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THE ANTI-APOPTOTIC POTENTIAL OF *Paederia foetida* L. LEAF EXTRACT THROUGH THE DOWNREGULATION OF CASPASE-3 EXPRESSION IN AN *ESCHERICHIA COLI*-INDUCED SEPSIS MICE MODEL

Potensi Anti-Apoptosis Ekstrak Daun Kentut (*Paederia foetida* L.) melalui Penurunan Ekspresi Caspase-3 pada Model Sepsis Mencit yang Diinduksi *Escherichia Coli*

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ABSTRACT

Sepsis is a life-threatening condition characterized by an abnormal immune response to infection, leading to high mortality rates in intensive care units (ICUs) worldwide. Caspase-3, a crucial enzyme in the apoptosis pathway, plays a significant role in sepsis-related cellular damage. This study investigates the anti-apoptotic potential of *Paederia foetida* L. leaf extract by examining its effect on Caspase-3 expression in an *Escherichia coli*-induced sepsis mice model. Male Balb/c mice were divided into six groups, including positive control, negative control, and treatment groups receiving varying doses of the extract (100 mg/kgBW, 300 mg/kgBW, and 500 mg/kgBW). Caspase-3 expression in the spleen was measured after 24 hours of treatment. The results demonstrated a significant reduction in Caspase-3 expression, particularly in the group treated with 500 mg/kgBW of the extract, indicating its anti-apoptotic effect. These findings suggest that *P. foetida* leaf extract may serve as a potential therapeutic agent for reducing cell apoptosis in sepsis, warranting further investigation into its mechanisms and clinical pharmacological field.

***Keywords**: Spleen, Caspase-3, Sepsis, Paederia foetida L., Escherichia coli*

ABSTRAK

Sepsis adalah kondisi yang mengancam jiwa yang ditandai dengan respons imun yang abnormal terhadap infeksi, yang menyebabkan tingginya angka kematian di unit perawatan intensif (ICU) di seluruh dunia. Caspase-3, enzim penting dalam jalur apoptosis, berperan signifikan dalam kerusakan sel yang terkait dengan sepsis. Penelitian ini mengkaji potensi anti-apoptosis dari ekstrak daun *Paederia foetida* L. dengan mengamati efeknya terhadap ekspresi Caspase-3 pada model mencit sepsis yang diinduksi *Escherichia coli*. Mencit jantan Balb/c dibagi menjadi enam kelompok, yaitu kelompok normal, kelompok kontrol positif, kelompok kontrol negatif, dan kelompok perlakuan yang menerima dosis ekstrak yang berbeda (100 mg/kgBB, 300 mg/kgBB, dan 500 mg/kgBB). Ekspresi Caspase-3 pada limpa diukur setelah 24 jam perlakuan. Hasil penelitian menunjukkan

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penurunan signifikan ekspresi Caspase-3, terutama pada kelompok yang menerima dosis 500 mg/kgBB ekstrak, menunjukkan efek anti-apoptosisnya. Temuan ini mengindikasikan bahwa ekstrak daun P. foetida berpotensi menjadi agen terapeutik untuk mengurangi apoptosis sel pada sepsis, sehingga memerlukan penelitian lebih lanjut mengenai mekanisme dan aplikasi farmakologinya.

Kata kunci: Limpa, Caspase-3, Sepsis, *Paederia foetida* L., *Escherichia coli*

INTRODUCTION

Sepsis is a serious medical condition that occurs when the immune system responds abnormally to an infection or injury (Frederico et al., 2024; Singer et al., 2016). It represents a significant public health issue and is a leading cause of death in intensive care units (ICUs) globally. The mortality rate for sepsis varies between 11% and 40%, depending on the severity of the condition (Peng et al., 2023). Besides its high mortality, sepsis is also linked to considerable morbidity. Research indicates that prompt recognition and swift treatment can save lives and reduce the disease's economic burden, which exceeds $17 billion annually in the U.S. (Prescott et al., 2018). Caspase-3 is an essential executor in apoptosis, and cleavage of Caspase-3 has been regarded as a biomarker of cell apoptosis (Liu et al., 2023).

Systemic inflammation and the subsequent activation of endothelial cells lead to the disruption of proteins and tight junctions in the blood-brain barrier (BBB), increasing its permeability and allowing activated immune cells to enter the brain (Barichello et al., 2011; Danielski et al., 2018a). These immune cells can produce reactive oxygen species (ROS), which heighten oxidative stress and cause neuronal damage in the brain (Cancelier et al., 2009; Danielski et al., 2018b; Réus et al., 2015).

Sepsis is characterized by an abnormal host response to infection, primarily caused by bacteria (Kevin et al., 2023). Due to its complexity, sepsis is believed to result from a multifactorial mechanism involving various pro- and anti-inflammatory mediators. Impaired microcirculation, cellular function, and coagulation lead to tissue hypoperfusion and subsequent tissue damage (Al-Sadi et al., 2016). Research has shown that sepsis reduces blood flow to digestive organs, rapidly causing ischemia and acute intestinal injury, particularly in the colon. Inflammatory cytokines, especially TNF-α and Caspase-3, play a major role in sepsis (Alvarez et al., 2011)). Inhibiting Caspase-3 has been found to lower TNF-α production in various cell types, suggesting it could be a promising therapeutic target for treating inflammatory diseases (Freedman et al., 2018; Billmeier et al., 2016). These findings suggest that targeting TNF-α and Caspase-3 could offer potential benefits in reducing sepsis-related morbidity and mortality. Traditional medicines derived from botanical sources are also being explored for this purpose (Marshall et al., 2022).

Plants are a rich source of natural compounds vital for the development of new pharmaceuticals, especially those with antioxidant and anticancer properties. *Paederia foetida Linn*, commonly referred to as "daun kentut" in Indonesia, is a perennial aromatic climbing plant frequently eaten as a leafy vegetable, either raw or cooked. This plant contains various phytochemicals, including steroids, saponins, alkaloids, flavonoids, vitamin C, and volatile oils (Mazumder et al., 2018; Patel, 2017; Rosli et al., 2013). Extracts from *P. foetida* have been extensively researched for their biological activities, such as antibacterial and antibiofilm properties (Priyanto et al., 2022), antifungal effects (Morshed et al., 2012), antihyperlipidemic, antihyperglycemic, and antioxidant activities (Kumar et al., 2014), anti-melanogenic potential (Chung et al., 2021), chemoprotective properties (Li et al., 2021), and cytotoxic effects on human prostate cancer cells (Pradhan et al., 2019).

Previous studies on fresh and dried *P. foetida* leaves from Malaysia revealed significant antioxidant activity, with 68% and 76% levels, respectively, which correlated with their total phenolic content (Osman et al., 2009). Additionally, the plant's

methanolic extract exhibited antioxidant activity with IC50 values between 538.97 and 859.20 µg/ml. Moreover, a study on the effect of *P. foetida* leaves on interleukin-6 (IL-6) levels in a mouse sepsis model induced by *Escherichia coli* showed that a 500 mg/kgBW dose was most effective in reducing IL-6 levels (Savitri and Kasimo, 2022). However, variations in plant growing conditions and extraction methods could result in differences in the composition and solubility of active compounds.

MATERIALS AND METHODS

Materials

The materials used in this research include disposable syringes (1 mL, 3 mL, 5 mL), a 35 cm feeding tube, a 60 mL urine container, a micropipette, a freezer, a rotary or sliding microtome, a brush, a water bath, glass slides, an Eppendorf pipette (1500 µL) along with blue, yellow, and white tips (10 µL), an oven, a digital scale, a surgical board, a surgical instrument set (scissors, tweezers, lancets), gloves, tissues, masks, a binocular light microscope, and a microscope camera.

The study also involved 24 male Balb/c mice, aged 4-8 weeks and weighing 20-30 grams, sourced from the Veterinary Center in Surabaya, East Java. Additional materials included *Paederia foetida* leaf extract from Materia Medika Batu, East Java; ciprofloxacin; a clinical isolate of *E. coli* ($1x10^5$ CFU/mL) ATCC 25922 PK/5 from Nano Laboratory with accession number 501192 and product number R4607050; physiological saline solution (PZ); mouse feed; wood powder (kawol); methanol; Giemsa stain; distilled water; 37% formaldehyde solution (H_2CO); formalin buffer; sodium hydrogen phosphate dibasic (Na_2HPO_4) (6.5 grams in 900 mL distilled water); 37-40% formaldehyde; ethanol (80%, 95%, and absolute); xylene; clearing solution; paraffin; and poly-L-lysine.

Method

Male mice were weighed and housed in standard polypropylene cages with wood powder bedding for a two-week acclimatization period. The bedding was changed daily. Approximately 90 grams of softened feed per cage (with six mice) was provided ad libitum, along with water, both of which were replenished daily. After the two-week acclimatization period, the mice were divided into six treatment groups.

The acclimatized mice underwent a 14-day treatment as follows: 1) Group 1, the normal control (N), received no gastric intubation; 2) Group 2, the negative control (K-), received 0.5 mL of distilled water; 3) Group 3, the positive control (K+), was given ciprofloxacin at 500 mg/kgBW (0.26 mL); 4) Group 4 received 100 mg/gBW of *Paederia foetida* leaf extract (P1); 5) Group 5 was administered 300 mg/gBW of the extract (P2); and 6) Group 6 received 500 mg/gBW of the extract (P3).

Mice receiving treatment were injected intraperitoneally with *E. coli* at a dose of $1x10^5$ CFU/mL. After 24 hours of exposure to polymicrobial sepsis, apoptosis was observed in the spleen. If a mouse died before the 24-hour mark, immediate surgery was performed to remove the spleen to prevent autolysis. Spleen samples were collected from the middle, left, and right regions.

The spleen tissue was preserved in formalin buffer to maintain cellular structure, prevent autolysis, and inhibit microbial growth. The tissue was then embedded in paraffin blocks, sectioned into 4-6 micron slices using a rotary or sliding microtome, placed in a water bath, adhered to slides coated with tissue adhesive, air-dried at room temperature, and dried overnight in an oven. Immunohistochemistry (IHC) is a valuable technique to assess the expression and localization of specific proteins, such as cleaved caspase-3, in tissue sections. In your experiment examining the anti-apoptotic potential of *Paederia foetida* L. leaf extract, IHC can be employed to detect and visualize caspase-3 expression at the cellular level in tissues of the *E. coli*-induced sepsis mice model. The procedure involves preparing tissue sections from organs such as the liver, spleen, or lungs, which are then incubated with a primary antibody specific to cleaved caspase-3. This antibody binds to the protein of interest, and a secondary antibody, usually conjugated with an enzyme such as horseradish peroxidase (HRP), is added to amplify the signal.

After applying a substrate that reacts with the enzyme, a colored or fluorescent signal is produced where caspase-3 is expressed. The intensity and distribution of this signal can be examined under a microscope to assess the extent of caspase-3 activation and apoptotic activity in the tissue. To quantify the results, image analysis software like ImageJ or Fiji can be used to measure the staining intensity or the percentage of positive cells in the tissue sections. These quantitative data can then be compared between treatment groups (e.g., *Paederia foetida* extract vs. control) to determine if the extract downregulates caspase-3 expression and thus exhibits an anti-apoptotic effect. The tissue-specific expression of cleaved caspase-3 and the number of apoptotic cells can provide important insights into how the extract modulates apoptosis in response to sepsis.

RESULTS AND DISCUSSION

The anti-apoptotic testing of *P. foetida* L. leaf extract was conducted by observing Caspase-3 expression in the spleen of *E. coli*-induced sepsis mice models. The results of Caspase-3 expression in the spleen of the sepsis-induced mice can be seen in Figure 1. The findings for the normal group show an average Caspase-3 expression in the spleen of 7.09%±0.06 (Figure 2), the positive control group at 26.36%±0.02, the negative control group at 72.60%±0.05 (Figure 3), the treatment group I (100mg/kgBW) at 71.04%±0.04 (Figure 4), the treatment group II (300mg/kgBW) at 62.22%±0.02 (Figure 5), and the treatment group III (500mg/kgBW) at 40.92%±0.01 (Figure 6).

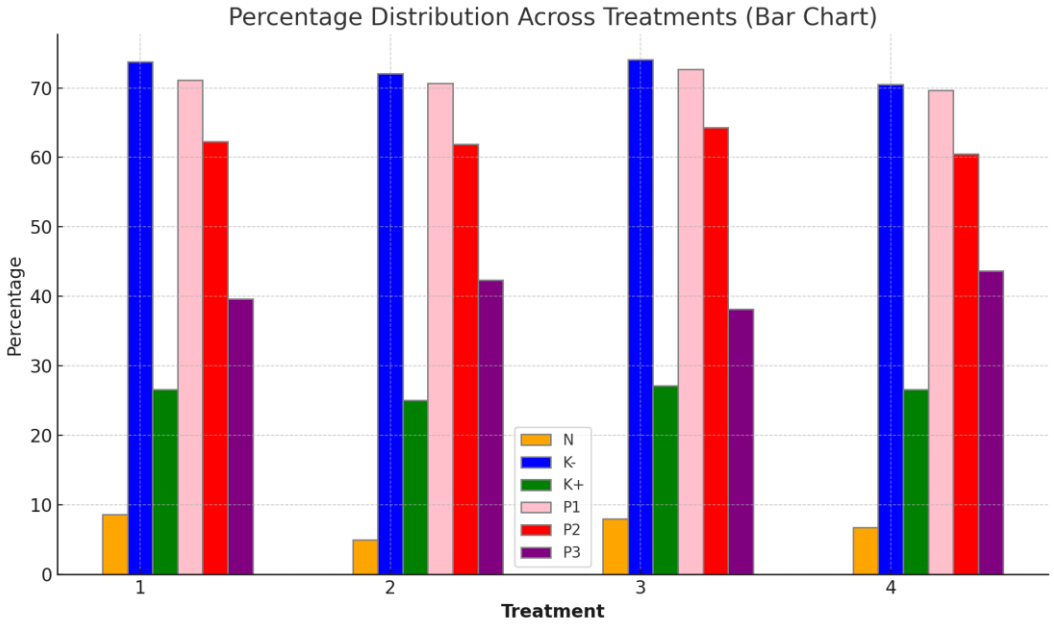

Figure 1. Diagram of Observed Caspase-3 Expression in the Spleen of Sepsis Mice Models

Based on the ANOVA analysis, an F-statistic value of 1,031.29 was obtained, indicating high variability between the observed groups. The p-value resulting from the ANOVA analysis is 1.83×10^{-21}, which is extremely small compared to the commonly used significance level (e.g., 0.05). This indicates that there is a statistically significant difference in the reduction of Caspase-3 expression between the groups treated with *P. foetida* L. leaf extract and the control group. The leaf extract significantly reduces Caspase-3 expression in sepsis-induced mice, demonstrating its anti-apoptotic potential.

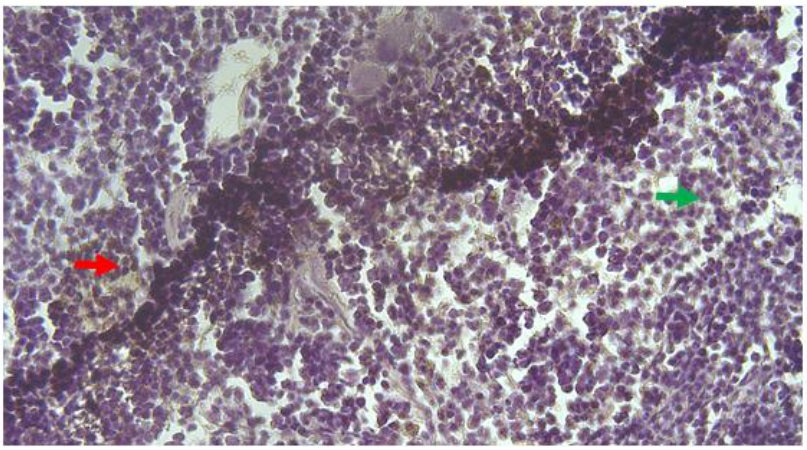

Figure 2. Spleenocyte Cells in the Normal Group (red arrow: expressed Caspase-3, green arrow: un-expressedCaspase-3) at 400x magnification

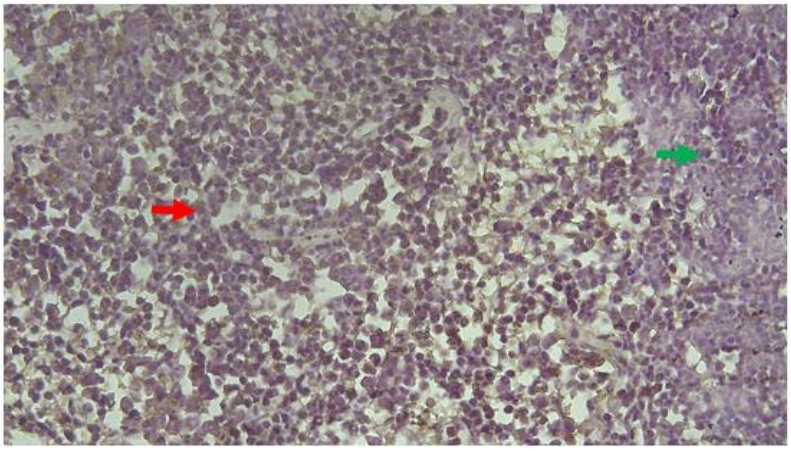

Figure 3. Spleenocyte Cells in the Positive Control Group (red arrow: expressed Caspase-3, green arrow: unexpressed Caspase-3) at 400x magnification

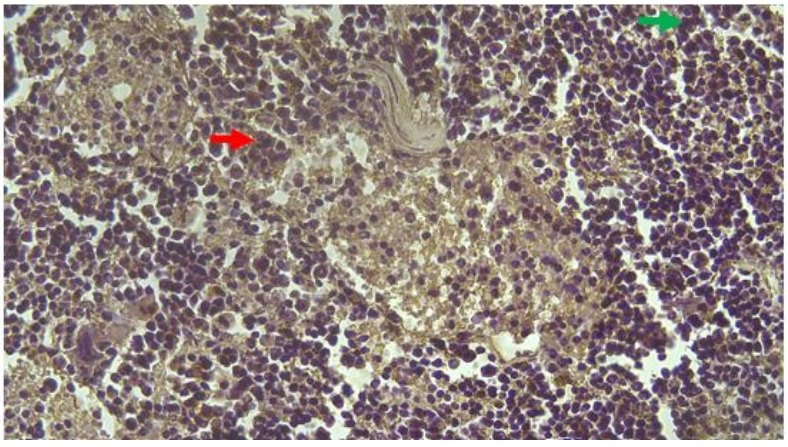

Figure 4. Spleenocyte Cells in the Negative Control Group (red arrow: expressed Caspase-3, green arrow: unex-pressed Caspase-3) at 400x magnification

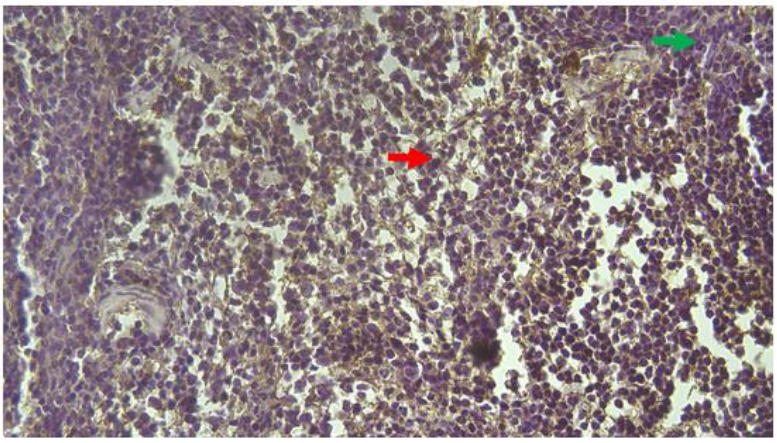

Figure 5. Spleenocyte Cells in the Treatment 1 Group (red arrow: expressed Caspase-3, green arrow: unex-pressed Caspase-3) at 400x magnification

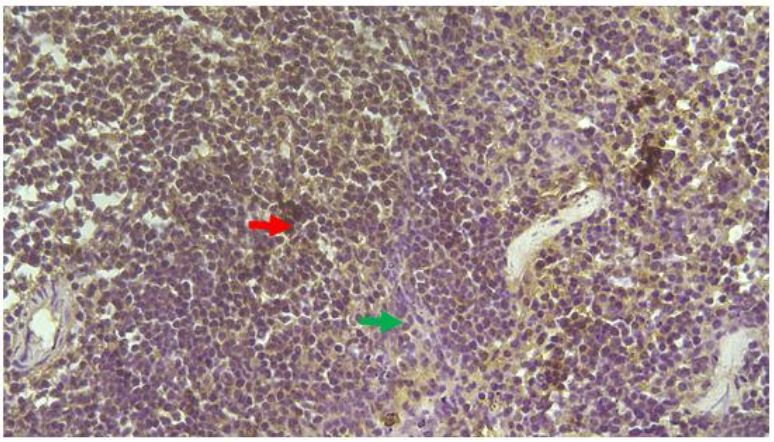

Figure 6. Spleenocyte Cells in the Treatment 2 Group (red arrow: expressed Caspase-3, green arrow: unex-pressed Caspase-3) at 400x magnification

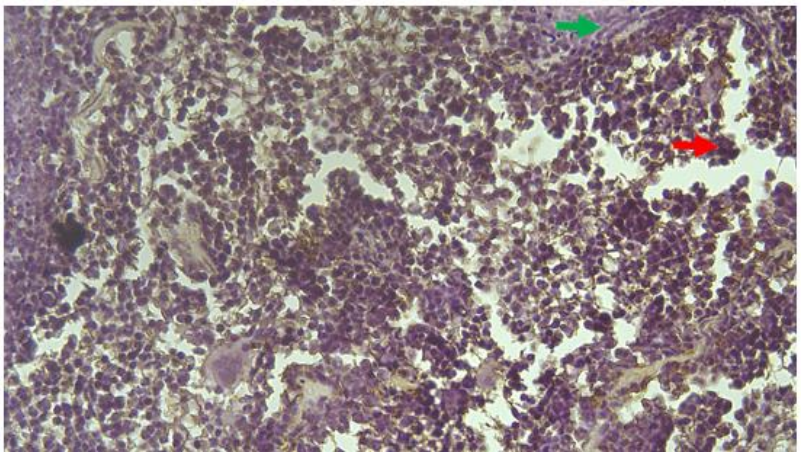

Figure 7. Spleenocyte Cells in the Treatment 3 Group (red arrow: expressed Caspase-3, green arrow: unex-pressed Caspase-3) at 400x magnification

Based on the research results, it was found that a significant increase in caspase-3 expression occurred in the negative control group. The function of the spleen is centered on systemic circulation. The spleen has two compartments that are functionally and morphologically distinct: the red pulp and the white pulp. The red pulp is a blood filter that removes foreign materials and damaged erythrocytes; it also serves as a

storage site for iron, erythrocytes, and platelets. The spleen is a site of hematopoiesis in mice, especially in fetuses and neonatal animals. It is also the largest lymphoid organ, containing about a quarter of the body's lymphocytes and initiating immune responses to blood-borne antigens (Nolte et al., 2002; Balogh et al., 2004).

Apoptosis appears to be a key mechanism that regulates specific cell populations. Cytosolic cytochrome is required for the initiation of the apoptosis program and shows a possible connection between Bcl-2 and cytochrome c, which is typically located in the mitochondrial intermembrane space. Immune dysfunction is associated with increased oxidative stress and mitochondrial dysfunction. The regulation of mitochondrial Bax-mediated cytochrome c may involve the activation pathway of Caspase-3. Bax is part of the BH3 domain protein that facilitates the assembly of pro-apoptotic proteins. Bax is located in the outer pores of the mitochondrial membrane and functions to alter mitochondrial permeability, releasing various apoptosis-inducing factors, including cytochrome c, through mitochondrial permeability transition. In general, a reduction in mitochondrial transmembrane potential is followed by the release of cytochrome c, which binds to Apaf-1 and promotes the activation of caspase-9 and caspase-3 (Green and Reed, 1998).

Active caspase-3 recognizes specific short peptide cleavage motifs (DXXD) and cleaves cellular proteins where these motifs are present and accessible. The potential of caspase-3 to induce apoptosis means that once activated, its activity must be tightly controlled. This control is achieved through constant enzyme turnover, ensuring that the threshold for enzyme activation is not reached without an apoptotic stimulus (Lai et al., 2011). Additionally, eukaryotic cells have been documented to have low levels of caspase-3 activity in non-apoptotic states, suggesting that sub-apoptotic levels of the enzyme are expressed independently of apoptosis (Connolly et al., 2014).

Caspase-3 activity during bacterial infection plays a role in fundamental processes beyond apoptosis, most notably in cell proliferation and differentiation, as well as in immunomodulation, signal transduction, and cell migration. Therefore, disruption of caspase-3 by pathogenic bacteria may have consequences beyond simply determining the fate of infected cells. Apoptosis is also considered a deliberate host response to bacterial infection, ultimately leading to the removal of compromised cells. Apoptosis has been described as a fundamental pathway in bacterial-host interactions, but its role in inhibiting or facilitating infection has not yet been clearly defined. While apoptosis can remove infected and compromised cells to benefit the host, the induction of apoptosis can achieve this in a non-inflammatory manner, while also potentially disrupting, for example, epithelial barriers to infection or removing circulating immune cells (Peters et al., 2013).

The research findings indicate a significant increase in caspase-3 expression in the negative control group, highlighting its crucial role in regulating apoptosis, especially in immune and hematopoietic functions of the spleen. These results suggest that apoptosis, through mechanisms like caspase-3 activation, plays an essential role in immune responses to bacterial infections, with potential implications for controlling infection and cell proliferation. Further investigation into the modulation of apoptosis in response to pathogens could provide valuable insights into therapeutic strategies for immune modulation.

CONCLUSION

Paederia foetida leaf extract has shown potential in reducing Caspase-3 expression in sepsis models, particularly at a dose of 500 mg/kgBW, indicating its anti-apoptotic effects. The findings suggest that *P. foetida* could be explored further as a natural treatment for sepsis. Future research should focus on refining extraction methods and understanding its mechanisms in reducing cell damage. Furthermore, the leaf extract of *P. foetida* is a promising target for therapy.

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