Print ISSN: 2085-3297, Online ISSN: 2355-9179

# The Indonesian BIOMEDICAL JOURNAL

Volume 16 Number 1
February 2024

Published by:

PERI
The Prodia Education & Research Institute

Secretariat: Prodia Tower 9th Floor
Jl. Kramat Raya No.150,
Jakarta 10430, Indonesia
Tel.: +62-21-3144182
Fax.: +62-21-3144181
E-mail: Secretariat@InaBJ.org
Website: www.InaBJ.org

#200/M/KPT/2020

Included in:
Scopus DOAJ DIRECTORY OF OPEN ACCESS JOURNALS
EMERGING SOURCES CITATION Clarivate Analytics

## REVIEW ARTICLES



## RESEARCH ARTICLES

Print ISSN: 2085-3297, Online ISSN: 2355-9179

# The Indonesian BIOMEDICAL JOURNAL

Volume 16 Number 1, February 2024




## Contact Address
**Secretariat of The Indonesian Biomedical Journal**
Prodia Tower 9th Floor
Jl. Kramat Raya No.150, Jakarta 10430, Indonesia
Tel.: +62-21-3144182, ext. 3872
Fax.: +62-21-3144181
WhatsApp No.: +62 877-3616-3117
E-mail: Secretariat@InaBJ.org
Website: www.InaBJ.org


## Focus & Scope
The Indonesian Biomedical Journal (InaBJ) is an open access, peer-reviewed journal that encompasses all fundamental and molecular aspects of basic medical sciences, emphasizing on providing the molecular studies of biomedical problems and molecular mechanisms.

InaBJ is dedicated to publish original research and review articles covering all aspects in biomedical sciences. The editors will carefully select manuscript to present only the most recent findings in basic and clinical sciences. All professionals concerned with biomedical issues will find this journal a most valuable update to keep them abreast of the latest scientific development.

## Section Policies
**Review Article**
Review Article should consist of no more than 10,000 words, not including the words in abstract, references, table, figure, and figure legend. The manuscript should have no more than eight figures and/or tables in total and no more than 250 references. Only invited authors are allowed to submit review article.

**Research Article**
Research Article should consist of no more than 3,500 words, not including the words in abstract, references, table, figure, and figure legend. The manuscript should have no more than six figures and/or tables in total and no more than 40 references.

## Peer Review Process
All manuscripts submitted to InaBJ will be selected and double-blind peer-reviewed by two or more reviewers to present valuable and authentic findings in biomedical sciences. At least, an external reviewer will be included as the reviewer in each manuscript reviewing process.

Author can suggest reviewer/s that not having publication together within five years and should not be member/s of the same research institution. However, reviewers will be selected independently by Section Editor based on their expertise, specialties, and independencies to fit the topic. Section Editor will ensure that the reviewers will be not from the same institution as the author.

Manuscript will be reviewed comprehensively, including appropriate title; content reflecting abstract; concise writing; clear purpose, study method and figures and/or tables; and summary supported by content. Supplementary data will also be sent to reviewer. The reviewing process will take generally 2-3 months depends on sufficiency of information provided.

Decisions are ultimately made by the Section Editor based on the peer-reviewing results. Therefore, Section Editor will consider thoroughly, if necessary Section Editor can invite another one or more reviewer/s to conclude the final decision.

## Publication Frequency
InaBJ is published bimonthly (in February, April, June, August, October, and December).

## Open Access Policy
InaBJ provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.

Print ISSN: 2085-3297, Online ISSN: 2355-9179

# Content

*The Indonesian Biomedical Journal*
*Volume 16 Number 1, February 2024*

## REVIEW ARTICLE



## RESEARCH ARTICLE



## RESEARCH ARTICLE

RESEARCH ARTICLE

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

Ryco Giftyan Ardika[1], Bernardus Parish Budiono[2], Nyoman Suci Widiastiti[3], Nani Maharani[4], Neni Susilaningsih[5], Ferry Sandra[6,*]

[1]Biomedical Science Postgraduate Program, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[2]Digestive Surgery Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[3]Clinical Pathology Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[4]Pharmacology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[5]Histology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[6]Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

*Corresponding author. Email: ferry@trisakti.ac.id

Received date: Oct 15, 2023; Revised date: Feb 13, 2024; Accepted date: Feb 15, 2024

## 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 Capsase-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 µm thick sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated with 0.5% $H_2O_2$ 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/kgBW/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/kgBW/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

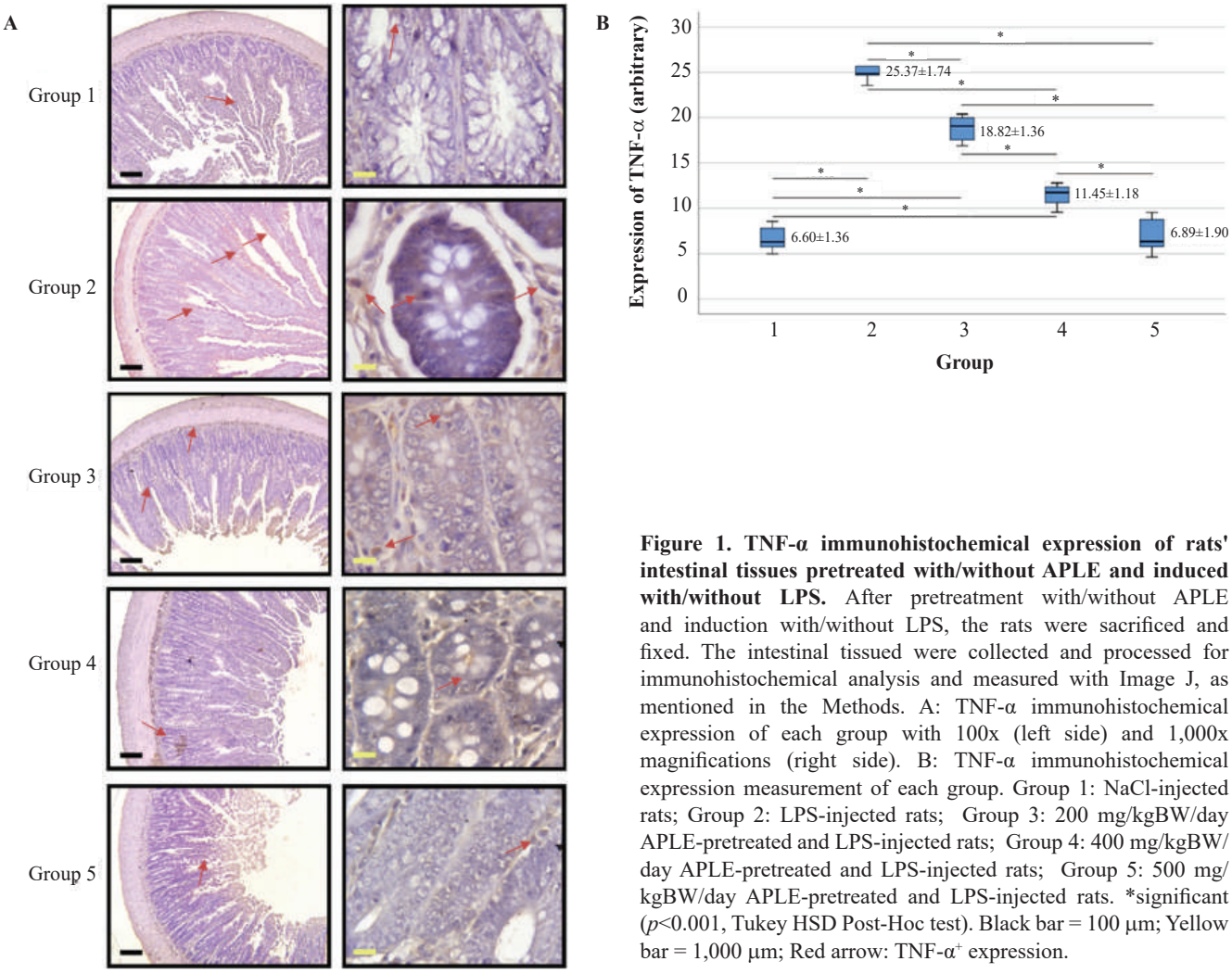



**Figure 1. TNF-α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for immunohistochemical 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 µm; Yellow bar = 1,000 µm; 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-κB), 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

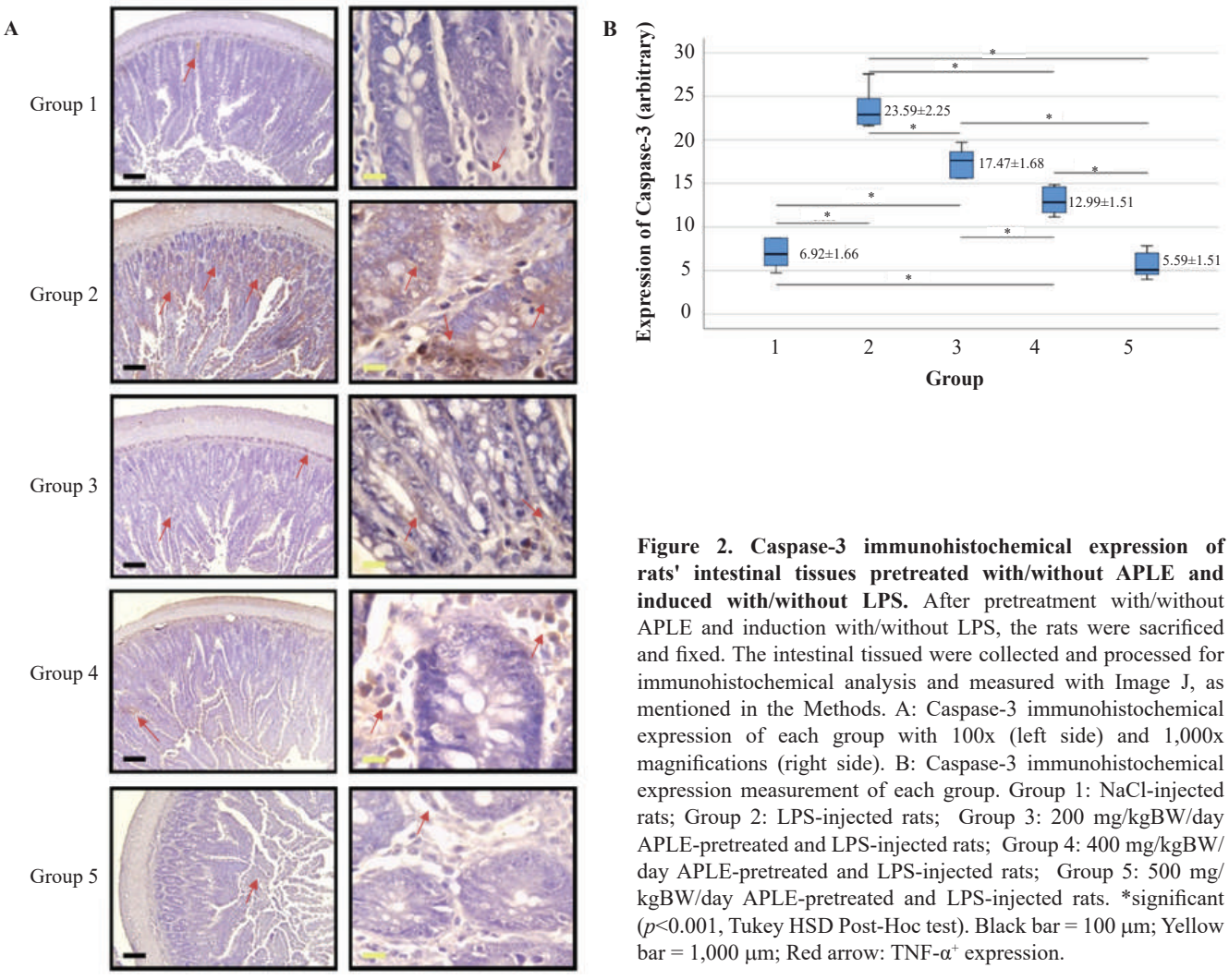



**Figure 2. Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for 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 µm; Yellow bar = 1,000 µm; Red arrow: TNF-$\alpha^{+}$ 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.

## Acknowledgments

The authors would like to thank the Prodia Education and Research Institute (PERI) as well as Clara and Ardi Prasetio from Stem Cell and Cancer Research Indonesia for the support in this study.

## 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.

## References

1. World Health Organization. Global Report on the Epidemiology and Burden of Sepsis: Current Evidence, Identifying Gaps and Future Directions. Geneva: World Health Organization; 2020.
2. Yealy DM, Mohr NM, Shapiro NI, Venkatesh A, Jones AE, Self WH. Early care of adults with suspected sepsis in the emergency department and out-of-hospital environment: A consensus-based task force report. Ann Emerg Med. 2021; 78(1): 1–19. doi: 10.1016/j.annemergmed.2021.02.006.
3. Kevin M, Widyastiti NS, Budijitno S, Prajoko YW, Susilaningsih N. The protective effect of Andrographis paniculata against lipopolysaccharide-induced sepsis in lung tissues of a rat model through the decrease of ICAM-1 and E-selectin expression. Indones Biomed J. 2023; 15(6): 411–9.
4. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet. 2018; 392(10141): 75–87.
5. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α modulation of intestinal tight junction permeability is mediated by NIK/IKK-α axis activation of the canonical NF-κB pathway. Am J Pathol. 2016; 186(5): 1151–65.
6. Alvarez S, Blanco A, Fresno M, Muñoz-Fernández MÁ. TNF-α contributes to caspase-3 independent apoptosis in neuroblastoma cells: Role of NFAT. PLoS One. 2011; 6(1): e16100. doi: 10.1371/journal.pone.0016100.
7. Freedman JC, Navarro MA, Morrell E, Beingesser J, Shrestha A, McClane BA, *et al.* Evidence that Clostridium perfringens enterotoxin-induced intestinal damage and enterotoxemic death in mice can occur independently of intestinal caspase-3 activation. Infect Immun. 2018; 86(7): e00931–17.
8. Billmeier U, Dieterich W, Neurath MF, Atreya R. Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases. World J Gastroenterol. 2016; 22(42): 9300–13.
9. Marshall JC, Leligdowicz A. Gaps and opportunities in sepsis translational research. eBioMedicine. 2022; 86: 104387. doi: 10.1016/j.ebiom.2022.104387.
10. Sandra F. Role of herbal extract in stem cell development. Mol Cell Biomed Sci. 2018; 2(1): 19–22.
11. Qurrotuaini SP, Wiqoyah N, Mustika A. Antimicrobial activity of ethanol extract of Centella asiatica leaves on Proteus mirabilis, Proteus vulgaris, and Yersinia enterocolitica in vitro. Mol Cell Biomed Sci. 2022; 6(3): 135–40.
12. Suastika LOS, Oktaviono YH, Soemantri D, Sandra F. Purple sweet potato extract and vitamin C increase the proliferation of endothelial progenitor cells from stable coronary artery disease patients. Bali Med J. 2021; 10(1): 243–8.
13. Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (Sauropus androgynus) leaves as an anti-inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. Mol Cell Biomed Sci. 2022; 6(3): 129–34.
14. Ranggaini D, Sandra F, Halim J, Ichwan SJA, Djamil MS. Curcuma xanthorrhiza rhizome extract induces apoptosis in HONE-1 nasopharyngeal cancer cells through bid. Indones Biomed J. 2023; 15(1): 100–5.
15. Sikumalay A, Suharti N, Masri M. Efek antibakteri dari rebusan daun sambiloto (Andrographis paniculata Nees) dan produk herbal sambiloto terhadap Staphylococcus aureus. J Kesehat Andalas. 2014; 5(1): 196–200.
16. Lin HC, Li CC, Yang YC, Chiu TH, Liu KL, Lii CK, *et al.* Andrographis paniculata diterpenoids and ethanolic extract inhibit TNFα-induced ICAM-1 expression in EA.hy926 cells. Phytomedicine. 2019; 52: 157–67.
17. Astuti NT, Novitasari PR, Tjandrawinata R, Nugroho AE, Pramono S. Anti-diabetic effect of andrographolide from Sambiloto herbs (Andrographis paniculata (Burm.f.) Nees) through the expression of PPARγ and GLUT-4 in adipocytes. Indones J Biotechnol. 2022; 27(4): 203–11.
18. Yu L, Liu Y, Cao C, Yang L, Liu H, Wang C. Andrographolide attenuates inflammation due to intra-abdominal sepsis by enhancing bacterial clearance in mice. J Inflamm Res. 2023; 16: 4413–23.
19. Kusmardi K, Ariffandi B, Lubis NS, Lestari TW, Intan PR, Pakpakan A. Effects of sambiloto (Andrographis paniculata) extract and spirulina (Spirulina platensis) administration on Ki-67 protein expression in the colon epithelial cells of Plasmodium berghei-infected mice. Indones Biomed J. 2022; 14(1): 84–90.
20. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E. Andrographolide reverses doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene expressions. Indones Biomed J. 2023; 15(5): 288–96.
21. Pourdast A, Sanaei M, Jafari S, Mohammadi M, Khalili H, Shafiee G, *et al.* Effect of SeptimebTM as a new natural extract on severe sepsis: A randomized clinical trial. Caspian J Intern Med. 2017; 8(1): 35–43.
22. Lee YY, Yuk HJ, Saba E, Kim SD, Kim DS, Kopalli SR, *et al.* Duchesnea indica extract ameliorates LPS-induced septic shock in mice. Evid Based Complement Alternat Med. 2022: 2022: 5783867. doi: 10.1155/2022/5783867.
23. Williams JM, Duckworth CA, Watson AJM, Frey MR, Miguel JC, Burkitt MD, *et al.* A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Dis Model Mech. 2013; 6(6): 1388–99.
24. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines. 2017; 5(4): 34. doi: 10.3390/vaccines5040034.
25. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013; 14(1): 32. doi: 10.1186/1471-2121-14-32.
26. Lin BF, Chao WW, Kuo YH, Hsieh SL. Inhibitory effects of ethyl acetate extract of Andrographis paniculata on NF-κB trans-activation activity and LPS-induced acute inflammation in mice. Evid Based Complement Altern Med. 2011; 2011: :254531. doi: 10.1093/ecam/nep120.
27. Guo W, Liu W, Chen G, Hong S, Qian C, Xie N, *et al.* Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways. Int Immunopharmacol. 2012; 14(4): 613–9.
28. Low M, Khoo CS, Münch G, Govindaraghavan S, Sucher NJ. An in vitro study of anti-inflammatory activity of standardised Andrographis paniculata extracts and pure andrographolide. BMC Complement Altern Med. 2015; 15: 18. doi: 10.1186/s12906-015-0525-7.

# Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

| | |
|---|---|
| Submission author: | Ferry Sandra |
| Assignment title: | SIJALI 1 |
| Submission title: | InaBJ V16N1A7 - Andrographis paniculata Leaves Extract Inh... |
| File name: | V16N1A7.pdf |
| File size: | 2.09M |
| Page count: | 6 |
| Word count: | 3,946 |
| Character count: | 22,141 |
| Submission date: | 29-Feb-2024 03:24PM (UTC+0700) |
| Submission ID: | 2307765485 |

The Indonesian Biomedical Journal, Vol.16, No.1, February 2024, p.1-93 Print ISSN: 2085-3297, Online ISSN: 2355-9179

RESEARCH ARTICLE

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

Ryco Giftyan Ardika[1], Bernardus Parish Budiono[2], Nyoman Suci Widiastiti[3], Nani Maharani[4], Neni Susilaningsih[5], Ferry Sandra[6,*]

[1]Biomedical Science Postgraduate Program, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[2]Digestive Surgery Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[3]Clinical Pathology Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[4]Pharmacology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[5]Histology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[6]Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

*Corresponding author. Email: ferry@trisakti.ac.id

Received date: Oct 15, 2023; Revised date: Feb 13, 2024; Accepted date: Feb 15, 2024

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***

# InaBJ V16N1A7 - Andrographis paniculata Leaves Extract Inhibit TNF-α and Caspase-3 Expression of Septic Rats' Intestinal Tissues

*by* Ferry Sandra

**Submission date:** 29-Feb-2024 03:24PM (UTC+0700)
**Submission ID:** 2307765485
**File name:** V16N1A7.pdf (2.09M)
**Word count:** 3946
**Character count:** 22141

The Indonesian Biomedical Journal, Vol.16, No.1, February 2024, p.1-93 Print ISSN: 2085-3297, Online ISSN: 2355-9179

RESEARCH ARTICLE

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

Ryco Giftyan Ardika[1], Bernardus Parish Budiono[2], Nyoman Suci Widiastiti[3], Nani Maharani[4], Neni Susilaningsih[5], Ferry Sandra[6,*]

[1]Biomedical Science Postgraduate Program, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[2]Digestive Surgery Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[3]Clinical Pathology Department, Faculty of Medicine, Universitas Diponegoro/Kariadi General Hospital, Jl. Dr. Sutomo No.16, Semarang 50244, Indonesia
[4]Pharmacology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[5]Histology Department, Faculty of Medicine, Universitas Diponegoro, Jl. Prof. Sudarto No. 13, Semarang 50275, Indonesia
[6]Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

*Corresponding author. Email: ferry@trisakti.ac.id

Received date: Oct 15, 2023; Revised date: Feb 13, 2024; Accepted date: Feb 15, 2024

## 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 Capsase-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 μm thick sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated with 0.5% $H_2O_2$ 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/kgBW/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/kgBW/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

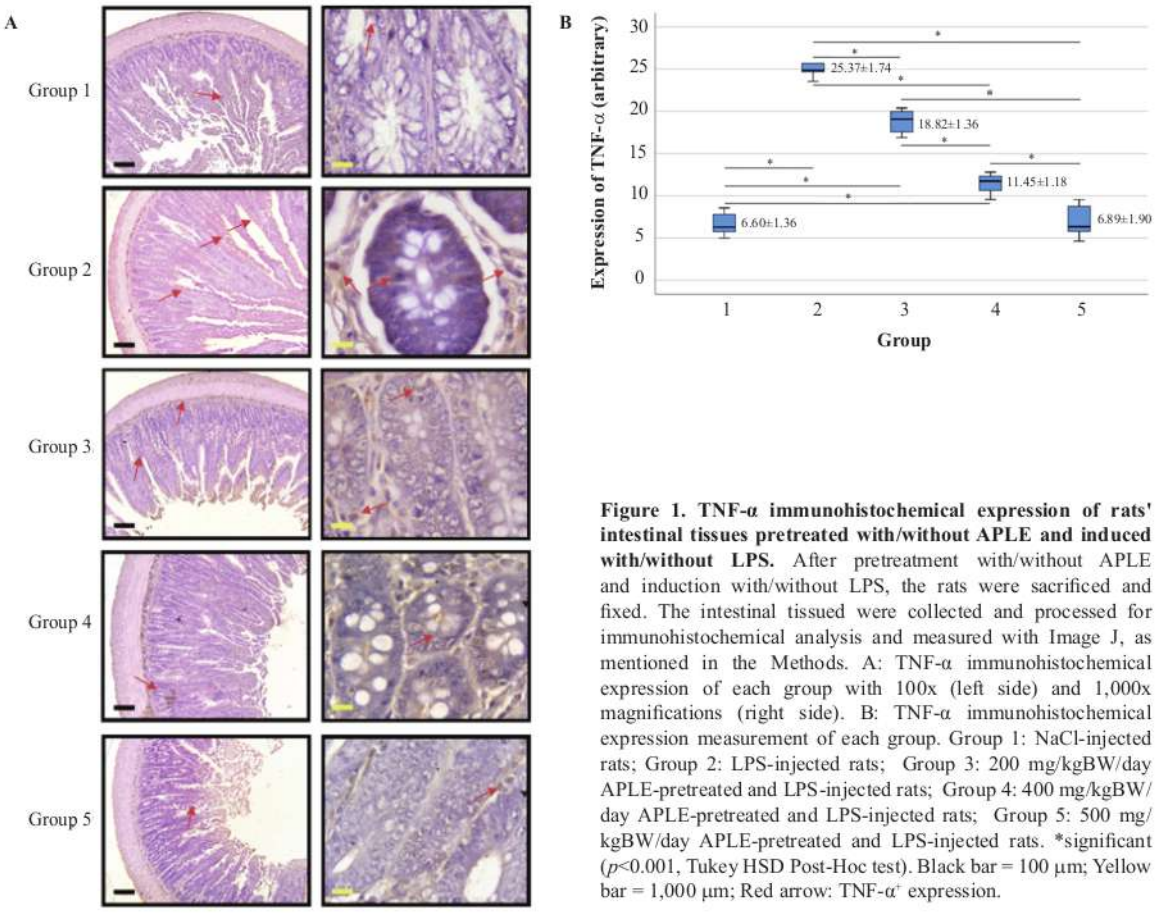



**Figure 1. TNF-α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for immunohistochemical 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 µm; Yellow bar = 1,000 µm; 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-κB), 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

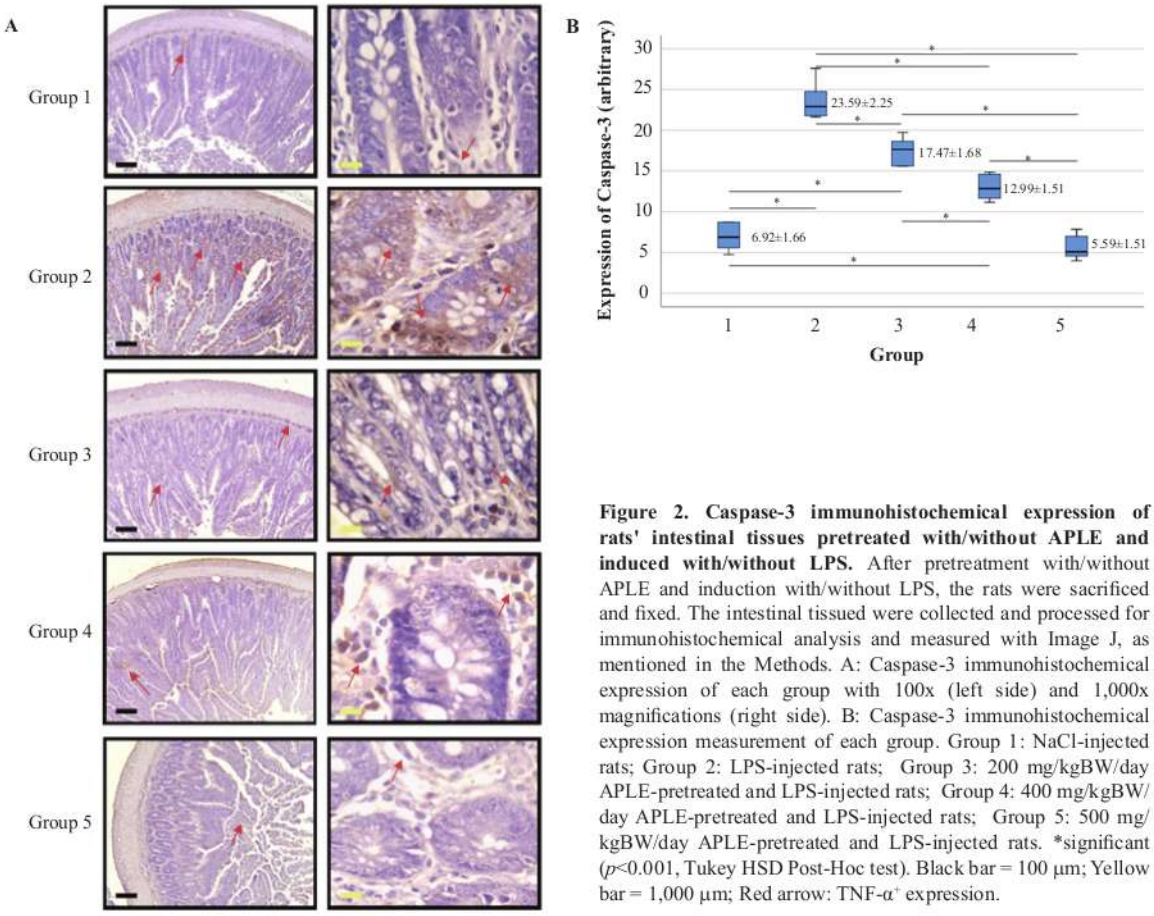



**Figure 2. Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for 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 µm; Yellow bar = 1,000 µm; Red arrow: TNF-$\alpha^+$ 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.

## Acknowledgments

The authors would like to thank the Prodia Education and Research Institute (PERI) as well as Clara and Ardi Prasetio from Stem Cell and Cancer Research Indonesia for the support in this study.

## 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.

## References


1. World Health Organization. Global Report on the Epidemiology and Burden of Sepsis: Current Evidence, Identifying Gaps and Future Directions. Geneva: World Health Organization; 2020.
2. Yealy DM, Mohr NM, Shapiro NI, Venkatesh A, Jones AE, Self WH. Early care of adults with suspected sepsis in the emergency department and out-of-hospital environment: A consensus-based task force report. Ann Emerg Med. 2021; 78(1): 1–19. doi: 10.1016/j.annemergmed.2021.02.006.
3. Kevin M, Widyastiti NS, Budijitno S, Prajoko YW, Susilaningsih N. The protective effect of Andrographis paniculata against lipopolysaccharide-induced sepsis in lung tissues of a rat model through the decrease of ICAM-1 and E-selectin expression. Indones Biomed J. 2023; 15(6): 411–9.
4. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet. 2018; 392(10141): 75–87.
5. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α modulation of intestinal tight junction permeability is mediated by NIK/IKK-α axis activation of the canonical NF-κB pathway. Am J Pathol. 2016; 186(5): 1151–65.
6. Alvarez S, Blanco A, Fresno M, Muñoz-Fernández MÁ. TNF-α contributes to caspase-3 independent apoptosis in neuroblastoma cells: Role of NFAT. PLoS One. 2011; 6(1): e16100. doi: 10.1371/journal.pone.0016100.
7. Freedman JC, Navarro MA, Morrell E, Beingesser J, Shrestha A, McClane BA, *et al.* Evidence that Clostridium perfringens enterotoxin-induced intestinal damage and enterotoxemic death in mice can occur independently of intestinal caspase-3 activation. Infect Immun. 2018; 86(7): e00931–17.
8. Billmeier U, Dieterich W, Neurath MF, Atreya R. Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases. World J Gastroenterol. 2016; 22(42): 9300–13.
9. Marshall JC, Leligdowicz A. Gaps and opportunities in sepsis translational research. eBioMedicine. 2022; 86: 104387. doi: 10.1016/j.ebiom.2022.104387.
10. Sandra F. Role of herbal extract in stem cell development. Mol Cell Biomed Sci. 2018; 2(1): 19–22.
11. Qurrotuaini SP, Wiqoyah N, Mustika A. Antimicrobial activity of ethanol extract of Centella asiatica leaves on Proteus mirabilis, Proteus vulgaris, and Yersinia enterocolitica in vitro. Mol Cell Biomed Sci. 2022; 6(3): 135–40.
12. Suastika LOS, Oktaviono YH, Soemantri D, Sandra F. Purple sweet potato extract and vitamin C increase the proliferation of endothelial progenitor cells from stable coronary artery disease patients. Bali Med J. 2021; 10(1): 243–8.
13. Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (Sauropus androgynus) leaves as an anti-inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. Mol Cell Biomed Sci. 2022; 6(3): 129–34.
14. Ranggaini D, Sandra F, Halim J, Ichwan SJA, Djamil MS. Curcuma xanthorrhiza rhizome extract induces apoptosis in HONE-1 nasopharyngeal cancer cells through bid. Indones Biomed J. 2023; 15(1): 100–5.
15. Sikumalay A, Suharti N, Masri M. Efek antibakteri dari rebusan daun sambiloto (Andrographis paniculata Nees) dan produk herbal sambiloto terhadap Staphylococcus aureus. J Kesehat Andalas. 2014; 5(1): 196–200.
16. Lin HC, Li CC, Yang YC, Chiu TH, Liu KL, Lii CK, *et al.* Andrographis paniculata diterpenoids and ethanolic extract inhibit TNFα-induced ICAM-1 expression in EA.hy926 cells. Phytomedicine. 2019; 52: 157–67.
17. Astuti NT, Novitasari PR, Tjandrawinata R, Nugroho AE, Pramono S. Anti-diabetic effect of andrographolide from Sambiloto herbs (Andrographis paniculata (Burm.f.) Nees) through the expression of PPARγ and GLUT-4 in adipocytes. Indones J Biotechnol. 2022; 27(4): 203–11.
18. Yu L, Liu Y, Cao C, Yang L, Liu H, Wang C. Andrographolide attenuates inflammation due to intra-abdominal sepsis by enhancing bacterial clearance in mice. J Inflamm Res. 2023; 16: 4413–23.
19. Kusmardi K, Ariffandi B, Lubis NS, Lestari TW, Intan PR, Pakpakan A. Effects of sambiloto (Andrographis paniculata) extract and spirulina (Spirulina platensis) administration on Ki-67 protein expression in the colon epithelial cells of Plasmodium berghei-infected mice. Indones Biomed J. 2022; 14(1): 84–90.
20. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E. Andrographolide reverses doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene expressions. Indones Biomed J. 2023; 15(5): 288–96.
21. Pourdast A, Sanaei M, Jafari S, Mohammadi M, Khalili H, Shafiee G, *et al.* Effect of SeptimebTM as a new natural extract on severe sepsis: A randomized clinical trial. Caspian J Intern Med. 2017; 8(1): 35–43.
22. Lee YY, Yuk HJ, Saba E, Kim SD, Kim DS, Kopalli SR, *et al.* Duchesnea indica extract ameliorates LPS-induced septic shock in mice. Evid Based Complement Alternat Med. 2022: 2022: 5783867. doi: 10.1155/2022/5783867.
23. Williams JM, Duckworth CA, Watson AJM, Frey MR, Miguel JC, Burkitt MD, *et al.* A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Dis Model Mech. 2013; 6(6): 1388–99.
24. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines. 2017; 5(4): 34. doi: 10.3390/vaccines5040034.
25. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013; 14(1): 32. doi: 10.1186/1471-2121-14-32.
26. Lin BF, Chao WW, Kuo YH, Hsieh SL. Inhibitory effects of ethyl acetate extract of Andrographis paniculata on NF-κB trans-activation activity and LPS-induced acute inflammation in mice. Evid Based Complement Altern Med. 2011; 2011: :254531. doi: 10.1093/ecam/nep120.
27. Guo W, Liu W, Chen G, Hong S, Qian C, Xie N, *et al.* Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways. Int Immunopharmacol. 2012; 14(4): 613–9.
28. Low M, Khoo CS, Münch G, Govindaraghavan S, Sucher NJ. An in vitro study of anti-inflammatory activity of standardised Andrographis paniculata extracts and pure andrographolide. BMC Complement Altern Med. 2015; 15: 18. doi: 10.1186/s12906-015-0525-7.

# InaBJ V16N1A7 - Andrographis paniculata Leaves Extract Inhibit TNF-α and Caspase-3 Expression of Septic Rats' Intestinal Tissues

ORIGINALITY REPORT

| 10% | 8% | 6% | 0% |
| --- | --- | --- | --- |
| SIMILARITY INDEX | INTERNET SOURCES | PUBLICATIONS | STUDENT PAPERS |

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

1%

★ Hartono Yudo, Wilma Amiruddin, Ari Wibawa Budi Santosa, Ocid Mursid, Tri Admono. "Study on the characteristics of pipe buckling strength under pure bending and external stress using nonlinear finite element analysis", Journal of Mechatronics, Electrical Power, and Vehicular Technology, 2021

Publication

| | | | |
| --- | --- | --- | --- |
| Exclude quotes | On | Exclude matches | < 15 words |
| Exclude bibliography | On | | |

# InaBJ V16N1A7 - Andrographis paniculata Leaves Extract Inhibit TNF-α and Caspase-3 Expression of Septic Rats' Intestinal Tissues

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

# [InaBJ] M2023322 Editor Decision - Resubmit for Review

**Secretariat of InaBJ** <secretariatinabj@gmail.com> Tue, Nov 28, 2023 at 11:26 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, 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**
Prodia Tower 9th Floor
Jl. Kramat Raya No.150, Jakarta 10430, Indonesia
Phone. +62-21-3144182 ext. 3872
Fax. +62-21-3144181
https://www.inabj.org

**3 attachments**

- **Reviewer 2 - Manuscript Review Form.pdf** 114K
- **Reviewer 1 - Manuscript Review Form.pdf** 168K
- **Reviewer 2 - Manuscript.docx** 4027K

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


## Manuscript Review Form

| | | |
|---|---|---|
| Reviewer | : | Reviewer 1 |
| Manuscript # | : | M202333 |
| Manuscript Title | : | Sambiloto Leave (Andrographis paniculata) 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 | √ | |

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


| | | | |
|---|---|---|---|
| | 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.

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


| |
|---|
| |

| **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) | |
| 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:**

Manuscript is acceptable with some minor corrections.

Date and Sign:
November 27, 2023

Reviewer 1

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


## Manuscript Review Form

| | | |
|---|---|---|
| Reviewer | : | Reviewer 2 |
| Manuscript # | : | M2023322 |
| Manuscript Title | : | **Sambiloto Leave (*Andrographis paniculata*) 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? | | **V** |
| | Notes:<br>A lot of research has been done on bitter leaves, especially those related to infection and inflammation | | |
| 2. | Are the title and abstract of the manuscript appropriate? | | V |
| | Notes:<br>The abstract already describes the title but needs to be sharpened further regarding the reasons for the urgency of this research | | |
| 3 | Do the title and abstract reflect the study result/content? | | V |
| | Notes: The conclusions drawn were too early because the dose given was too large and did not explain the toxic effects | | |
| 4. | Is the significance of the study well explained at the Background? | V | |
| | Notes: | | |
| 5. | Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists? | V | |
| | Notes: | | |

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


| | | | |
|---|---|---|---|
| | | | |
| 6. | Are the results, ideas, and data presented in this manuscript important enough for publication? | | V |
| | Notes:<br>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

# The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia
Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181
Email: Secretariat@InaBJ.org, Website: www.InaBJ.org


| |
|---|
| |

| **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

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


## Abstract

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

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

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

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


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

**Commented [HI1]:** 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?

## Introduction

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

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

**Commented [HI2]:** Add some types of bacteria that cause sepsis

as caspase-9 and caspase-3.[10] Thus, this study aims to determine the effect of *Andrographis paniculata* (Sambiloto) leave extract on the expression of TNF-α and caspase-3 in the colon tissues of *Rattus norvegicus* mice in the sepsis model.

## Methods

### *Animal Study*

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

### *Sambiloto Leave Extract Preparation*

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

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

***Induction of Sepsis***

Sepsis was induced at the 22nd day after all the mice were given the assigned diet for 14 days. Induction was done by injecting lipopolysaccharide (LPS) intraperitoneally with a dose of 1 mg/200 gBW. The rat's head were positioned lower than the abdomen when injecting the needle at approximately 100 degrees from the surface. The injection was given slightly away from the midline to avoid hitting the bladder and lower to avoid the liver. Sham-operated mice were given intraperitoneal injection of 0,5 mL NaCl.

**Commented [HI3]:** 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?

***Outcome Analysis***

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

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

***Statistical Analysis***

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

## Results

***Sepsis Model***

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

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

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

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

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

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

## Discussion

This current study showed that the expression levels of TNF-α and caspase-3 from the healthy control group were the lowest. It indicated that the induction using LPS injection was proven to resemble sepsis conditions, which increased the expression of TNF-α and caspase-3. This study does not use positive control group because the objective was to study the preventive effect of Sambiloto leave extract on sepsis. A similar result has 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 .[11] Another study also reports a successful sepsis induction in BALB/c mice using sub-lethal dose of LPS, proven by the increase in TNF-α, Nf-kB, and nitrate concentrations that were measured using real-time polymerase chain reaction.[12]

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

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

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

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

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

## Conclusion

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

## Acknowledgments

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

## Authors' Contributions

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

## References


1. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet. 2018;392(10141):75–87.
2. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α Modulation of Intestinal Tight Junction Permeability Is Mediated by NIK/IKK-α Axis Activation of the Canonical NF-κB Pathway. Am J Pathol. 2016 May;186(5):1151.
3. Alvarez S, Blanco A, Fresno M, Muñoz-Fernández MÁ. TNF-α contributes to caspase-3 independent apoptosis in neuroblastoma cells: role of NFAT. PLoS One. 2011 Jan;6(1):e16100.
4. Freedman JC, Navarro MA, Morrell E, Beingesser J, Shrestha A, McClane BA, et al. Evidence that Clostridium perfringens Enterotoxin-Induced Intestinal Damage and Enterotoxemic Death in Mice Can Occur Independently of Intestinal Caspase-3 Activation. Infect Immun. 2018 Jul;86(7).
5. Billmeier U, Dieterich W, Neurath MF, Atreya R. Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases. World J Gastroenterol. 2016 Nov;22(42):9300.
6. Sikumalay A, Suharti N, Masri M. Efek Antibakteri dari Rebusan Daun Sambiloto (Andrographis paniculata Nees) dan Produk Herbal Sambiloto Terhadap Staphylococcus Aureus. J Kesehat Andalas. 2014;5(1):196–200.
7. Lin H, Li C, Yang Y, Chiu T, Liu K, Lii C. Andrographis paniculata diterpenoids and ethanolic extract inhibit TNFα-induced ICAM-1 expression in EA.hy926 cells. Phytomedicine. 2018;
8. Yu L, Liu Y, Cao C, Yang L, Liu H, Wang C. Andrographolide Attenuates Inflammation Due to Intra-Abdominal Sepsis by Enhancing Bacterial Clearance in Mice. J Inflamm Res. 2023;16(October):4413–23.

9. Kusmardi K, Ariffandi B, Lubis NS, Lestari TW, Intan PR, Pakpakan A. Effects of Sambiloto (Andrographis paniculata) Extract and Spirulina (Spirulina platensis) Administration on Ki-67 Protein Expression in the Colon Epithelial Cells of Plasmodium berghei-infected Mice. Indones Biomed J. 2022;14(1):84–90.
10. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E. Andrographolide Reverses Doxorubicin Resistance in Human Breast Cancer Stem Cells by Regulating Apoptotic Gene Expressions. 2023;15(5).
11. Pourdast A, Sanaei M, Jafari S, Mohammadi M, Khalili H, Shafiee G, et al. Effect of Septimeb$^{TM}$ as a new natural extract on severe sepsis: A randomized clinical trial. Casp J Intern Med. 2017;8(1):35–43.
12. Lee YY, Yuk HJ, Saba E, Kim SD, Kim DS, Kopalli SR, et al. Duchesnea indica Extract Ameliorates LPS-Induced Septic Shock in Mice. Evidence-based Complement Altern Med. 2022;2022.
13. Williams JM, Duckworth CA, Watson AJM, Frey MR, Miguel JC, Burkitt MD, et al. A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Dis Model Mech. 2013 Nov;6(6):1388–99.
14. Kuzmich NN, Sivak K V., Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines. 2017;5(4):1–25.
15. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol [Internet]. 2013;14(1):32. Available from: https://doi.org/10.1186/1471-2121-14-32
16. Lin BF, Chao WW, Kuo YH, Hsieh SL. Inhibitory effects of ethyl acetate extract of andrographis paniculata on NF-κB trans-activation activity and LPS-induced acute

inflammation in mice. Evidence-based Complement Altern Med. 2011;2011.

17. Guo W, Liu W, Chen G, Hong S, Qian C, Xie N, et al. Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways. Int Immunopharmacol. 2012;14(4):613–9.

## Tables

**Table 1.** TNF-α Expression Analysis.

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

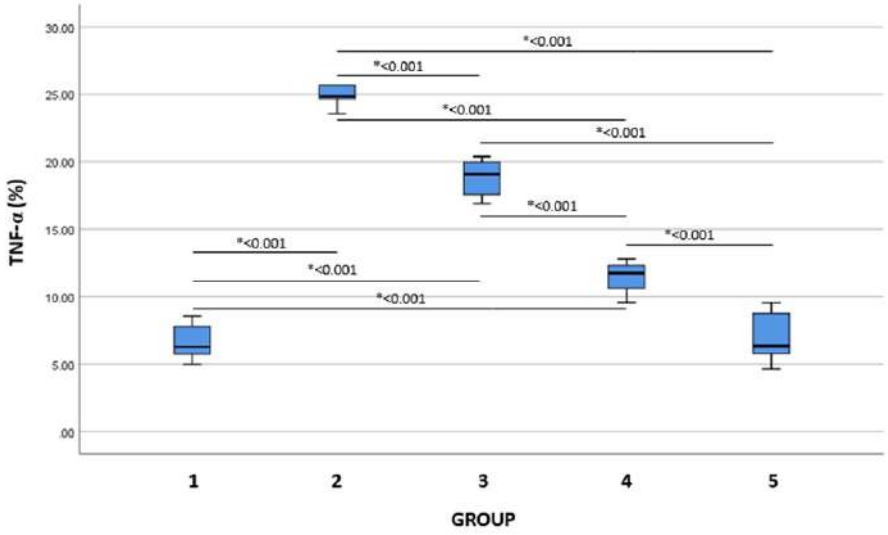
Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day. *Normal distribution ($p$>0,05)

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

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

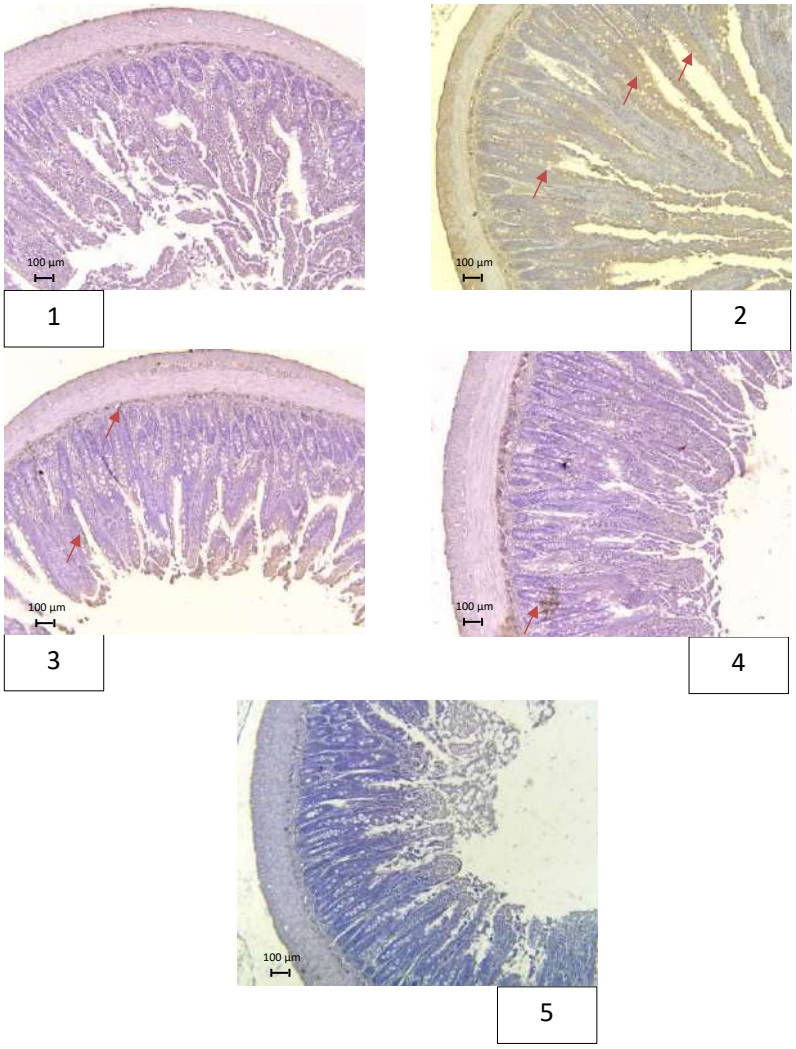
Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day. *Normal distribution ($p$>0,05)

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



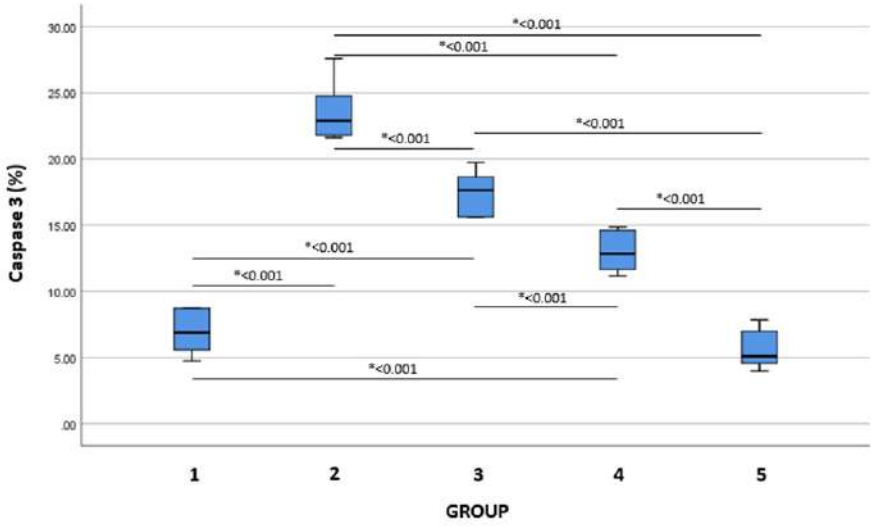

Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day. * Significant (p<0,05)

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



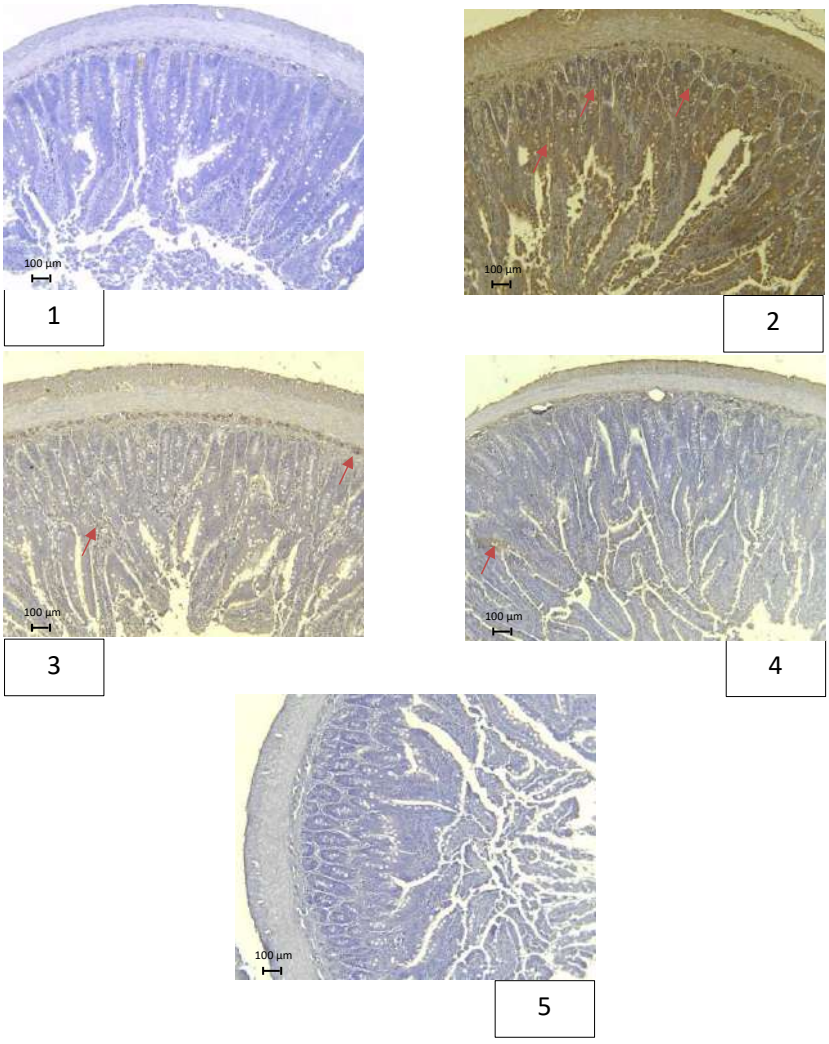

Description: (1) Sham; (2) sepsis induced; (3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day.

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



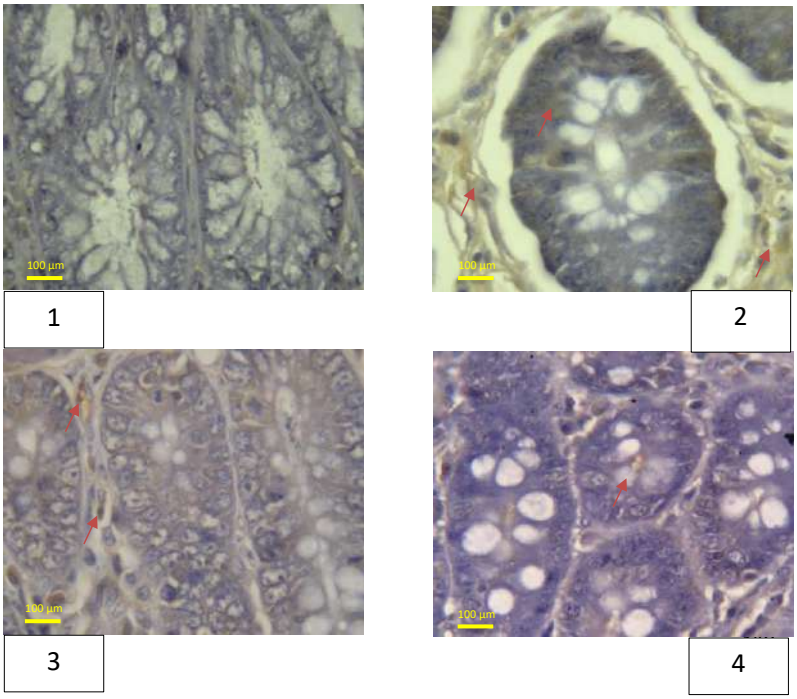

Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day. * Significant ($p$<0,05)

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



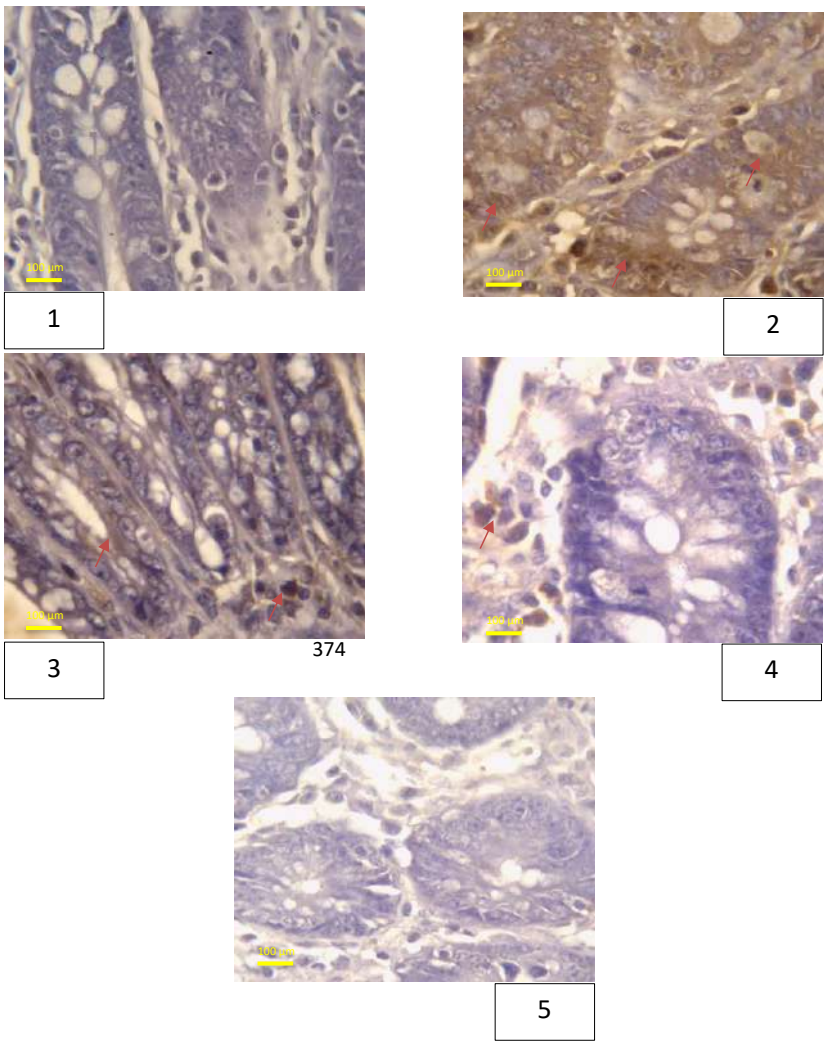

Description: (Group 1) Sham; (Group 2) sepsis induced; (Group 3) sepsis induced and Sambiloto leave extract 200 mg/kgBW/day; (Group 4) sepsis induced and Sambiloto leave extract 400 mg/kgBW/day; and (Group 5) sepsis induced and Sambiloto leave extract 500 mg/kgBW/day.

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




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

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




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

UNIVERSITAS TRISAKTI

**Ferry Sandra <ferry@trisakti.ac.id>**

# [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]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

**M2023322 Manuscript - FS 07022024 FINAL REV.docx**
2208K

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


## 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. paniculate* extract's effect on sepsis have not been commonly studied, especially in intestinal tissues. Therefore, this study was conducted to determine *A. paniculate* leave extract (APLE) effect in sepsis-induced intestinal tissues of rats by examining the expression of inflammatory cytokines involved in sepsis, namely 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

## 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 Capsase-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. paniculate* 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* Leave 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 grams, 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 µm thick sections, deparaffinized, rehydrated, antigen-retrieved with citrate buffer (pH 6.0), incubated with 0.5% $H_2O_2$ 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/kgBW/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 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 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/kgBW/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 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-κB), 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. paniculate,* 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) Another phytochemical study has shown that *A. paniculate* 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 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.

## Acknowledgments

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

## Authors' Contributions

RGA was involved in 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.

## References


1. World Health Organization. Global Report on the Epidemiology and Burden of Sepsis: Current Evidence, Identifying Gaps and Future Directions. Geneva: World Health Organization; 2020.
2. Yealy DM, Mohr NM, Shapiro NI, Venkatesh A, Jones AE, Self WH. Early care of adults with suspected sepsis in the emergency department and out-of-hospital environment: A consensus-based task force report. Ann Emerg Med. 2021; 78(1): 1–19. doi: 10.1016/j.annemergmed.2021.02.006.
3. Kevin M, Widyastiti NS, Budijitno S, Prajoko YW, Susilaningsih N. The protective effect of andrographis paniculata against lipopolysaccharide-induced sepsis in lung tissues of a rat model through the decrease of ICAM-1 and E-selectin expression. Indones Biomed J. 2023; 15(6): 411–9.
4. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet. 2018; 392(10141): 75–87.
5. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α modulation of intestinal tight junction permeability is mediated by NIK/IKK-α axis activation of the canonical NF-κB pathway. Am J Pathol. 2016; 186(5): 1151–65.
6. Alvarez S, Blanco A, Fresno M, Muñoz-Fernández MÁ. TNF-α contributes to caspase-3 independent apoptosis in neuroblastoma cells: role of NFAT. PLoS One. 2011; 6(1): e16100. doi: 10.1371/journal.pone.0016100.
7. Freedman JC, Navarro MA, Morrell E, Beingesser J, Shrestha A, McClane BA, et al. Evidence that clostridium perfringens enterotoxin-induced intestinal damage and enterotoxemic death in mice can occur independently of intestinal caspase-3 activation. Infect Immun. 2018; 86(7): e00931–17.
8. Billmeier U, Dieterich W, Neurath MF, Atreya R. Molecular mechanism of action of

anti-tumor necrosis factor antibodies in inflammatory bowel diseases. World J Gastroenterol. 2016; 22(42): 9300–13.

9. Marshall JC, Leligdowicz A. Gaps and opportunities in sepsis translational research. EBioMedicine. 2022: 86: 104387. doi: 10.1016/j.ebiom.2022.104387.

10. Sandra F. Role of herbal extract in stem cell development. Mol Cell Biomed Sci. 2018; 2(1): 19–22.

11. Qurrotuaini SP, Wiqoyah N, Mustika A. Antimicrobial activity of ethanol extract of Centella asiatica leaves on Proteus mirabilis, Proteus vulgaris, and Yersinia enterocolitica in vitro. Mol Cell Biomed Sci. 2022; 6(3): 135–40.

12. Suastika LOS, Oktaviono YH, Soemantri D, Sandra F. Purple sweet potato extract and vitamin C increase the proliferation of endothelial progenitor cells from stable coronary artery disease patients. Bali Med J. 2021; 10(1): 243–248

13. Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (Sauropus androgynus) leaves as an anti-inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. Mol Cell Biomed Sci. 2022; 6(3): 129–34.

14. Ranggaini D, Sandra F, Halim J, Ichwan SJA, Djamil MS. Curcuma xanthorrhiza rhizome extract induces apoptosis in HONE-1 nasopharyngeal cancer cells through bid. Indones Biomed J. 2023; 15(1): 100–5.

15. Sikumalay A, Suharti N, Masri M. Efek antibakteri dari rebusan daun sambiloto (Andrographis paniculata Nees) dan produk herbal sambiloto terhadap Staphylococcus aureus. J Kesehat Andalas. 2014; 5(1): 196–200.

16. Lin HC, Li CC, Yang YC, Chiu TH, Liu KL, Lii CK, et al. Andrographis paniculata diterpenoids and ethanolic extract inhibit TNFα-induced ICAM-1 expression in EA.hy926 cells. Phytomedicine. 2019; 52: 157–67.

17. Astuti NT, Novitasari PR, Tjandrawinata R, Nugroho AE, Pramono S. Anti-diabetic

effect of andrographolide from Sambiloto herbs (Andrographis paniculata (Burm.f.) Nees) through the expression of PPARγ and GLUT-4 in adipocytes. Indones J Biotechnol. 2022; 27(4): 203–11.

18. Yu L, Liu Y, Cao C, Yang L, Liu H, Wang C. Andrographolide attenuates inflammation due to intra-abdominal sepsis by enhancing bacterial clearance in mice. J Inflamm Res. 2023; 16: 4413–23.

19. Kusmardi K, Ariffandi B, Lubis NS, Lestari TW, Intan PR, Pakpakan A. Effects of sambiloto (Andrographis paniculata) extract and spirulina (Spirulina platensis) administration on Ki-67 protein expression in the colon epithelial cells of plasmodium berghei-infected mice. Indones Biomed J. 2022; 14(1): 84–90.

20. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E. Andrographolide reverses doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene expressions. Indones Biomed J. 2023; 15(5): 288–96.

21. Pourdast A, Sanaei M, Jafari S, Mohammadi M, Khalili H, Shafiee G, et al. Effect of Septimeb™ as a new natural extract on severe sepsis: A randomized clinical trial. Casp J Intern Med. 2017; 8(1): 35–43.

22. Lee YY, Yuk HJ, Saba E, Kim SD, Kim DS, Kopalli SR, et al. Duchesnea indica Extract Ameliorates LPS-Induced Septic Shock in Mice. Evid Based Complement Alternat Med. 2022: 2022: 5783867. doi: 10.1155/2022/5783867.

23. Williams JM, Duckworth CA, Watson AJM, Frey MR, Miguel JC, Burkitt MD, et al. A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. Dis Model Mech. 2013; 6(6): 1388–99.

24. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and

sepsis. Vaccines. 2017; 5(4): 1–25.

25. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013; 14(1): 32. doi: 10.1186/1471-2121-14-32.

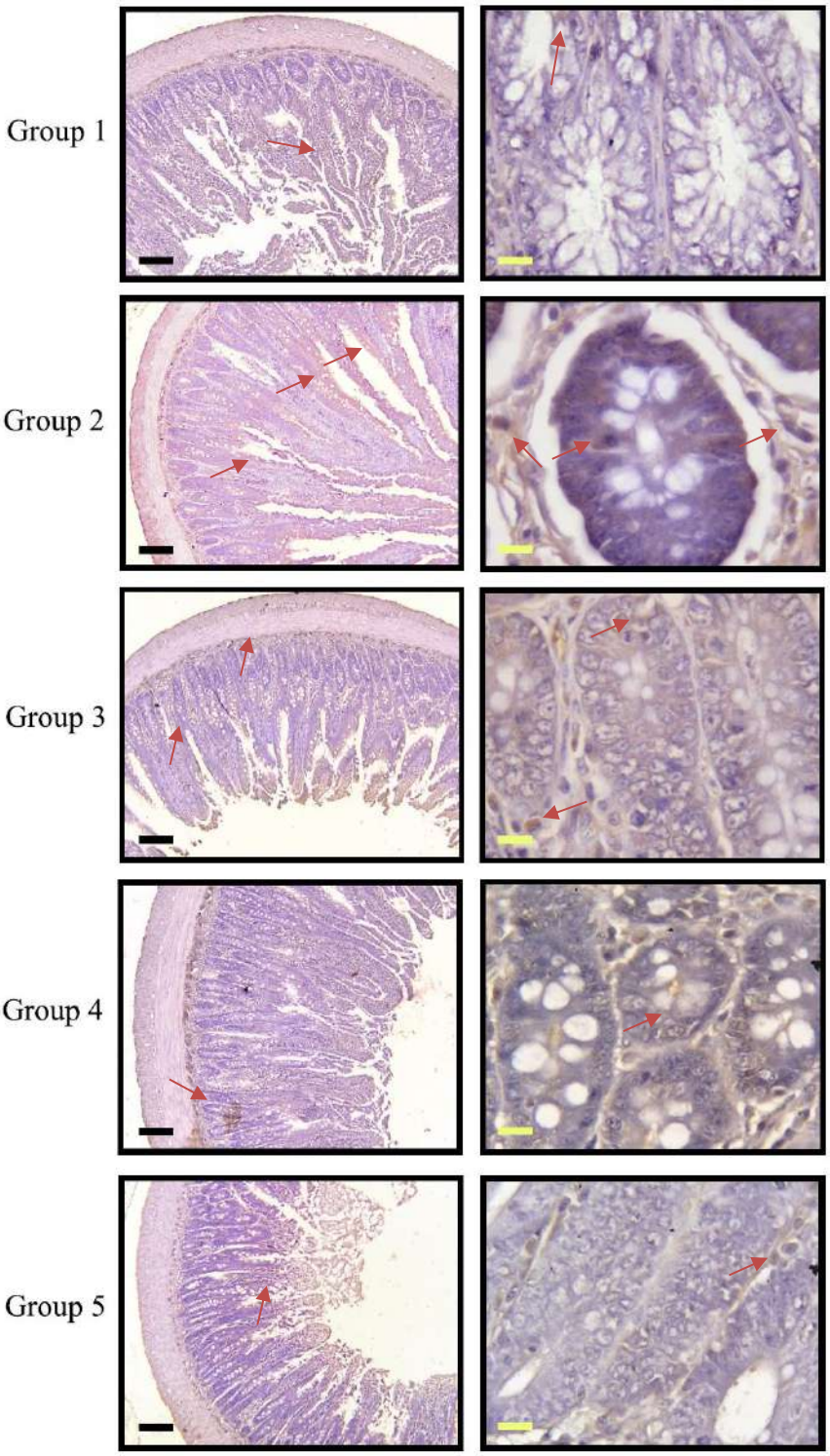
26. Lin BF, Chao WW, Kuo YH, Hsieh SL. Inhibitory effects of ethyl acetate extract of andrographis paniculata on NF-κB trans-activation activity and LPS-induced acute inflammation in mice. Evidence-based Complement Altern Med. 2011; 2011: :254531. doi: 10.1093/ecam/nep120.

27. Guo W, Liu W, Chen G, Hong S, Qian C, Xie N, et al. Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways. Int Immunopharmacol. 2012; 14(4): 613–9.

28. Low M, Khoo CS, Münch G, Govindaraghavan S, Sucher NJ. An in vitro study of anti-inflammatory activity of standardised Andrographis paniculata extracts and pure andrographolide. BMC Complement Altern Med. 2015; 15(1): 1–9.

## Figures

A



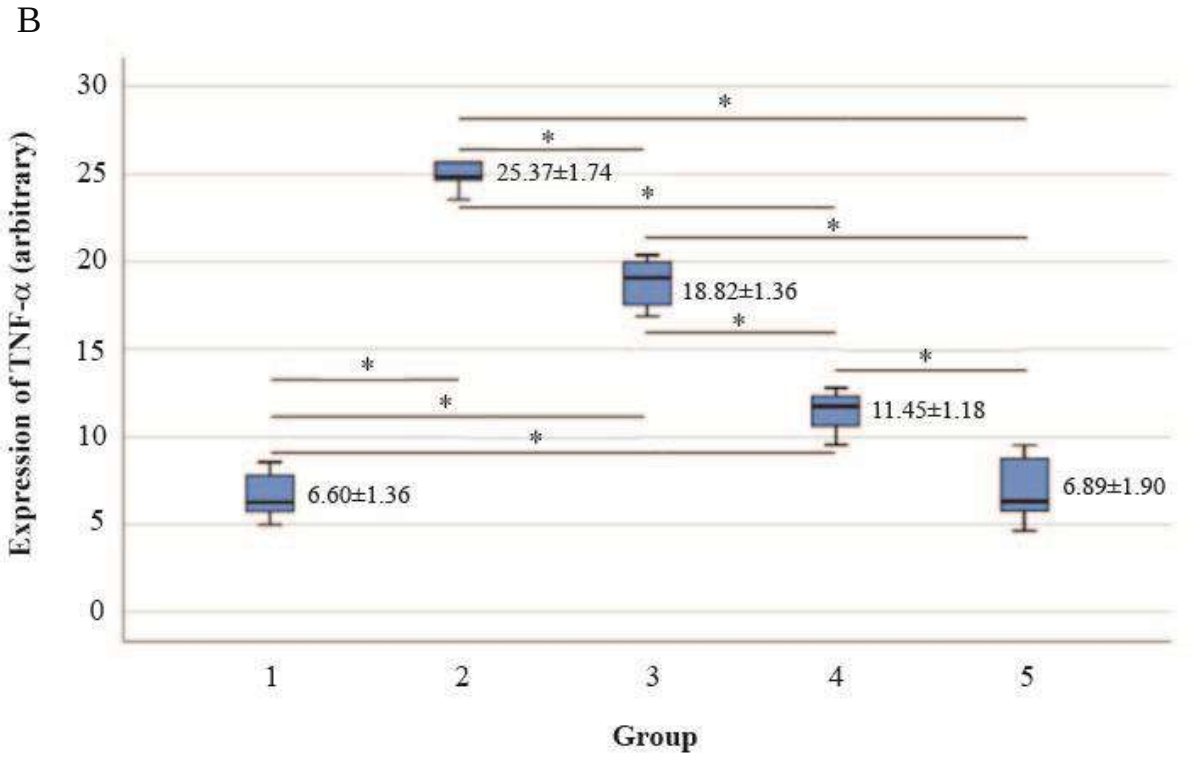
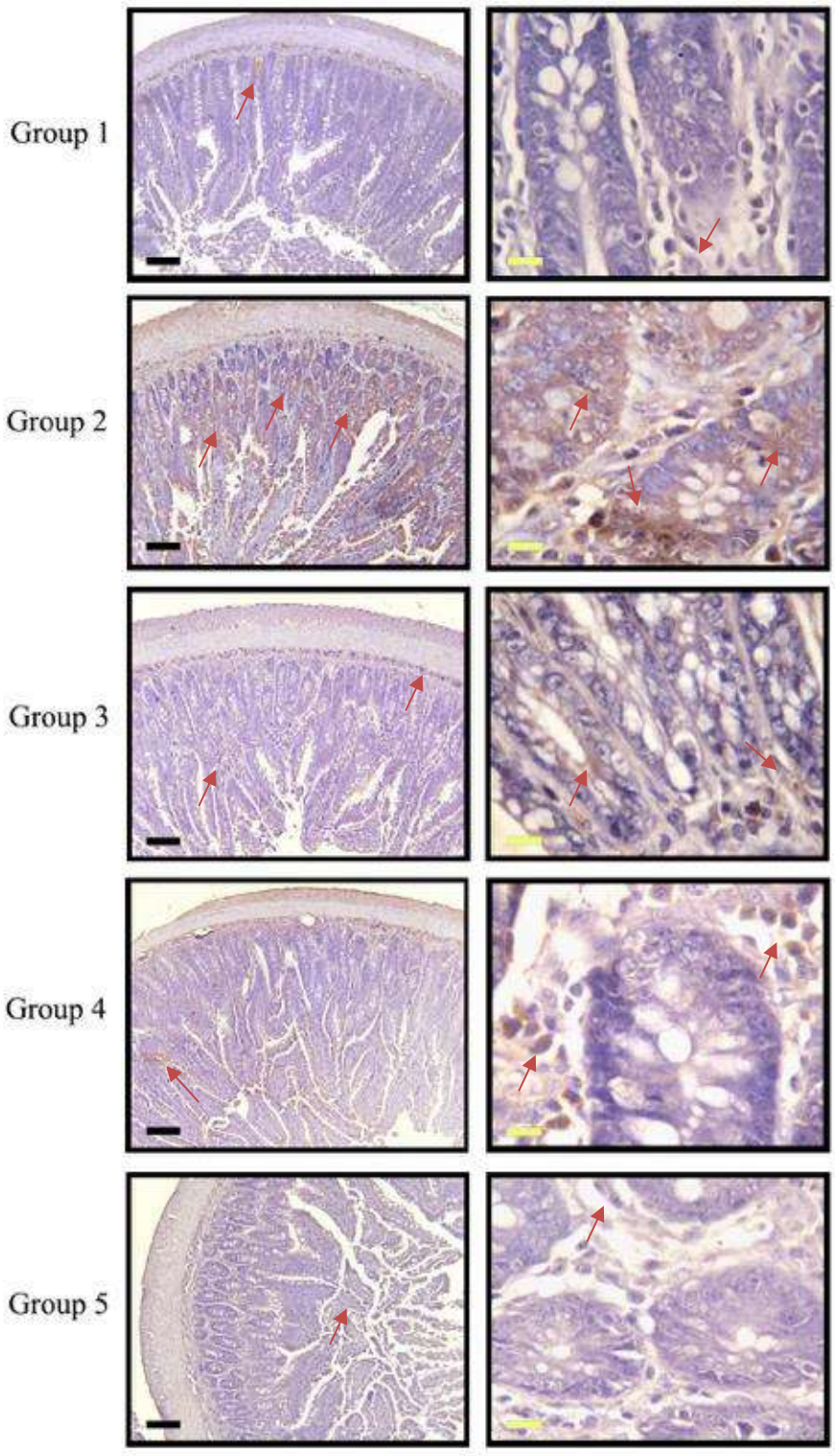



**Figure 1.** TNF-α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS. After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for immunohistochemical 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 = 100μm; Yellow bar = 1,000μm; Red arrow: TNF-$\alpha^{+}$ expression.

307

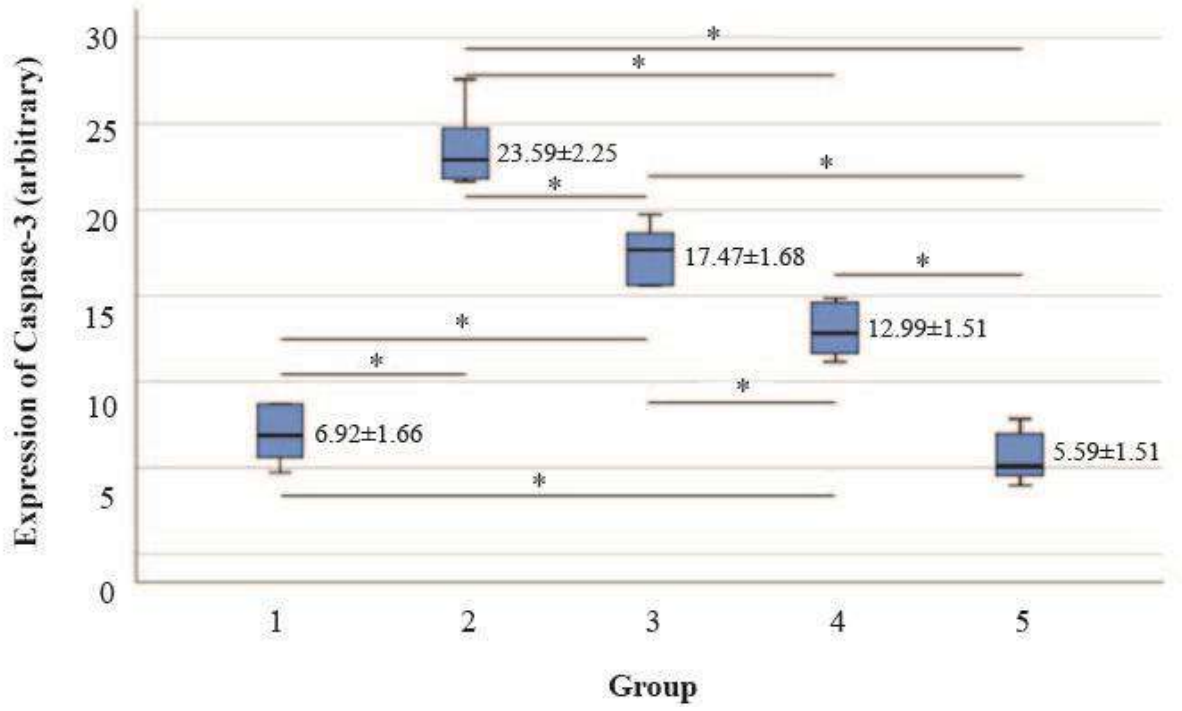
308 A




309

310

311 B




312

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 tissued 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 = 100μm; Yellow bar = 1,000μm; Red arrow: TNF-$\alpha^+$ expression.

324

UNIVERSITAS TRISAKTI

**Ferry Sandra <ferry@trisakti.ac.id>**

# [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,

--
**Secretariat of The Indonesian Biomedical Journal**
Prodia Tower 9th Floor
Jl. Kramat Raya No.150, Jakarta 10430, Indonesia
Phone. +62-21-3144182 ext. 3872
Fax. +62-21-3144181
https://www.inabj.org