The Potency of Polyphenols Extract of Robusta Coffee Bean (Coffea robusta) on COX-2 Inhibition in Neutrophil Cells

Potensi Ekstrak Polifenol Biji Kopi Robusta (Coffea robusta) terhadap Penghambatan COX-2 pada Sel Neutrofil

ABSTRACT

Inflammation is a physiological response to various stimuli including infection and tissue injury. One cause of inflammation is gram-negative bacteria that release various toxins, e.g. lipopolysaccharide endotoxin (LPS). The first body defense response is neutrophils. Body defense response is expressed by the cyclooxygenase (COX) enzyme that functions to transform arachidonic acid into prostaglandin. Polyphenols extract from Robusta coffee bean is known to play an anti-inflammatory role, but the mechanism for inhibiting COX-2 remains unknown. This study aimed to determine the potential of polyphenols extracted from Robusta coffee beans on COX-2 inhibition in neutrophils exposed to E. coli LPS. The research design was experimental in vitro with a post-test-only control group design. The sample consisted of 6 treatment groups, neutrophil isolates was incubated in concentrations of Robusta coffee polyphenols extract of 3.13%, 6.25%, 12.5%, and 25% and exposed to E. coli LPS. Measurement of COX-2 expression was conducted using immunohistochemical methods. ANOVA and LSD test results showed COX-2 expression in the treatment groups, namely neutrophils which were incubated with Robusta coffee polyphenols extract and exposed to E. coli LPS, was lower than the neutrophil group exposed only to E. coli LPS. The conclusion of this study is that the polyphenols extract of the Robusta coffee bean can inhibit COX-2 expression on neutrophils exposed to E. coli LPS.

Keywords: COX-2, LPS, neutrophils, polyphenols, robusta coffee

ABSTRAK


Kata Kunci: COX-2, kopi robusta, LPS, neutrofil, polifenol

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INTRODUCTION

Inflammation is a complex biological response of vascular tissue to harmful stimuli, such as pathogens or irritants (1). In the cellular phase of the inflammatory process, the first cells chemically attracted to the area of inflammation are neutrophils. Neutrophil cells are phagocytic cells that become a factor in the naural immune system and function in pathogenic microbes' phagocytosis process. Neutrophils have a short life span, are active in the inflammatory process, and are responsible for tissue damage (2-4). Increased vascular permeability, vasodilation, edema formation, and pain in the inflammatory process are caused by the influence of prostaglandins as inflammatory mediators (5).

Inflammatory reactions can be caused by bacterial invasion and its products. Bacteria will produce endotoxin in the form of lipopolysaccharide (LPS), a large molecule containing lipids and carbohydrates (6). LPS induces the production of local factors, which are proinflammatory cytokines, such as interleukin 1α (IL-1α), interleukin-1-beta (IL-1β), IL-6, TNF-α, and prostaglandins (7).

Cyclooxygenase (COX) is an enzyme that catalyzes the formation of prostaglandins, an inflammatory mediator, and a product of arachidonic acid metabolism. The two COX isoenzymes in the body are COX-1 and COX-2. COX-1 is a constitutive enzyme that catalyzes the formation of regulatory prostanoïds in various tissues, especially in the mucous membranes of the gastrointestinal tract, kidney, platelets, and vascular epithelium. COX-1 is almost entirely expressed in most cells and tissues. Cyclooxygenase 2 (COX-2) is a family of myeloperoxidases located on the luminal side of the endoplasmic reticulum and nuclear membrane. In general, COX-2 is undetectable but is rapidly induced when cells receive inflammatory stimuli. COX-2 also acts at inflammation sites (8-10).

In inflammatory conditions, the standard therapy is the administration of non-steroidal anti-inflammatory drugs (NSAIDs). Pharmacological studies have shown that non-steroidal anti-inflammatory drugs on the market can inhibit both forms of COX enzymes. Some medicines used to reduce pain are aspirin, indomethacin, ibuprofen, and piroxicam (11). Administration of indomethacin in rats could significantly decrease COX-2 expression (12,12). On the contrary, long-term use of NSAIDs has a toxic effect, especially on the liver, kidneys, and gastrointestinal tract (11,14-17).

Coffee, which is currently favored by the public and has become a lifestyle, is a beverage beneficial for health (18,19). Study results show that coffee intake modifies various immune functions (20-22). One kind of coffee that is widely consumed is Robusta. Robusta coffee beans naturally contain caffeine, phenolic compounds, trigonelline, and chlorogenic acid with anti-bacterial and anti-inflammatory activities (23). Polyphenols have health benefits (24-26). In recent decades, several benefits of polyphenols from plants as antioxidants and anti-inflammatory have been recognized. Research conducted by Willenberg et al., stated that polyphenols could reduce COX-2 expression in monocyte cells exposed to LPS (27). Polyphenols in coffee plants can be used to inhibit the development of diseases, such as cancer, cardiovascular disease, diabetes, osteoporosis, and neurodegenerative diseases (22,28). However, little research has been done on the potency of polyphenol extract of Robusta coffee beans on COX-2 expression in neutrophils. Therefore, this study aimed to determine the potency of the polyphenol extract of Robusta coffee beans on COX-2 inhibition in neutrophils exposed to LPS E.coli.

METHOD

Experimental Design

The Invitro laboratory experimental with research design The Post Test Only Control Group Design. The independent variable in this study is the polyphenol extract of robusta coffee beans. The dependent variable is the amount of COX-2 expression of neutrophil cells. The controlled variables in this study were the concentration of robusta coffee bean polyphenol extract, neutrophil isolate, and LPS E.Coli 0111: B4 (List Biology Lab).

Extraction of Robusta Coffee Bean Polyphenols

The extraction used the sonication method. The procedure for making the extract is as follows. 721.1 grams of coffee beans were baked at 60°C for 2 to 3 days. The coffee beans were put in a hammer mill to become coffee powder. The sonication process was carried out by adding 96% ethanol solvent and rotating with 80KHz vibration for one hour. Mixing with ethanol was done 3 times to obtain pure flavonoid content, then centrifuged at 4000 rpm for 5 minutes to precipitate particles from coffee powder so that an oil-like liquid was obtained. The liquid is dried in an oven at 60°C so that the ethanol evaporates and coffee bean polyphenol extract is obtained.

Neutrophil Preparation

Neutrophil isolation was performed using a modified gradient density technique using Double Ficoll Hypaque Centrifugation material (29). Six ml of cubital venous blood was added to the heparin tube and mixed thoroughly. Pipette 3ml histopage M119 into a sterile falcon tube and add 3ml lymphoprep slowly through the tube wall, and 2 layers were formed. Pipette 6ml of blood into the 2-layer tube, slowly through the wall of the falcon tube, and 3 layers are formed (Histopage 119, Lymphoprep, and blood). Centrifuge at 700 g for 30 minutes at 20°C. Carefully pipette the 4th layer of Polymorphonuclear (fog ring) into a sterile tube. Dilute the Mononuclear sample using HBSS (1:1) homogenize. Centrifuge at 700g (gravity) for 10 minutes at 20°C do 3 repetitions. Add 1ml of HBSS pH 7.4 to the supernatant obtained. Add 5μl fungizone and 20μl Penicilin- Streptomycin. Prepare Well Plate Culture, and insert sterile poly L-lysin coverslip in each well as needed. Drip 100μl of cell isolation supernatant on the prepared coverslip. Incubate for 20-30 minutes at 37°C. Take and add 1ml of RPMI culture medium, and incubate again for 20-30 minutes at 37°C. Observe under an inverted microscope, by gently shaking to see the cell attachment. Wash using RPMI media 3 times, carefully to release cell contamination. Once the sterile cells are free from contamination. Replace the culture medium using M.199 culture medium, the cells are ready for cell treatment.

Treatment and Immunocytochemical Assay

Neutrophils were resuspended with 1000μl RPMI, then discarded. Add 100μl of polyphenol extract with a certain concentration (3.13%, 6.25%, 12.5%, 25%) to the cell culture well according to the treatment and homogenize. Each treatment group was slowly added with 100μl of E.coli LPS and homogenized. Incubate for 2 hours at 37°C and 5% CO2. Observed the changes and development of neutrophils every hour during the incubation time with LPS E.Coli. 0111: B4 (List Biology Lab).
LPS for 4 hours. After incubation of LPS exposure for 4 hours, the immunostaining procedure was continued using COX-2 monoclonal antibody. COX-2 expression was analyzed by immunocytochemical method. COX-2 expression was shown by neutrophils whose cell membranes were brown in color, observations were made under a microscope with 400 magnification. The study data was the average number of neutrophils expressing COX-2 counted per 100 cells.

**Statistical Analysis**

The data obtained were analyzed using the Kolmogorov-Smirnov test for the normality test and the Levene test for the homogeneity test. The normality test was performed with the parametric statistical test, namely One Way ANOVA, and continued with the LSD test. All tests used a significance level of 95% (α=0.05).

**RESULTS**

The results showed that there were differences in COX-2 expression between the control and treatment groups. The average number of neutrophil cells expressing COX-2 in the control and treatment groups can be seen in Figure 1.

![Figure 1. Graph of the mean COX-2 expression](image)

The lowest mean COX-2 expression (3.9±1.26) was found in the treatment group with exposure to polyphenol extract with a concentration of 3.13%, and the highest COX-2 expression (22.65±6.65) was found in the positive control group (LPS). These results indicate that polyphenol incubation treatment show lower COX2 expression in neutrophils than those in positive controls, nearly the same as normal conditions (control).

The neutrophils expressing COX-2 can be seen in Figure 2. COX-2 expressions yield a dark or brown color. The results of the Kolmogrov-Smirnov test showed that the data were normally distributed (p>0.05). Based on the Levene test, it had a homogeneous variance (p=0.118), meaning that the data obtained was homogeneous. One-way ANOVA test showed a significant difference in COX-2 between the control and treatment groups (p=0.00). LSD analysis showed a significant difference between the negative control group (neutrophil cells exposed to LPS) and the treatment groups (neutrophil cells incubated with polyphenol extracts and exposed to LPS). There were no significant differences between treatment groups given different doses of coffee extract. These results indicate that polyphenol extract of coffee beans can reduce COX-2 levels in neutrophil cells exposed to LPS, and increasing the dose did not provide a significant difference.

**DISCUSSION**

This research was an in vitro experimental study using neutrophil cells and aimed to know the potency of polyphenol extract of Robusta coffee beans as an anti-inflammatory agent by determining COX-2 expression. Neutrophils are immune cells in the body that act as the...
first line of defense against injuries, including bacteria and their products, such as LPS (2, 7). Neutrophils migrate to the inflamed area by releasing elastase to kill bacteria, but it results in tissue damage if elastase is produced in large quantities (30). Neutrophils use three ways to fight and kill pathogenic microbes and their products, namely phagocytosis, degranulation, and Neutrophil Extracellular Trap (NET) formation (31-35). Based on research conducted by Pieterse et al., LPS E. coli exposed to neutrophil cells was shown to release NET production as a defense response against damaging agents (36). In the phagocytosis process, neutrophils engulf microbes into phagosomes, while in the degranulation process, neutrophils release protease granules, which can cause damage to host cells (36-38).

The results showed that the mean COX-2 expression in the LPS-induced and polyphenol extracts groups tended to decrease compared to the group only exposed to LPS. LPS exposure to neutrophils showed the highest COX-2 expression compared to the treatment and control groups. It is because LPS is a potent endotoxin that is responsible for inflammation. LPS E. coli is a bacterial product that can bind to the CD14/Toll-like receptor 4 (TLR4) on the cell surface of macrophages and monocytes. The binding between TLR4 and LPS activates inflammatory signaling pathways. The inflammatory response results in the secretion of proinflammatory cytokines from several cell types. Macrophages that bind to bacteria due to TLR4 will secrete cytokines (IL-1α, IL-1β, TNF-α, and prostaglandins (PGE)) (39). These results are in line with studies conducted by Nieves et al., (39) that LPS exposure in astrocyte cell culture was proven to increase inflammatory properties, marked by a decrease in COX-2 expression in neutrophil cells exposed to LPS. Polyphenols with various concentrations tend to reduce COX-2 expression. The group given polyphenols extract of Robusta coffee beans with 3.13% concentration showed the lowest COX-2 expression compared to higher treatment doses. The potential of polyphenols in suppressing inflammation is by blocking the cyclooxygenase (COX) and lipoxygenase (LOX) cycles. Polyphenols inhibit cell membranes that trigger the release of phospholipase enzymes and will inhibit the formation of arachidonic acid so that COX-2 expression and the formation of prostaglandins can be suppressed. Protein kinase C (PKC) activates phospholipase A1 (PLA1) in an inflammatory state and releases arachidonic acid from membrane phospholipids. Arachidonic acid is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) to produce eicosanoids, including prostaglandins and leukotrienes (40). Flavonoids, such as quercetin and myricetin, are COX and LOX inhibitors (40-44). The results obtained are in accordance with findings from Yue et al., (45) that polyphenol extracts from pomace apple can reduce COX-2 expression in rat macrophage cell culture.

Polyphenol extracts are shown to decrease the expression of pro-inflammatory cytokines IL-6, TNF-α, PGE2, and COX-2 in LPS-induced macrophage culture cells (46). The results of the LSD test showed that the variation of the polyphenol extract with higher doses (3.13%, 6.25%, 12.5%, and 25%) did not show significant differences in COX-2 expression. Polyphenols have different chemical structure characteristics. In addition, polyphenols have different mechanisms for controlling neutrophils depending on the receptors that act on the cells. This study proves that the polyphenol extract of Robusta coffee beans has anti-inflammatory properties, marked by a decrease in COX-2 expression in neutrophil cells. Therefore, polyphenols of Robusta coffee beans can be developed as alternative medicine in the health sector.

REFERENCES


