RESEARCH ARTICLE

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

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## Abstract

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

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

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

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

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

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## 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

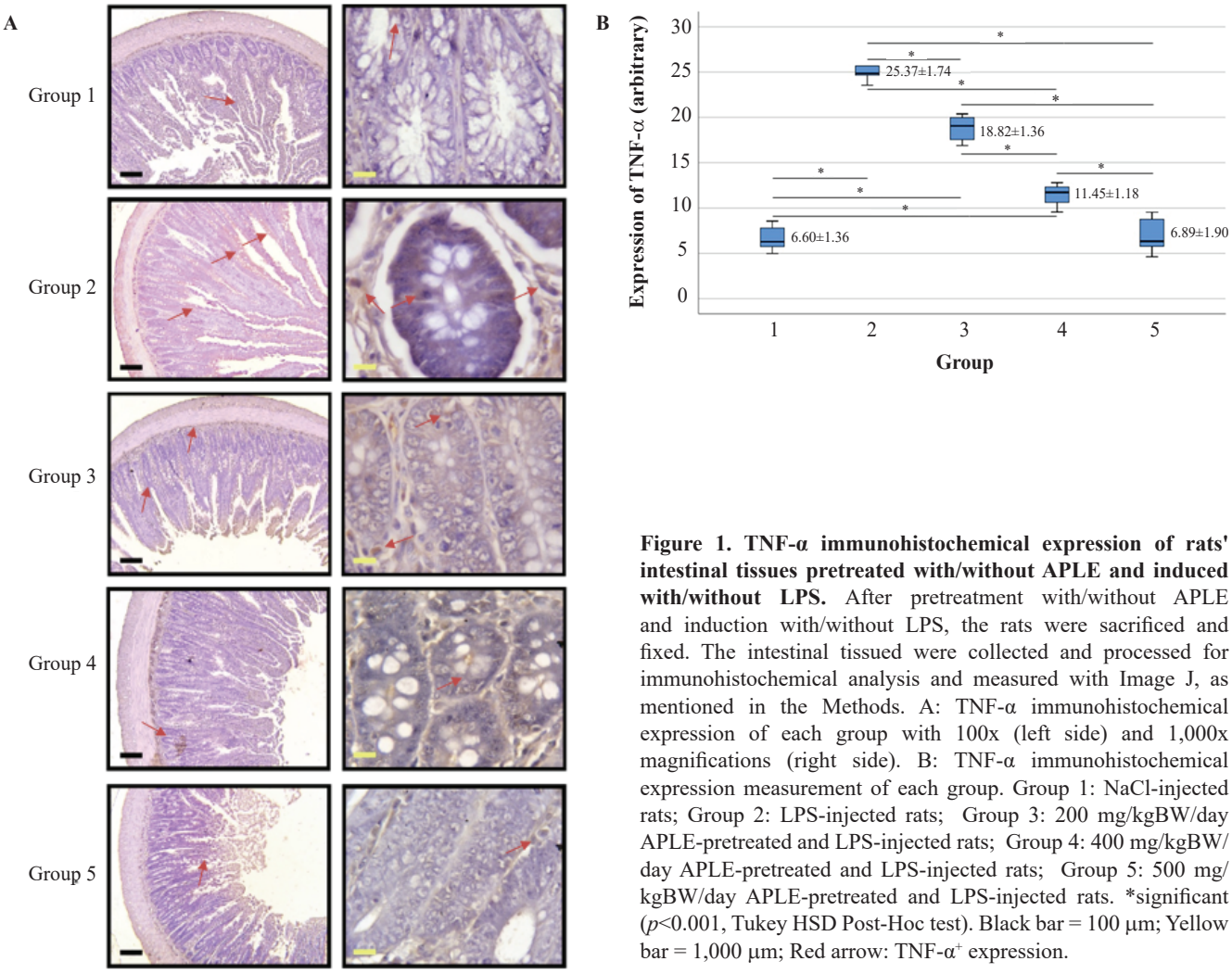


**Figure 1. TNF-α immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for immunohistochemical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

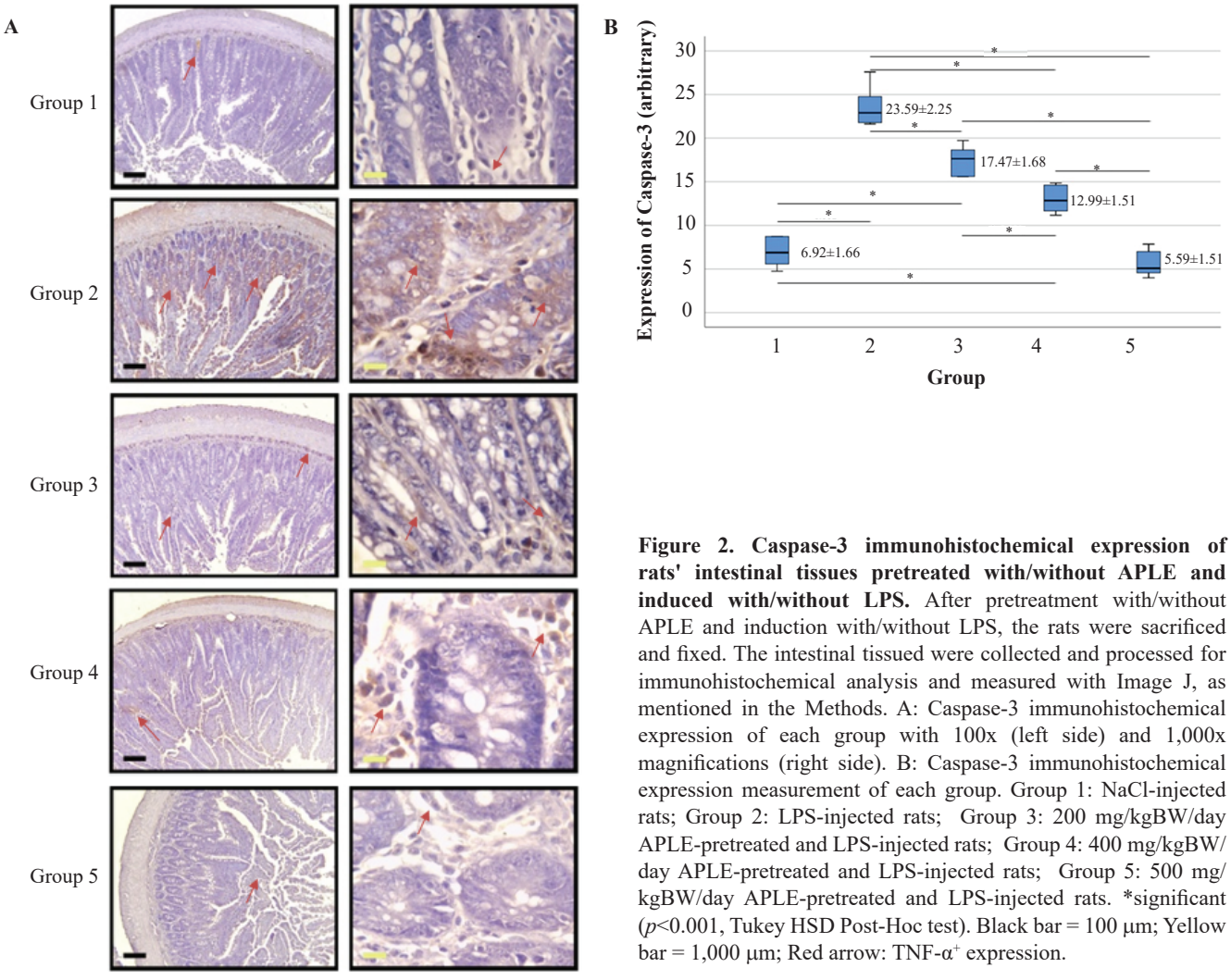


**Figure 2. Caspase-3 immunohistochemical expression of rats' intestinal tissues pretreated with/without APLE and induced with/without LPS.** After pretreatment with/without APLE and induction with/without LPS, the rats were sacrificed and fixed. The intestinal tissued were collected and processed for 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.

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## Authors Contribution

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

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