

## Green Tea Extract Reduced Lipopolysaccharide-Induced Inflammation in L2 Cells as Acute Respiratory Distress Syndrome Model Through Genes and Cytokine Pro-Inflammatory

Didik Priyandoko <sup>1</sup>, Wahyu Widowati <sup>2\*</sup>, Lenny Lenny <sup>3</sup>, Sintya Novianti <sup>3</sup>, Revika Revika <sup>3</sup>, Hanna Sari Widya Kusuma <sup>4</sup> and Ika Adhani Sholihah <sup>4,5</sup>

1. Biology Study Program, Faculty of Mathematics and Natural Sciences, Indonesia University of Education, Bandung 40154, Indonesia

2. Faculty of Medicine, Maranatha Christian University, Bandung 40164, Indonesia

3. Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, BSD Campus, Tangerang 15345, Indonesia

4. Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung 40163, Indonesia

5. School of Life Sciences and Technology, Bandung Institute of Technology, Bandung 40132, Indonesia

### Abstract

**Background:** Acute Respiratory Distress Syndrome (ARDS) is a severe lung inflammatory condition that has the capacity to impair gas exchange and lead to hypoxemia. This condition is found to have been one of the most prevalent in patients of COVID-19 with a more serious condition. Green tea (*Camellia sinensis* L.) contains polyphenols that possess many health benefits. The purpose of this study was to assess the anti-inflammatory activities of green tea extract in Lipopolysaccharide (LPS)-induced lung cells as ARDS cells model.

**Methods:** In this study, rat lung cells (L2) were induced by LPS to mimic the inflammation observed in ARDS and later treated with green tea extract. Pro-inflammatory cytokines such as Interleukin (IL)-12, C-Reactive Protein (CRP) as well as Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) were investigated using the ELISA method. Gene expression of NOD-Like Receptor Protein 3 (*NLRP-3*), Receptor for Advanced Glycation End-product (RAGE), Toll-like Receptor-4 (*TLR-4*), and Nuclear Factor-kappa B (*NF- $\kappa$ B*) were evaluated by qRT-PCR. Apoptotic cells were measured using flow cytometry.

**Results:** The results showed that green tea extract treatment can reduce inflammation by suppressing gene expressions of *NF- $\kappa$ B*, *NLRP-3*, *TLR-4*, and *RAGE*, as well as pro-inflammatory cytokines such as IL-12, TNF- $\alpha$ , and CRP, an acute phase protein. Apoptosis levels of inflamed cells also found to be lowered when green tea extract was administered; thus, also increasing live cells compared to non-treated cells.

**Conclusion:** These findings could lead to the future development of supplements from green tea to help alleviate ARDS symptoms, especially during critical moments such as the current pandemic.

**Keyword:** Acute respiratory distress syndrome, *Camellia sinensis*, Cytokines, Inflammation, Tea

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### Introduction

Systemic inflammation of the lungs of the body causes Acute Respiratory Distress Syndrome (ARDS). ARDS is often associated with diseases such as traumatic brain injury, burn injury, immune system systemic suppression, and bacterial or viral infections, as recently found out in the case of SARS-CoV-2 <sup>1-3</sup>. ARDS is characterized by very poor oxygenation, reduced pulmonary infiltrates, and bilateral radiographic

infiltrates <sup>4</sup>. Various studies have shown that the death of patients with ARDS is common <sup>5</sup>. Previous studies have shown that there are similarities between the pathological anatomy of patients who died from COVID-19 and ARDS. The pathological anatomy of ARDS can aid in the understanding and treatment of COVID-19 patients <sup>6</sup>.

ARDS is usually caused and worsened by a phe-

nomenon known as the Cytokine Storm Syndrome (CS). CS is a phenomenon in which the immune cells, as a direct response to injury or pathogens, release a huge amount of pro-inflammatory cytokine and chemokine like Interleukin (IL)-6, IL-12, IL-1 $\beta$ , and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) in an attempt to attract other immune cells to migrate to the site of the injury<sup>5,7,8</sup>. Moreover, C-Reactive Protein (CRP), which is also released in reaction to inflammation and primarily functions to aid in opsonization as well as the release of other pro-inflammatory cytokines, is also produced<sup>9</sup>. The genes that are responsible for producing and playing a role in its cascade in the inflammatory pathways are also diverse. These inflammatory cytokines would then be recognized by receptors which are regulated by genes such as Toll-Like Receptor-4 (*TLR-4*) and Receptor for Advanced Glycation End products (*RAGE*)<sup>10</sup>. The gene that is also responsible for the production of cytokines would include Nod-Like Receptors-3 (*NLRP-3*) which also plays a role in the pathway of "Nuclear Factor-kappa B (*NF-kB*)"<sup>11,12</sup>. Accordingly immune cells, whether innate or adaptive, will migrate and destroy the inflammation trigger and protect infected or injured cells<sup>13,14</sup>. At an appropriate amount, these immune cells will help to cure, but when there is too much destruction our body will not be able to handle it. Especially in the case of ARDS where the destruction happens in one of the most critical tissues in the body, the alveoli which help with gas exchange. Once the epithelial cells are destroyed, leakage of high-protein fluids will lead to edema. Over time, this edema can cause hypoxia due to its impact on gas exchange<sup>1,3</sup>.

Treatment for patients usually include the usage of low tidal mechanical ventilators in hospitals. However, other than synthetic drugs such as simvastatin, and in the case of COVID-19, methylprednisolone and dexamethasone, an alternative cure has yet to be found<sup>15,16</sup>. The problem with synthetic drugs is that they often cause negative side consequences. Non-Steroidal. Anti-Inflammatory Drugs (NSAIDs), generally also used to cure inflammations, are known to increase the risk of gastrointestinal illnesses like dyspepsia and esophagitis<sup>17,18</sup>. Various plants and herbs have been investigated as the source of anti-inflammatory agents. Tea (*Camellia sinensis* L.) is a renowned plant that contains large amounts of polyphenols and can act as an anti-inflammatory agent. Tea contains several types of compound groups such as phenolics, alkaloids, polysaccharides, and saponins<sup>19</sup>. The main polyphenol compound in green tea that is thought to help with its anti-inflammation activities are flavonoids such as quercetin and myricetin. A previous study has also detected 8-prenylaringenin (8-PN) and 6-prenylaringenin (6-PN) in the Green Tea Extract (GTE) used<sup>17,20</sup>.

The aim of this study was to evaluate the effect of GTE on Lipopolysaccharide (LPS)-induced lung cells as ARDS cells model by measuring gene expression of

*NF-kB*, *NLRP-3*, *RAGE*, and *TLR-4* by RT-PCR and examination of proinflammatory cytokine such as cytokines TNF- $\alpha$ , CRP, and IL-12 using sandwich Enzyme-Linked Immunosorbent Assay (ELISA). Flow cytometry was utilized to examine the apoptotic activity of the cells to determine their condition.

## Materials and Methods

### Green tea extract preparation

The GTE was processed by PT. Fathonah Amanah Shidiq Tabligh (Depok, Indonesia) (Batch No. 00107201057) and the process based on Good Manufacturing Practice (GMP) standard by The Indonesian Food and Drug Authority (BPOM). The extraction process of GTE used 70% ethanol as its solvent. Before GTE was stored at 25 $\pm$ 2  $^{\circ}$ C, lactose was added to GTE first for getting the powder form.

### L2 cells-LPS induced as ARDS cells model

Rat lung (L2) cells (ATCC $\text{\textcircled{R}}$  CCL-149<sup>TM</sup>) was obtained from the Biomolecular and Biomedical Research Center, Aretha Medika Utama which is located in Bandung, West Java, Indonesia. T75 flask with a complete medium was used for cell growth until confluent. The complete medium composition was Dulbecco's Modified Eagle's Medium (DMEM) High-glucose (Biowest, L1003-500), 10% Fetal Bovine Serum (FBS) (Biowest, S1810-5000), 1% Antibiotic-antimycotic (ABAM) 100 $\times$  (Biowest, L0009), and 0.1% Gentamicin (Gibco, 15750078). Confluent cells were induced with 4  $\mu$ g/ml LPS *Escherichia coli* O55:B5 (Sigma-Aldrich L2880) to produce an inflammation model and incubated for 18 hr at 37 $^{\circ}$ C in a 5% CO<sub>2</sub> incubator. Various concentrations of GTE (1.56, 3.13, and 6.25  $\mu$ g/ml) were applied to the cells and incubated for 24 hr in a 5% CO<sub>2</sub> incubator (37 $^{\circ}$ C). The levels of IL-6, CRP and TNF- $\alpha$  in the harvested supernatant cells were measured using the ELISA method, while the cell pellets were collected for RNA isolation to be used for testing *TLR-4*, *RAGE*, *NF-kB*, and *NLRP-3* gene expression using qRT-PCR<sup>8</sup>.

### Quantification of TNF- $\alpha$ , CRP, and IL-12 levels in the L2 cells using ELISA

The supernatant cells were used to determine the levels of pro-inflammatory cytokine expression in the L2 cells, such as TNF- $\alpha$ , CRP, and IL-12. The TNF- $\alpha$ , CRP, and IL-12 levels were determined by the ELISA kit (Elabscience E-EL-R0019, E-EL-R0506, and E-EL-R0064, respectively). The process was carried out according to the manufacturer's guidelines. The sample absorbances were tested at a wavelength of 450 nm<sup>8</sup>.

### Quantification of NF-kB, NLRP-3, RAGE, and TLR-4 gene expression in the L2 cells using qRT-PCR

The levels of *NF-kB*, *NLRP-3*, *RAGE*, and *TLR-4* gene expression were determined from pellet cells. Direct-zol RNA Miniprep Plus Kit (Zymo, R2073) was applied to isolate total RNA, the procedure was according to the protocol of manufacture. Then, the Sensi-

FAST cDNA synthesis kit was used to synthesize complementary DNA and the procedure was according to the protocol of manufacture. AriaMx 3000 Real-Time PCR System (Agilent) was used to measure quantitative gene expression, primer sequence (Table 1), and Evagreen master mix (Bio-Rad, 1725200) for the reaction mixture. The procedure was undertaken based on the manufacturer's protocol <sup>21</sup>. Real-time PCR was run for 40 cycles using *GAPDH* as reference gene, and the annealing temperatures was 54°C, 56°C, and 57°C for *TLR-4*, *RAGE*, and *NF-κB*, *NLRP-3*, respectively.

**Apoptosis assay using flow cytometry analysis**

Annexin V-FITC/PI Apoptosis Detection Kit (Elabsci, E-CK-A211) was utilized to assess the cells apoptosis in line with the manufacturer's instructions, with slight modification. Cells incubated for 2 hr on a 6-well plate (n=500,000). After 4 days, the growth medium was removed, harvested and centrifuged at 1,600 rpm for 5 min, and then 500 μl FACS buffer added on pellet and centrifuged. Additionally, annexin and Propidium Iodide (PI) staining was performed on the pellets after the addition of 100 μl FACS buffer. MACS-Quant Analyzer. 10 Flow Cytometer (Miltenyi, Bergisch Gladbach, Germany) was used to evaluate the stained cells that have been incubated for 1 hr <sup>22</sup>.

**Statistical analysis**

The data analysis was conducted using SPSS 26.0 software, and experimental results are shown as mean±SD. The comparison between groups was assessed using a one-way ANOVA and the post hoc test was Dunnett's test and Tukey HSD test with p<0.05. Analysis using Kruskal-Wallis and Mann-Whitney. post hoc with p<0.05 was used for non-parametric.

**Results**

**The influence of GTE on TNF-α, CRP, and IL-12 levels**

Pro-inflammatory cytokine was increased by LPS induction. Figure 1 shows the effect of GTE toward IL-12, TNF-α, and CRP levels. Compared to the negative control, the level of IL-12, TNF-α, and CRP increased significantly in positive control (p<0.05). Treatment

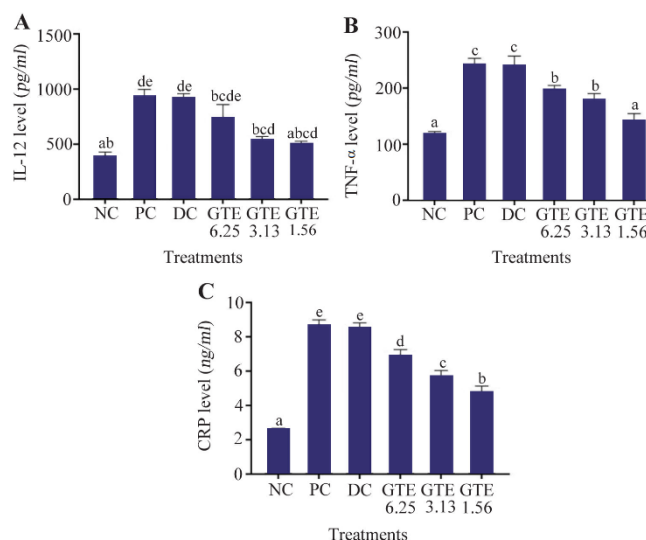


Figure 1. Effect of GTE toward IL-12, TNF-α, CRP on LPS-induced L2 cells. A) IL-12 level (pg/ml) on LPS-induced L2 cells, B) TNF-α level (pg/ml) on LPS-induced L2 cells, C) CRP level (ng/ml) on LPS-induced L2 cells.

\* The data is presented as means value ± standard deviation. NC: untreated cell, PC: LPS-induced cell, DC: PC+DMSO1%, GTE 6.25: PC + GTE 6.25 μg/ml, GTE 3.13: PC + GTE 3.13 μg/ml, GTE 1.65: PC + GTE 1.65 μg/ml. A different mark (alphabetical) indicates a significant difference among treatments toward CRP level based on Kruskal-Wallis and Mann-Whitney post hoc test (p<0.05). A different letter (alphabetical) indicates a significant difference among treatments based on Anova and Tukey HSD post hoc test for TNF-α level and Dunnett T3 post hoc for IL-12 level at Anova and Tukey HSD post hoc test p<0.05.

with GTE on L2 cells reduced pro-inflammatory cytokine levels compared to positive control. The results showed that GTE reduced CRP and TNF-α levels significantly in the rat lung cell at concentration of 1.56 μg/ml (p<0.05). Meanwhile, the IL-12 level did not show any significant reduction in all concentration of GTE.

**Effect of GTE toward TLR-4, RAGE, NF-κB, and NLRP-3 genes expression**

LPS-induced L2 cells significantly increased *TLR-4*, *RAGE*, *NF-κB*, and *NLRP-3* genes expression (p<0.05).

Table 1. Primer sequence

Gene	Primer sequence (5'-3')	Product length (bp)	Reference
	Forward/Reverse		
<i>TLR-4</i>	AGTGAGAATGCTAAGGTTGG	289	NCBI Reference Sequence: NM_019178.2
	ATTAGGAAGTACCTCTATGCAG		
<i>RAGE</i>	CGAGTCTACCAGATTCCTG	163	NCBI Reference Sequence: NM_053336.2
	CTTTGCCATCAGGAATCAG		
<i>NF-κB</i>	GGACTATGACTTGAATGCGG	230	NCBI Reference Sequence: NM_199267.2
	ACACCTCAATGTCTTCTTCTG		
<i>NLRP-3</i>	CAGATGCTGGAGTTAGACAACCTG	153	NCBI Reference Sequence: NM_001191642.1
	TTCAGAACCCTCACAGAGCGT		
<i>GAPDH</i>	TCAAGATGGTGAAGCAG	217	NCBI Reference Sequence: NM_001289726.1
	ATGTAGGCCATGAGGTCCAC		

As shown in figure 2, GTE treatments downregulated the expression of *TLR-4*, *RAGE*, *NF- $\kappa$ B*, and *NLRP-3* genes expression ( $p < 0.05$ ). The results showed that *TLR-4*, *RAGE*, and *NLRP-3* genes expression were significantly decreased with GTE treatment at the concentration of 1.56  $\mu\text{g/ml}$  ( $p < 0.05$ ). In contrast, the 6.25  $\mu\text{g/ml}$  GTE showed the most effective concentration in suppressing *NF- $\kappa$ B* gene expression but did not show any significant difference with the positive control.

#### Effect of GTE toward cells apoptosis

The effect of GTE was analyzed by flow cytometry in triplicate using concentrations of 6.25, 3.13, and 1.56  $\mu\text{g/ml}$  respectively. Figure 3 shows that LPS induction increased the number of cells in necrosis, early, and late apoptosis, and also reduces the number of live cells quantity compared to the negative control. The dot blot of the effect of GTE on cell apoptosis can be seen in figure 4. The number of necrosis, early and late apoptosis were decreased following GTE treatment, while the number of viable cells increased.

According to figure 4, the GTE treatment increased the percentage of live cells compared to the positive control. Moreover, depending on the concentration, treatments with GTE decreased the percentage of necrosis cells with concentration dependent manner, and GTE treatment reduced the percentage of apoptotic cells. The highest GTE concentration (6.25  $\mu\text{g/ml}$ ) had the smallest apoptosis percentage compared to other GTE treatments with the apoptosis percentage of 10.34%.

### Discussion

One of the life-threatening illnesses observed in severely ill COVID-19 patients is ARDS. ARDS, just

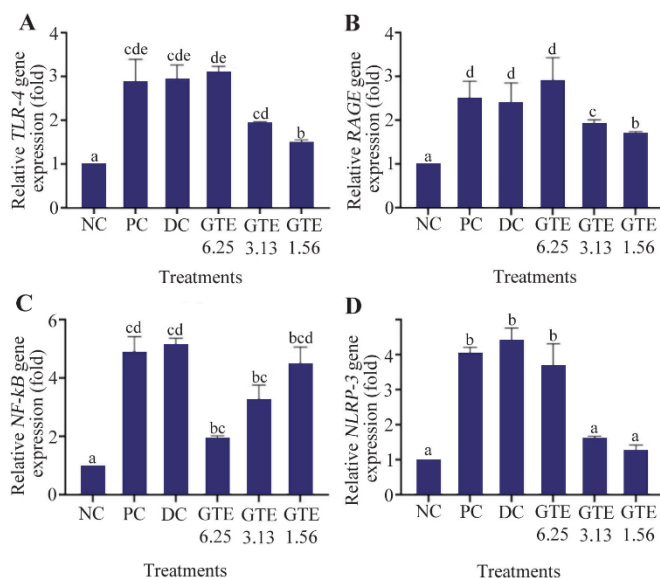


Figure 2. Effect of GTE toward *TLR-4*, *RAGE*, *NF- $\kappa$ B*, and *NLRP-3* gene expression on LPS-induced L2 cells. A) *TLR-4* gene expression on LPS-induced L2 cells, B) *RAGE* gene expression on LPS-induced L2 cells, C) *NF- $\kappa$ B* gene expression on LPS-induced L2 cells, D) *NLRP-3* gene expression on LPS-induced L2-cells.

\* The data is presented as means value  $\pm$  standard deviation. NC: untreated cell, PC: LPS-induced cell, DC: PC+DMSO 1%, GTE 6.25: PC + GTE 6.25  $\mu\text{g/ml}$ , GTE 3.13: PC + GTE 3.13  $\mu\text{g/ml}$ , GTE 1.65: PC + GTE 1.65  $\mu\text{g/ml}$ . A different mark (alphabetical) indicates a significant difference among treatments toward *RAGE* gene expression, based on Kruskal-Wallis and Mann-Whitney post hoc test ( $p < 0.05$ ). A different letter (alphabetical) indicates a significant difference among treatments based Anova and Tukey HSD post hoc test for *NLRP-3* gene expression and based Anova and Dunnett T3 post hoc test for *TLR-4* and *NF- $\kappa$ B* gene expression ( $p < 0.05$ ).

like any other inflammation can be induced by different agents such as protein in viruses or LPS of bacteria.

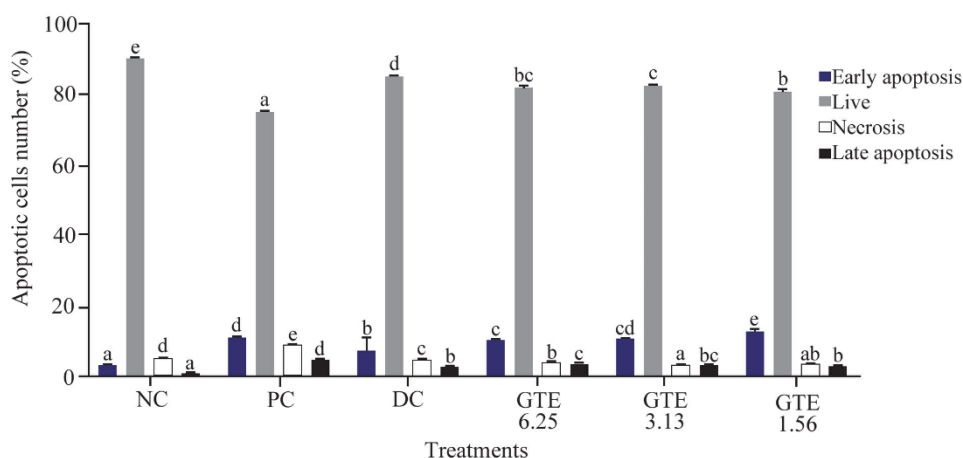


Figure 3. Effect of GTE toward early apoptosis, live cells, necrosis cells, late apoptosis on LPS-induced L2 cells.

\* The data is presented as means value  $\pm$  standard deviation. NC: untreated cell, PC: LPS-induced cell, DC: PC+DMSO 1%, GTE 6.25: PC + GTE 6.25  $\mu\text{g/ml}$ , GTE 3.13: PC + GTE 3.13  $\mu\text{g/ml}$ , GTE 1.65: PC + GTE 1.65  $\mu\text{g/ml}$ . The number of early apoptosis, live, necrosis, and late apoptosis cells was measured in triplicate for each sample. A) Early apoptosis on LPS-induced L2 cells, B) Live cells on LPS-induced L2 cells, C) Necrosis cells on LPS-induced L2 cells, D) Late apoptosis on LPS-induced L2 cells. The data is presented as means value  $\pm$  standard deviation. NC: untreated cell, PC: LPS-induced cell, DC: PC+DMSO 1%, GTE 6.25: PC + GTE 6.25  $\mu\text{g/ml}$ , GTE 3.13: PC + GTE 3.13  $\mu\text{g/ml}$ , GTE 1.65: PC + GTE 1.65  $\mu\text{g/ml}$ . A different mark (alphabetical) indicates a significant difference among treatments toward early apoptosis, live cells, necrosis cells, late apoptosis based on Anova and Tukey HSD post hoc test ( $p < 0.05$ ).



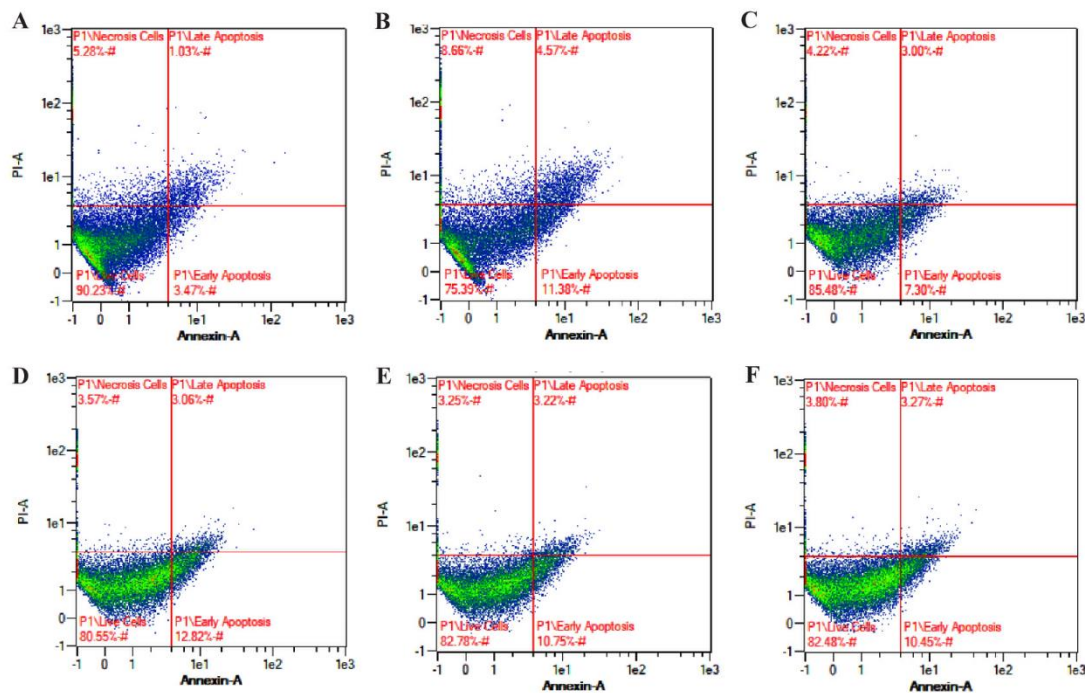


Figure 4. The representative dot blots of different GTE concentrations on LPS-induced L2 cells toward percentage of apoptosis by flow cytometry. \* A) Negative control (untreated cell): live cells 90,23%, early apoptosis 3.47%, late apoptosis 1.03%, necrosis cells 5.28%. B) Positive control (LPS-treated cells): live cells 75.39%, early apoptosis 11.38%, late apoptosis 4.75%, necrosis cells 8.66%. C) DMSO control (untreated cells+DMSO): live cells 85.48%, early apoptosis 7.30%, late apoptosis 3.00%, necrosis 4.22%. D) GTE 1.56  $\mu\text{g/ml}$ : live cells 80.55%, early apoptosis 12.82%, late apoptosis 3.06%, necrosis cells 3.57%. E) GTE 3.13  $\mu\text{g/ml}$ : live cells 82.78%, early apoptosis 10.75%, late apoptosis 3.22%, necrosis cells 3.25%. F) GTE 6.25  $\mu\text{g/ml}$ : live cells 82.48%, early apoptosis 10.45%, late apoptosis 3.27%, necrosis cells 3.08%.

LPS administration in both *in vivo* and *in vitro* leads to inflammation and causes the release of inflammatory cytokines<sup>23,24</sup>. LPS induction led to an increase in CRP levels as well as pro-inflammatory cytokines as TNF- $\alpha$  and IL-12, as shown in figure 1. This is also proven by a previous study, who also found that LPS induction releases uncontrolled cytokines that could lead to lung injury<sup>25</sup>.

In order to compare the cytokine or expression levels of inflamed cells with those of healthy cells, negative controls were required for all assays in which LPS or the extract was not added to the cells. Additionally, both positive and DMSO controls were established. To confirm that the solvent for the GTE had no significant effect on the inflamed cells, DMSO was used as a control. This would allow us to conclude that the difference in results between treated and untreated cells was due to the GTE treatment and not the solvent used.

In the absence of a stimulus, healthy cells should have lower levels of pro-inflammatory cytokines and expression of genes related to inflammation than inflamed cells<sup>26</sup>. When pro-inflammatory triggers such as LPS are identified, pro-inflammatory cytokines begin to be produced. In both animal models and patient studies, the *TLR-4* has been shown to be a receptor for the response to LPS<sup>27</sup>. *RAGE* receptors, receptors highly expressed in skin and lung tissue, are also

involved in the LPS response. *RAGE* expression can be increased in the presence of inflammatory conditions, aging, and at the time of injury. *RAGE* and *TLRs* are crucial components of the natural immune system; as these compounds can interact with various microbial products and molecules (one of them is LPS) in inflammation and tissue injury<sup>28</sup>. The *NF- $\kappa$ B* signaling pathway can be activated by either *TLR-4* or *RAGE* receptors. Activated *NF- $\kappa$ B* controls the synthesis of proinflammatory cytokines and chemokines during inflammation<sup>29</sup>. Previous research has shown that *NF- $\kappa$ B* signaling is involved in activating *NLRP-3*<sup>30</sup>. An immunological complex called the *NLRP-3* inflammasome is made up of *NLRP-3*, an apoptosis-associated protein with a pro-caspase-1 and a C-terminal caspase recruitment domain (ASC)<sup>31</sup>. *NLRP-3* also recognizes Reactive Oxygen Species (ROS) and Damage-Associated Molecular Patterns (*DAMPs*) in inflammation cells (LPS-induced cells)<sup>32</sup>.

Figures 1-4 show that DMSO controls had insignificant levels of pro-inflammatory cytokines compared to positive controls. Based on the results of DMSO control for the gene expression, levels of *TLR4*, *RAGE*, *NF- $\kappa$ B*, and *NLRP3* were comparable with figure 3; where the DMSO control had insignificant results with positive control. Thus, it could be assumed that all the results from treatments are purely based on the

treatment of GTE.

Green tea contains many beneficial polyphenols such as catechins, quercetin, myricetin, and kaempferol<sup>33</sup>. These polyphenols have made green tea a well-known beverage with many therapeutic benefits and are often used in many traditional therapies. Additionally, green tea has been reported to have antioxidant and anti-inflammatory properties that can help decrease inflammation by lowering inflammatory markers. Its antioxidant activity helps to scavenge ROS which can lead to the suppression of *NF-κB* activity<sup>34,35</sup>. Figure 2 shows that there was an increase in the expression of *TLR-4*, *RAGE*, *NF-κB*, and *NLRP-3* genes. LPS induction on human dental pulp stem cells upregulated *TLR-4* gene expression<sup>36</sup>. The findings of this study demonstrate that *TLR-4* is a particular receptor for inducing LPS in cells<sup>37</sup>. In addition, in this study, the increase of *TLR-4* gene expression was in line with *NF-κB* gene expression in response to LPS. These results are consistent with previous studies which LPS induction increased *NF-κB* gene expression<sup>37</sup>. Moreover, LPS induction also increases the *NLRP-3* expression on RAW 264.7 cells and is in line with the *NF-κB* expression<sup>38</sup>. The results of this research indicate that GTE down-regulated the expression of *TLR-4*, *RAGE*, *NF-κB*, and *NLRP-3* mRNA levels in LPS-induced L2 cells as ARDS cells model.

The findings in figure 2 concur with those of the previous investigations. From figure 2, it can be inferred that GTE has the ability to suppress the mRNA expression of *TLR-4*, *RAGE*, *NF-κB*, and *NLRP-3* in the LPS-induced L2 cells as ARDS cells model. The expression of proinflammatory cytokines including TNF-α and IL-12 was also decreased by reducing *NF-κB* and other gene expressions (Figure 2).

Besides *NF-κB*, green tea catechin also inhibits the pro-inflammatory mediators' secretion through *NLRP-3* inflammasome, a multiprotein complex that activates inflammatory responses<sup>39</sup>. In prior research, treatment with quercetin, one of the components in green tea, was shown to be able to decrease both the mRNA level of *NLRP-3* and the amount of the pro-inflammatory cytokine<sup>40</sup>. Epigallocatechin-3-gallates (EGCG), in particular, was discovered in a different study to have the ability to suppress and lower the expression of *TLR-4* and *RAGE* following a pre-treatment of EGCG in rat LPS-induced inflammation<sup>41-43</sup>. Inhibiting those gene expressions also resulted in a reduction of pro-inflammatory cytokines TNF-α and IL-12<sup>43,44</sup>.

Precisely in this study, GTE treatment at all three concentrations significantly reduced TNF-α levels as compared to positive controls. These matched previous findings of GTE and their ability to lower inflammation by modulating pro-inflammatory cytokines like TNF-α, is especially due to the presence of flavonoids such as myricetin and quercetin<sup>45</sup>. Treatment with 1.56 μg/ml GTE showed the most active to lower TNF-α

level compared to other GTE treatments and significantly decreased TNF-α level compared to positive control. Moreover, GTE 1.56 μg/ml also decreased IL-12 level significantly compared to positive control.

The CRP levels were also found to be highly associated to immunological alterations as a marked rise in pro-inflammatory biomarkers<sup>46</sup>. Although CRP is mostly produced in the liver, its mRNA has been found in respiratory tract epithelial cells and T-lymphocytes<sup>47</sup>. The cytokine storm in ARDS induces macrophage activation syndrome and results in a pro-inflammatory hypercytokinemia profile, which increases CRP generation by hepatocytes<sup>48,49</sup>.

Based on the results shown in figure 2C, administration of GTE showed a significantly reduced level of CRP. The decreased level of CRP is in correlation with IL-6 and TNF-α levels. CRP, an acute phase protein, was induced by both IL-6 and TNF-α in inflammation. It was shown that by decreasing these inflammatory cytokines, the level of CRP was reduced<sup>9</sup>. As discussed previously, GTE reduced the level of inflammatory mediators in an inflammation cells model. The most effective result was seen in 1.56 μg/ml GTE for all parameters, except for the expression of *NF-κB* in which 6.25 μg/ml GTE was the most active to decrease *NF-κB* gene expression. Therefore, further research is required to establish the excellent concentration of GTE for its effectiveness in treating ARDS.

One other marker of inflammation is apoptosis or programmed cell death<sup>50</sup>. Apoptosis is very crucial when it comes to the host defense system as it would allow the damaged or infected cells to die and end further damage inside the host<sup>50</sup>. There are several ways apoptosis can be activated and in inflammation, apoptosis could happen due to both internal aspects such as mitochondrial dysfunction and stress, along with external factors including induction by LPS<sup>50,51</sup>, both of which activate caspase to start the apoptosis<sup>52</sup>. This is why in most cases of inflammation the apoptosis level of cells is usually increased<sup>51</sup>.

In case of ARDS, the death of a large number of cells could also lead to the destruction of vital tissues, causing what would ideally be a preventative process of a deadly damage rapidly<sup>50,51,53</sup>. Apoptosis suppression has played a crucial role in alleviating inflammation symptoms. The result of the apoptosis assay by flow cytometry indicated that all three-concentrations managed to decrease apoptosis levels of inflamed cells as could be seen in the significant difference of live cells compared to the positive controls. The results of the study were consistent with previous study<sup>54</sup> which found that quercetin, one of the constituents of green tea can suppress apoptosis in LPS-induced inflammation. Moreover, all GTE concentration treatments showed significantly reduced cell death, early and late apoptosis levels.

### Conclusion

In this study, it is proven that GTE can help reduce the inflammation by modulation of TNF- $\alpha$ , CRP and IL-12 pro-inflammatory marker as well as suppressing inflammation-related genes expression including *TLR-4*, *RAGE*, *NF- $\kappa$ B*, and *NLRP-3*. Furthermore, GTE was able to reduce the apoptosis in inflamed cells.

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### Conflict of Interest

The authors declare that they have no competing interest.

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