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Anti-Inflammatory Effect of Isolate Compounds from Ethyl Acetate Fraction of *Piper crocatum* Ruiz and Pav Leaves on Lipopolysaccharide-induced RAW 264.7 Cells

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ABSTRACT

Red betel (*Piper crocatum* Ruiz and Pav) is a traditional Indonesian plant. The ethyl acetate fraction of red betel leaf has the potential as an anti-inflammatory. This research aimed to determine the anti-inflammatory effect of isolate compound from ethyl acetate fraction of *Piper crocatum* leaves on the production of cytokine pro-inflammatory TNF-α and ICAM-1 in lipopolysaccharide-induced RAW 264.7 cells. Cell viability was determined by the MTT method in the concentration range of 1.25; 2.5; 5; 10; 20, and 40 µg/ml. The TNF-α and ICAM-1 level was determined by the TNF-α and ICAM-1 assay kit as measured by an ELISA plate reader. Cell viability test of isolate compound from ethyl acetate fraction, the concentrations that gave viability percentages above 80% were 10; 5; 2.5 and 1.25 ug/ml. Isolate compound at concentrations of 10, 5, 2.5 and 1.25 ug/ml significantly inhibited TNF-α and ICAM-1 production with $p<0.05$ ($p = 0.000$).

1. Introduction

Inflammation involves the innate and adaptive immune systems as a normal response to injury and infection. Inflammation that is allowed to continue can cause autoimmune disorders, auto-inflammation, neurodegenerative diseases, and cancer (Dinarello 2010). The emergence of a varied inflammatory response depends on various factors, namely the initial stimulus or trigger, the type of cell involved in the response, and the specific effector cytokines produced (Yang *et al.* 2007).

Cytokines are soluble glycoproteins that are produced by and mediate communication between and within immune and nonimmune cells, organs and organ systems throughout the body. Pro- and anti-inflammatory mediators constitute the inflammatory cytokines, which are modulated by various stimuli, including physical activity, trauma and infection. Physical activity affects local and systemic cytokine production at different levels, often exhibiting striking similarity to the cytokine response to trauma and infection. TNF-α is primarily a product of mononuclear phagocytes, but is also produced by T lymphocytes, Kupffer cells, nerve cells and endothelium (Moldoveanu *et al.* 2001). Tumour necrosis factor-alpha (TNF-α) is a well-known pro-inflammatory cytokine responsible for modulating the immune system. TNF-α plays an important role in almost every type of inflammatory disorder (Subedi *et al.* 2019).

Intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule that is increased by inflammatory stimuli. ICAM-1 is well known for mediating leukocyte adhesion to endothelial cells and guiding leukocytes across the vessel wall. Macrophages have been shown to express ICAM-1. ICAM -1 expression is induced during the polarization of inflammatory macrophages. Many macrophages expressing ICAM-1 are noted in inflamed colon tissue in models of murine colitis and human inflammatory bowel disease (Wiesolek *et al.* 2020).

Red betel (*Piper crocatum*) is a traditional Indonesian plant. In previous studies, it has been reported that red betel leaf extract has been shown to have anti-inflammatory effects (Wahjuni and Astawa 2016), (Ginting *et al.* 2021), antidiabetic (Wahjuni *et al.* 2017), antioxidants (Alfarabi *et al.* 2010), antirheumatism (Maslikah *et al.* 2019). From identifying phytochemical

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components, red betel leaf is reported to contain flavonoid, alkaloid, tannin-polyphenolic compounds, steroids, terpenoids and saponins (Lely *et al.* 2021). Compounds that have been isolated from red betel leaf include pipercroside A and B phenolic glycosides (Li *et al.* 2019); sitosterol and 2-(5', 6' dimethoxy-3', 4'-methylenedioxyphenyl)-6-(3", 4", 5"-trimethoxyphenyl)-3,7-dioxabicyclo [3,3,0] octane (Emrizal *et al.* 2014), compound bicyclo[3.2.1]octanoid neolignans of guianin-type named as, (1′R, 2′R, 3′S, 7S, 8R)-Δ5′, 8′-2-acetoxy-3, 4, 5, 3′, 5′-pentamethoxy-4′-oxo-8.1′,7.3′-neolignan (crocatin A) and (1′R, 2′R, 3′S, 7S, 8R)-Δ5′, 8′-2′-hydroxy-3, 4, 5, 3′, 5′-pentamethoxy-4′-oxo-8.1′,7.3′-neolignan (crocatin B) (Arbain *et al.* 2018).

2. Materials and Methods

2.1. Plant Material

Daun *Piper crocatum* Ruiz and Pav were taken on Sukarami, 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.

2.2. Chemicals and Reagents

Methanol, *n*-hexane, ethyl acetate, *n*-butanol, silica gel (63-200 µm particle size) (Merck), TLC plate, DMSO, RAW 264.7 cells, Dulbecco's Modified Eagle Medium (DMEM) (Gibco), penicillin-streptomycin (Gibco), Fetal Bovine Serum (FBS) (Sigma Aldrich), Phosphate buffered saline (PBS) (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (eBioscience), dimethylsulfoxide, LPS form *Escherichia coli* (eBioscience Cat. No 00-4976-93). wash buffer (eBioscience), dexamethasone (Dexamedica), Mouse TNF-α ELISA Kit (BT LAB Cat.NoE0117Mo), Mouse ICAM-1 ELISA Kit (BT LAB Cat.NoE0039Mo.

2.3. Isolation

Red betel leaves for 2.5 kg were dried, then powdered and macerated with methanol (5 L) for 48 h. The macerate was evaporated using a rotary vacuum evaporator. The viscous extract was fractionated with *n*-hexane, ethyl acetate, and *n*-butanol as solvents, and each solvent was evaporated using a rotary vacuum evaporator. The separation of the main compound from the ethyl acetate fraction (15 g) was carried out using column chromatography. The stationary phase used silica gel (63-200 µm particle size) (Merck), and the mobile phase used a combination of *n*-hexane and ethyl acetate with a step gradient polarity system. Recrystallization using ethyl acetate and *n*-hexane. The isolated compound obtained is 510 mg.

2.4. Isolate Characterization

TLC examination was carried out to show the purity and determine the value of the isolated compound's retention factor (Rf) with the appropriate mobile phase. The physicochemical examination includes the examination of compounds with melting points, ultraviolet spectrophotometry, infrared spectrophotometry, High-Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance spectrophotometry (^{13}C NMR and ^{1}H NMR) (Silverstein *et al.* 2005).

2.5. Cell Culture

RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS and 1% penicillin-streptomycin solution. Cell cultures were incubated at 37°C, with a humidified atmosphere and 5% CO_2 until the cells were confluent. RAW 264.7 cells were 70% confluent, and then the old medium was discarded and washed two times using 5 ml of phosphate buffer (PBS). Two ml of growth medium was added to the cells, and the resuspended cells were then transferred to a 15 ml tube. Cells were centrifuged at 1,000 rpm for 5 minutes. The supernatant was then discarded, and the pellet was resuspended with 1 ml of growing medium and then counted using an automatic cell counter (Jeong and Lee 2018).

2.6. Compound Isolate Viability Test

A cell viability test was carried out using the MTT method to determine the concentration of the test, which was not toxic to RAW 264.7 cells. A total of 1 x 10^4 per well was planted in 96 wells and then incubated for 24 hours at 37°C. After 24 hours, it was replaced with a new one, then a solution of the isolate compound with a concentration of was added 40; 20; 10; 5; 2.5; 1.25 µg/ml and dexamethasone (10; 5; 2.5; 1.25 µg/ml). Incubate at 37°C in a 5% CO_2 incubator for 24 hours. At the end of incubation, the test medium and solution were removed, and the cells were washed

with PBS. Add 100 µl of culture medium and 10 µl of 5 mg/ml MTT solution to each well and incubate again for 4-6 hours. The formazan crystals formed were dissolved in 100 µl DMSO. Absorption was measured using a microplate reader at a wavelength of 570 nm. Cell viability was expressed in per cent by comparing the uptake of the test material group with the control group. Each concentration was replicated three times and the data presented was the mean percentage viability ± standard deviation (SD) (Huang *et al.* 2018).

2.7. Inflammatory Activation (LPS induction)

RAW 264.7 cells were grown as much as 1 x 10^4 cells per well in a 96-well plate and incubated for 24 hours at 37°C. The medium was discarded, and a new medium was added to the cells, and 200 L of isolate compound was added at a concentration of 10; 5; 2.5 and 1.25 µg/ml, dexamethasone 5; 2.5 and 1.25ug/ml in the medium and cells without adding the test compound as a negative control, then incubated for 2 hours. A total of 200 µl of LPS solution with a 1 µg/ml concentration was added to each well and incubated again for 24 hours. The medium was taken and centrifuged at 2,000 x g for 20 minutes at 2-8°C (Cheng *et al.* 2017). The supernatant portion was taken and stored at -80°C for measurement of TNF-α and ICAM-1 levels.

2.8. Measurement of TNF-α and ICAM-1 Levels

Cytokine levels were measured by the sandwich ELISA method using the BT Lab ELISA kit. Add 50 µl of the standard antibody solution to each well plate (unless the standard solution wells already contain biotinylated antibodies, add 40 µl of the sample to each pre-coated well, and add 10 µl of the specific antibody. (TNF-α and ICAM-1), then added 50 µl of streptavidin-HRP (horseradish peroxidase) to each sample and standard well, except control and mixed. Covered and incubated at 37°C for 60 minutes. Liquid from each well was discarded, then washed with wash buffer solution five times. Soak each well with 0.35 ml of wash buffer solution for 30 seconds to 1 minute for each wash. Add 50 µl of substrate solution A to each well and then add 50 µl of substrate solution B. Incubate the plates for 10 minutes at 37°C in the dark. Add 50 µl of stop solution to each well. After 10 minutes, determine the optical value for each well's density (OD) was measured immediately using a microplate reader at a wavelength of 450 nm. The test was replicated three times and the data presented was the mean ± SD (BT Lab).

2.9. Statistical Analysis

Statistical analysis was conducted using SPSS software (version 26.0). Significant differences between the groups were determined using the analysis of variance (ANOVA) followed by Duncan post hoc test.

3. Results

The results of the characterization of isolate compounds:

a. TLC examination

The results of the TLC examination of silica gel GF 254 with a mixed mobile phase of ethyl acetate: *n*-hexane (3:2), the value of the retention factor (R_f) of the isolated compound was 0.403.

b. Physicochemical Examination

The results of HPLC examination of compound isolates with a mixed mobile phase of acetonitrile: H_2O (70:30) showed a retention time of 2,564 minutes with a per cent area of 98%. The UV-Vis spectrum in methanol showed an absorption peak at 265.4, 236.6, and 205.8 nm. The infrared spectrum shows the absorption peaks at wavenumbers 3431, 2937, 1616, 1588, 1507, 1683, 1375, 1616 and 623 cm^{-1}.

c. Examination of ^{13}C NMR and ^{1}H NMR

The interpretation results of the ^{13}C NMR and ^{1}H NMR spectrum of the isolated compound from the ethyl acetate fraction of red betel leaf showed that the chemical shift of ^{13}C NMR and ^{1}H NMR was relatively the same as the chemical shift of ^{13}C NMR and ^{1}H NMR of crocatin B compounds that had been previously reported (Table 1).

Table 1. Chemical shift values of ^{13}C NMR and ^{1}H NMR isolate compound and crocatin B

Position	^{13}C NMR isolate compound	^{1}H NMR isolate compound	^{13}C NMR crocatin B	^{1}H NMR crocatin B
4′	192.2		192.1	
3	152.9		152.8	
5	152.9		152.8	
5′	152.5		152.4	
4	137.0		136.9	
8′	134.4	5.90 (^{1}H, m)	134.3	5.90 (^{1}H, m)
1	133.6		133.5	
6′	125.3	6.17 (^{1}H, s)	125.2	6.17 (^{1}H, s)
9′	118.8	5.30 (^{1}H, d, Jtrans = 17.0 Hz) 5.21 (^{1}H, d, Jcis = 10.0 Hz)	118.7	5.30 (^{1}H, d, Jtrans = 17.0 Hz) 5.21 (^{1}H, d, Jcis = 10.0 Hz)
2	106	6.26 (^{3}H, s)	105.9	6.26 (^{3}H, s)
6	106	6.26 (^{3}H, s)	105.9	6.26 (^{3}H, s)
3′	95.8		95.6	
2′	78.9	4.01 (^{1}H, s)	78.8	4.01 (^{1}H, s)
4-OCH3	60.8	3.77 (^{3}H, s)	60.7	3.77 (^{3}H, s)
7	59.5	3.33 (^{1}H, d, J = 6.5 Hz)	59.4	3.33 (^{1}H, d, J = 6.5 Hz)
3-OCH3	55.9	3.78 (^{3}H, s)	55.8	3.78 (^{3}H, s)
5-OCH3	55.9	3.78 (^{3}H, s)	55.8	3.78 (^{3}H, s)
3′-OCH3	55.4	3.68 (^{3}H, s)	55.3	3.68 (^{3}H, s)
5′-OCH3	54.6	3.43 (^{3}H, s)	54.5	3.43 (^{3}H, s)
8	49.1	2.24 (^{1}H, m)	49.0	2.24 (^{1}H, m)
1′	49.0		48.9	
7′	34.8	2.77 (^{1}H, m) 2.41 (^{1}H, m)	34.7	2.77 (^{1}H, m) 2.41 (^{1}H, m)
9	17.5	1.30 (^{3}H, d, J = 7.0 Hz)	17.4	1.30 (^{3}H, d, J=7.0 Hz)

3.1. Viability Isolate Compound

The data of optical density measurement of red betel leaf compound isolates in RAW 264.7 cells at a concentration of 40; 20; 10; 5; 2.5 and 1.25 µg/m and the measurement data of dexamethasone as a positive control with a concentration of 5; 2.5; 1.25 µg/mL. Cell viability is determined based on the formula.

$$\text{Viability cell} = \frac{\text{Cell absorbance by treatment} - \text{Absorbance control media}}{\text{Cell control absorbance} - \text{Media control absorbance}} \times 100\%$$

The data from the calculation of cell viability of isolated compounds from red betel leaf in RAW 264.7 cells at a concentration of 40; 20; 10; 5; 2.5 and 1.25 µg/ml and cell viability calculation data of dexamethasone at a concentration of 10; 5; 2.5 and 1.25 µg/ml (Table 2).

3.2. TNF-α and ICAM-1 Levels

Measurements were carried out on isolate compounds at a concentration of 10; 5; 2.5, and 1.25 µg/ml, dexamethasone concentrations of 5 and 2.5 µg/ml, normal cells, and cells induced by LPS. The results of the TNF-α and ICAM-1 level from isolate compounds, dexamethasone, normal cells, and cells induced by LPS (Table 3).

4. Discussion

The results of the HPLC chromatogram of compound isolates from red betel leaf using a mixed mobile phase of acetonitrile:H20 (70:30) showed a retention time of 2,564 minutes with a purity level of 98%. The interpretation of the ultraviolet spectrum of the compound isolate in methanol solvent showed the maximum absorption at a wavelength of 265.4; 236.6, and 205.8 nm. The maximum absorption

Table 2. The average value of the percentage of live cells from the administration of compound isolates from the ethyl acetate fraction of red betel leaves and dexamethasone in RAW 264.7 cells

Concentration of isolate compound (µg/ml)	Per cent of viability cells± standard deviation	Concentration of dexamethasone µg/ml	Per cent of viability cells± standard deviation
40	9.173±2.321	10	71.163±4.787
20	45.095±6.155	5	86.149±2.349
10	80.654±4.819	2.5	95.096±5.785
5	89.192±6.664	1.25	102.134±2.345
2.5	95.005±5.235		
1.25	99.909±2.636		

Table 3. The average level of TNF-α and ICAM-1 from the effect of administration compound isolates from the ethyl acetate fraction of red betel leaves and dexamethasone, normal and LPS-induced cells on RAW 264.7 cells

Treatment Group (µg/ml)	Average level TNF-α (ng/L)	Average level ICAM-1 (ng/L)
Isolate compound 10	305.287±26.892^{c}	289.653±12.891^{b}
Isolate compound 5	439.269±17.218^{d}	374.041±45.117^{c,d}
Isolate compound 2,5	557.787±23.156^{e}	386.699±43.099^{d}
Isolate compound 1,25	627.509±21.669^{f}	471.087±17.567^{e}
dexamethasone 5	168.528±8.902^{b}	275.588±20.814^{b}
dexamethasone 2.5	338.620±19.113^{c}	334.660±12.891^{c}
Cell + LPS	802.694±26.005^{g}	821.298±21.652^{f}
Normal cell	26.398±4.252^{a}	50.553±11.164^{a}

The superscript letter ($^{a-g}$) in each column indicates significance different among treatments based on Duncan pos hoc test with $p<0.05$ is considered as significantly different

wavelength of the UV spectrum indicates that the isolated compound isolated from red betel leaf has several chromophore groups responsible for electron excitation (Silverstein *et al.* 2005). The interpretation of the infrared spectrum of the red betel leaf isolate showed an absorption band at 3431 cm^{-1} indicating the presence of an OH group, an absorption band at 2937 cm^{-1} indicating the presence of a CH group, absorption bands at 1616, 1588, and 1507 cm^{-1} indicating the presence of a C group. = C aromatic, the absorption band at 1683 cm^{-1} indicates the presence of C = O groups, the absorption band at 1375 cm^{-1} indicates the presence of aromatic CH groups, the absorption band at 1616 cm^{-1} indicates the presence of C = C alkenes. The absorption band at 623 cm^{-1} indicates the presence of an aromatic CH group (Silverstein *et al.* 2005). Meanwhile, from the ^{13}C NMR and ^{1}H NMR spectrums of isolates from red betel leaf, the chemical shifts of the ^{13}C NMR and ^{1}H NMR spectra showed relatively the same values as the chemical shifts of the ^{13}C NMR and ^{1}H NMR spectra of crocatin B compounds, which are compounds belonging to the neolignan group (Table 1) (Arbain *et al.* 2018). Neolignans are compounds that have the potential as anti-inflammatory compounds (Santos *et al.* 2019; Shih *et al.* 2016).

Cell viability test for compound isolates was carried out using the MTT method (3, (4, 5-dimethyltiazo l-2-1-il)-2,5-diphenyl tetrazolium bromide) assay with the aim of determining the concentration that is not toxic to RAW 264 cells, 7. MTT assay is one of the quantitative tests to determine the concentration that is safe and non-toxic to the cells used in the test. This test is based on the colour intensity (colourimetry) formed as a result of substrate metabolism by living cells into coloured products. The MTT salt will be reduced to formazan by the tetrazolium succinate reductase system and the mitochondria of living cells. Only metabolically active living cells can reduce MTT to form formazan. The greater the colour intensity of the medium, the more viable cells (Zheng *et al.* 2017). The formazan crystals formed were dissolved in 100 µl DMSO. Absorption was measured using a microplate reader at a wavelength of 570 nm (Huang *et al.* 2018).

The concentration of the isolate compound used in the viability test was 40; 20; 10; 5; 2.5 and 1.25 µg/ml and dexamethasone as positive controls at concentrations of 10; 5; 2.5; 1.25 µg/ml (Table 2). The results obtained showed a correlation between the increase in the concentration of isolate compound with the effect of cell viability caused. The viability test results showed that the higher the concentration of the isolate compound, the smaller the cell viability value. The results of the viability test of the compound isolates showed that the concentration that gave the

percentage of viability above 80% was concentration 10; 5; 2.5 and 1.25 µg/ml. This concentration was used to test the inhibition of TNF-α and ICAM-1 production. Dexamethasone as a positive control, the concentrations selected were 5 and 2.5 µg/ml because they showed viability above 80%. Testing of TNF-α levels in RAW 264.7 cells was carried out by inducing RAW 264.7 cells with lipopolysaccharide (LPS). Lipopolysaccharide is a substance derived from gram-negative bacteria's outer membrane, an endotoxin that activates macrophages to release proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 (Maldonado *et al.* 2016). TNF-α is a major cytokine in the acute inflammatory response to gram-negative bacteria and other microbes. Severe infections can trigger the production of large amounts of TNF-α causing systemic reactions. LPS is a potent stimulus for macrophages to secrete TNF-α. TNF-α levels in RAW 264.7 cells induced by LPS showed an increase in TNF-α compared to normal cells group. Administration of isolated compounds significantly reduced TNF-α levels at a concentration of 2.5; 5, and 10 µg/ml compared with LPS-induced cell groups.

Examination of ICAM-1 levels in RAW 264.7 cells was also carried out by inducing RAW 264.7 cells with lipopolysaccharide (LPS). Lipopolysaccharides increase the expression of the adhesion molecule ICAM-1 on the surface of endothelial cells and cause leukocyte migration in the inflammatory reaction (Wiesolek *et al.* 2020). The calculation data for ICAM-1 levels in RAW 264.7 cells induced by LPS showed an increase in the number of ICAM-1, which was significant compared to normal cells. The administration of isolate compound A at concentrations of 10; 5; 2.5, and 1.25 µg/ml in LPS-induced RAW 264.7 cells could reduce ICAM-1 levels compared to RAW 264.7 cells induced only by LPS.

Administration of isolated compounds from red betel leaf can significantly reduce levels of TNF-α and ICAM-1 compared to the group of cells induced by LPS. The percentage decrease in TNF-α and ICAM-1 level against the group of cells induced by LPS (Figure 1) indicates that the isolated compound treatment group had lower TNF-α and ICAM-1 level. Decreased levels of TNF-α and ICAM-1, which are pro-inflammatory cytokines, indicating that the isolated compound can inhibit the production of pro-inflammatory cytokines TNF-α and ICAM-1 and have potential as anti-inflammatory compounds.

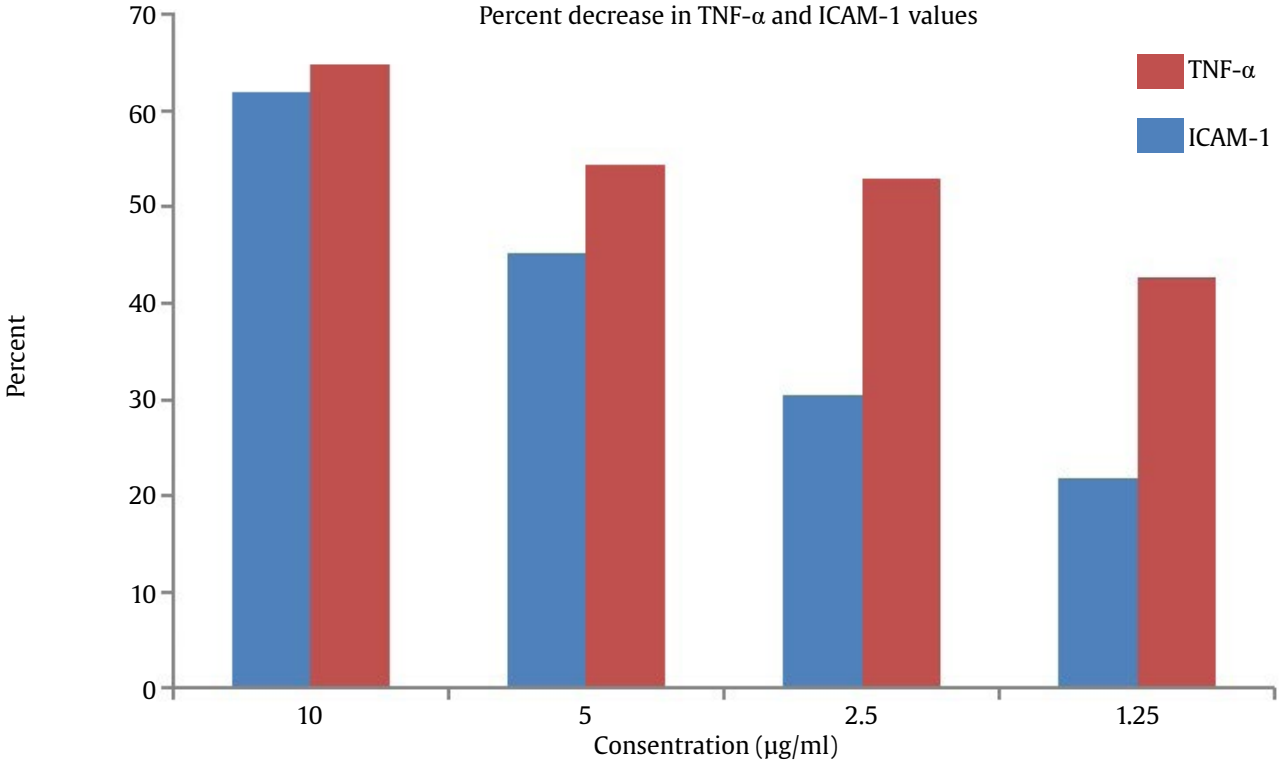

Figure 1. Diagram of the percentage decrease in the average value of TNF-α and ICAM-1 levels against cells induced with LPS

In conclusion, administration of isolated compounds from the ethyl acetate fraction of red betel leaf can reduce the production of TNF-α and ICAM-1 in RAW cells induced by LPS.

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