Anti-inflammatory Activities of Pineapple *(Ananas comosus)* Core Extract in Lipopolysaccharide-induced RAW264.7 Cell Line

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Abstract: Inflammation is a biological response process by the immune system that may induce acute/chronic inflammatory and leading tissue damage or diseases. Pineapple *(Ananas comosus)* cores that have been investigated for anti-inflammatory properties and immunomodulator. This research aims to evaluate the anti-inflammatory potency of pineapple core extract (PCE) in lipopolysaccharide-induced macrophage cells (RAW 264.7). The viability assay of PCE was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to ensure the safe and non-toxic concentration in RAW 264.7 cells. The pro-inflammatory induction of cells using 200 µL of lipopolysaccharide (LPS). Levels of PGE-2 and pro-inflammatory cytokines IL-1β and TNF-α levels were measured using enzyme-linked immunosorbent assay. RESULTS: PCE 4 and 20 µg/mL showed high viability (>90%) with the values 95.03% and 92.94%, respectively. PCE 20 µg/mL showed the lower of PGE-2 and TNF-α levels (507.68 pg/mL; 345.90 pg/mL) compared to PCE 4 µg/mL (795.37 pg/mL; 474.19 pg/mL) and positive control (870.48 pg/mL; 581.71 pg/mL). In IL-1β level, PCE 20 µg/mL showed the lower (217.63 pg/mL) compared to PCE 4 pg/mL (350.78 pg/mL) and positive control (433.53 pg/mL). Pineapple core extract has beneficial for anti-inflammatory by downregulating inflammatory mediators including PGE-2, TNF-α, and IL-1β in LPS-induced RAW 264.7 cell lines.

1 INTRODUCTION

Inflammation is an immune system reaction that can be caused by a several factors, including damaged cells, pathogens, and toxic substances. These factors can cause acute and/or chronic inflammatory

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responses in the pancreas, liver, heart, lung, brain, kidney, reproductive system, and intestinal tract, which could result in tissue damage ordiseases (Chen et al., 2018).

Inflammatory cytokines are produced by activated macrophages, such as interleukins (ILs), tumor necrosis factor (TNF-α), inflammatorymediators like nitric oxide (NO), and prostaglandins (PGs), which play a protective role for the host in inflammatory conditions and also preserving normal cellular conditions (Lee et al., 2017; Saanin et al., 2020). The activation of macrophages causes the release of a variety of chemicals, including Nitric Oxide (NO), reactive oxygen species (ROS), prostaglandin E (PGE), and Interleukin (IL)-1β, Interleukin (IL)-6, cyclooxygenase-2 (COX-2), and tumor necrosis factor (TNF)-α (Widowati et al., 2016; Novilla et al., 2017; Lee et al., 2017; Laksmitawati et al., 2017). Many chronic diseases have been linked to the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), which are responsible for raising NO and prostaglandins (PGs) levels, respectively (Widowati et al., 2021). Thus, the suppression of pro-inflammatory mediators can be an effective indicator of anti-inflammatory drugs (Saanin et al., 2020; Widowati et al., 2021).

Anti-inflammatory medications, both steroidal and non-steroidal, are used in conventional treatment for inflammatory diseases. However, the limitations and risks associated with conventional therapy have led people to explore alternative measures such as medicinal plants for the treatment of inflammatory diseases (Kargutkar and Brijesh, 2017).

Ananas comosus (L.) Merr. which is known as pineapple is a species of tropical plant that belongs to the Bromeliaceae family (Rahman et al., 2020). *A. comosus* has a various compounds from several parts of the plant, including alkaloids, anthraquinones, bromelain, cardiac glycoside, coumarins, flavonoids, glycoside, inulin, naphthoquinones, phenols, phytosterols, polyphenols, quinine, saponin, steroids, sterols, tacorins, terpenoids, tannins, and triterpenes (Rahman et al., 2020). *A. comosus* can prevent undesirable inflammatory processes and also has anti-inflammatory activity (Yatoo et al., 2018). Bromelain is a bioactive compound and as a major protease enzyme found in *A. comosus* stems that demonstrate anti-thrombotic, anti-inflammatory, and anti-edematous (Ramli et al., 2018). Bromelain also has anti-cancer properties and facilitates cell death by apoptosis (Pavan et al., 2012). Pineapple core extract (PCE) exhibit as antioxidant potent by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) scavenging activity (Vrianty et al., 2019).

However, the previous studies were focused on the fruit, peels, and leaves of pineapple (*A. comosus)* but there were limited studies on the core of the fruit.

This study was purpose to evaluates the anti-inflammatory activity of PCE through the inhibitory activity of pro-inflammatory mediators including PGE-2, IL-1β, and TNF-α on LPS-induced RAW 264 cells.

2 METHODS AND MATERIALS

2.1 Extract Preparation

A. comosus plants were obtained from Tambaksari village, Cagak district, Subang, West Java, Indonesia. Plant identification was done at Herbarium Bandung Laboratory, School of Biological Sciences, Bandung Institute of Technology. The preparation of *A. comosus* ethanolic extract based on Vrianty et al. (2019) method. *A. comosus* were sorted, washed, weighed in wet weight, dried in a food dehydrator, weighed in dry weight, and then crushed into powder form (core crude drug).And then, the core crude drug was extracted using maceration techniques with a 70% ethanol solvent. Every 24-hour, the filtrate was until the ethanol filtrate turned colorless. Before being used for assays,PCE was stored at 20 ºC (Vrianty et al., 2019).

2.2 Raw264.7 Cells Culture

The RAW 264.7 (ATCC®TIB-71™) murine macrophage cell line was obtained from the Biomolecular and Biomedical Research Center, Aretha Medika Utama. RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0104) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810) and 1% antibiotic–antimycotic (Gibco, 15240062). The cells were incubated at 37 ºC and 5% CO_2 in the humidified atmosphere until confluent (80%–90%). Trypsin-EDTA 0.25% (Gibco, 25200072) was used to harvest the cells which were then seeded on plates for the assays (Sandhiutami et al., 2017; Laksmitawati et al., 2016; 2017; Saanin et al., 2020; Widowati et al., 2016; 2021).

2.3 Viability Assay

The cytotoxicity of PCE was determined by the viability of RAW 264.7 cells using MTS assay (Promega, G3580). This method is used to assess the sample concentration that is both safe and non-toxic

for the next assay. The cells density of 5×10^3 cells per well were plated in medium (DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic in a 96-well plate and incubated for 24 hours at 37°C in a humidified atmosphere incubator with 5% CO_2. The medium was washed and replaced with 99 µL of fresh medium and 1 µL of PCE in two concentrations (4, 20, 100 µg/mL), and 1% DMSO, then the plate was incubated for 24 hours. The negative control group consisted of cells that had not been treated. The twenty microlitres of MTS were added to each well. For 4 hours, the plate was incubated in 5% CO_2 at 37 °C at an incubator. The absorbance was quantified at 490 nm using a microplate reader (Multiskan Go, Thermoscientific) (Widowati et al., 2016; 2021; Laksmitawati et al., 2016; 2017; Saanin et al., 2020).

2.4 Pro-inflammatory Activation of RAW264.7 Cells

The cells were seeded at a density of 5×10^5 cells per well in a 6 well-plate and incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2. The DMEM was washed and supplemented with a 1,600 µL growth medium and 200 µL PCE (4,20 µg/mL) after being supplemented with 10% FBS and 1% antibiotic–antimycotic. After 1-2 hours, 200 µL lipopolysaccharide (1 µg/mL) from *Escherichia coli* (Sigma Aldrich, L2880) was added to the medium and incubated for 24 hours at 37°C, 5% CO_2. The medium was then taken for PGE-2, TNF-α, and IL-1β levels quantification, centrifuged at 2000 g for 10 minutes, and the supernatant was stored at -80^0 C. (Widowati et al., 2016; 2021; Laksmitawati et al., 2016; 2017; Sandhiutami et al., 2017; Novilla et al., 2017; Saanin et al., 2020).

2.5 Quantification of PGE-2, TNF-α, IL-1β Levels in RAW264.7 Cells

The measurement of PGE-2, TNF-α, and IL- 1β levels were conducted based on the ELISA method, using PGE-2 ELISA Kit (E-EL-M0052), TNF-α ELISA Standard Kit (Elabscience, E-EL- M0049), and IL-1β ELISA Kit (Elabscience, E-EL- M0037), respectively according to the manufacturer's instructions. The inhibition activity was calculated based on the percentage of a PGE-2, TNF-α, and IL- 1β levels (Widowati et al., 2016; 2021; Laksmitawati et al., 2016; 2017; Saanin et al., 2020).

2.6 Statistical Analysis

All data were obtained after doing it in triplicate. The data were presented as mean ± standard deviation. The data were analyzed using ANOVA and Tukey HSD Post Hoc Test with $p < 0.05$ using SPSS software (version 20.0).

3 RESULTS AND DISCUSSION

3.1 Viability RAW264.7 Cells

The viability assay was performed to determine the safe and nontoxic concentration for the next assay, which was assessed using the MTS assay (Widowati et al., 2016; 2021). The viability of RAW264.7 cell lines has been presented in Table 1.

Table 1: The effect of various concentrations of PCE towards the viability of RAW264.7 cells.

Sample	Viability cells (%)
Negative Control	100.00 ±3.54^c
PCE 4 µg/mL	95.03 ±2.07^b
PCE 20 µg/mL	92.94 ±1.04^b
PCE 100 µg/mL	76.86 ±4.09^a

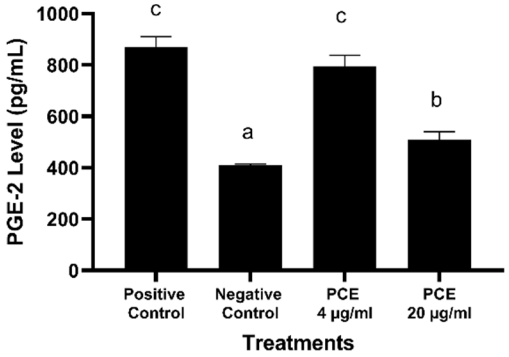
*The data was presented as mean ± standard deviation from 3 replications. Different superscript letters in the same column (a, b, c) showed significant differences among treatments at p <0.05 (Tukey HSD post hoc test).

The increased concentration was correlated with increased toxicity (<90% viability cells). Table 1 shows the cytotoxicity of PCE concentration on RAW264.7 cell lines. The viability of cells was decreased in a concentration-dependent manner. In concentration 100 µg/mL demonstrated the lowest viability of cells by PCE with a value of 76.86 ± 4.09%. Based on the data (Table 1), the safe and un-toxic of PCE in murine macrophage cells were 4, 20 µg/mL, these concentrations were used for the treatment in LPS-induced RAW264.7 cells as inflammation cells model.

3.2 Effect of PCE toward PGE-2 Level in LPS-induced RAW264.7 Cells

Inhibitory activity of PGE-2 generation from COX-2 also causes an anti-inflammatory effect (Mahesh et al., 2021). The RAW 264.7 murine macrophage cell line is commonly used as an *in vitro* inflammatory model (Widowati et al., 2016; 2021). The

inflammatory response is marked releasing of the PGE-2 level. The anti-inflammatory activity was showed by decreasing PGE-2 level in LPS-induced RAW264.7 cells as inflammatory cells model.



*The data was presented as mean ± standard deviation from 3 replications. Negative control: untreated cell; Positive control: LPS-induced RAW264.7 cells. Different superscript letters (a, b, c) showed significant differences among treatments at p <0.05 (Tukey HSD post hoc test).

Figure 1: Effects of PCE toward PGE-2 level in LPS-induced RAW264.7 cells.

PCE 20 µg/mL has a better ability to suppress PGE-2 level (507.68 pg/mL) compared to PCE 4 µg/mL (795.37 pg/mL) and positive control (LPS-induced cells) with value 870.48 ± 39.54 pg/mL (Figure 1). Our findings provide evidence that PCE significantly inhibits the production of PGE-2 without affecting the cell viability in LPS-induced RAW 264.7 cells.

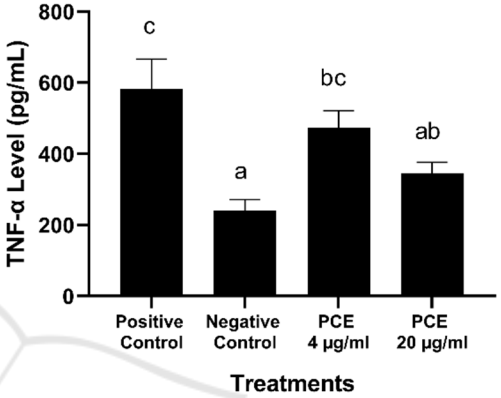
A. comosus leaf extract has an ability to inhibit PGE2 production in a dose-dependent manner with a value of 2198.83 ± 280.87 pg/mL in the highest concentration (500 µg/ml). The inhibition of COX-2 enzyme induction and subsequent inhibition of COX-2 mRNA expression may be the mechanism behind some phytoconstituents anti-inflammatory activity (Yatoo et al., 2018). It has also been discovered that inhibiting COX-2 development results in a lowerlevel of PGE **(**Kasemsuk et al., 2018).

The methanol extract of fruit peel (MEFP) of *A. comosus* has anti-inflammatory potential through decreasing the level of PGE-2, so it has the potential to protect cartilage from damage caused during rheumatoid arthritis (Kargutkar and Brijesh, 2016).

The anti-inflammatory effect of bromelain also was correlated with reduced LPS-induced nuclear factor-kappaB (NF-κB) activity and cyclooxygenase 2 (COX-2) mRNA expression in rat livers **(**Kasemsuk et al., 2018). The roles of bromelain are also well recognized in activating the healthy immune system with the rapid response to cellular stress (Rathnavelu et al., 2016).

3.3 Effect of PCE towards TNF-α Level in LPS-induced RAW264.7 Cells

TNF-α is one of the most important pro-inflammatory cytokines and is mainly produced by monocytes and macrophages. These pro- inflammatory cytokines influence the proliferationand death of cells and it is secreted during the early phase of acute and chronic inflammatory diseases such rheumatoid arthritis (Wang and He, 2018; Saanin et al., 2020).



*The data was presented as mean ± standard deviation from 3 replications. Negative control: untreated cell; Positive control: LPS-induced RAW264.7 cells. Different superscript letters (a, ab, bc, c) showed significant differences among treatments at p <0.05 (Tukey HSD post hoc test).

Figure 2: Effects of PCE toward TNF-α level in LPS-induced RAW264.7 cells.

Treatment with PCE 20 µg/mL potentially inhibit TNF-α production (345.90 pg/mL) compared to PCE 4 µg/mL (474.19 pg/mL) and also positive control with value 581.71 pg/mL (Figure 2). This treatment indicates that PCE had anti-inflammatory activity. Based on another study, *A. comosus* extract has a significant antioxidant activity, as well as anti-inflammatory ability. It is thought that molecules with both an anti-oxidative effect and an anti-inflammatory effect should be effective in treating diseases caused by oxidative stress as a result of ROS generation as well as in inflammatory diseases (Lee et al., 2018; Vrianty et al., 2019).

Kargutkar and Brijesh's (2017) study showed that *A. comosus* leaf extract (ALE) has anti-inflammatory activity due to significantly decreasing the release of TNF-α, IL-1β, PGE-2, and ROS by LPS-stimulated macrophages in a dose-dependent manner. ALE can decrease the secretion of TNF-α at a concentration of 500 µg/mL with a maximum reduction of 409.89 pg/mL (Kargutkar and Brijesh, 2017).

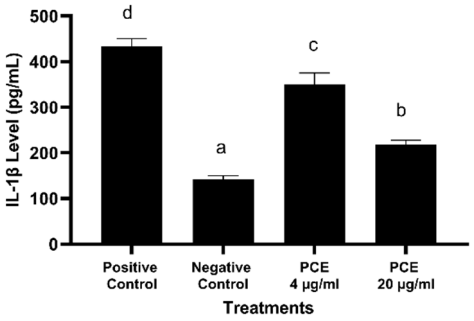
The phytoconstituents prevent inflammation and apoptosis by inhibiting TNF-α in human macrophages (Wee et al., 2020). Bromelain in *A. comosus* potentially inhibits the pro-inflammatory mediators productions of NFκB, IL-1β, IL-6, TNF-α, PGE-2 (Bakare et al., 2021). In another study was reported that Bromelain can inhibit the secretion of IL-1, IL-6, and TNF by peripheral blood mononuclear cells (PBMCs) and modulate surface adhesion molecules on T cells, macrophages, and natural killer cells (Pavan et al., 2012). Bromelain inhibits the Raf-1/extracellular-regulated-kinase- (ERK-) 2 pathways by inhibiting T cell signaling (Kwatra, 2019). Bromelain also suppressed the LPS-activated extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) (Insuan et al., 2021). However, the anti-inflammatory effects of the bromelain preparation in vitro and in vivo studies suggest its therapeutic potentials (Kasemsuk et al., 2018).

3.4 Effect of PCE towards IL-1β Level in LPS-induced RAW264.7 Cells

IL-1β is a potent immunomodulator that regulates a variety of immune and inflammatory responses, including B and T cells activation (Rapsinski et al., 2015).

PCE 20 µg/mL decreased the IL-1β level (217.63 pg/mL) compared to PCE 4 µg/mL (350.78 pg/mL) and also positive control with value 433.53 pg/mL. PCE has anti-inflammatory properties through inhibitory of IL-1β level among treatments.

Bromelain is a crude, aqueous extract derived from the stem and fruit of the pineapple plant, which contains a variety of proteolytic enzymes and has anti-inflammatory and analgesic properties (Cai et al., 2017). Bromelain from *A. comosus* also has ability to decreases NO, PGE-2, TNF-α, IL-1β and IL-6 production by downregulation of the NF-κB, AP-1, and JAK/ STAT signaling pathways in LPS-induced RAW 264.7 macrophages (Lee et al., 2017; Kargutkar and Brijesh, 2016).



*The data was presented as mean ± standard deviation from 3 replications. Negative control: untreated cell; Positive control: LPS-induced cell. Different superscript letters (a, b, c, d) showed significant differences among treatments at $p < 0.05$ (Tukey HSD post hoc test).

Figure 3: Effects of PCE toward IL-1β level in LPS-induced RAW264.7 cells.

Bromelain has the ability to modulate the immune response to reduce the allergic reaction and to modulate macrophages, NK cells, and T cells. It also increases the secretion of IL-1β, IL-6, and TNF-α (Cai et al., 2017). Flavonoids and tannins compounds in *A. comosus* can be attributed in anti- inflammatory properties (Jiang et al., 2014).

4 CONCLUSIONS

PCE has the potential as an anti-inflammatory by decreasing PGE2, TNF-α, and IL-1β levels.However, PCE protects against LPS-induced RAW264.7 cells via inhibiting oxidative stress and inflammation.

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