Anti-Inflammatory Effects of Methanol Extract, Hexane, Ethyl Acetate, and Butanol Fraction of *Piper crocatum* Ruiz & Pav Leaves on Lipopolysaccharide-induced RAW 264.7 Cells

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ABSTRACT

*Piper crocatum* Ruiz & Pav is a traditional Indonesian plant that is empirically used to treat various diseases. Several studies have stated that *Piper crocatum* leaves extract has anti-inflammatory effects. *Piper crocatum* leaves contain different secondary metabolites alkaloids, flavonoids, tannin-polyphenols, steroids, terpenoids, and saponins. The purpose of this study was to determine the anti-inflammatory effect of *Piper crocatum* leaves extract and fraction on the production of nitric oxide in lipopolysaccharide-induced RAW 264.7 cells. Anti-inflammatory effect of methanol extract of *Piper crocatum* leaves (MEPC), n-hexane fraction of *Piper crocatum* leaves (HFPC), ethyl acetate fraction of *Piper crocatum* leaves (EAFPC), and n-butanol fraction of *Piper crocatum* leaves (BFPC) against RAW 264 cell macrophages, 7 Lipopolysaccharide-induced (LPS). Extraction of *Piper crocatum* leaves was the maceration method. Cell viability was determined by the MTT method in the concentration range of 12.5;25;50;100;200 µg/mL. The anti-inflammatory effects of MEPC, HFPC, EAFPC and BFPC were tested against nitric oxide (NO) production inhibition in LPS-induced RAW 264.7 macrophage cells. NO levels were determined by the ELISA plate reader. The viability test of MEPC, HFPC, EAFPC, and BFPC from *Piper crocatum* leaves showed the concentrations that gave the percentage of viability above 80% were concentrations of 100, 50, and 25 µg/mL. MEPC, HFPC, EAFPC, and BFPC at concentrations of 100, 50, 25 µg/mL could significantly inhibit NO production with *p*<0.05 (*p*=0.000).

The highest NO production inhibition effect was on EAFPC. MEPC, HFPC, EAFPC, and BFPC have anti-inflammatory effects, with the highest effect on EAFPC.

Key words: *Piper crocatum*, Anti-inflammatory effect, LPS, Nitric oxide.

INTRODUCTION

Inflammation is a physiological response to stimuli such as infection and tissue injury. This stimulation causes the release of inflammatory mediators such as nitric oxide, histamine, serotonin, Bradykinin, and prostaglandins which cause an inflammatory reaction in the form of heat, pain, redness, swelling, and accompanied by impaired function.¹

Lipopolysaccharide is a component of the cell wall of gram-negative bacteria, the polyclonal activator of the immune system, stimulates the release of various inflammatory mediators, including nitric oxide (NO), prostaglandin E₂, pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, TNF-α, and TNF-β. Activated macrophages are the main effector cells in the host defense against bacteria through the production of nitric oxide (NO), which is cytotoxic to parasites. Nitric oxide (NO) and reactive oxygen species exert several modulating effects on inflammation and play a key role in regulating the immune response. NO is the main product controlled by nitric oxide synthase (NOS), such as iNOS (inducible nitric oxide synthase). iNOS is induced in macrophages so that its activation leads to organ destruction in several inflammatory and autoimmune diseases.²,³

*Piper crocatum* is one of the plants used by the people of Indonesia as traditional medicine. Empirically red betel is often used to treat diabetes mellitus, hepatitis, kidney stones, lowering cholesterol, preventing stroke, gout, hypertension, coronary heart disease, uterine cancer, breast cancer, hemorrhoids, tuberculosis, toothache medicine, canker sores, body odor, venereal disease, inflammation of the liver, inflammation of the prostate, inflammation of the eye, vaginal discharge, ulcers, fatigue, joint pain, smoothing the skin, inflammation of the ear, cough medicine, inflammation of the lungs, inflammation of the throat, inflammation of the gums, inflammation of the breast, nose bleed, and coughing up blood.⁴

Research conducted stated that the methanol extract of *Piper crocatum* leaves showed anti-inflammatory effects.⁵,⁶ The ethanol extract of *Piper crocatum* has an antioxidant effect.⁷ *Piper crocatum* contains various secondary metabolites. Identifying phytochemical components from red betel leaf contains flavonoids, alkaloids, tannin-polyphenols, steroids, terpenoids and saponins.⁸,⁹

MATERIALS AND METHODS

Chemicals and Reagents

Methanol, n-hexane, ethyl acetate, n-butanol, RAW cells 264.7 (CCRC UGM), DMSO, dulbecco’s Modified Eagle Medium (DMEM) (Gibco), penicillin-streptomycin (gibco), Fetal Bovine Serum (FBS) (Sigma), Phosphate buffered saline (PBS) (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (eBioscience), dimethylsulfoxide, wash buffer lipopolysaccharide (eBioscience), dexamethasone (Dexamedia), NO Colorimetric Assay Kit (Elabscience).

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Figure 1: The mean percentage of live cells of MEPC, HFPC, EAFPC, and BFPC of Piper crocatum Ruiz & Pav leaf and dexamethasone in RAW cells 264.7.

Figure 2: NO levels of MEPC, HFPC, EAFPC, and BFPC of red betel leaf, dexamethasone, negative control, in LPS-induced RAW 264.7 cells, and normal cells.
Table 1: NO levels of MEPC, HFPC, EAFPC and BFPC of red betel leaf, dexamethasone, negative control, in LPS-induced RAW 264.7 cells, and normal cells.

| Sample       | Average (μmol/L) |
|--------------|------------------|
| 1 Methanol 100 µg/mL | 28.04 ± 5.268     |
| 2 Methanol 50 µg/mL  | 35.42 ± 1.909     |
| 3 Methanol 25 µg/mL  | 44.58 ± 1.909     |
| 4 n-Hexane 100 µg/mL | 32.08 ± 0.722     |
| 5 n-Hexane 50 µg/mL  | 39.58 ± 2.602     |
| 6 n-Hexane 25 µg/mL  | 48.75 ± 1.250     |
| 7 Ethyl Acetate 100 µg/mL | 15.42 ± 0.722     |
| 8 Ethyl Acetate 50 µg/mL | 20.42 ± 1.909     |
| 9 Ethyl Acetate 25 µg/mL | 24.58 ± 0.722     |
| 10 n-Butanol 100 µg/mL | 37.92 ± 4.018     |
| 11 n-Butanol 50 µg/mL  | 48.75 ± 1.250     |
| 12 n-Butanol 25 µg/mL  | 58.33 ± 1.443     |
| 13 Dexta 5 µg/mL     | 1.25 ± 1.250      |
| 14 Dexta 2.5 µg/mL   | 3.75 ± 1.250      |
| 15 Control negative (LPS) | 63.75 ± 1.250   |
| 16 Control normal    | 0.83 ± 0.722      |

Sample and Plant Identification

_Piper crocatum_ Ruiz and Pav were taken on Kebun Bunga street Palembang, carried out identification of red betel plants at the Andalas University Herbarium (ANDA) Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University Padang with collection number 106/K-ID/ANDA/II/2020

Extraction and Fractionation

_Piper crocatum_ Ruiz and Pav dry powder (600 g) were macerated with methanol (MeOH) (3x2L) solvent for 48 hours. The macerate was evaporated in vacuo, and 93 g of thick extract was obtained. Eighty-three grams of the viscous extract was fractionated with n-hexane, ethyl acetate and n-butanol as solvents, and each solvent was evaporated in vacuo with a rotary evaporator to obtain the thick fraction of n-hexane (22 g), ethyl acetate fraction (20 g) and viscous fraction n-butanol (8 g).

Cell Culture

RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. Cell cultures were incubated at 37°C in, humidified atmosphere and 5% CO2 until the cells were confluent. Cells were then washed and harvested using trypsin-EDTA.

Cell Viability

Cell viability test was carried out using the MTT test method on 264.7 RAW cells. MTT assay is one of the quantitative tests to determine safe concentrations and is not toxic to cells used in research. The viability test results from MEPC, HFPC, EAFPC, and BFPC from red betel leaf showed a decrease in the number of live cells with increasing concentration. The concentration of the test substance is: 200; 100; 50; 25 and 12.5 g/mL. Dexamethasone as a positive control with a concentration of 20; 10; 5 and 2.5 g/mL.

Production of Nitric Oxide (NO)

The NO production test was carried out using the Nitric Oxide (NO) Colorimetric Assay Kit. Tests were carried out on MEPC, HFPC, EAFPC, and BFPC from red betel leaf at concentrations of 100, 50 and 25 g/mL, dexamethasone as a positive control at concentrations of 5 and 2.5 g/mL, negative control (LPS-induced cells), and standard control. (cells without LPS induction).

Graph of NO levels calculated from the n-hexane fraction, ethyl acetate fraction, butanol fraction, red betel leaf methanol extract and dexamethasone, normal and LPS-induced cells in RAW 264.7.

DISCUSSION

Results: Cell Viability

Cell viability test was carried out using the MTT method (3, 4, 5-dimethylthiazolo 1-2-1-il)-2,5-diphenyl tetrazolium bromide assay to determine the non-toxic concentration of the n-butanol, ethyl acetate, n-hexane and methanol extract of _Piper crocatum_ leaves Ruiz and Pav against 264.7 RAW cells. MTT assay is one of the quantitative tests to determine safe concentrations and is not toxic to cells used in research. The viability test results from MEPC, HFPC, EAFPC, and BFPC from _Piper crocatum_ Ruiz and Pav leaves showed that the concentrations that gave the viability percentage above 80% were 100, 50 and 25 g/mL were used as concentrations for the inhibition test of NO production. Dexamethasone as a positive control, the concentrations were chosen were 5 and 2.5 g/mL because they showed viability above 80%.

Production of Nitric Oxide (NO)

The test of NO production in RAW 264.7 cells was carried out by inducing RAW 264.7 cells with lipopolysaccharide (LPS). Lipopolysaccharide is a substance derived from the outer membrane of gram-negative bacteria, which is an endotoxin that stimulates the release of inflammatory mediators such as NO, prostaglandins, cyclooxygenase. NO is a product produced by macrophages activated by LPS. The resulting NO production is measured as the concentration of nitrite in the culture medium, and NO is very easily oxidized to nitric (NO2) in the solution. The formed NO2 is added with diazotizing reagent (sulfanilamide) in an acidic medium to form a diazonium salt that reacts with a coupling reagent to form a stable colored azo compound. Cells induced with LPS showed that the concentrations that gave the viability percentage above 80% were 100, 50 and 25 g/mL were used as concentrations for the inhibition test of NO production. The treated cells showed a decrease in the amount of NO. The treated cells showed a decrease in the amount of NO and BFPC with concentrations of 100, 50, 25 g/mL and positive control (dexamethasone) with the concentration of 5, 2.5 g/mL, negative control, and normal control. Then it was stimulated using LPS (1 g/mL) and then incubated again for 24 hours in a 5% CO2 incubator at 37°C. The amount of nitrite in the culture medium was measured as an indicator of NO production. The amount of nitrite was calculated using NO assay kit reagent (Catalog No: E-BC-K035-M). Then, 100 L of the culture supernatant was added to 100 L of NO reagent and then incubated for 10 minutes in a dark room. The absorbance was measured at 550 nm on a microplate reader. The nitrite concentration was calculated using the sodium nitrite standard curve.
The ethyl acetate fraction showed the most significant decrease in all the fractions tested for NO production in LPS-induced RAW 264.7 cells. The analyzed measurement data from each fraction was analyzed with one-way ANOVA statistics, showing different responses between treatment groups p<0.05 (p=0.000). The analysis continued with Duncan’s test showing the ethyl acetate fraction with 100, 50 and 25 g/mL concentration, giving the highest effect. This shows the ability of FEAPC at concentrations of 100, 50 and 25 g/mL to reduce NO production induced by larger LPS compared with EMPC, HFPC and FBPC at the same concentration.

CONCLUSION

Giving MEPC, HFPC, EAPC, and BFPC of red betel leaf to RAW 264.7 cells induced by lipopolysaccharide at concentrations of 100 g/mL, 50 g/mL, 25 g/mL can reduce NO levels.

FEAPC from red betel leaf gave the best effect in reducing NO levels in RAW 264.7 cells induced by lipopolysaccharide.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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GRAPHICAL ABSTRACT

Leaves of *Piper crocatum*

Cell viability
MTT
Non-toxic concentration

MEPC, HFPC, EAFPC and BFPC
LPS-induced RAW 264.7 macrophage cells.
Nitric oxide (NO) production inhibition

NO levels of MEPC, HFPC, EAFPC, and BFPC, dexamethasone, and normal cell in LPS-induced RAW 264.7 cells
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