Effect of *Hedyotis corymbosa* (pearl grass) extract on pro-inflammatory cytokines IL10, IL6, and TNFα in mice with acute lung inflammation induced by lps

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Abstract. Pro-inflammatory cytokines such as IL10, IL6, and TNFα are key players in the inflammatory response associated with acute lung injury. Excessive levels of these cytokines can worsen lung conditions, making their suppression a potential treatment strategy. Previous research suggests that pearl grass extract has the potential to alleviate inflammation. In the present study, we evaluated the effects of ethanol extract from pearl grass on the TNFα, IL-6, and IL-10 levels in mice with lung inflammation induced by lipopolysaccharide (LPS). Twenty-five mice were served as a normal group (received distilled water), a negative control group (administered LPS without the Extract), and groups that received LPS along with 750, 500, and 250 mg kg -1 BW pearl grass extract. The test preparation was given intragastrium for seven days, followed by intranasal LPS on day eight. Seventy-two hours after LPS exposure, BAL fluid was collected, and BAL fluid cytokine levels were measured using ELISA. Pearl grass extract 250 mg kg-1 BW remarkably reduced TNFα, and IL6 levels but did not affect IL10. These findings suggest that pearl grass extract could help alleviate acute lung inflammation by regulating pro-inflammatory cytokines, indicating a potential treatment approach to reduce disease severity

1 Introduction

Acute lung inflammation often initiates a series of more severe lung diseases. If not treated adequately, acute lung inflammation can progress to more serious respiratory problems,

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including acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). This syndrome is marked by widespread lung inflammation and damage, leading to fluid accumulation, severe hypoxemia, and disrupted carbon dioxide levels [1]. The prevalence of ARDS is remarkably elevated in sufferers of ARDS with a poor prognosis, contributing to its high mortality rate. Studies estimate the annual incidence of ARDS to be around 59 cases per 100,000 people [2].

As a systemic inflammatory disorder, ARDS shows a reciprocal relationship between lung function and other organ systems. Inflammatory cytokines like IL6, TNFα, IL1β, and IL8 are found in greater quantities in Bronchoalveolar lavage fluid (BALF) and plasma samples from individuals diagnosed with ARDS [3]. Thus, these cytokines are key contributors to the onset of ARDS, making the inhibition of cytokine upregulation, particularly TNFα and IL6, valuable in preventing and managing the inflammation associated with ARDS. Various herbs, including Genistein, Echinacea, Curcumin, Eugenol, Allicin, Quercetin, and 6-Gingerol, have shown a potential to regulate cytokine levels and exert significant anti-inflammatory effects [4].

Recently, pearl grass (*Hedyotis corymbosa (L.) Lamk*), which belongs to the Rubiaceae family and the genus Oldenlandia [5], has demonstrated anti-inflammatory properties in addition to its antibacterial, antipyretic, diuretic, and detoxifying effects. This plant contains diverse bioactive compounds, including organic acids, flavanols, flavones, monoterpenes, triterpenes, cyclo terpenes, sesquiterpenes, and phenolics [6].

A prior study found that a 70% ethanol extract of pearl grass at 400 mg kg^{-1} body weight (BW) effectively inhibited hind paw edema in rats [7]. Additionally, pearl grass extract reduced NO, IL-1β, and TNF-α concentration in RAW264.7 cells [8].

Previous studies well-supported pearl grass's role in modulating immune response and controlling inflammation. However, its impact on lung inflammation remains unexplored. The present study aimed to determine the effect of pearl grass 70% ethanol extract on IL10, IL6, and TNFα levels in mice suffering from acute lung inflammation induced by LPS.

2 Research method

2.1 Raw material preparation

Pearl grass was obtained from the Bekasi District in West Java, Indonesia. Dried Healthy samples were crushed into a fine powder with a grinder (IKA®, Malaysia) and then passed through a 40-mesh sieve. Five hundred grams of pearl grass powder were soaked in 2 liters of 70% ethanol (One Med, Indonesia) and allowed to stand at room temperature for 24 hours, with shaking conducted every hour during the initial six hours. The macerate was separated from the solid residues by filtering through a batis cloth. The residue underwent maceration 3 times using the same procedure, the first with 2 L and then with 1 L of 70% ethanol. In total, 5 L of 70% ethanol was used to extract 500 g of pearl grass powder (1:10 ratio). All filtrates were combined and concentrated using a vacuum evaporator (Ogawa, Japan) to obtain the dry extract. The extract underwent phytochemical screening to determine alkaloids, flavonoids, phenols, tannins, saponins, and terpenoids. The remaining extract was utilized to evaluate its efficacy in preventing lung inflammation in mice induced by LPS.

2.2 Phytochemical Test

Phytochemical analysis was carried out to determine the various secondary metabolite groups present in the pearl grass extract, such as flavonoids, alkaloids, saponins, tannins, and

terpenoids, following the methodology outlined by Hanani [9]. For the alkaloid test, pearl grass powder (0.5 g) was mixed with 2N sulfuric acid (Merck, Germany) and distilled water. This mixture was then heated for two minutes, cooled, and filtered. The filtrate was treated with Dragendorff's, Mayer's, or Buchardt's reagents. The formation of a red-to-orange precipitate, a yellowish-white precipitate, and a blackish-brown precipitate after the addition of Dragendorff's, Mayer's, and Buchardt's reagents, respectively, demonstrated the existence of alkaloids.

In the flavonoid assay, an extract weighing 0.5 g was mixed with 5 mL of 96% ethanol (Brataco, Indonesia), followed by the addition of 0.1 g of magnesium powder (Merck, Germany) and 10 drops of concentrated HCl (Merck, Germany). The development of a red-orange/purple-red color demonstrated flavonoids, while an orange-yellow color suggested the presence of flavones. The saponin assay required shaking 0.5 g extract in 10 mL of water for 10 minutes. A positive outcome was demonstrated by the development of stable foam that lasted for several minutes following a single drop of 2N HCl (Merck, Germany). Steroids and triterpenoids were identified through the maceration of 1 g of the pearl grass extract with N-hexane (Merck, Germany) for 2 hours, followed by filtration. The filtrate was then evaporated in an evaporator cup. To the remaining filtrate, acetic anhydride ($C_4H_6O_3$) (Merck, Germany) and one drop of concentrated sulfuric acid (Merck, Germany) were added. The formation of either a blue or green color indicated terpenoids, while red, pink, or purple colors signified the presence of triterpenoids.

2.3 Animal model and experimental design

The research protocol had been approved by the Animal Ethics Commission of IPB University's veterinary and biomedical school (SVBS) with approval number 176/KEH/SKE/I/2024. Furthermore, the overall care for the experimental animals was under the Animal Welfare Act. A total of 25 male mice were acclimated for seven days at the Biopharma Tropica Laboratory Animal Research Center. They were kept in cages located in temperature-regulated rooms, where the temperatures ranged from 21 to 23 °C and humidity levels were sustained between 40% and 60%, all while adhering to a 12-hour light-dark cycle. The mice had unlimited access to both tap water and food. After acclimatization, they were randomly divided into different groups (n=5). The normal group received distilled water without LPS (from Sigma-Aldrich, USA), while the Negative Control group was given distilled water combined with LPS. The treatment groups were administered pearl grass extract at doses of 750 mg, 500 mg, and 250 mg per kg of body weight, along with LPS. The pearl grass extract was dissolved in 0.5% carboxymethyl cellulose (CMC) from Brataco, Indonesia.

All tested preparations were administered orally for seven days. On the eighth day, a combination of ketamine (Agrovetmarket, Canada), xylazine (Xyla, Holland), and acepromazine (Castran, Holland) at doses of 70, 10, and 10 mg kg-1 BW, respectively, were injected intramuscularly into the mice. Lipopolysaccharide (20 µL LPS solution containing 20 µg) was then administered intranasally to the anesthetized mice. Seventy-two hours later, the mice underwent a bronchoalveolar lavage procedure and were subsequently euthanized with an intraperitoneal overdose of an anesthetic cocktail.

2.3.1 Lavage fluid collection from Bronchoalveoli (BALF)

The anesthetized mice were promptly subjected to a tracheotomy, and a catheter with a diameter of 0.18 mm was placed into the trachea. The lungs were then rinsed in situ through the catheter four times with a Phosphate-Buffered Saline (PBS) solution (Oxoid, UK). A total

of 1 mL of PBS was used across the four rinses. The collected bronchoalveolar lavage fluid (BALF) was later analyzed to measure the levels of the cytokines IL10, IL6, and TNFα.

2.3.2 Pro-inflammatory cytokines determination

BAL fluid was homogenized following the addition of a cold PBS solution. The supernatant was then separated using a centrifuge at 12,000 g (Therm Fodher Scientific, USA) for 30 minutes at 4 °C. The concentration of IL10, IL6, and TNFα were determined using commercial ELISA kits: Mice TNFα (Tumor Necrosis Factor Alpha), Mice IL6 (Interleukin 6) (Elabscience®, USA), and Mice IL10 ELISA Kit respectively. All procedures were carried out following the manufacturer's instructions.

2.3.3 Statistical analysis of collected dara

This research utilized an experimental approach featuring a completely randomized design (CRD). Data was analyzed quantitatively using IBM SPSS software, version 26. The homogeneity of the data was evaluated through Levene's test, while the assessment of normality was carried out using the Kolmogorov-Smirnov and Shapiro-Wilk tests, followed by the Kruskal-Wallis test. A p-≤ 0.05 is considered a statistically significant difference.

3 Experimental Result

3.1 Phytochemical screening of secondary metabolites of pearl grass

A summary of the results from the phytochemical screening of pearl grass extract is presented in Table 1. The analysis revealed the presence of various metabolites in the extract. In the Buchardt and Dragendorff assays, the formation of distinct blackish-brown and blackish-orange precipitates confirmed the presence of alkaloids. Tannins were indicated by a green or blackish-blue coloration when treated with $FeCl_3$. The extract's ability to produce stable foam upon mixing with water confirmed the presence of saponins. Moreover, adding chloroform and sulfuric acid (H_2SO_4) resulted in a brown coloration, suggesting the presence of terpenoids. Flavonoids were detected by an orange-red color observed in tests using ethanol and hydrochloric acid (HCl) combined with either magnesium (Mg) or zinc (Zn) powder. Overall, these results confirm the presence of alkaloids, tannins, flavonoids, saponins, and terpenoids in the pearl grass extract.

Table 1. Secondary Metabolites Identified in the 70% Ethanol Extract of Pearl Grass.

Metabolite	Reagent	Result	Parameter
Alkaloid	*Buchardt*	+ (Positive)	Blackish brown
	Dragendorff	+ (Positive)	Blackish orange precipitate
	Mayer	- (Negative)	White precipitate
Tannin	Gelatin 10% in NaCl 10%	- (Negative)	White precipitate
	FeCl 3%	+ (Positive)	Green/Blackish blue

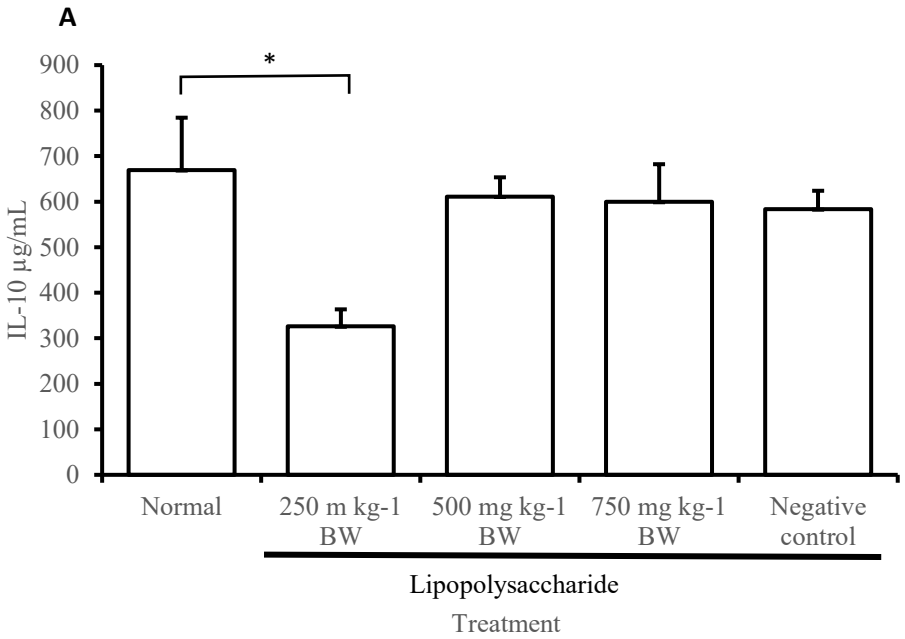
Table 1. Secondary Metabolites Identified in the 70% Ethanol Extract of Pearl Grass (continue).

Saponin	*Aquadest*	+ (Positive)	Foam does not disappear
Terpenoid	Chloroform+H_2SO_4	+ (Positive)	Brown color

Flavonoid	Ethanol, $HCl_{(p)}$ and Mg powder	+ (Positive)	Orange/red color
	Ethanol, $HCl_{(p)}$ and Zn powder	+ (Positive)	Orange/red color

3.2 Impact of pearl grass ethanol extract on the lung of pro-inflammatory cytokines

The impact of the pearl grass ethanol extract on IL10, IL6, and TNFα levels is presented in Figure 1 A-C. At 72 hours following LPS induction, the cytokines IL10, IL6, and TNFα levels of LPS-treated mice demonstrated no significant differences from the cytokines of the normal group ($p>0.05$). In contrast, the mice administered 250 mg kg -1 BW of pearl grass ethanol extract alongside LPS showed significantly lower levels of IL-6 and TNF-α compared to the negative control group. Furthermore, while the IL-10 concentration in this treatment group was notably lower than that of the normal control group, it was not significantly different from the levels observed in the negative control group.



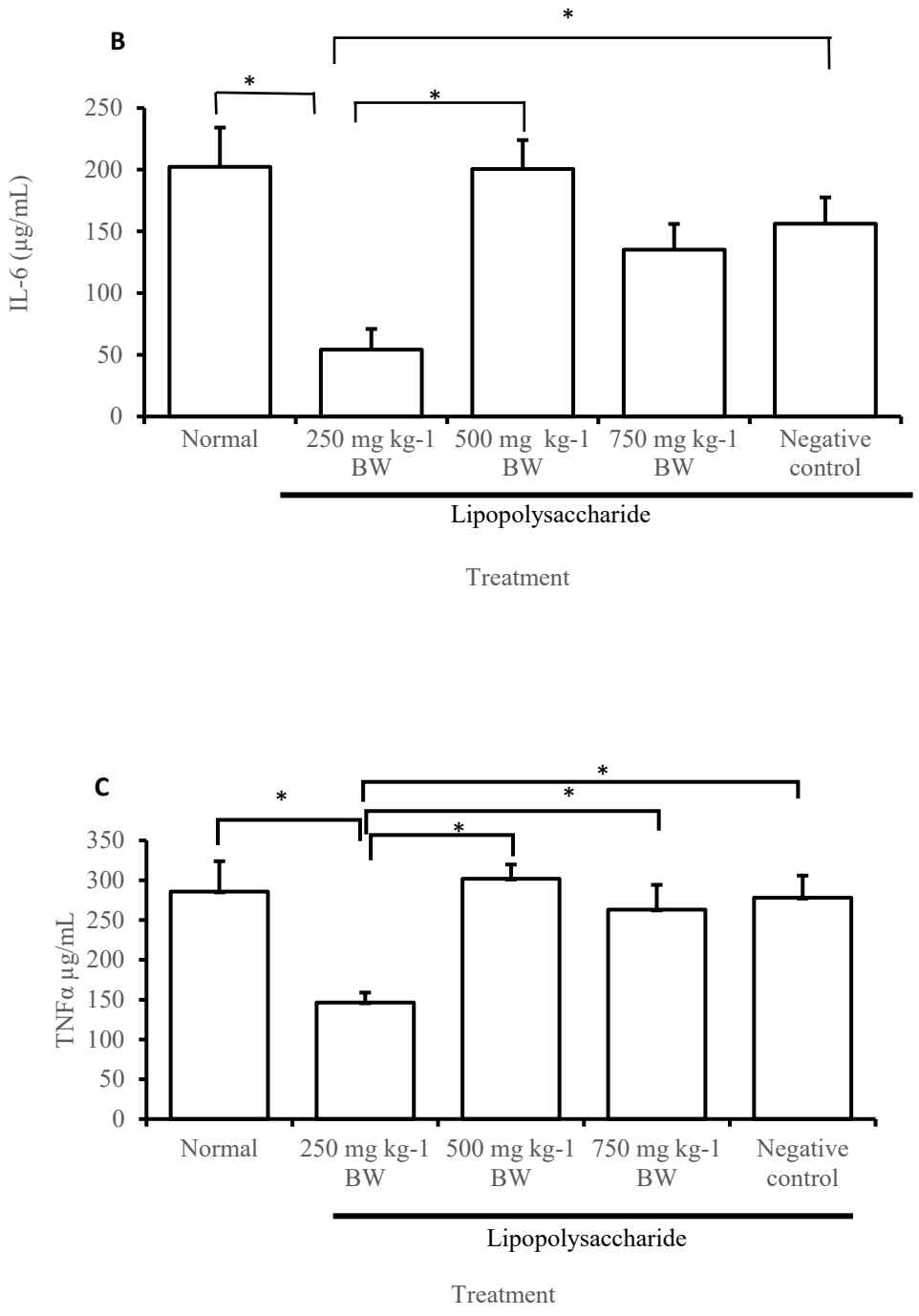


Fig. 1. Level of IL10 (A), IL6 (B), and TNFα (C) in the lungs of mice stimulated by LPS when treated with 750, 500, and 250 mg kg^{-1} BW pearl grass extract.

4 Discussion

Anti-inflammatory medications play a vital role in managing diseases related to cellular and humoral immune responses, as well as chemokine activity. Because the inflammatory response triggered by injury or infection involves multiple inflammatory pathways, these medications can target various mechanisms to help reduce inflammation.

A glycolipid derived from the membranes of cellular Gram-negative bacteria, lipopolysaccharide (LPS), is an inflammatory inducer able to influence the permeability of the alveolar membrane, leading to the recruitment of activated macrophages, lymphocytes, and neutrophils into the lungs [10]. Many proinflammatory cytokines, including IL6 and tumor necrosis factor (TNFα), are also frequently released in the lungs after exposure to LPS, thereby contributing to the onset of acute lung inflammation [10].

Present findings indicate that cytokines IL10, IL6, and TNFα levels did not show statistically significant changes 72 hours after LPS was administered intranasally ($P < 0.05$) (Figure 3). This study does not support the upregulation effect of LPS on IL10, IL6, and TNFα in the homogenate of mouse lungs induced by LPS. In a previous study, researchers observed a significant increase in cytokines IL-6 and TNF-α levels in the lavage fluid of bronchoalveolar mice treated with either 1 or 75 μg μL-1 of LPS, 24 hours after intranasal instillation. However, at days 4, 7, and 28 post-treatments of LPS [11], the levels of IL6 and TNFα were not significantly different compared to the control group. The mild pro-inflammatory cytokine response observed following LPS induction suggests a resolution of the acute phase of inflammation, leading to a return to normal cytokine levels.

Despite LPS not significantly affecting IL6 and TNFα levels during the resolution phase, it was interesting to observe that administering 250 mg pearl grass ethanol extract per kg body weight caused a remarkable decrease in LPS-induced IL6 and TNFα ($P<0.05$) (Figure 3). When comparing the cytokines levels, TNFα levels in LPS-treated mice appeared to decrease relative to those of IL10 and IL6. Nevertheless, this change was not statistically significant when contrasted with the normal group (Figure 3). The pearl grass extract at 250 mg kg-1 BW seemed to lower the levels of IL10 induced by LPS. A similar reduction in IL10 levels was noted in leukocyte cytokine release in chronic systemic patients receiving tricetin treatment [12].

The lungs host two distinct types of macrophages: alveolar and interstitial macrophages, whose properties and roles are shaped by the local environment [13]. Additionally, macrophages can be categorized into two types based on their gene expression patterns and roles in immune defense: classically activated (M1) and alternatively activated (M2) macrophages [13]. M1 macrophages are stimulated by T-helper 1 cytokines, including TNFα and IFN γ, as well as LPS. When activated, these macrophages produce and release pro-inflammatory substances like TNFα, IL1β, and iNOS. Meanwhile, M2 macrophages are activated by cytokines released from Th2 cells, specifically IL4 and IL13. These macrophages produce and release anti-inflammatory molecules, including IL10 and transforming growth factor-β (TGF-β) [13]. Based on the observed effects of pearl grass extract, which showed a more significant impact on TNFα and IL6 compared to IL10, it can be inferred that the extract may have a stronger effect on Th1 cytokines than on Th2 cytokines under the conditions tested.

Analysis of the pearl grass extract for phytochemicals content demonstrated the existence of alkaloids, saponins, flavonoids, tannins, and terpenoids (Table 1). Flavonoids exhibit anti-inflammatory effects by targeting multiple pathways depending on the chemical structure of flavonoids. Tricetin and fisetin, for example, can reduce IL10, IL8, IL6, and TNFα. Levels. However, many flavonoids have an anti-inflammatory impact by disrupting the inflammatory mediators production like IL6, TNFα, and IL1β in various cell lines via the Mitogen-Activated Protein Kinase (MAPK) signaling pathway [14].

The more pronounced anti-inflammatory effect seen at the lower dosage (250 mg kg^{-1} BW) compared to the higher doses (500 and 750 mg kg^{-1} BW) may be attributed to the presence of biphasic or hormetic response. A biphasic or hormetic response refers to a phenomenon where low doses of a substance show beneficial effects (e.g., anti-inflammatory). In contrast, greater doses can diminish this effectiveness or even result in negative outcomes, such as fostering inflammation. The hormetic dose response is also evidenced by phytoestrogen compounds, including polyacetylenes falcarinol, falcarrindiol, isoquinoline alkaloid berberine, and prenylated saponins [15].

Plant extracts contain diverse bioactive compounds that can activate multiple receptors simultaneously, each with varying sensitivity. At low concentrations, receptors promoting the desired effect may be predominantly activated, leading to synergistic effects as opposing receptors remain inactive. However, at higher concentrations, opposing receptors may also be activated, antagonizing the main effect and reducing activity. This phenomenon highlights the importance of receptor sensitivity and selective activation in determining the dose-dependent effects of plant extracts [15].

The biphasic effect may also occur due to receptor saturation, leading to the activation of different Pathways at high doses. Furthermore, toxicity or cellular stress effects at higher doses can induce toxic or stressful effects on cells, potentially stimulating the production of inflammatory mediators as a stress response. These findings emphasize the importance of determining an optimal dose for the desired anti-inflammatory effect. A lower dose does not always imply a weaker effect, as biological effects often depend on the body's complex regulatory mechanisms [15].

5 Conclusion

Our research findings indicated that 250 mg of a 70% ethanol extract from pearl grass per kilogram body weight, demonstrated potential as an anti-inflammatory therapy. This extract significantly reduced the levels of pro-inflammatory cytokines TNFα and IL6 in the lung tissues of mice. Thus, the ethanol extract of pearl grass may serve as a preventative approach to combat lung inflammation that could progress into more severe lung diseases.

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Conflict of interest
To ensure transparency in their research contributions, the authors declare that they have no conflicting interests.

Ethical considerations
With clearance number 176/KEH/SKE/I/2024, the Animal Ethics Committee at IPB University's School of Veterinary Medicine and Biomedical Sciences approved the study protocol.

References

1. G.U. Meduri, D. Annane, G.P. Chrousos, P.E. Marik, S.E. Sinclair. Activation and regulation of systemic inflammation in ARDS: rationale for prolonged glucocorticoid therapy. Chest. 136,1631–1643 (2009). https://doi.org/10.1378/chest.08-2408

2. World Health Organization. WHO - The top 10 causes of death. 24 Maggio [Internet]. 1–7 (2018). Available from: http://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death.
3. M. Bhatia, S. Moochhala. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J Pathol. 202(2),145–56 (2004). https://doi.org/10.1002/path.1491
4. M. Barakat, N.K.Syed, E. Hasen, S.B. Abdulrazzaq, S. Thiab, M.A. Al-Najjar,A. Omar, T.T., Lucy, A.N.M. Mamun-Or-Rashid, M.Yagi, and Y. Yonei, Phytomedicine Plus.The effect of natural products on inflammatory cytokines production and secretion. Phytomedicine Plus, p.100488 (2023). https://doi.org/10.1016/j.phyplu.2023.100488
5. S. Dalimartha. Atlas Tumbuhan Obat Indonesia Jilid 5. Jakarta Indonesia: Pustaka Bunda, 2008.
6. T.Wijayanti . Skrining senyawa metabolit sekunder ekstrak rumput mutiara (Hedyotis corymbosa (l.) Lamk.) Dengan metode GC-MS. Florea. J. Biol. dan Pembelajarannya. 2017;4(1):24.
7. R. Ardiani. Study the anti-Inflammatory activity of the ethanol extract of pear grass (hedyotis corymbosa l. lamk) on white rats. J. Penelit Farm. Herb. **5**(1), 5–9 (2022). DOI: https://doi.org/10.36656/jpfh.v5i1.858.
8. 8..L.M.Lin, S.H. Xiong, L.J. Zhao, J. Tang, Z.M. Zhang,Y.M. Li, T. Zheng, B.H., Xia. Extraction, characterization, antioxidant, and immunostimulatory activities of polysaccharides from hedyotis corymbosa. Evidence-Based Complementary and Alternative Medicine, 2018(1), p.8617070..(2018.). https://doi.org/10.1155/2018/8617070 .
9. Hanani E. Analisis Fitokimia. Jakarta: Penerbit Buku Kedokteran EGC., 2015.
10. M. Bhatia, S. Moochhala. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J. Pathol. **202**(2),145–56 (2004). https://doi.org/10.1002/path.1491.
11. S.T. Tsikis, S.C. Fligor, T.I. Hirsch, A. Pan, L.J. Yu, H. Kishikawa, M.M. Joiner, P.D. Mitchell, M. Puder. Lipopolysaccharide-induced murine lung injury results in long-term pulmonary changes and downregulation of angiogenic pathways. Sci Rep. **12**(1),1–12 (2022). https://doi.org/10.1038/s41598-022-14618-8
12. A.R. Weseler, L. Geraets, H.J.J. Moonen, R.J.F. Manders, L.J.C. Van Loon, H.J. Pennings, E.F.Wouters, A. Bast, G.J. Hageman, Poly (ADP-ribose) polymerase-1-inhibiting flavonoids attenuate cytokine release in blood from male patients with chronic obstructive pulmonary disease or type 2 diabetes. J Nutr. **139**(5), 952–7 (2009). https://doi.org/10.3945/jn.108.102756
13. N., Partida-Zavala, M.A. Ponce-Gallegos, I. Buendía-Roldán, and R.Falfán-Valenci, Type 2 macrophages and Th2 CD4+ cells in interstitial lung diseases (ILDs): an overview. Sarcoidosis, Vasculitis, and Diffuse Lung Diseases, 35(2), p.98-108. (2018).
14. K.C. Li, Y.L. Ho, W.T. Hsieh, S.S. Huang, Y.S. Chang, G.J. Huang. Apigenin-7-glycoside prevents LPS-induced acute lung injury via downregulation of oxidative enzyme expression and protein activation through inhibition of MAPK phosphorylation. Int. J. Mol. Sci. **16**(1), 1736-1754 (2015) https://doi.org/10.3390/ijms16011736.
15. J. Jodynis-Liebert, M. Kujawska. Biphasic dose-response induced by phytochemicals: experimental evidence. J. Clin. Med. **9**(3), 718 (2020).; https://doi.org/10.3390/jcm9030718.