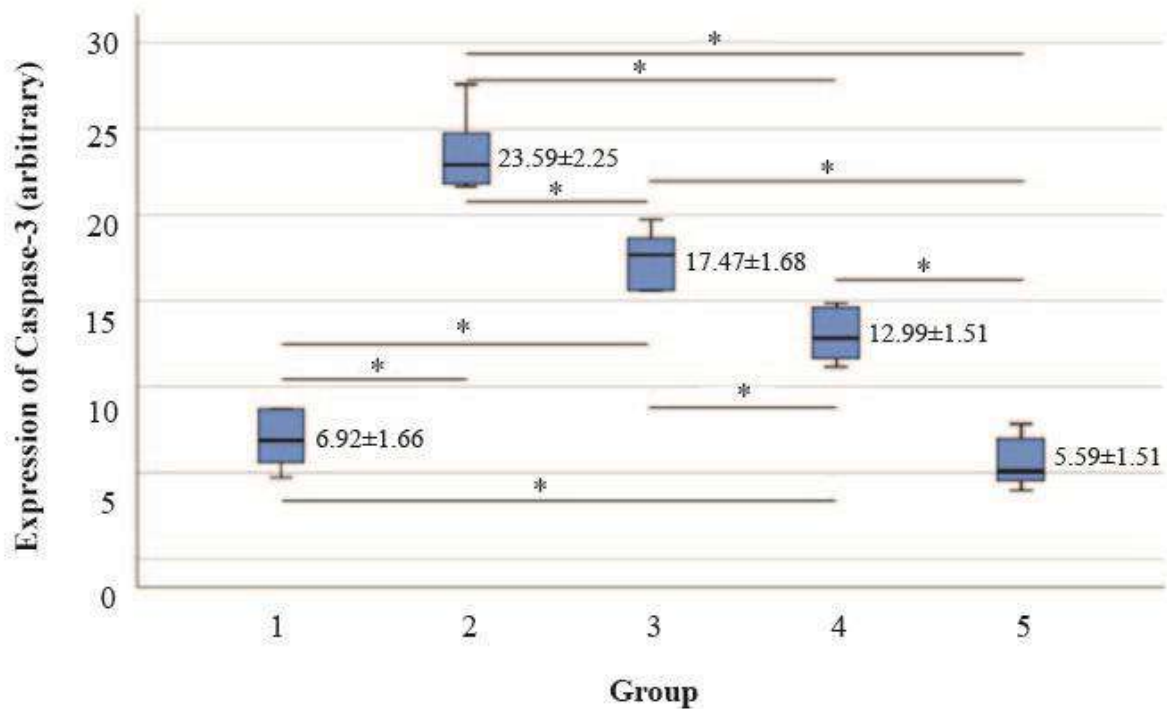
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311 B



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313 **Figure 2.** Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated
 314 with/without APLE and induced with/without LPS. After pretreatment with/without APLE and
 315 induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissues were
 316 collected and processed for immunohistochemical analysis and measured with Image J, as
 317 mentioned in the Methods. A: Caspase-3 immunohistochemical expression of each group with
 318 100x (left side) and 1,000x magnifications (right side). B: Caspase-3 immunohistochemical
 319 expression measurement of each group. Group 1: NaCl-injected rats; Group 2: LPS-injected
 320 rats; Group 3: 200 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 4: 400
 321 mg/kgBW/day APLE-pretreated and LPS-injected rats; Group 5: 500 mg/kgBW/day APLE-
 322 pretreated and LPS-injected rats. *significant ($p < 0.001$, Tukey HSD Post-Hoc test). Black bar
 323 = 100 μ m; Yellow bar = 1,000 μ m; Red arrow: TNF- α ⁺ expression.

324



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[InaBJ] M2023322 Editor Decision - Manuscript Accepted

Secretariat of InaBJ <secretariatinabj@gmail.com>

Thu, Feb 15, 2024 at 10:12 AM

To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Andrographis paniculata* Leaves Extract Inhibit TNF- α and Caspase-3 Expression of Septic Rats' Intestinal Tissues.**"

Our decision is to: **Accept Manuscript.**

Your manuscript will be sent to our publisher for typesetting and you should receive the proofreading in due course.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

--

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