Luteolin Possess Anti-inflammatory Effect on LPS Induced RAW 264,7 Cell Lines

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Keywords: Luteolin, Anti-inflammatory, PGE-2, TNF-α and IL-1β.

Abstract: BACKGROUND: Inflammation is a natural human reaction to potentially harmful effects such as tissue stress, trauma, and microbial infection. Extended inflammation is believed related to several chronic conditions, involving asthma, rheumatoid arthritis and even cancer. To avoid immune cells from causing more tissue damage, inflammatory responses must be regulated. Anti-inflammatory agents are particularly beneficial for these purposes. Luteolin is flavonoid and has potent anti-inflammatory effects. OBJECTIVE: The study aimed to determine anti-inflammatory effect of luteolin on LPS induced RAW 264,7 cell lines. METHOD: The MTS assay was used to determine the viability of cells and the nontoxic concentration of cell lines. The anti-inflammatory activity was assessed with Elisa assay of inflammatory parameters including PGE-2, TNF-α, and IL-1β using secreted cytokine levels in culture supernatants of RAW 264,7 cell line. RESULT: The toxic concentration of luteolin was 100 µM/mL, so that the concentration was not used for treatment. Concentrations of 4 and 20 µM/mL demonstrated high viability (>90%), they were suitable for treatment. Luteolin 4 µM/mL significantly increased the inhibition of inflammatory cytokines PGE-2, TNF-α and IL-1β compared to positive control. CONCLUSION: The research reported that Luteolin possesses the anti-inflammatory effect indicated by properties of inflammatory inhibition toward PGE-2, TNF-α and IL-1β.

1 INTRODUCTION

Inflammation is a critical biological reaction to damage that is linked to a variety of disorders including, inflammatory bowel disease, rheumatoid arthritis, Alzheimer's disease, cancer and atherosclerosis (Laksmitawati et al., 2016)

The cell line model for inflammation using macrophage cell (RAW 264.7) which is triggered by exposure to interferon (IFN), pro-inflammatory

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Afifah, E., Tiono, H., Onggowidjaja, P., Obeng, S., Widowati, W., Wahyuni, C., Wijayanti, C., Maulana, M., Handayani, T. and Rizal, R.
Luteolin Possess Anti-inflammatory Effect on LPS Induced RAW 264,7 Cell Lines.
DOI: 10.5220/0010744500003113
In *Proceedings of the 1st International Conference on Emerging Issues in Technology, Engineering and Science (ICE-TES 2021)*, pages 74-80
ISBN: 978-989-758-601-9

cytokines, or bacterial lipopolysaccharide (LPS) (Duque & Descoteaux, 2014; Laksmitawati et al., 2016). LPS has been shown to enhance cytokine activity as an inflammatory mediator (Widowati et al., 2016; 2019; 2021). LPS contains pro-inflammatory glycolipids, which make up the gram negative bacterial cell wall (Boots et al., 2011). LPS-activated macrophages and inflammatory processes are good candidate anti-inflammatory drug development (Laksmitawati et al., 2016; 2017; Novilla et al., 2017; Widowati et al., 2016; 2019; 2021)

Activated macrophages produce inflammatory mediators and pro-inflammatory cytokines including interleukin (IL-1β) and tumor necrosis factor alpha (TNF-α) (Duque & Descoteaux, 2014; Widowati et al., 2016; 2019; 2021). TNF-α activates and controls the inflammatory mechanism at the multicellular level through the production of pro-inflammatory cytokines such as IL-1β and IL-6.(Wang & Tan, 2015). Prolonged inflammation can result in the overproduction of inflammatory mediators and cytokines, which can cause cellular and tissue disruption (Lee and Surh, 2012). Furthermore, IL-1β and TNF-α can trigger transcriptional factors of NFKB. Elevated NFKB activation is linked to increased COX-2 levels, which provide a significant role in the synthesis of prostaglandin E2 (PGE-2). PGE-2 upregulation can result in acute inflammatory and contribute in tumorigenesis (Bustami et al., 2020; Widowati et al;, 2021).

Anti-inflammatory medications are essential to treat the risk of persistent inflammation associated with chronic illness. Over several years, natural phytochemicals were used therapeutically, leading to the development of anti-inflammatory medication including non-steroid anti-inflammatory drugs (NSAIDs) (Laksmitawati et al., 2016; 2017; Novilla et al., 2017; Widowati et al., 2016; 2019; 2021; Girsang et al, 2019)

Plant extracts contain bioactive compounds, the majority of which have been shown to be free of side effects (Mehta et al., 2010). These chemical compounds are often used to treat inflammation. Flavonoids, which are present in plants, have a high anti-inflammatory ability (Novilla et al., 2017).

Luteolin (3',4',5,7-tetrahydroxyflavone) is a flavonoid that is commonly found in edible tropical fruits such as belimbii and pineapple (Asif et al, 2013; Vrianty et al., 2019).

The aim of this study is to determine the antiinflammatory ability of luteolin by measuring TNF-α, IL-1β, and PGE-2 levels in LPS-induced murine macrophage cell line (RAW 264.7) model.

2 METHODS

2.1 RAW 264.7 Cells Culture

Mouse macrophage cell line (RAW264.7) (ATCC®TIB-71T^{M}) was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center Bandung. The macrophage cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0416-500) enriched with 10% Fetal Bovine serum (FBS) (Biowest, S1810-500) 1% Antibiotic/antimycotic (ABAM) (Biowest, L0010100), 1% Nanomycopulitine (Biowest, L-X16-100), 1% Amphotericin B (Gibco, 1%), 0.1% Gentamicin (Gibco, 15750045). The cells were incubated at 37^{o}C in a humidified atmospheric incubator of 5% CO_2 until they reached confluence. The cells were drained, harvested with Trypsin-EDTA (Biowest, L0931-500), and centrifuged for 4 minutes at 2500 rpm (Laksmitawati et al., 2016; 2017; Novilla et al., 2017; Widowati et al., 2019; 2021).

2.2 Viability Assay

The viability assay was carried out to assess the non - toxic concentration for the following assay, which was tested using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) assay. In brief, 5 x10^3 cells per well were cultured to 96-well plates in DMEM combined with 1% pennicilin-streptomcycin 10% FBS and incubated at 37°c for 24 hours in a humidified atmosphere incubator with 5% CO_2. The medium was then washed and 180 µL of fresh medium and 20 µL of luteolin in various concentrations were applied in triplicate to the plate, which was then incubated for 24 hours. The untreated cells acted as the control. In a brief, each well received 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, G3582). For 3 hours, the plate was incubated in a 5% CO_2 incubator at 37^0 C. A microplate reader was used to test the absorbance at 490 nm (Laksmitawati et al., 2016; 2017; Novilla et al., 2017; Widowati et al., 2016; 2019; 2021).

2.3 Cell Treatment and Induction for Proinflammatory Activation

Laksmitawati et al (2016) and a modified procedure is used to induce cells for pro-inflammatory purposes. The cells were seeded in a 6-well plate at a density of

$5x10^5$ cells per well and incubated for 24 hours at 37ºc in a humidified atmosphere of 5% CO_2. The medium (DMEM combined with 10% FBS and 1% penicillin streptomycin) was then washed and supplemented with 1.600 µL growth medium and 200 µL (Luteolin 4 and 20 µM/mL). After around 1-2 hours, the medium was supplemented with 200 µL LPS (L4516) and incubated for 24 hours at 37 ºC in a humidified atmosphere with 5% CO_2. The RAW 264.7 cells were incubated with LPS for 24 hours before being tested (Widowati et al., 2016; 2019; 2021; Sandhiutami et al., 2017)

2.4 Quantitative Analysis of IL-1β, TNF α, and PGE-2 Concentrations

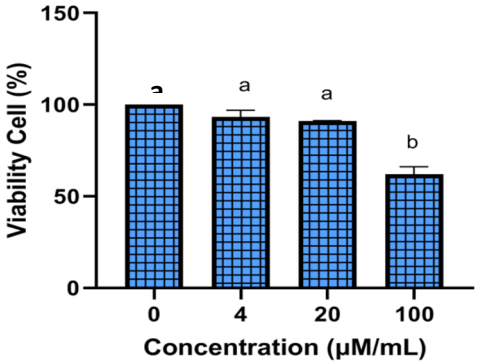
The ELISA Kit Elabscience was used to determine the concentrations of IL-1β (E-EL-M0037), TNF-α (E-EL-M0049) and PGE-2 (E-EL-0034) in the cell-free supernatant. Regarding that, 50 µL of stop solution was applied, and the absorbance was read at 450 nm in a spectrophotometer (Widowati et al., 2016; 2019; 2021; Laksmitawati et al., 2016; 2017)

2.5 Statistical Analysis

SPSS software (version 20.0) was used for data analysis . The data where provided in the form of mean standard deviation. Significant variations between groups were calculated using the Analysis of Variance (ANOVA) followed by the Tukey's Post Hoc Test, with $P < 0.05$ found statistically.

3 RESULTS AND DISCUSSION

The preliminary study to assess the effect of Luteolin on RAW 264.7 cell viability was using MTS assay. The assay aimed to decide the safe and non-toxic concentration for the following assay. The MTS assay was used to determine viability by converting yellow tetrazolium salt into a purple formazan substance. The percentage of viable cells was calculated by comparing the treatment's cell value to the control. The viability assays revealed that luteolin in the given concentrations was still accessible for normal RAW 264.7 cells (Figure 1).



*The data was presented as mean ± standard deviation. The viability of 0 µM/mL was 100%, 4 µM/mL was 93,32%, 20 µM/mL was 91,15% and 100 µM/mL was 62,02 %. Different letter (a, b) shows significantly differences among luteolin concentrations (4 µM , 20 µM, 100 µM) based on Tukey's HSD post hoc test ($p<0.05$).

Figure 1: Effect various concentration of Luteolin toward RAW 264.7 cells viability.

The toxic concentration of luteolin 100 µM/mL with viability 62,02 % was not used in this treatment. Luteolin concentrations of 4 µM/mL with viability 93,32% and 20 µM/mL with 91,15% viability showed good results and also no toxic on RAW 264.7. The non-toxicity of that compound was shown by the fact that over 90% of cells were viable in viability test using the MTS assay. The viability test is a significant feature of pharmacology that deals with the adverse impact of a bioactive agent on living organisms before use as a medication or chemical in clinical use (Jothy et al., 2011; Widowati et al., 2016; 2019; 2021; Laksmitawati et al., 2016; 2017).

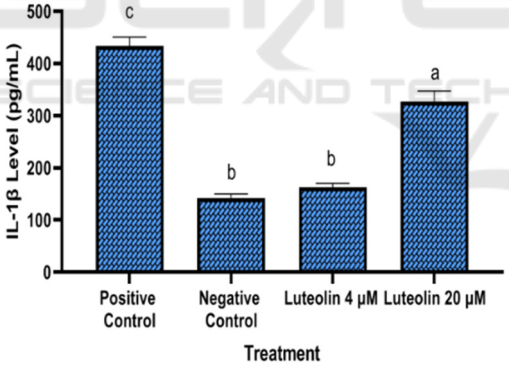
LPS is a pro-inflammatory glycolipid part of Gram-negative bacteria's cell wall that has been shown to stimulate macrophages and increase the synthesis of pro-inflammatory mediators such as nitric oxide (NO), IL-1β, IL-6, and TNF-α.(Saanin et al, 2020; Widowati et al, 2019; 2021). This condition was shown in the present research, which showed that the positive control (RAW 264.7 cells induced by LPS) had significantly higher TNF-α, IL-1β and PGE-2 concentrations than the negative control (RAW 264.7 cells not induced by LPS) showing that LPS is effective in increasing pro-inflammatory mediators.

IL-1β is a powerful pro-inflammatory cytokine released by macrophages during systemic inflammatory responses that regulate the inflammatory (Widowati et al., 2021). Inhibiting pro-inflammatory mediator agent needs to discover for further inflammatory medication. Luteolin has strong anti-inflammatory activity.

This study indicated that LPS caused inflammation and elevated IL-1β levels in RAW264.7, as shown by a high level of IL-1β in the positive control and a significant difference as opposed to the negative control. IL-1β levels in the Luteolin treatment were lower and significantly different from the positive control. These findings suggest that luteolin can reduce IL-1β levels in inflammation-induced cells. Luteolin at concentration of 4 µM/mL greatly reduced PGE-2 levels and was significantly different from the positive control (Figure 2).

IL-1β plays a role in homeostatic processes. IL-1β overproduction results in physiologic changes. IL-1β is expressed by both immune and non-immune cells and is involved in inflammation and pain via Caspase-1 via the inflammasomes. IL-1β may trigger the release and/or activation of nociceptors molecules including IL-6, prostaglandins, and MMP-9 (Goldring, et al., 2011) Inhibiting the synthesis of IL-1β was critical in the discovery of the anti-inflammatory drug (Widowati et al., 2018)

Based on Lami et al (2015), luteolin blocked IL-1β mediated phosphorylation of inhibitor of NF^{k}B, unclear transcription factor-B (NF^{k}B) p65, extracellular signal-regulated kinase-1/2, and c-Jun amino-terminal kinase (Lamy et al, 2015).

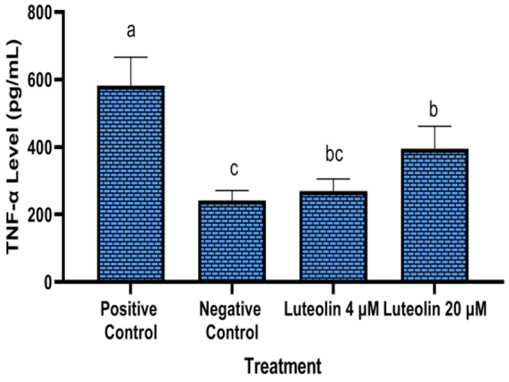


*The data was presented as mean ± standard deviation. Different letter (a, b, c) shows significantly differences among treatment (positive control, negative control, 4 µM, 10 µM luteloin) based on Tukey's HSD post hoc test ($p<0.05$)

Figure 2: Effect of Luteolin toward IL-1β level in LPS-induced RAW264.7 cell.

TNF-α is a multipurpose cytokine that has regulatory and inflammatory effects on a variety of lymphoid and non-lymphoid cells, as well as tumor cells (Stamatkina et al, 2011).

Based on a lower concentration of luteolin, it was shown that luteolin has an inhibitory effect against TNF-α synthesis as opposed to the positive control (LPS-stimulated cells free supernatant without luteolin). The elevated TNF-α inhibitory effect shown by the negative control was given the low concentration of TNF-α in the normal cell and used as a negative control (Figure 3).



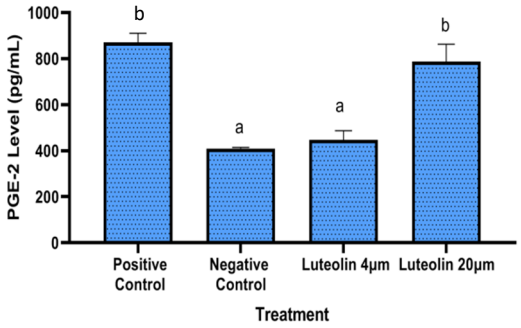
*The data was presented as mean ± standard deviation. Different letter (a, b, bc, c) shows significantly differences among treatment including positive control, negative control, 4 µM, 10 µM) based on Tukye's HSD post hoc test ($p<0.05$)

Figure 3: Effect of Luteolin toward TNF-α level in LPS-induced RAW264.7 cells.

TNF- and IL-1β act as endogenous pyrogens, causing fever during infection by increasing inflammatory responses and promoting the development of chronic diseases (Damte et al, 2011). Inhibiting TNF-α may have beneficial effects for further inflammatory medication, according to the previous study that was conducted by Jinxia et al (2018), TNF-α was inhibited by luteolin as flavonoid in RAW 264.7 macrophages. Luteolin inhibits TNF-α production by blocking the MAPK and InB/NF^{k}B signal pathways.

Prostaglandins (PGE-2) promote cell growth and tissue regeneration. Pro-inflammatory prostaglandins contribute to tumor development in a variety of ways, including cell proliferation, immunosuppression, and angiogenesis. PGE-2 is commonly regarded as the main target of NSAID anti-inflammatory action (Lalier et al., 2011).

In this study, low concentration of luteolin can inhibit the synthesis of PGE-2. The low luteolin concentration at 4 µM exhibited the greatest inhibitory effect, with significant differences compared to 10 µM and it was comparable with negative control (Figure 4).



*The data was presented as mean ± standard deviation. Different letter (a, b) shows significantly differences among treatment (positive control, negative control 4 µM, 10 µM luteloin) based on Tukye's HSD post hoc test ($p<0.05$)

Figure 4: Effect of Luteolin toward PGE-2 level in LPS-induced RAW264.7 cells.

PGE-2 was the extremely abundant prostaglandin found in the human body. PGE-2 is involved in nearly all inflammatory signals, such as redness, swelling, and discomfort, during the inflammatory phase. Reduced PGE-2 activity would minimize inflammation and promote healing (Ricciotti & Fitzgerald, 2011). Anti-inflammatory to inhibit PGE-2 was needed to discover. Jin et al (2017) was reported that flavonoid significantly reduced production of PGE-2 in RAW 264.7 cells.

According to recent and previous study, luteolin has strong anti-inflammatory activity because it is a flavone compound present in many medicinal plants. Flavones are a form of flavonoid that is one of the most prevalent secondary metabolites in plants and is commonly considered to be involved in a variety of pharmacological activities (Aziz et al, 2018).

Flavons serve as an anti-inflammatory agent by modulating the expression of pro-inflammatory genes such as cyclooxigenase-2 (COX-2) and nitric oxide synthase (NOS), as well as other cytokines. During the inflammatory process, cyclooxygenases and lipooxygenases play essential roles. These enzymes are involved in the production of arachidonic acid, which is the first step in the inflammatory process. Since this activity produces cytokines, inhibiting these enzymes will decrease the development of inflammatory metabolites (Masuoka et al., 2011; Panche et al., 2016).

Luteolin has a hydroxyl (-OH) group bound to the flavone backbone structure at the 5-, 7-, 3′ -, and 4'-places. The existence of a hydroxyl group at the 3′ -position separates this flavone from the long-studied apigenin. Flavones are distinguished by the presence of a double bond between C2 and C3, which follows a ketone at the C-4-position. ring's Flavones are differentiated from flavonols by the lack of a hydroxyl group on C3. Article presents the chemical structure of luteolin in Figure 5 (Aziz et al., 2018).

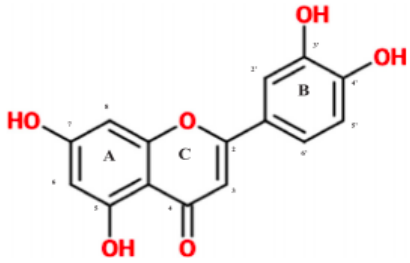


Figure 5: The chemical structure of luteolin (Aziz et al., 2018).

These findings contribute to the investigation of the pharmacological application of luteolin in an in vitro laboratory model of inflammation. Based on this study and literature review, we summarized the mechanism of how luteolin could act as anti-inflammatory against LPS induced RAW 264,7 Cell Lines (Figure 6).

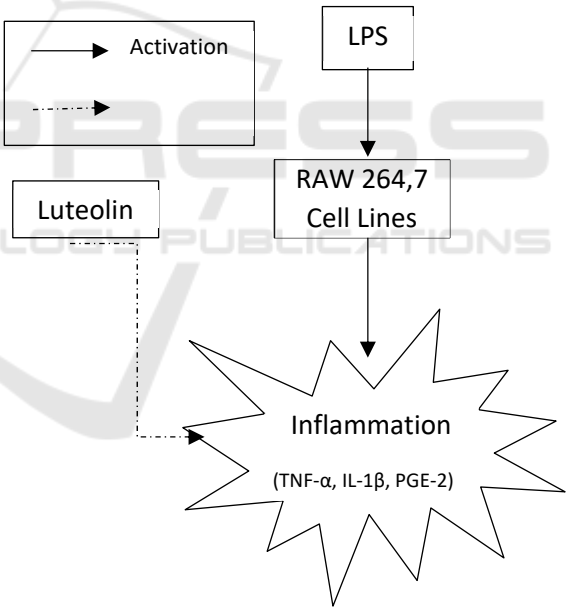


Figure 6: The mechanism of luteolin as anti-inflammatory against LPS induced RAW 264,7 Cell Lines.

4 CONCLUSIONS

This study discovered that Luteolin possesses anti-inflammatory effects as shown by its inhibitory action of IL-1β, TNF-α and PGE-2 secretion. The inhibitory process by luteolin was best against PGE-2 with 62.51% over positive control. Nevertheless,

additional tests such as clinical and preclinical trials should be conducted before pharmaceutical applications.

ACKNOWLEDGEMENTS

We surely appreciate that the work was carried out successfully with the support of the Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia. We are thankful to Hanna Sari Widya Kusuma, Seila Arumwardana from the Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia, for their valuable assistance.

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