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Guava (*Psidium guajava* L.) extract inhibits cytokine storm in acute respiratory distress syndrome rats model

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ABSTRACT: COVID-19 infection is always accompanied by a cytokine storm that causes acute respiratory distress syndrome (ARDS). The reduction of pro-inflammatory cytokines is expected to be used for treating COVID-19 patients. Guava (*Psidium guajava* L.) was believed to have properties as an antioxidant and anti-inflammatory. This study aims to determine the effect of guava extract (PGE) on the ARDS rats model so that it can be used as a PGE treatment for the effects caused by ARDS. This study was conducted on 25 Sprague Dawley rats model in 5 treatment groups. Lipopolysaccharides (LPS) induction was carried out to create ARDS rats model. Determination of pro-inflammatory cytokines in lung Interleukin-18 (IL-18), IL-12 and serum IL-1β, Tumour Necrosis Factor alpha (TNF-α) was performed by ELISA, NOD-, LRR- and Pyrin domain-containing protein 3 (NRLP3) expressions were determined by the quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) method and IL-6, Nuclear factor kappa B (NF-κB) were analysed by immunohisto-chemistry assay. The results showed that PGE contained phenolic compound, daidzin and genistin. The IL-18, IL-12, Il-1β, TNF-α, IL-6, NF-kB and NRLP3 in ARDS model rats were decreased by PGE treatment. In conclusion, PGE can be used as a therapy for treating ARDS by decreasing pro-inflammatory marker.

KEYWORDS: Antioxidant; anti-inflammatory; ARDS; COVID-19; *Psidium guajava* L..

1. INTRODUCTION

Corona virus-19 (COVID-19) is a new outbreak since 2019. The RNA virus SARS-Cov-2 is responsible for COVID-19. Infection of COVID-19 is caused by an aggressive inflammatory response that involves the production of large amounts of pro-inflammatory cytokines, known as a "cytokine storm." [1]. A cytokine storm is an exaggerated immune response that is common in viral infections [2]. One of the consequences of cytokine storms is lung injury, which can rapidly evolve into severe acute lung injury, known as acute respiratory distress syndrome (ARDS) [3,4]. ARDS is a dead-cause disease that endangers human life and health [5]. After onset, it may cause an imbalance in the body's oxygen metabolism, an inflammatory cascade reaction, and the activation of the body's stress response system, resulting in the impairment or dysfunction of the immune system, coagulation system, cardiovascular system, liver, and kidney function [6], eventually leading to the occurrence of multiple organ failure [7]. During ARDS, cell damage occurs in the lungs. This is caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) reactions [8,9,10]. Many studies have been conducted to determine the mechanism of ARDS, but there is no effective treatment for this disease [11].

During inflammation, several cytokines were secreted. In the COVID-19 cases, Interleukin-2 (IL-2), IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, Tumor Necrosis Factor (TNF-α), Nuclear factor kappa B (NF-kB), and Metalloproteinase (MMP-6) were increased [12]. Another study reported increasing plasma levels of IL-1β, IL-8, IL-9, Granulocyte-Colony Stimulating Factor (G-CSF), Granulocyte Macrophage- Colony-Stimulating Factor (GM-CSF), Interferon-γ (IFN-γ), Interferon-Inducible Protein-10 (IP-10), NOD-, LRR- and Pyrin domain-containing protein 3 (NLRP3), Macrophage Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory

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Protein-1α (MIP-1α), MIP-1β, Platelet Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF) in both Intensive Care Unit (ICU) patients and non-ICU patients [13]. Thus, the inhibition of pro-inflammatory cytokines is expected to treat the ARDS of COVID-19.

Guava (*Psidium guajava* L.) belongs to the Myrtaceae family and contains quercetin, catechin, gallic acid, ursollic acid, caryophyllene, β-sitosterol, saponins, and avicularin as its major constituent [14]. Guava fruits are high in vitamins and minerals, as well as secondary compounds such as flavonoids, phenolic acids, and triterpenoids. Thus, it has anticancer, anti-inflammatory, hepatoprotective, and antioxidant properties [15,16]. This study aims to determine the properties of *P. guajava* extract (PGE) on ARDS model rats so it can be an alternative for COVID-19-induced inflammation treatment.

2. RESULTS

2.1. *LC-MS/MS*

The analysis results from LC-MS/MS show various compounds contained in PGE. The compounds appearing at each peak of this chromatogram are the ratio of mass to ion charge shown in Figure 1. Based on the results of the LC-MS/MS analysis, the total number of ions detected at each retention time is shown in Figure 1A, while the intensity specific ion signals in PGE are shown in Figures 1B and 1C. In Figures 1B and 1C, the chromatogram peaks appeared at a retention time of 0.91 minutes with m/z = 252.50-253.50, which was identified as a daidzin compound, and a retention time of 1.21 minutes with m/z = 267.50-268.50, which was identified as a genistin.

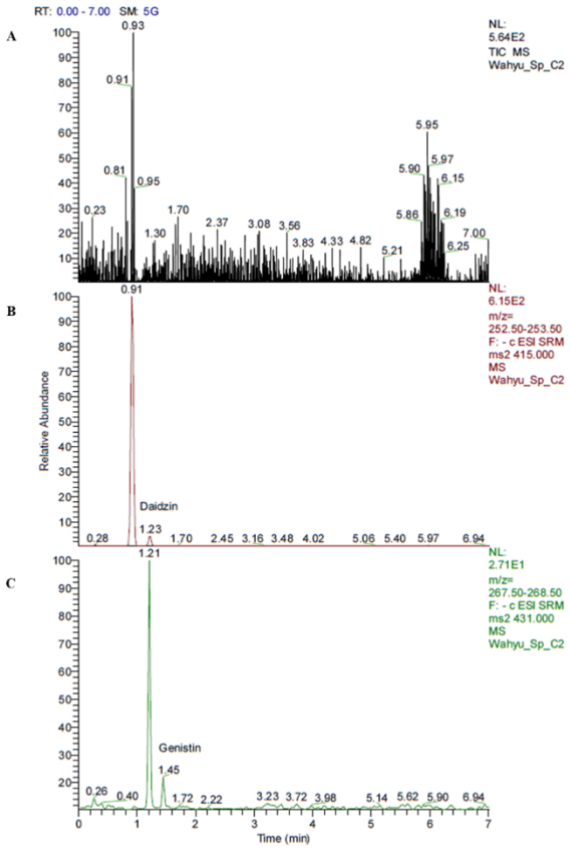

Figure 1. Total ion chromatogram of standard compound (A) and ion chromatogram of PGE (B and C) using LCMS/MS method

2.2. Quantification of lung IL-18, IL-12 and serum IL-1β, TNF-α

Figure 2 shows the lung IL-18, IL-12, and serum IL-1β and TNF-α levels of ARDS rats treated with PGE. LPS induction causes increased pro-inflammatory cytokine production in the serum and lung. PGE treatment can significantly decrease ($p<0.05$) the pro-inflammatory cytokines in the lung and serum of ARDS rats model compared to the positive control. Based on the data, it was found that PGE decreased significantly ($p<0.05$) pro-inflammatory cytokine of lung TNF-α, IL-1β levels and IL-18, IL-12 serum level.

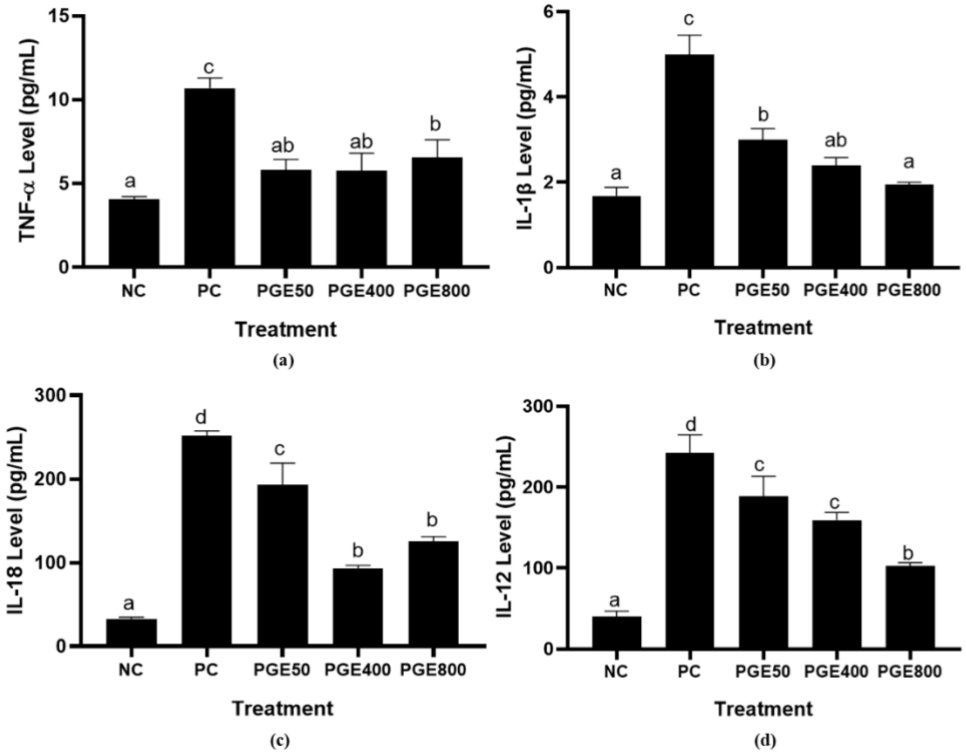

Figure 2. Effect of various PGE doses on level of inflammation markers in ARDS model rats
(a) TNF-α lung level of ARDS rat (b) IL-1β lung level of ARDS (c) IL-18 serum level ARDS rat (d) IL-12 serum level of ARDS rat. Data are presented as mean ± standard deviation. NC: Negative Control (untreated rat), PC : Positive Control (LPS-induced rats), PGE50 : ARDS rats model treated with PGE 50 mg/kg BW daily; PGE200 : ARDS rats model treated with PGE 400 mg/kg BW daily; PGE800 : ARDS rats model treated with PGE 800 mg/kg BW daily. Figure 2a. different mark (a,ab,b,c) shows the difference among PGE doses toward TNF-α lung level. Figure 2b. different mark (a,ab,b,c) shows the difference among PGE doses toward IL-1β lung level. Figure 2c. different mark (a,b,c,d) shows the difference among PGE doses toward IL-18 serum level. Figure 2d. different mark (a,b,c,d) shows the difference among PGE doses toward IL-12 serum level according Tukey Post Hoc Test at $p<0.05$ significance.

2.3. Gene expression of NLRP3

Figure 3 shows the NLRP3 gene expression of ARDS rats treated with PGE. Induction of LPS enhances NRLP3 gene expression significantly ($p<0.05$) compared to the negative control. The NLRP3 gene expression decreased significantly ($p<0.05$) with the increase in PGE dose. The most effective dose of PGE was 800 mg/kg BW daily.

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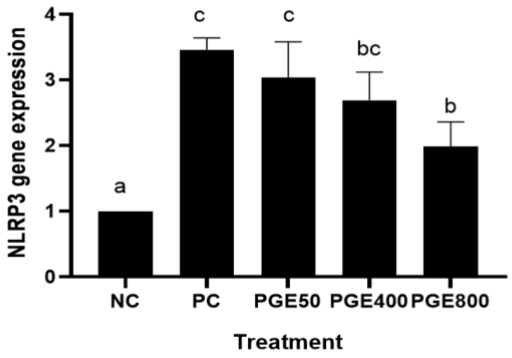
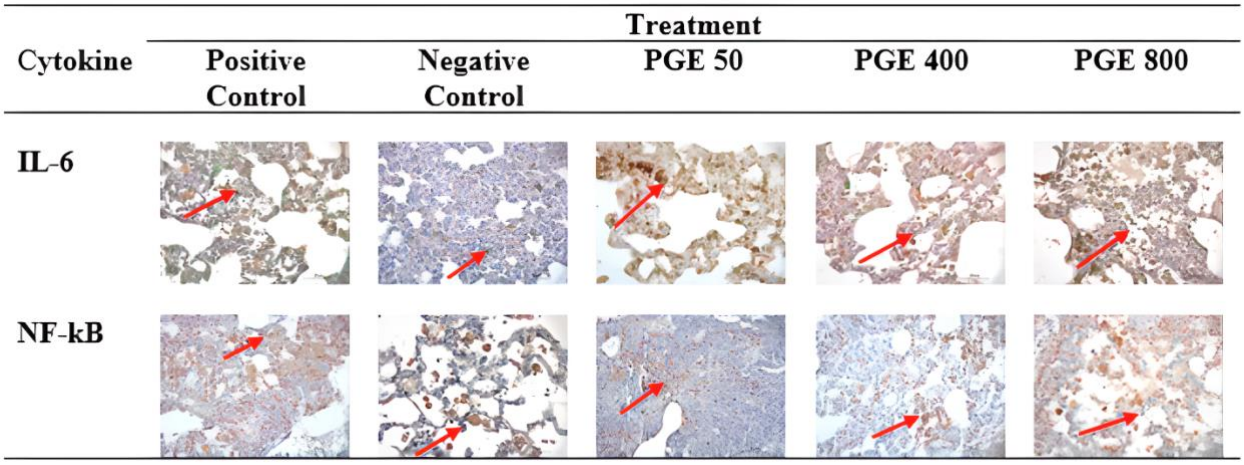


Figure 3. Effect of various PGE doses on NLRP3 gene expression of ARDS rats model
Data are presented as mean ± standard deviation. NC : Negative Control (untreated rat), PC : Positive Control (LPS-induced rats), PGE50 : ARDS rats model treated with PGE 50 mg/kg BW daily; PGE200 : ARDS rats model treated with PGE 400 mg/kg BW daily; PGE800 : ARDS rats model treated with PGE 800 mg/kg BW daily. The different mark (a,b,bc,c) shows the difference among NLRP3 expressions among treatments according Tukey Post Hoc Test at $p<0.05$ significance.

2.4. Immunohisto-chemistry of IL-6 and NF-κB

Figure 4 shows the effect of PGE on IL-6 and NF-kB protein expression of the lung in ARDS rats model. The brown colour determines the existence of IL-6 and NF-kB cytokines. IL-6 and NF-kB indicate the damage to cells due to LPS induction significantly ($p<0.05$). The positive control of IL-6 staining (Table 3 and Figure 5) was 402.6 ± 35.71 per view, and PGE treatment significantly decreased ($p<0.05$) IL-6 of ARDS rats' lung. The most effective dose was PGE at 800 mg/kg BW daily, with a total of IL-6 staining were 172.8 ± 35.71 per view. A similar phenomenon happened to the number of NF-kB stained. The decrease in the number of the NF-kB-stained expressions was accompanied by an increase in the PGE dose significantly ($p<0.05$). The highest NF-kB staining value was positive control (414.80 ± 24.52), which then significantly decreased ($p<0.05$) by the 800



mg/kg BW of PGE treatment to 179.4 ± 24.15 per view.

Figure 4. Effect of various PGE doses on IL-6 and NF-kB expression of lung in ARDS rats model
*IL-6, NF-kB expression at magnification 400x. The spot red color with red arrow are positive IL-6 expression and spot green colour with redrow are NF-kB expression
*NC : Negative Control (untreated rat), PC : Positive Control (LPS-induced rat), PGE50 : ARDS rat model treated with PGE 50 mg/kg BW daily; PGE200 : ARDS rat model treated with PGE 400 mg/kg BW daily; PGE800 : ARDS rat model treated with PGE 800 mg/kg BW daily. .

Table 3. Effect of various PGE doses toward IL-6 and NF-kB expression

Treatment	IL-6	NF-κβ
PC	402.6 ± 35.71^{c}	414.80 ± 24.52^{e}
NC	66.6 ± 17.11^{a}	89.0 ± 20.24^{a}
PGE50	229.6 ± 47.08^{b}	317.8 ± 18.23^{d}

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PGE400	168.2 ± 35.84[b]	258.4 ± 34.37[c]
PGE800	172.8 ± 35.71 [b]	179.4 ± 24.15[b]

*Data are presented as mean ± standard deviation. NC : Negative Control (untreated rat), PC : Positive Control (LPS-induced rats), PGE50 : ARDS rats model treated with PGE 50 mg/kg BW daily; PGE200 : ARDS rats model treated with PGE 400 mg/kg BW daily; PGE800 : ARDS rats model treated with PGE 800 mg/kg BW daily. Figure 5a and Table 3, the different mark (a,b,c) show the significant difference among treatment on lung IL-6 expression. Figure 5b and Table 3, the different mark (a,b,c,d,e) show the significant difference among treatment on lung NF-kB expression according Tukey Post Hoc Test at $p<0.05$ significance.

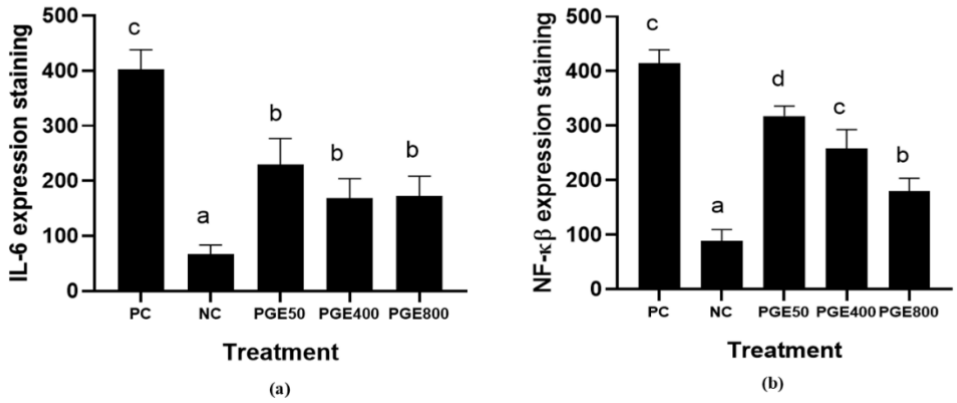

Figure 5. Effect of various PGE doses on IL-6 and NF-kB expression of lung in ARDS rats model
*(a) IL-6 expression staining of lung ARDS rats (b) NF-κβ expression staining of lung ARDS rats
Data are presented as mean ± standard deviation. NC : Negative Control (untreated rat), PC : Positive Control (LPS-induced rats), PGE50 : ARDS rats model treated with PGE 50 mg/kg BW daily; PGE200 : ARDS rats model treated with PGE 400 mg/kg BW daily; PGE800 : ARDS rats model treated with PGE 800 mg/kg BW daily.
Figure 5a. the different mark (a,b,c) shows the difference among treatments on IL-6 expression according Tukey Post Hoc Test at $p<0.05$ significance. Figure 5b. the different mark (a,b,c,d) shows the difference among treatments on NF-kB expression according Tukey Post Hoc Test at $p<0.05$ significance.

3. DISCUSSION

COVİD-19 is a disease caused by the SARS-CoV virus infection. This virus causes damage to cells in the body, thereby increasing pro-inflammatory cytokines. The effect of cell damage causes lung injury, commonly known as ARDS. The LPS induction in rats as an ARDS model was carried out to damage cells in the lungs of rats so that they suffered ARDS. Induction of LPS in animals can cause lung injury, which shows the effects of ARDS such as leukocyte accumulation in lung tissue, pulmonary oedema, severe pulmonary inflammation, and death [22]. LPS is a major constituent of the outer membrane of Gram-negative bacteria [23]. LPS is generally used as an inflammatory starter because it can trigger the production of pro-inflammatory cytokines against pathogens [24]. LPS-induced mice macrophage cells trigger inflammation cells and upregulate various inflammatory markers such as IL-1β, TNF-α, Prostaglandin E2 (PGE2), Cyclooxygenase2 (COX2), inducible Nitric Oxide Synthase (iNOS), and Nitric Oxide (NO) compared to LPS-uninduced macrophage cells [25]. LPS-stimulated microgial cells increased Toll-Like Receptor 4 (TLR4) and NRLP3 gene and protein levels [26]. Overactivation of the NLRP3 inflammasome triggers the abundant secretion of IL-1β and IL-18, induces pyroptosis, and promotes the release of a swathe of proinflammatory proteins, all of which contribute to fibrogenic processes in multiple organs [27]. Based on the research that has been done, LPS induction as a positive control has shown significant increase in pro inflammatory activity compared to the negative control.

Phenolic compounds were analyzed by the LC-MS/MS method. The result showed that PGE contains genistin and daidzin. Various fruits contain isoflavones such as genistin, daidzin, biochanin A, and formononetin [28]. Previous studies have shown that the phenolic compounds in PGE act as antioxidant and anti-inflammation agents [29,30]. Daidzin and genistin have antioxidant properties [19,31]. This previous research was confirmed by decreasing levels of pro-inflammatory cytokines in serum and lung by PGE treatment in an ARDS rats model [32]. Daidzin and genistin 40, 200 µg/mL act as anti-inflammatory by downregulating PGE2, IL-1β, and TNF-α levels in LPS-stimulated macrophage cells [33].

Molecular mechanisms of polyphenol anti-inflammatory activities include inhibition of enzymes associated with proinflammatory properties such as COX2, Lipoxygenase (LOX), and iNOS, inhibition of NF-kB [34]. During inflammation, ROS and RNS production is increased, so it enhances the activity of pro

inflammatory agents [35]. The anti-inflammation activity of phenolic compounds is inhibiting the process of inflammation, which include ROS, and working against pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, iNOS, COX2, and leukotrienes. Moreover, the phenolic compound also regulates the expression of NK-κB [36,37]. Phenolic compound from Moraiolo Virgin Olive Oil have the anti-inflammatory ability to downregulate NLRP3, IL-6, IL-1β, TNF-α gene, and protein level in LPS-microgial cells [26]. Phenolic compound widely found in vegetables, tea, and fruits suppress NRLP3 inflammasome activation and decrease IL-1β and IL-18 in various fibrotic diseases of Sprague-Dawley rats [26]. Isoflavone can downregulate inflammatory cytokine production (IL-6, IL-8, TNF-α, IL-12) in several different immune cell subtypes [38]. Genistin has immunomodulatory effects to inhibit proinflammatory cytokines IL-6, IL-1β, TNF-α, NF-kB in LPS-induced monocytes [39]. A previous study also showed that ARDS causes excessive production of free radicals that cause oxidative stress in the body [40]. Polyphenolic compounds from PGE act as antioxidants to scavenge free radicals and prevent the cell damage.

NLRP3 gene expression decreased with increasing PGE dose. Activation of NRLP3 via the central mediator NF-kB finally activates Caspase 1 (CASP1) leads the release of IL-18 and IL-1β in the inflammation process [41]. The results were simillar to previous studies, which stated that natural products such as phenolic and isoflavones compounds were able to reduce the expression of the NLRP3 [27]. The proposed mechanism of this study is described in Figure 6.

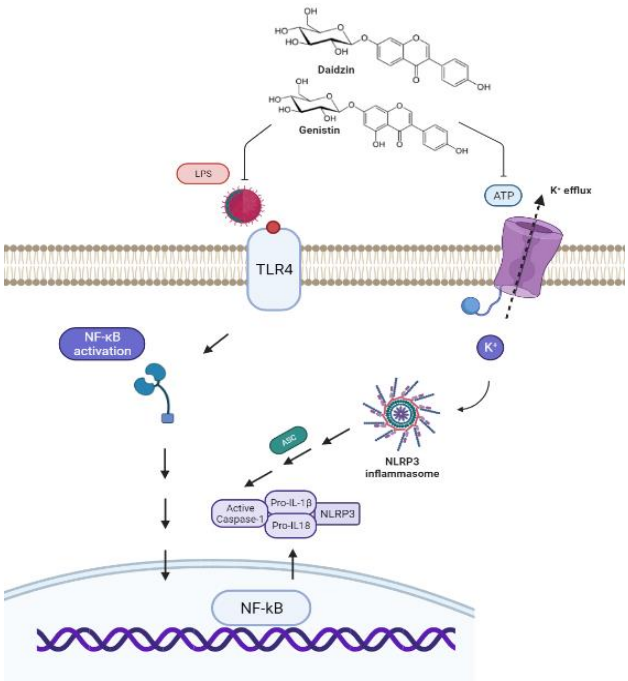

Figure 6. The proposed mechanism of PGE potential in inhibiting ARDS
*The LPS induction in rats caused oxidative stress and the production of TLR4 that leading to enhance the production of pro inflammatory cytokines such as NF-kB, TNF-α, IL-18, IL-1β, IL-6, NF-kB, NLRP3. The phenolic compound in PGE inhibit oxidative stress and inhibit the production of TLR4, thus it lowers the pro-inflammatory cytokines production.

4. CONCLUSION

This *Psidium guajava* L. extract contain phenolic compounds, namely genistin and daidzin, based on LC-MS/MS analysis. The PGE has anti-inflammatory activity by inhibiting the production of IL-18, IL-12, IL-1β, TNF-α, NF-kB, IL-6 and NLRP3 of ARDS rats model. In conclusion, PGE can be used as therapy for treating cytokine storms in the ARDS rats model.

5. MATERIALS AND METHODS

5.1. Ethical approval

The experimental study was carried out under a protocol approved by the Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia (No.: 099/KEP/VII/2020).

5.2. Sample preparation

PGE was processed by Traditional Medicine Industry PT. Fathonah Amanah Shiddiq Tabligh (batch no. 00107201056), Depok, West Java, Indonesia. The maceration process used aquademineral as solvent with lactose as an additional substance, and the extraction process followed Good Manufacturing Practices (GMP) standards set by the National Agency of Drug and Food Control of the Republic of Indonesia. The PGE was analyzed by the LC-MS/MS method to determine the polyphenol compound in the extract. Columns are used with Hypersil Gold specifications (150mm x 2.1mm x 1.9µm). The use of an MS/MS Triple Q (quadrupole) TSQ Quantum Access mass spectrometer with Electrospray Ionization (ESI) ionization source is controlled by TSQ Tune software which is operated with a positive charge [17-18].

5.3. Animals and experimental design

The study was conducted using 25 male Sprague Dawley rats for 8 weeks, weighing 115-135 g. There were 5 treatment groups with 5 rats each, namely negative control (without treatment), positive control (LPS-induced rats as ARDS rat model), PGE100 (rats were given PGE 100 mg/kg BW daily), PGE 400 (rats were given PGE 400 mg/kg BW daily), and PGE 800 (rats were given PGE 100 mg/kg BW daily) for 28 days. After one week of acclimatization, rats were intratracheal injected with LPS (5 µg/mg BW) as an ARDS model. Then the ARDS model rats were treated with various doses of PGE (PGE100, PGE400, and PGE800). On day 29, the rats were euthanized using ketamine (Ikapharmindo Putramas) 100 mg/kg BW and xylazine (361453, Interchemie) 15 mg/kg BW via intraperitoneal injection, and the blood was collected for serum preparation. The lungs were then snap-frozen in liquid nitrogen and stored at -80°C until used. Lungs were stored and dissected for histopathological analysis and immunohisto-chemistry [18-20].

5.4. Quantification of lung IL-18, IL-12, and serum IL-1β, TNF-α

The ELISA kit was used to determine the pro-inflammatory cytokine expression in the lung and serum of ARDS rats model. IL-18 and IL-12 in the lung of ARDS model rats were determined by an ELISA kit (Elabscience, E-EL-R0567, E-EL-R0064). The IL-1β and TNF-α in the serum were determined by an ELISA kit (Elabscience, E-EL-R0012, E-EL-R2856). The sample analysis was according to the protocol of manufacture with a modified method. The absorbance was read triplicate at 450 nm (Multiskan GO, Thermo Scientific) [18,20].

5.5. qRTPCR NRLP3 expression

The total mRNA of the treated cells was extracted using TRI reagent (Zymo, R2050-1-200) from the lung according to the manufacturer's protocol and purified with RNA isolation KIT (Zymo, R2073). Subsequently, iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad, 170-8841) was used to create complementary-DNA using a manufactured protocol. The expression of the gene was analyzed by qRT-PCR (Clever, GTC96S) triplicate with SsoFast Evagreen Supermix (Bio-Rad, 172-5200) [18-21]. The primer sequence (Macrogen) is shown in Table 1. The concentration and purity of RNA are shown in Table 2.

Table 1. The qRT-PCR detail of NLRP3 gene

Gene	Primer Sequence	Product Size	Annealing	Cycle	Reference
Rat NLRP3	5'-CAGATGCTGGAGTTAGACAACTG-3' 5'-TTCAGAACCTCACAGAGCGT-3'	153	57	40	NCBI Reference Sequence: NM_001191642.1
Rat GAPDH	5′-TCAAGATGGTGAAGCAG-3′ 5′-ATGTAGGCCATGAGGTCCAC-3′	217	57	40	NCBI Reference Sequence: NM_001289726

Table 2. Concentration and purity of RNA

Treatment	RNA concentration (ng/ml)	RNA purity (λ260/λ280 nm)
PC	228.64	2.2636
NC	260.48	2.1947
PGE50	75.12	2.2704
PGE400	119.64	2.0479
PGE800	81.64	2.0907

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Author contributions: Study conception or design – D.P., W.W.; Experimental research – A.N., R.R.; Data analysis and draft manuscript preparation – H.S.W.K., V.A.Y.; Final approval of the version to be published (the names of all authors must be listed) – D.P., W.W., A.N., H.S.W.K., V.A.Y., R.R.

Conflict of interest statement: None.

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