

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

Nilda Lely^{1,3}, Helmi Arifin², Yufri Aldi², Fatma Sri Wahyuni^{2,*}

Nilda Lely^{1,3}, Helmi Arifin², Yufri Aldi², Fatma Sri Wahyuni^{2,*}

¹Postgraduate Student, Faculty of Pharmacy, Universitas Andalas, Padang, Sumatera Barat 25163, INDONESIA.

²Faculty of Pharmacy, Universitas Andalas, Padang, Sumatera Barat 25163, INDONESIA.

³STIFI Bhakti Pertiwi Palembang. Jl. Ariodillah III No.22A, RT.27/RW.9, 20 Ilir D. IV, Ilir Tim. I, Kota Palembang, Sumatera Selatan 30128, INDONESIA.

Correspondence

Fatma Sri Wahyuni

Faculty of Pharmacy, Universitas Andalas, Kampus Limau Manis, Padang 25163, INDONESIA.

E-mail: fatmasriwahyuni@phar.unand.ac.id

History

- Submission Date: 26-07-2021;
- Review completed: 12-08-2021;
- Accepted Date: 23-08-2021.

DOI : 10.5530/pj.2021.13.169

Article Available online

<http://www.phcogj.com/v13/i6>

Copyright

© 2021 Phcogj.Com. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

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 ug/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 NO Colorimetric Assay Kit as measured 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 g/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, proinflammatory 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.^{5,6,7} The ethanol extract of *Piper crocatum* has an antioxidant effect.⁸ *Piper crocatum* contains various secondary metabolites. Identifying phytochemical components from red betel leaf contains flavonoids, alkaloids, tannin-polyphenols, steroids, terpenoids and saponins.^{9,10}

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 (Dexamedica), NO Colorimetric Assay Kit (Elabscience),

Cite this article: Lely N, Arifin H, Aldi Y, Wahyuni FS. 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. Pharmacogn J. 2021;13(6): 1341-1346.

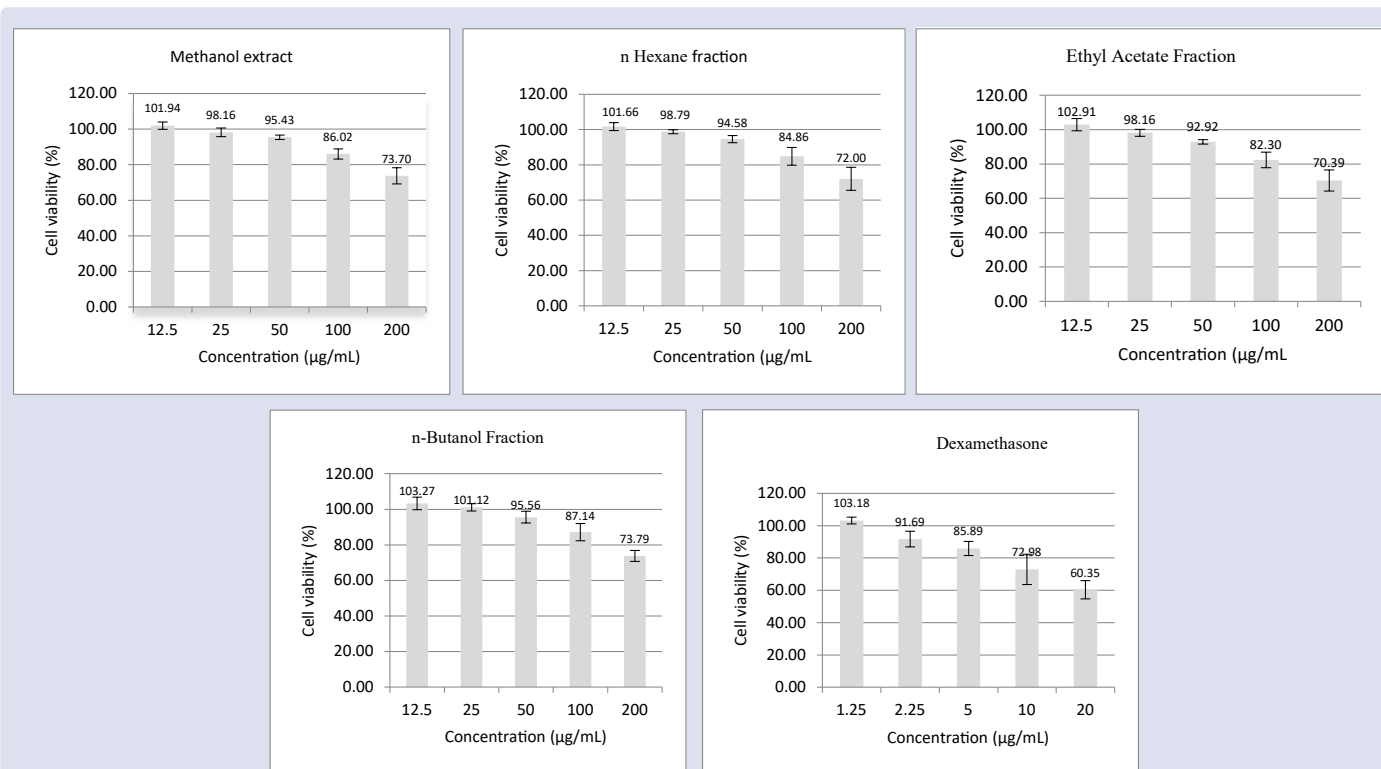


Figure 1: The mean percentage of live cells of MEPC, HFPC, EAFPC, and BFPC of *Piper crocatum* Ruiz & Pav leaf and dexamethasone in RAW cells 264.7.

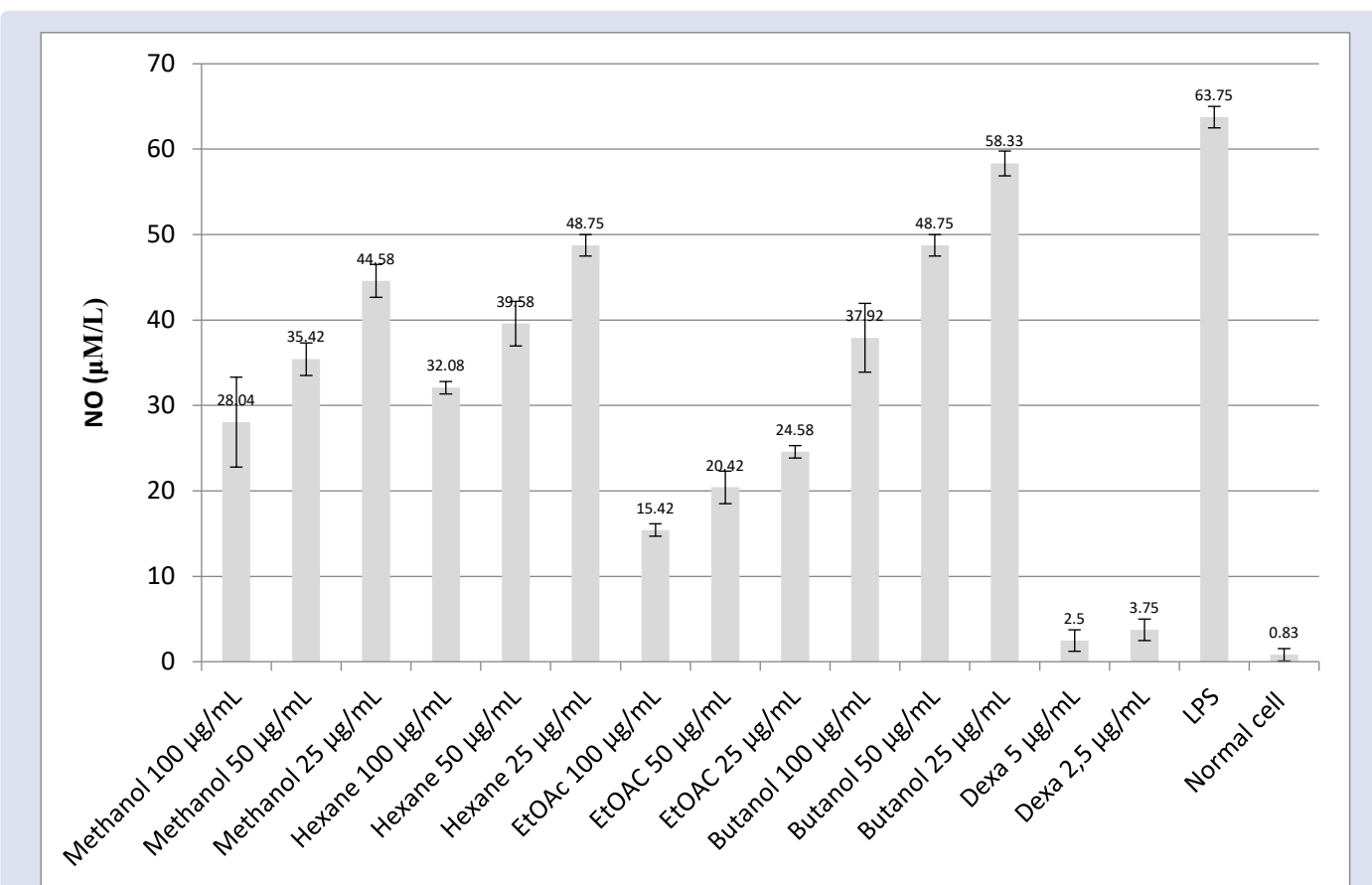


Figure 2: NO levels of MEPC, HFPC, EAFPC, and BFPC of red betel leaf, dexamethasone, negative control, in LPS-induced RAW 264.7 cells, and normal cells.

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 Dexa 5 μg/mL	1,25 ± 1,250
14 Dexa 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.¹¹

Cell Viability

A cell viability test was carried out using the MTT test method on 264.7 RAW cells. A total of 1 x 10⁴ cells per well were plated in 96 wells and then incubated for 24 hours at 37°C. After 24 hours the medium was replaced with a new one and then added test solution (MEPC, HFPC, EAFPC, and BFPC), with a concentration of 200; 100; 50; 25; 12.5 g/mL and dexamethasone (20; 10; 5; 2.5; 1.25 g/mL). Incubate for 24 hours at 37°C in a 5% CO₂ incubator. At the end of incubation, the test medium and solution were removed, and the cells were washed with PBS. To each well, add 100 l of culture medium and 10 L of 5 mg/mL MTT to each solution and incubate again for 4 hours. The formazan crystals formed were dissolved in 100 L DMSO. Absorption was measured using a microplate reader at a wavelength of 570 nm.^{12,13}

Nitric Oxide (NO) production test

RAW 264.7 cells (1x10⁴ cells/well) were grown on 96-well plates and incubated for 24 hours, added test solutions (MEPC, HFPC, EAFPC,

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.¹⁴

RESULTS

Cell Viability

Cell viability test of RAW 264.7 on the administration of MEPC, EAFPC, HFPC 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 cells 264.7.

DISCUSSION

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%.^{15,16}

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.¹⁷ Cells induced with LPS released higher NO into the medium than cells not treated with LPS.¹⁸ The treated cells showed a decrease in the amount of NO. Treatment of cells with MEPC, HFPC, EAFPC, and BFPC at concentrations of 100, 50 and 25 g/mL showed the ability to reduce NO production.

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, FHPC and FBPC at the same concentration.

CONCLUSION

Giving MEPC, HFPC, EAFPC, 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.

ACKNOWLEDGEMENTS

This research was funded by the Directorate of Resources, Directorate General of Higher Education, Ministry of Education, Culture, Research and Technology through the multiple year basic research and capacity building in Higher Education 2021 with decree no 09/E1/KPT/2021 with main contracts no 104/E4.1/AK.04.PT/2021 and derivative contract T/5/UN.16.17/PT.01.03/PDD-Kesehatan/2021

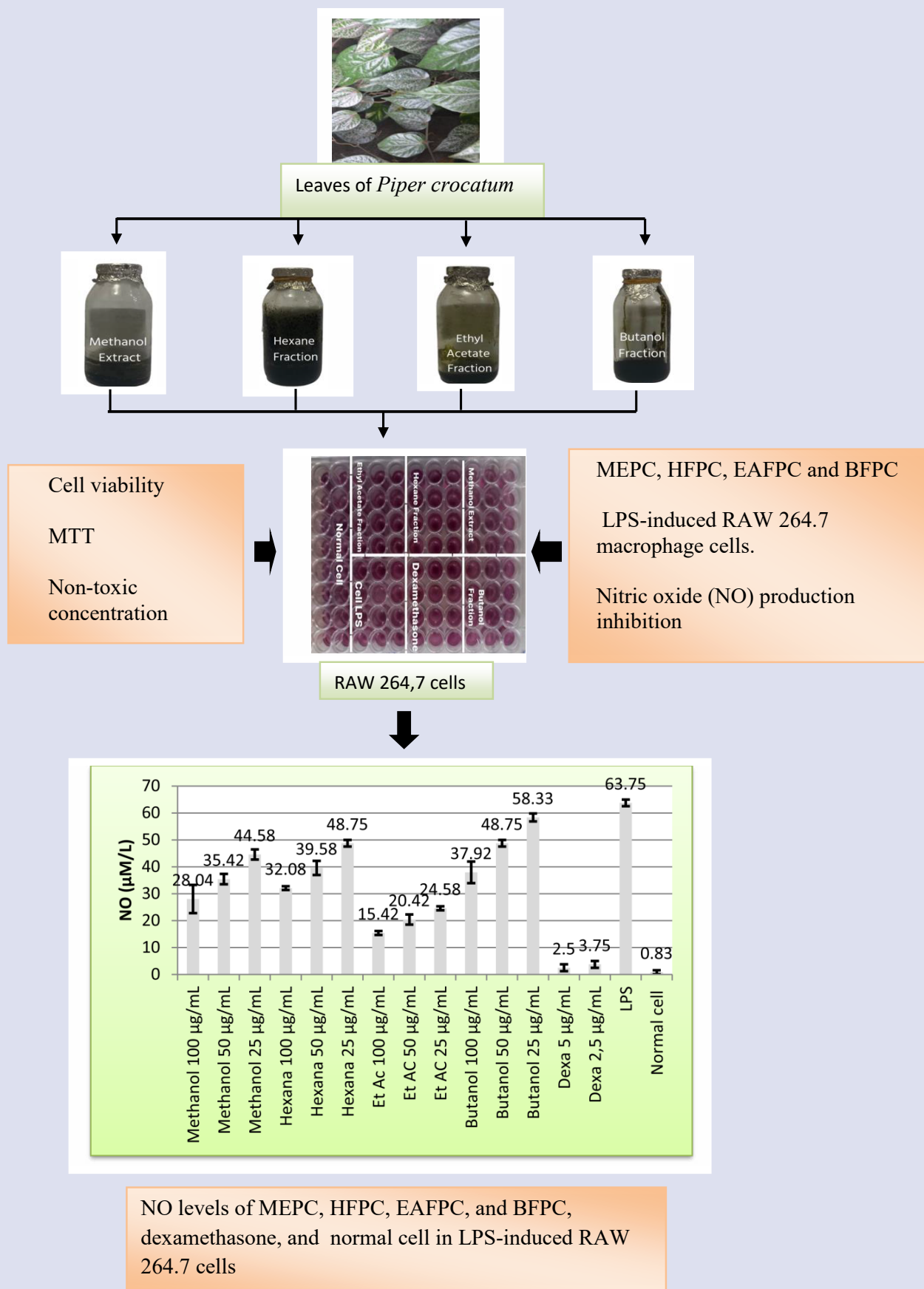
CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

1. Katzung BG. *Basic & Clinical Pharmacology*. 14th ed. New York, 2018.
2. Maldonado RF, Isabel S, and Valvano MA. Lipopolysaccharide modification in Gram-negative. *FEMS Microbiol Rev*. 2016; 40(4): 480-493.
3. Guzik TJ, Korbut R, and Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol*. 2003; 54(4): 469-487.
4. Manoi F. Sirih Merah sebagai Tanaman Obat Multifungsi. *War. Puslitbangbun*. 2007; 13(2): 275.
5. Fitriyani A, Winarti L, Muslichah S, and Nuri D. Uji antiinflamasi ekstrak metanol daun sirih merah (*Piper crocatum* Ruiz & Pav) pada tikus putih. *Maj Obat Tradis*. 2011; 16(1): 2011.
6. Wahjuni S, Wita IW, and Astawa M. Anti-Inflammatory Effect of Red Piper Crocatum Leaves Extract Decrease TNF- α and IL-6 Levels in Wistar Rat with Atherosclerosis. *Bali Med J*. 2016; 5(2): 51-56.
7. Ehrich IN, Novalinda C, Girsang E, Dea E, Mardhotillah A, and Widowati W. Hepatoprotective properties of red betel (*Piper crocatum* Ruiz and Pav) leaves extract towards H₂O₂-induced HepG2 cells via anti-inflammatory, antinecrotic, antioxidant potency. *Saudi Pharm J*. 2020; 28(10): 1182-1189.
8. Alfarabi M, Bintang M, Suryani, and Safithri MEGA. The Comparative Ability of Antioxidant Activity of *Piper crocatum* in Inhibiting Fatty Acid Oxidation and Free Radical Scavenging. *HAYATI J Biosci*. 2010; 17(4): 201-204.
9. Parfati N, Windono T. Sirih Merah (*Piper crocatum* Ruiz & Pav.) *Kajian Pustaka Aspek Botani, Kandungan Kimia, dan Aktivitas Farmakologi*. *Media Pharmaceutica Indonesiana* 2016; 1(2): 106-115
10. Arbain D, Nofrizal, Syafni N, Ismed F, Yousuf S, and Choudhary MI. Bicyclo[3.2.1]octanoid neolignans from Indonesian red betel leaves (*Piper crocatum* Ruiz & Pav.). *Phytochem Lett*. 2018; 24: 163-166.
11. Hwang JH, Ma JN, Park JH, Jung HW, and Park YK. Anti-inflammatory and antioxidant effects of MOK, a polyherbal extract, on lipopolysaccharide-stimulated RAW 264.7 macrophages. *Int J Mol Med*. 2019; 43(1): pp 26-36.
12. Zheng L, Wang M, Peng Y, and Li X. Physicochemical Characterization of Polysaccharides with Macrophage Immunomodulatory Activities Isolated from Red Ginseng (*Panax ginseng* C. A. Meyer). *J Chem*. 2017: 1-8.
13. Fan C, Wu LH, Zhang GF, Xu F, Zhang S, Zhang X, Sun L, Yu Y, Zhang Y, Ye RD. 4'-Hydroxywogonin suppresses lipopolysaccharide-induced inflammatory responses in RAW 264.7 macrophages and acute lung injury mice. *PLoS One*. 2017; 12(8): 1-17.
14. He J, Lu X, Wei T, Dong Y, Cai Z, Tang L, Menghua Liu M. Asperuloside and Asperulosidic Acid Exert an Anti-Inflammatory Effect via Suppression of the NF- κ B and MAPK Signaling Pathways in LPS-Induced RAW 264.7 Macrophages. *Int J Molecular Sci*. 2018; 19: 2027
15. Hikariastri P, Winarno H, Kusmardi K, Laksmiawati DR, and Abdillah S. Aktivitas Antiinflamasi Crude Extract Fukoidan dari *Sargassum crassifolium* pada Sel RAW 264.7 yang Diinduksi LPS. *Jurnal Kefarmasian Indonesia*. 2019; 9(2): 97-105.
16. Laksmiawati DR, Widyastuti A, Karami, Afifah E, Rihiba DD, Nufus H, Woidowati W. Anti-inflammatory effects of *Anredera cordifolia* and piper crocatum extracts on lipopolysaccharide-stimulated macrophage cell line. *Bangladesh J Pharmacol*. 2017; 12(1): 35-40.
17. Sun J, Zhang X, Broderick M, Fein H. Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. *Sensors*. 2003; 3(8): 276-284
18. Joo T, Sowndhararajan K, Hong S, Lee J, Park SY, Kim S, Jhoo JW. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L. *Saudi J Biol Sci*. 2014; 21(5): 427-435.

GRAPHICAL ABSTRACT



ABOUT AUTHORS



Nilda Lely She is a student of the Faculty of Pharmacy, Andalas University who has been involved in research studying the activity of the 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.



Prof. Helmi Arifin, is a Professor of Pharmacology at the Faculty of Pharmacy, Andalas University, Indonesia. He is the former dean of the faculty of Pharmacy. His research is mostly focusing on the investigation of medicinal plants effect for disease complications.



Prof. Dr. Yufri Aldi, M.Si, Apt. He is as a lecturer at the Faculty of Pharmacy, Andalas University. Graduated from the Faculty of Pharmacy, Andalas University in 1989, then the Master's Program in 1994 at the Bandung Institute of Technology School of Pharmacy (ITB) and the Doctoral Program in the Biomedical Department, Faculty of Medicine, Andalas University in 2013. Research and expertise in Pharmacolmmunology. Currently working as Head of the Department of Pharmacy Doctoral Program, Faculty of Pharmacy, Andalas University



Prof. Dr. Fatma Sri Wahyuni, Apt. She is a lecturer at the Faculty of Pharmacy, Andalas University. Got her undergraduate degrees from Andalas University in 1998 and finished her PhD from University Putra Malaysia in 2010. Her research is focusing on pharmacological activity studies of Sumatran Plants.

Cite this article: MLely N, Arifin H, Aldi Y, Wahyuni FS. 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. *Pharmacogn J.* 2021;13(6): 1341-1346.