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Soybean Seeds (*Glycine max* L.) Extract Against Cytokine Storm in ARDS Rat Model through Inhibiting Inflammation Marker

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ABSTRACT

Several studies have suggested that "cytokine storms" are significant causes of the severity of COVID-19. Controlling and inhibiting the cytokine storm in COVID-19 could prevent the spread of COVID-19 and saves patient lives. Soybean (*Glycine max* L.) is known to have various biological activities. This study aims to examine bioactive compounds in SSE and the effect of SSE on the ARDS rats model. A total of 25 Sprague Dawley Lipopolysaccharide-induced rats were used. Determination of serum IL-1β, IL-12, and lung TNF-α levels was performed by ELISA method. NF-κB and IFN-γ expression were determined by the qRTPCR method. IL-6 expressions were analyzed by immunohistochemistry assay. The bleeding, inflammation, and alveolus collapse score were analyzed using the HE staining method. The results showed that SSE could decrease the level of IL-1β, IL-12, TNF-α, IL-6, NF-kB, and IFN-γ and improve the bleeding, inflammation, and alveolus score in the lung. SSE could decrease the pro-inflammatory cytokines and improve lung condition in ARDS rats model.

1. Introduction

In 2019, an outbreak caused a worldwide emergency because this disease led to a high death rate and spread very quickly. The coronavirus disease 2019, more commonly known as COVID-19, is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Pneumonia, severe symptoms of acute respiratory distress syndrome (ARDS), and multiple organ failure are complications in COVID-19 patients (Yang *et al.* 2020). Based on the research that has been performed, it is revealed that the level of inflammatory biomarkers and cytokines increases in COVID-19 patients. The results of several studies show that a "cytokine storm" is a primary cause of the severity of COVID-19 (Wilson *et al.* 2020).

In viral infections, a cytokine storm is an excessive immunological response (Kunnumakkara *et al.* 2021). Cytokine storms are required for the inflammatory response during coronavirus infection, but they can also trigger an inflammatory immune response that attacks healthy alveolar cells. This can progress to ARDS and other multi-organ dysfunction, which can end in severe illness and death (Azmi *et al.* 2020; Mehta *et al.* 2020; Ye *et al.* 2020).

Inflammatory cytokines such as interleukin (IL)-1β, IL-2, IL-6, IL-7, IL-8, IL-10, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-inducible protein-10 (IP10), monocyte chemotactic protein 1 (MCP1), macrophage inflammatory protein-1α, Interferon (IFN)-γ, and Tumor necrosis factor (TNF)-α are significantly elevated in most severe cases of COVID-19 (Chen *et al.* 2020; Huang *et al.* 2020; Liu *et al.* 2020; Qin *et al.* 2020). The three most increased cytokines in severe cases of COVID-19 are IL-1β, IL-6, and IL-10 (Diao *et al.* 2020; Wan *et al.* 2020). In addition, a study by Yang *et al.* (2020) showed that levels of cytokines, including IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ, were elevated in severe and critical cases of COVID-19. While in non-severe patients, levels

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of cytokines, including IL-1β, IL-1RA, IL-2R, IL-6, IL-7, IL-8, IL-9, IL-10, IFN-γ, TNF-α, G-CSF, GM-CSF, IP10, MCP1, are also upregulated in blood but significantly lower than in severe patients (Huang *et al.* 2020; Qin *et al.* 2020; Zhou *et al.* 2020).

Controlling and inhibiting the cytokine storm in COVID-19 is an approach to prevent COVID-19 from spreading and saving the patient's life. (Azmi *et al.* 2020). Since there is no cure for COVID-19 at present, new therapeutic approaches which are more inventive, safe, and effective in the treatment of ARDS are being critically developed.

Due to its great nutritional content, soybean (*Glycine max* L.) is one of the most popular plants for consumption. Soybean contains many compounds, such as saponins, isoflavones, phytosterols, phytic acid, trypsin inhibitors, and bioactive peptides. Meanwhile, soybean extract has a high content of polyphenol compounds, including isoflavones. The antioxidant properties of polyphenolic compounds contained in soybean cause soybean extract to enjoy biological activities, such as reducing the incidence of non-communicable diseases (NCD), including cancer and cardiovascular disease (Cabezudo *et al.* 2021). Therefore, this study aimed to determine the effect of soybean seed extract (SSE) on ARDS model rats as an alternative for the ARDS model by measuring levels of Il-1β, IL-12, TNF-α, IL-6, and NF-κB, IFN-γ expression.

2. Materials and Methods

2.1. Sample Preparation

Soybean seed extract (SSE) was produced based on current Herbal Good Manufacturing Practices of the National Agency of Drug and Food Control (NA-DFC) Republic of Indonesia. SSE was produced by FAST Co. (Depok, West Java, Indonesia) with CoA No. Batch 00107201055. SSE was extracted from the seeds using aquade-mineral with additional substance lactose.

2.2. LC-MS/MS Analysis

SSE was analyzed by applying the LC-MS/MS method to determine the contained compounds in the extract. The analysis was carried out using a column with Hypersil Gold specifications (150 mm × 2.1 mm × 1.9 µm). TSQ Quantum Access MS/MS Triple Q (quadrupole) mass spectrometer with ESI (Electrospray Ionization) was controlled by TSQ Tune software, which operated with a positive charge (Kaliawan and Danardono 2021).

2.3. Animal and Experimental Design

Twenty-five of 6 weeks-old white male Sprague Dawley (SD) rats (weighing 100-120 g) were supplied by iRATco Veterinary Laboratory Services (Bogor, Indonesia). The research was then approved by the Faculty of Medicine of Maranatha Christian University (No:099/KEP/VII/2020). The rats were housed in individually ventilated cages (IVC) and kept in an air-conditioned environment with a temperature of 20–24°C and a 12-hour light–dark cycle. During the procedure, the air humidity was maintained consistently (Gondokesumo *et al.* 2019). The rats were acclimatized for seven days. The animals were fed ad libitum meals containing 14% protein and 5% fat. Then, LPS dissolved in normal saline was induced once via intratracheally at a dose of 5 mg/kg body weight (BW). After eight h, the rats showed positive ARDS and were treated with SSE.

The rats were divided into five groups according to the number of treatments used (Jang *et al.* 2014; Ojewole *et al.* 2005).

I :negative control, normal rats treated with aquadest
II :positive control, LPS 5 mg/kg BW-induced rats + treated with aquadest
III :LPS-induced rats treated with SSE + 50 mg/kg BW of SSE
IV :LPS-induced rats treated with SSE 50 mg/kg BW LPS + 400 mg/kg BW of SSE
V :LPS-induced rats treated with SSE 50 mg/kg BW LPS + 800 mg/kg BW of SSE)

The number of animals used in this study was determined regarding Frederer's formula.
Frederer's formula (Ihwah *et al.* 2018).

$$t\,(r - 1) > 15$$

$$5\,(r - 1) > 15$$

$$r > \frac{15}{5} + 1$$

$$r > 4$$

$$r \approx 5$$

Where:
t = number of treatments
r = number of replications, defined as the number of animals in each group
r = number of replications, defined as the number of animals in each group

LPS was dissolved in normal saline, then administrated once via intra-trachea at a dose 5 mg/kg of body weight (BW). After 8 h, the rats showed positive ARDS, then were treated with SSE. SSE given twice, on the first and eight days and kept for 21 days, counted since the day after LPS induction (Hagawane *et al.* 2014). Normal rats (negative control) were induced with aquadest along LPS induction and treatment periods. After 21 days the rats suffered ARDS, their blood was collected through the retro-orbital plexus and kept in a coagulant containing tube. Furthermore, for the lung removal, the rats were anesthetized with ketamine (100 mg/kg BW) and xylazine (15 mg/kg BW) via intraperitoneal injection. A part of the lung was frozen in liquid nitrogen and stored at −80°C until it was used for the ELISA assay and qPCR, and the remaining part of the lung was fixed in 10% formalin (Gondokesumo *et al.* 2019).

2.4. Histopathological Analysis

The formalin-fixed lung specimens were dehydrated using alcohols, cleared in xylene, and embedded in paraffin. The paraffin slices were 5 µm thick which were stained with hematoxylin and eosin (H and E) according to the standard protocol (Tsai *et al.* 2020). Pathological changes were examined and scored using OriginPro 8.5.

2.5. Quantification of IL-6 Immunohistochemistry

The lung samples were fixed in 10% formalin and embedded in paraffin. After that, the lung samples were cut into 5 µm sections using a microtome (Leica, Leica Biosystem Nussloch GmbH, Wetzlar, Germany). Before antigen retrieval, the lung slices were de-paraffinized and re-hydrated. Antigen retrieval (Abcam; ab208572) was done/performed/conducted in citrate buffer (pH 6.0) for 10 minutes at 121°C. Endogenous peroxidase was blocked for 15 minutes at room temperature in methanol (Merck; 106009) and H_2O_2 3% (Merck, 107209). The primary rabbit-anti rat IL-6 (Elabsci; E-AB-40073) was incubated overnight at room temperature. Rabbit-Specific HRP/DAB (ABC) Detection IHC Kit (Abcam; ab64261) was used to visualize the target protein. The counterstaining agent used was hematoxylin, and stained tissues were examined by a PrimoStar (Zeiss) microscope equipped with a Lumenera Infinity 1-3c for photography (Gondokesumo *et al.* 2019; Tsai *et al.* 2020).

2.6. NF-kB and IFN-γ Genes Expressions

Total rat's lung RNA was isolated and purified according to the manufacturer's protocol using the Direct-zol RNA Miniprep Plus Kit (Zymo; R2073). Complementary DNA synthesis was carried out using the iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad; 170-8841). Quantitative gene expression was performed using the AriaMx 3000 Real-Time PCR System (Agilent; G8830A). The reaction mixture applied to perform qPCR was Evagreen master mix (Bio-Rad; 1725200) (Lister *et al.* 2020; Widowati *et al.* 2018a, 2018b). The primer sequence (Macrogen) can be seen in Table 1.

2.7. Quantification of lung TNF-α and Serum IL-1β, IL-12

The frozen rat lungs were homogenized and measured using the ELISA Kit TNF-α (Elabsci; E-EL-R0019). Rat serum was measured using Elisa Kit IL-1β (Elabsci; E-EL-R0012) and IL-12 (Elabcsi; E-EL-R0064). The procedure was performed according to the manufacturer's protocol. Sample absorption was read at 450 nm using a microplate reader (Multiskan GO, Thermo Scientific). The levels of TNF-α, IL-12, and IL-1β were calculated based on the protein standard curve (Prahastuti *et al.* 2019; Widowati *et al.* 2018a, 2018b).

2.8. Statistical Analysis

Experimental results are presented as (mean ± SD). The data obtained in this study were analysed using advantage SPSS 22.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used followed by Tukey Post Hoc test. P-values < 0.05 were considered significant.

Table 1. Primer sequence

Gene	Primer sequence (5'-3') forward reverse	Product size (bp)	Annealing (°C)	Reference
NF-κB rat	GGACTATGACTTGAATGCGG ACACCTCAATGTCTTCTTTCTG	230	57	NCBI Reference Sequence: NM_199267.2
IFN-γ rat	AAGTTCGAGGTGAACAACCC CCAGAATTCTTCTTATTGGCACAC	156	57	NCBI Reference Sequence: NM_138880.3
Rat GAPDH	TCAAGATGGTGAAGCAG ATGTAGGCCATGAGGTCCAC	217	57	NCBI Reference Sequence: NM_001289726

3. Results

3.1. LC-MS/MS Test of SSE

Figure 1 shows two chromatograms resulted from LC-MS/MS test. The first chromatogram is TIC (Total Ion Chromatogram) while the second chromatogram is EIC (Extracted Ion Chromatogram). TIC contains the target compound while EIC contains only the target compound. The results of the analysis showed that SSE compounds contain daidzein, daidzin, genistein, biochanin A, and glycitein.

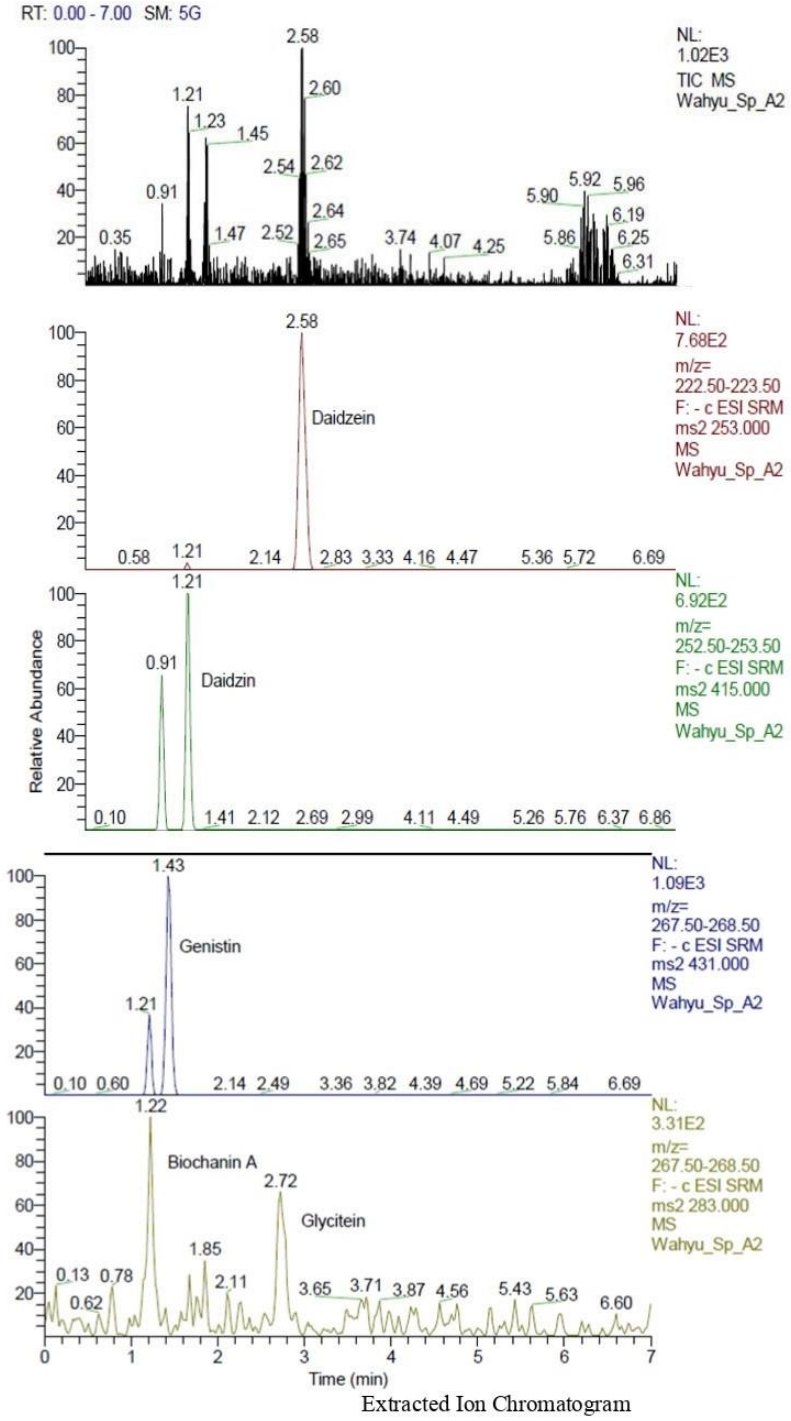


Figure 1. LC-MS/MS spectrum of SSE

3.2. Histopathological Analysis

Figure 2 shows the histopathological observations in LPS-induced rats. LPS could induce lung damage including bleeding, inflammation, and alveolus collapse. Based on the results of the study, the administration of SSE at a dose of 50 mg/kg BW, 400 mg/kg BW, and 800 mg/kg BW could improve lung damage caused by LPS induction. These results are supported by the ARDS scoring which can be seen in Figure 3. From the scoring results, the positive control had severe lung damage, while in the group receiving the SSE, it showed moderate damage. The negative control was not damaged and was normal. The results showed that the SSE 800 could reduce the bleeding, inflammation, and alveolar collapse scores to 1.60 when compared to the positive control, which was 2.67.

3.3. Effect of SSE on IL-6 Expression

Based on the results shown in Figure 4, the brown color indicates the presence of IL-6 cytokines in the lungs, which means cell damage due to LPS induction. SSE treatment, especially at a dose of 800 mg/kg BW, could improve damage to the lungs. This is supported by the results of the quantitative analysis shown in Figure 5. The IL-6 expression in positive control was 402.6±35.71, and SSE at a dose of 800 mg/kg BW could significantly reduce IL-6 expression to 102.0±32.01.

3.4. Effect of SSE on NF-κB and IFN-γ Gene Expression

LPS induction could increase the expression of NF-κB and IFN-γ genes. The effect of SSE administration on NF-κB and IFN-γ gene expression can be seen in Figure 6. Based on the results, SSE could significantly

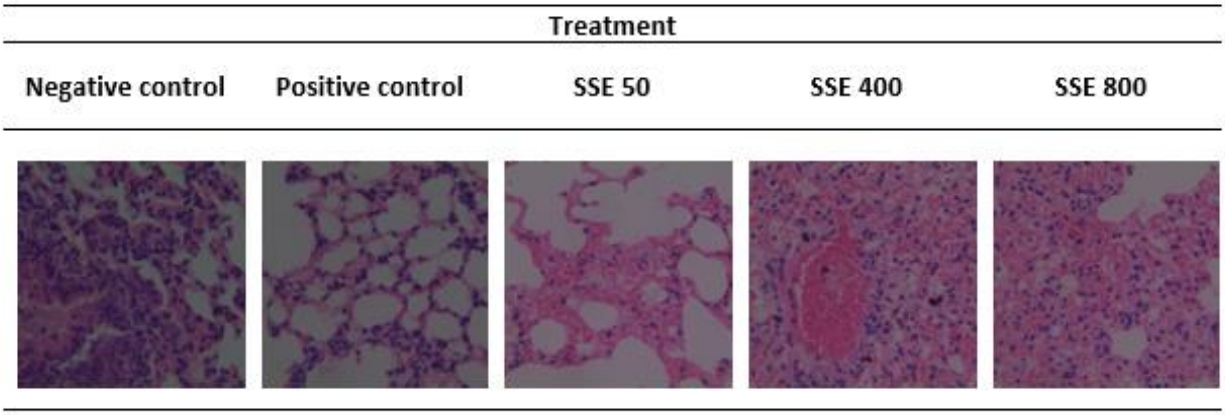


Figure 2. Alveolar histopathological features of ARDS rat model

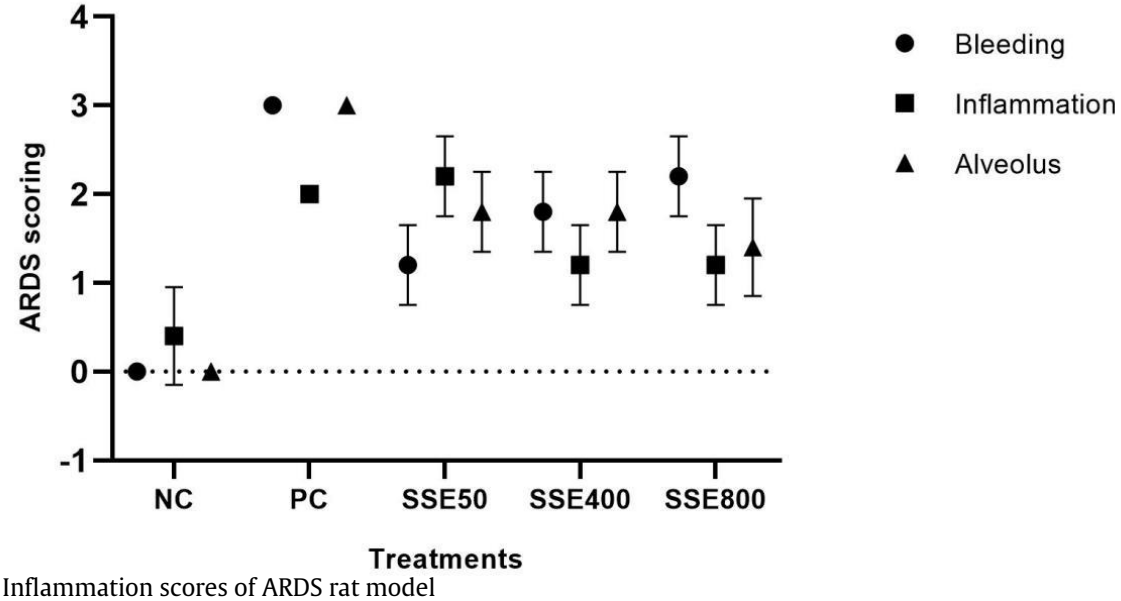


Figure 3. Inflammation scores of ARDS rat model

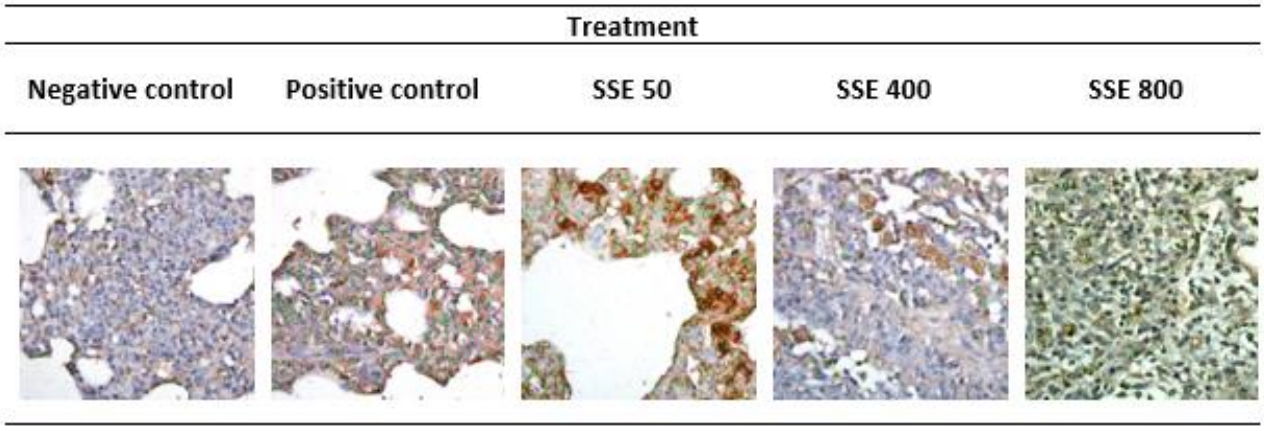


Figure 4. The effect SSE toward IL-6 expression on the rat lung. Cells positive IL-6 protein express brown colour as pointed by the arrow

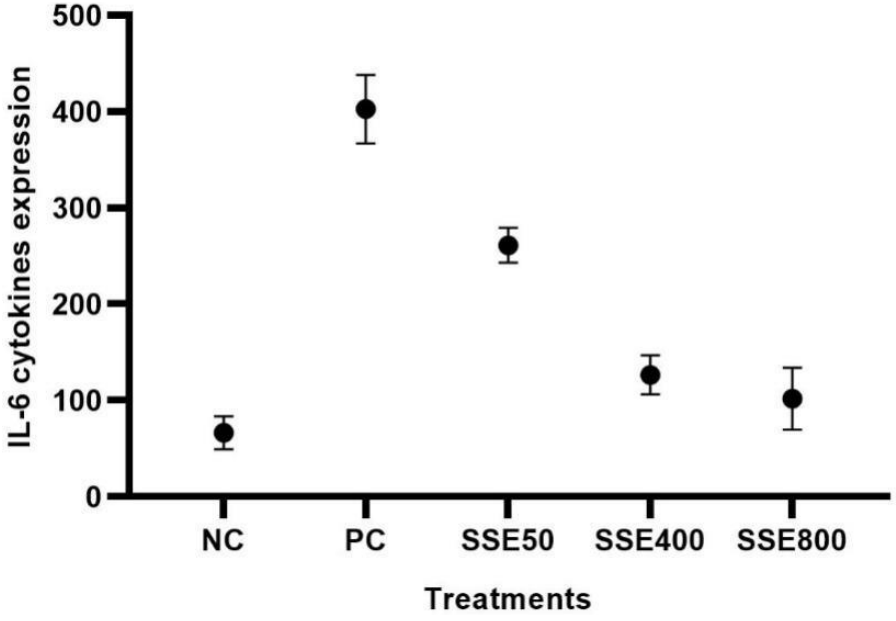
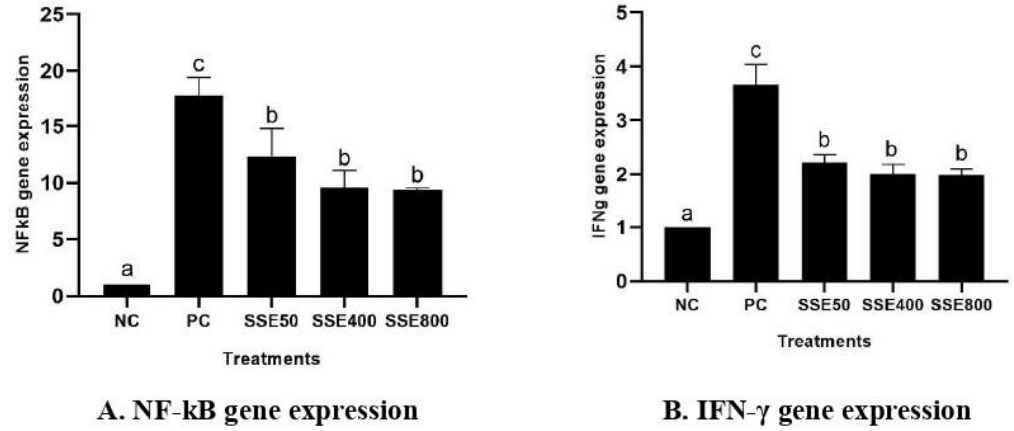


Figure 5. Effect soybean extract toward lung IL-6 expression in ARDS rats model. Data are presented as mean average ± SD from five replications. NC: negative control (normal rat + untreated SSE); PC: positive control (LPS-induced rat); SSE50: positive control + SSE 50 mg/kg BW; SSE400: positive control + SSE 400/kg BW; and SSE800: positive control + SSE 800 mg s/kg BW

reduce gene expression when compared to positive controls. SSE with a dose of 800 mg/kg BW was the most effective in reducing the expression of these two genes (Figure 6).

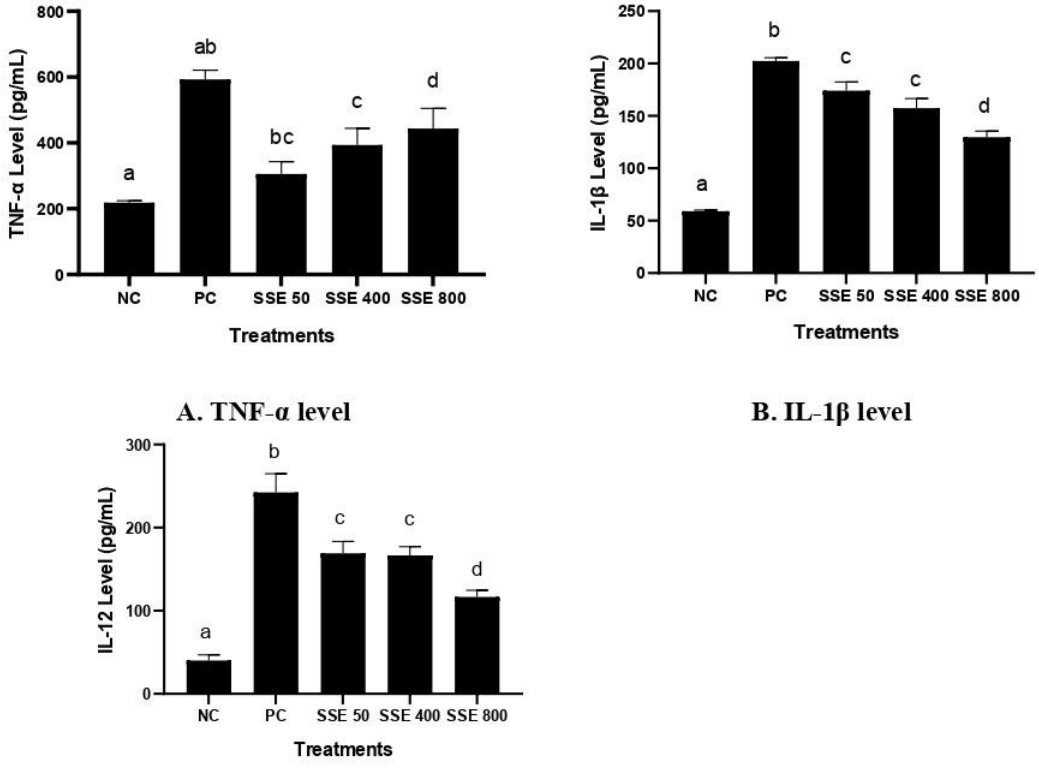
3.5. Effect of SSE on the Levels of TNF-α in the lung; IL-1β and IL-12 in serum

Induction of LPS leads to an increase in the production of pro-inflammatory cytokines in serum and lung, including TNF-α, IL-1β, and IL-12. Figure 7 shows TNF-α levels in the lungs and serum levels of IL-1β and IL-12 with SSE treatment. Compared with the positive control, SSE can decrease pro-inflammatory cytokines in the lung and serum of ARDS rat model. SSE could reduce serum levels of IL-1β and IL-12, with SSE at 800 mg/kg BW. Meanwhile, SSE at the dose of 50 mg/kg BW could reduce TNF-α levels in the lung; a dose of 50 mg/kg BW is a low dose that does not cause morbidity but affects the regulation of the immune system (Chen *et al.* 2004) (Figure 7).



A. NF-kB gene expression **B. IFN-γ gene expression**

Figure 6. Effect soybean extract toward pro-inflammatory genes expression in ARDS rat model. Data are presented as mean average ± SD from five replications: NC: negative control (normal rat + untreated SSE); PC: positive control (LPS-induced rat); SSE50: positive control + SSE 50 mg/kg BW; SSE400: positive control + SSE 400/kg BW; and SSE800: positive control + SSE 800 mg s/kg BW. Figure 3A. Different letters (a,b,c) show significant difference of NF-kB gene expression among treatments. Figure 2B. Different letters (a,b,c,) show significant difference of IFN-γ levels among treatments based on Tukey HSD post hoc test ($p<0.05$)



A. TNF-α level **B. IL-1β level**

C. IL-12 level

Figure 7. Effect soybean extract toward pro-inflammatory cytokines level. Data are presented as mean average ± SD from five replications: NC: negative control (normal rat + untreated SSE); PC: positive control (LPS-induced rat); SSE50: positive control + SSE 50 mg/kg BW; SSE400: positive control + SSE 400/kg BW; and SSE800: positive control + SSE 800 mg s/kg BW. Figure 2A. Different letters (a,ab,bc,c,d) show significant difference of TNF-α levels among treatments. Figure 2B. Different letters (a,b,c,d) show significant difference of IL-1β levels among treatments. Figure 2C. Different letters (a,b,c,d) show significant difference of IL-12 levels among treatments based on Tukey HSD post hoc test ($P<0.05$)

4. Discussion

This study aims to investigate SSE effects toward LPS-induced rats, as the model of ARDS rat. Lung damage, including hemorrhage, congestion, dust cell infiltration, and alveolus collapse, are obviously noticed in LPS-induced lung tissue (Figure 2). Moreover, lung inflammation is signified by the increases of pro-inflammatory cytokines, including IL-6, NF-kB, and IFN-γ (Figures 4, 5, and 6). Previous studies proposed LPS-induced rat as an ARDS model. Li *et al.* (2016) elucidated lung inflammation generated by LPS induction. Histological analysis demonstrated severe lung damage, indicated by an increase in inflammatory cell infiltration, edema, and hemorrhage in the alveolus. Additionally, ACE2 expression was decreased, which ACE2 knockdown resulted in a noticeable worsening of lung damage and increased cytokine release (Li *et al.* 2016). This result is also supported by a study by Liu *et al.* (2021) that LPS injection can cause severe acute lung injury and death in rats (Liu *et al.* 2021).

SSE contains isoflavone compounds, such as daidzein, daidzin, genistein, biochanin A, and glycitein, which have been proven from the results of the LC-MS/MS test in this study (Figure 1). Isoflavones could reduce pro-inflammatory secretions by inhibiting the NF-κB transcription system. Isoflavones also modulate arachidonic acid (AA) metabolism and NO production by inhibiting the activation of pro-inflammatory enzymes (Yu *et al.* 2016). A study by Arbenathy *et al.* (2017) conveyed that soy can prevent the infiltration and activation of neutrophils and macrophages that radiation causes in the lung parenchyma. In addition, the study suggested that soy isoflavones regulate the cellular mediators of the radiation-induced inflammatory response (Arbenathy *et al.* 2017).

Furthermore, SSE effects are investigated by assessing inflammation occurrences in lungs and blood. The levels of cytokines in lung, including TNF-α, IL-6, and NF-kB and IFN-γ, were analysed using ELISA method, IHC, and qPCR, respectively. Meanwhile the presence of cytokines in blood, including IL-1β and IL-12, were measured using ELISA method.

SSE alleviates lung injuries in ARDS rat lungs (Figure 2). The levels of bleeding, inflammation, and alveolar collapse were decreased as SSE treatments (Figure 3). Therapeutic effect of SSE on lung is resulted by the isoflavones. Abernathy *et al.* (2017) demonstrate alveolar repair after soy isoflavones treatment in radiation-exposed rat lung. The isoflavones contained in soybean acknowledged for their anti-inflammation in vary; for instance, daidzein and genistein on LPS-induced ALI (acute lung injury) rat, and biochanin A in rat adenocarcinoma (Aboushanab *et al.* 2021).

The effects of SSE on ARDS rat lung tissue are verified by the levels of pro-inflammatory cytokines (Cotogni *et al.* 2015). As presented on IHC tissue preparation, IL-6 expression on ARDS lungs is lowered as SSE treatments (Figure 4 and 5). Moreover, qPCR data demonstrates NF-kB and IFN-γ diminishes, and ELISA data shows TNF-α reduction on SSE-treated ARDS lung (Figure 6 and 7). Lung inflammation is associated with the increase of (TLR) 4 on cell membrane surface and downregulation of the angiotensin converting enzyme (ACE)-2 (Yu *et al.* 2021). TLR-4 activates mitogen activated protein kinase (MAPK) pathways, which leads to the activation of nuclear factor kappa B (NF-κB) and activation protein 1 (AP-1). NF-κB also has an important role in the expression of factors involved in inflammation, apoptosis, immune and stress responses, also adhesion (Giusti *et al.* 2017). During inflammation, NF-κB is activated by IkB kinase (IKK), which causes translocation of NF-κB into the nucleus and activates transcription of target genes, pro-inflammatory cytokines and chemokines, inducible nitric oxide synthases (iNOS), and cyclooxygenase 2 (COX-2) (Yu *et al.* 2016). These activations induce macrophage to produce pro-inflammatory cytokines, such as IL-6, tumor necrosis factor (TNF)-α, nitric oxide (NO), IL-1β, prostaglandin E (PGE)-2, inducible nitric oxide synthase (INOS), and COX-2 (Widowati *et al.* 2021; Laksmitawati *et al.* 2016). In addition, the activation of NF-κB can also lead to the activation of Th1 cells, inflammatory T cells, and the secretion of IFN-γ. IFN-γ is a cytokine that enhances cellular immunity and participates in the inflammatory process (Liu *et al.* 2017). Hence, the increases in IFN-γ level in alveolar cells was proportional to the increase of NF-κB. The study by Chen *et al.* (2019) elucidated glycitin anti-inflammatory activity by blocking the activation of TLR4-mediated NF-κB and MAPK signaling pathways, subsequently lower pro-inflammatory cytokines secretions (Chen *et al.* 2019). In virtue of this study, anti-inflammation generated by SSE possibly caused by its glycitin.

Besides, this study also examined the SSE effect toward blood immune activity by measuring IL-1β and IL-6. The results show that the cytokines secretions are diminished as SSE treatments. This finding implies that SSE potentially mitigate blood inflammation. A previous study suggested that this effect pertains to certain compounds in soybean. Genistein protects endothelial cells from damage induced by oxidative stress. LDL (Low-density lipoprotein) oxidation and plasma concentration of F2-isoprostanes (an in vivo marker of lipid peroxidation) in health volunteers ingested soybean (60 mg isoflavones in certain periods) are lower than baseline values (Rimbach *et al.* 2008). Genistein and daidzein are considered as important isoflavones for anti-inflammation. Both inhibit iNOS as well as NF-κB and STAT-1 pathways. The mentioned isoflavones also reduce IL-1β and TNF-α through the inhibition of TLR-4-LPS binding (Danciu *et al.* 2018).

The results in this study suggest SSE treatments in the ARDS rat model alleviate both lung and blood inflammations. Furthermore, SSE 800 µg/mL generates highest reduction of IL-6, NF-kB, and IFN-γ levels, while SSE 50 µg/mL results in the highest TNF-α decrease. Moreover, SSE contains bioactive compounds with specific effect. Hence the therapeutics effects are suggested for further study.

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