



In vitro evaluation of selligueain A effects on the pro-inflammatory mediators production in RAW264.7 murine macrophages

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ABSTRACT

Introduction: Selligueain A derived from the roots of *Polypodium feei* was shown to have anti-inflammatory activity, which was tested in vivo on the rats' paw edema induced by carrageenan. The aim of this study was to evaluate the anti-inflammatory mechanism of selligueain A in vitro against the production of pro-inflammatory mediators.

Methods: In this study, RAW264.7 cells were used as an inflammatory cell model, and observations were made on the inflammatory mediators nitric oxide (NO), inducible nitric oxide synthase (iNOS), and tumour necrosis factor- α (TNF- α). The NO concentration was measured by the Griess reaction, and the iNOS enzyme and the TNF- α concentrations were determined by the ELISA method. Cell viability was assessed by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] (MTS) test.

Results: Selligueain A at concentrations of 100 and 150 μ M suppressed the production of NO, iNOS, and TNF- α in RAW264.7 cells stimulated by lipopolysaccharide (LPS). The concentration of 150 μ M showed the highest inhibition of NO, iNOS, and TNF- α mediators with the percentage inhibition of 64.85, 55.01, and 48.54%, respectively.

Conclusion: This study shows that selligueain A has anti-inflammatory activity through inhibition of NO, iNOS, and TNF- α production in RAW264.7 macrophage cells.

Implication for health policy/practice/research/medical education:

Selligueain A of the *P. feei* roots had anti-inflammatory activity through the inhibition of the production of inflammatory mediators, NO, iNOS, and TNF- α . The results will encourage further researches to complement scientific evidence regarding the efficacy of Selligueain A, as an anti-inflammatory agent.

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Introduction

Inflammation is a physiological disorder of the body, which includes an increase in leukocytes and inflammatory mediators in certain tissues. Inflammation occurring for a long time will cause chronic inflammation, such as decreased immune system, degenerative diseases, and cancer (1). The response of inflammation has an important role in the pathogenesis of several diseases initiated by tissue injury and caused by the invasion of pathogenic microorganisms or free radicals that can cause vascular

and cellular reactions. Macrophages play an important role in the inflammatory response. They are the main source in producing pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), nitric oxide (NO), interleukins (IL) and inflammatory mediating enzymes such as cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS) (2). Overexpression of iNOS through activation of macrophages is the mechanism responsible for NO synthesis during inflammation. The free radical NO mediates the physiological and pathophysiological

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processes of inflammation. TNF- α is a cytokine that signals an inflammatory response. Inhibition of inflammation is an important target to prevent the occurrence of diseases in the body, especially diseases mediated by inflammation such as cancer, diabetes mellitus, neurological disease, autoimmune disease, and arthritis (1). One of the most common inflammatory diseases in the world is arthritis, which is characterised by pain, swelling and stiffness in the joints (3).

Polypodium feei METT., (synonym: *Selliguea feei*) is a plant of the genus of *Polypodium* in the *Polypodiaceae* family. This genus is widely spread throughout the world, with the highest species diversity in the tropics. *P. feei* is a fern plant that grows in forests, in open areas, among rocks, mostly at 900-3150 m altitude. In the Java islands, it grows especially on volcanoes, near craters, because it is resistant to volcanic fumes, and occurs in abundance around the crater of the Tangkuban Perahu mountain in West Java of Indonesia (4).

In traditional medicine, the roots or rhizomes of *P. feei* are commonly used to facilitate urination, treat rheumatism, lower blood pressure, or exert an aphrodisiac effect. Baek et al reported a trimeric proanthocyanidin constituent of the *P. feei* roots, named selligueain A, which was rated highly sweet by a taste panel and not toxic for mice in the preliminary acute toxicity test. In our previous study, selligueain A showed analgesic and anti-inflammatory activities in mice and rats. Also, its proanthocyanidin fraction inhibited COX activity (4). The molecular docking proved that proanthocyanidin afzelechin (a monomer of selligueain A) could interact with Met522 of *Cox-1* and *2* by the hydrogen binding (5).

The aim of this study was to evaluate anti-inflammatory mechanism of action of selligueain A isolated from the *P. feei* roots against the RAW264.7 cells stimulated by lipopolysaccharide (LPS). The macrophage RAW264.7 cells of LPS-stimulated mice were used as an inflammatory cell model.

Materials and Methods

Materials

Selligueain A was isolated from the *P. feei* roots. Aquabidest, Dulbecco's modified Eagle's medium/DMEM (Biowest[®]), 10% fetal bovine serum/FBS (Biowest[®]), 1% antibiotic and antimycotic/ABAM (Biowest[®]), sel RAW 264.7 (ATCC[®]TIB-71), mouse TNF- α ELISA kit (ElabScience[®]), mouse NOS2/iNOS ELISA Kit (ElabScience[®]), Kit NO assay (ElabScience[®]), micropipette (Finnpipette[™] F2), spectrophotometer (GO Thermo Scientific[®]), vortex (Wisemix[®]), Tips Blue, yellow, white (Neptune Scientific[®]), and microtube 1.5 mL (SPL) were used in this study.

Extraction and isolation of selligueain A

The dried powders of the *P. feei* roots (4.5 kg) were extracted with methanol by a maceration method at

room temperature. The extraction was carried out three times and each time of extraction was for 24 hours. The methanol extract was concentrated by evaporation of the solvent under lower pressure at the temperature of 50°C to get a concentrated extract (1232.7 g). The extract was fractionated by partitioning method using the *n*-hexane-water (3:1) and ethyl acetate-water (3:1) mixture successively to give the fractions of *n*-hexane, ethyl acetate, and water (60.12, 513.75, and 298.6 g, respectively). Based on our previous work (4), the targeted selligueain A compound was contained in the ethyl acetate fraction, so isolation work was conducted on the ethyl acetate fraction. The ethyl acetate fraction was column chromatographed using the mixture of *n*-hexane, ethyl acetate, and methanol as a mobile phase and silica gel 60 as a stationary phase to give 10 fractions. The fraction 6 that was predicted to contain selligueain A was further subjected to column chromatography using the mixture of chloroform-methanol (10:0, 9:1, 8:2, 7:3, and 6:4) as eluent to obtain the selligueain A. The spectroscopic spectral data (UV, IR, MS, and NMR) of the isolated compound were analysed to identify the compound which was confirmed by comparison of its data with those reported in the literature (6).

Cell culture

RAW264.7 cells of a murine macrophage were cultured in DMEM supplemented in 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were grown to a confluence of 80%–90% and applied to no more than 20 cell passages, afterward the cells were acclimatised for 12 hours before any use (7).

Measurement of RAW264.7 cell viability

Cell viability was determined by MTS assay. About 80%–90% of confluent cells were discarded and washed with PBS 1x. PBS for rinsing was removed and added 1 mL of Trypsin-EDTA 0.25%. The mixture was incubated at 37°C for 3 minutes, so that the cells were detached from the bottom of the flask. Checking was done using an inverted microscope to ensure that the cells have been completely detached from the bottom of the flask. Two mL of complete growth medium was added to stop the trypsinization process. The cells were put into a 15 mL centrifuge tube and centrifuged at 1600 rpm for 5 minutes. The supernatant was removed, and the pellet was resuspended with 1 mL growth medium. Cell resuspension was calculated using haemocytometer. After knowing the number of cells at 1 mL of previous cell resuspension, 5×10^3 cells/well cells were planted on a 96 well plate. The cells were incubated at 37°C, 5% CO₂ to 80%–90% confluent cells. If it was confluent, the samples at various doses (200, 150, 100, 50, 25, 12.5, and 6.25 μ M) were given and then incubated at 37°C, 5% CO₂ for 24 hours. After a 24 hours treatment, 20 μ L MTS was added, and incubated for 3 hours at 37°C, 5%

CO₂. The absorbance was recorded at a wavelength of 490 nm, and optical densities were determined at 490 nm by means of a microplate reader (8).

Measurement of NO

The RAW264.7 cells were seeded in 96-well plates (2×10^5 cells/mL) and incubated at 37°C in a humidified atmosphere with 5% CO₂. Twelve hours after treatment, the cells were treated with various concentrations of selligueain A (10, 100, and 150 µM) for 1 hours and afterward LPS (1 µg/mL) was added for 24 hours. Before heading to the NO test reaction, pre-treatment was carried out. In the standard and sample tubes, various concentrations of Sodium Nitrite Standard were added (To 150 µL was added 200 µL reagent 1 and 100 µL reagent 2). All reagents were mixed and incubated for 15 minutes at room temperature, then centrifuged (3100 g, 10 minutes). A total of 160 µL supernatant was inserted in 96 well-plates and added 80 µL chromogenic reagent. All reagents in the well were shaken for 2 minutes and incubated for 15 minutes at room temperature. The NO test results were read at OD 550 nm (9).

Determination of TNF-α by ELISA

TNF-α level in each sample was determined using a commercially available ELISA kit, and assays were conducted in accordance with the manufacturer's instructions. Briefly, the cells were cultured in 24-well plates (5×10^5 cell/mL) for 12 hours, pre-incubated for 1 hour with various concentrations of selligueain A, and stimulated with LPS (1 µg/mL) for 24 hours. Cell culture supernatants obtained were immediately quantified by means of ELISA kits based on the manufacturer's instructions. The standard solution was placed in each well twice as much as 100 µL, covered with a seal and incubated for 90 minutes at 37°C. Next, the solution was discarded and immediately added with biotinylated detection absorption of 100 µL/well, then covered and incubated for 1 hour at 37°C. The solution was discarded and washed with Wash Buffer, waited in 1-2 minutes then discarded-repeated three times. After cleaning, 100 µL/well HRP conjugate was added and the plate was covered and incubated for 30 minutes at 37°C. The plate was washed five times. Then, 90 µL/well substrate was added and incubated for 15 minutes at 37°C. If the colour was not changed, the incubation time was added but not until 30 minutes. The 50 µL/well stop solution was added to each well. Optical density (OD) was measured at 450 nm using a spectrophotometer (10).

Determination of iNOS by ELISA

iNOS level in each sample was measured using a commercially available ELISA kit, and assays were carried out based on the manufacturer's instructions. Briefly, the cells were cultured in 24-well plates for 12 hours,

pre-incubated for 1 hour with various concentrations of selligueain A, then stimulated with LPS (1 µg/mL) for 24 hours. Cell culture supernatants were collected and immediately quantified using ELISA kits in accordance with the manufacturer's instructions. The standard solution was placed in each well twice as much as 100 µL, then covered with a seal, and incubated for 90 minutes at 37°C. Next, the solution was discarded and a Biotinylated Detection Absolution of 100 µL/well was immediately added, covered, and incubated for 1 hour at 37°C. The solution was discarded and washed with Wash Buffer, then waited for 1-2 minutes and discarded-repeated three times. After cleaning, 100 µL/well HRP conjugate was added, and the plate was covered and incubated for 30 minutes at 37°C. The plate was washed five times. Then, 90 µL/well substrate was added and incubated for 15 minutes at 37°C. If the colour was not changed, incubation time was added but not until 30 minutes. The 50 µL/well stop solution was added to each well. OD was measured at 450 nm using a spectrophotometer (11).

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) and continued by a post hoc least-significant difference test. The data are presented as mean ± standard deviation. A *P* value of less than 0.05 was considered significantly different.

Results

Identification of selligueain A

Selligueain A is a known compound of the *P. feei* roots. The identification of this compound was just conducted by analyzing its spectroscopic spectral data (UV, IR, MS, and NMR) and comparing its data with those reported in the literature (6). Its structure is shown in Figure 1.

In vitro examination of the anti-inflammatory property of Selligueain A

An examination of selligueain A against the production of pro-inflammatory mediators on RAW 264.7 makrofag cells was initially conducted by determining cell viability, followed by measuring the NO, TNF, and iNOS levels.

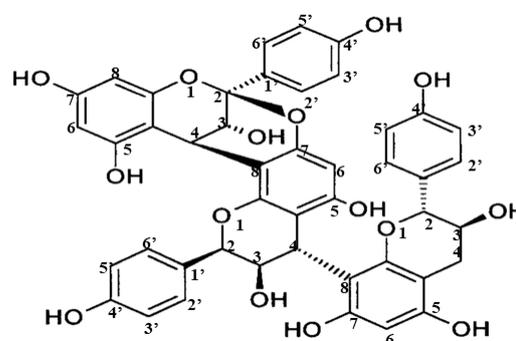


Figure 1. Structure of selligueain A.

Measurement of RAW264.7 cell viability

The data of the RAW264.7 cell viability measurement are shown in Figure 2. Selliguesin A did not cause cell death at any concentration used (6.25, 12.5, 25, 50, 100, 150, and 200 µM) on RAW264.7 cells.

Inhibition of NO release from LPS-stimulated RAW264.7 cells

Inhibition of selliguesin A against NO release from LPS-stimulated RAW264.7 cells is shown in Figure 3. Selliguesin A at the concentrations of 100 dan 150 µM decreased NO levels significantly as compared to the control ($P < 0.05$). The percentage inhibition of each concentration (10, 100, and 150 µM) showed a concentration-dependent manner (28.43, 57.30, and 64.85%, respectively).

Inhibition of iNOS enzyme in RAW264.7 cells

Selliguesin A was examined for its inhibition on the synthesis of iNOS enzyme, which is responsible as a provider and synthesizer of NO inflammatory mediators. The iNOS enzyme is a physiological element that can initiate inflammation. Selliguesin A at the concentrations of 100 dan 150 µM decreased iNOS levels significantly

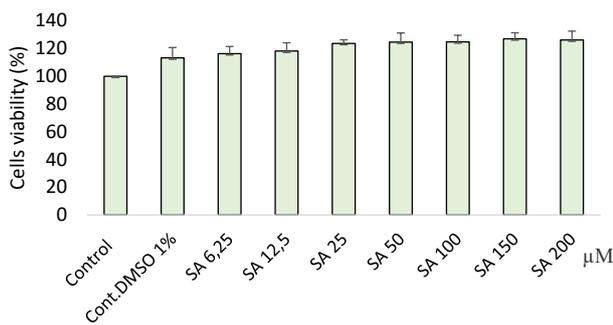


Figure 2. Viability of RAW264.7 cells. SA: Selliguesin A. Control: cells without lipopolysaccharide (LPS) and selliguesin A. Cont.DMSO 1%: cells with LPS and DMSO 1%. SA 6.25 to 200 µM: cells with LPS and 6.25 to 200 µM concentration of selliguesin A.

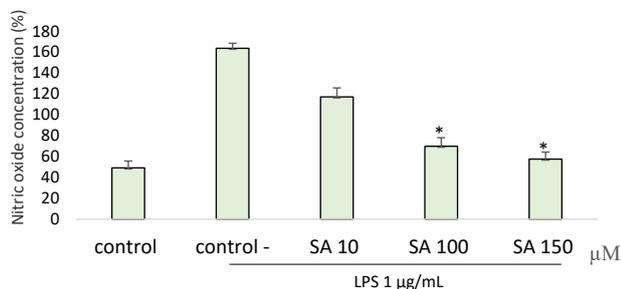


Figure 3. The concentration of Nitric oxide (NO) in RAW264.7 cells. SA: Selliguesin A. Control: cells without lipopolysaccharide (LPS) and selliguesin A. Control -: cells with LPS and DMSO 1%. SA 10: cells with LPS and selliguesin A of 10 µM. SA 100: cells with LPS and selliguesin A of 100 µM. SA 150: cells with LPS and selliguesin A of 150 µM. * Significantly different from the negative control according to ANOVA test ($P < 0.05$).

as compared to the control ($P < 0.05$) (Figure 4). The percentage inhibition of each concentration (10, 100, and 150 µM) showed a concentration-dependent manner (26.35, 30.89 and 55.01%, respectively).

Inhibition of TNF production in LPS-stimulated RAW264.7 cells

The inhibition of selliguesin A against TNF-α production in LPS-RAW264.7 cells is shown in Figure 5. Among the examined concentrations (10, 100, and 150 µM), the concentration of 150 µM decreased TNF-α production significantly as compared to the control ($P < 0.05$). The percentage inhibition of each concentration was 11.41, 27.78, and 48.54%.

Discussion

The evaluation of anti-inflammation of a test compound in vitro could be conducted by examining its inhibitory

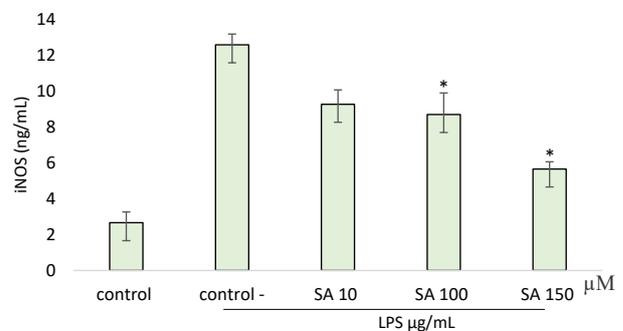


Figure 4. The concentration of nitric oxide synthase (iNOS) in RAW264.7 cells. SA: Selliguesin A. Control: cells without lipopolysaccharide (LPS) and selliguesin A. Control -: cells with LPS and DMSO 1%. SA 10: cells with LPS and selliguesin A of 10 µM. SA 100: cells with LPS and selliguesin A of 100 µM. SA 150: cells with LPS and selliguesin A of 150 µM. * Significantly different from the negative control according to ANOVA test ($P < 0.05$).

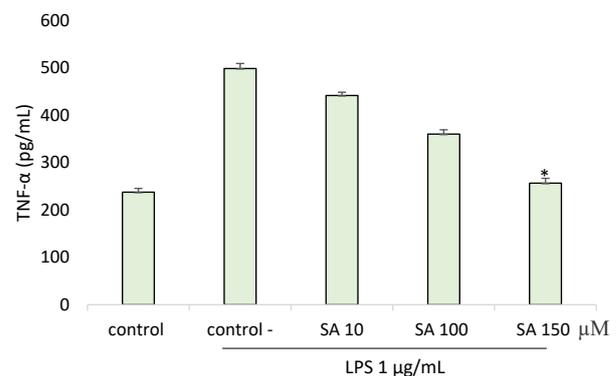


Figure 5. The concentration of tumour necrosis factor-α (TNF-α) in RAW264.7 cells. SA: Selliguesin A. Control: cells without lipopolysaccharide (LPS) and selliguesin A. Control -: cells with LPS and DMSO 1%. SA 10: cells with LPS and selliguesin A of 10 µM. SA 100: cells with LPS and selliguesin A of 100 µM. SA 150: cells with LPS and selliguesin A of 150 µM. * Significantly different from the negative control according to ANOVA test ($P < 0.05$).

activity against the inflammatory mediator production, such as NO, iNOS, and TNF- α on RAW 264.7 cells induced by LPS. The existence of macrophage cells in the inflammatory process is one of the cellular responses that play a role in body defense. Macrophages participate in initiating the inflammatory response during inflammation. The process involves the production of inflammatory mediators such as cytokines TNF- α , interleukins, prostaglandins, NO, and the enzymes such as COX and iNOS. These enzymes are useful in protecting the body from the infection or tissue damage (1,12). These mediators have an important role in the pathogenesis of acute and chronic inflammation (13). The production of cytokines and pro-inflammatory enzymes can be stimulated by LPS, an endotoxin component of gram-negative bacteria (14).

In this study, the selligueain A inhibition against the inflammatory mediator production was firstly examined by the toxicity test on the RAW264.7 cells using an MTS method. The test indicated no cell death caused by selligueain A at any concentration used.

Further testing was conducted on the inhibitory activity of selligueain A against the production of NO, iNOS, and TNF- α in LPS-induced RAW 264.7 cells. Inhibition of the production of these mediators is one of the targets to explore the mechanisms of action of anti-inflammatory drugs. Selligueain A at the concentrations of 100 and 150 μ M inhibited NO production (indicated by the decrease in NO levels) was significantly different when compared to the control group. The inflammatory mediator NO is derived from the amino acid L-arginine by the iNOS enzyme. Drugs that can inhibit NO production can treat acute and chronic inflammatory symptoms (15,16).

In line with the inhibitory activity on NO production, the iNOS enzyme inhibition test was also carried out. Selligueain A at the concentrations of 100 and 150 μ M reduced levels of iNOS enzyme protein, which was different from the control group. The RAW 264.7 cells, when induced by LPS, produced the iNOS enzyme to synthesize the inflammatory mediator NO.

Subsequent tests carried out on TNF- α showed that selligueain A at the concentration of 150 μ M could significantly reduce the levels of TNF- α cytokines with the percentage inhibition value of 48.54%. TNF- α is a cytokine that can bind to receptors on the surface of target cells that play a role in ongoing inflammation. TNF- α can activate macrophages/monocytes, fibroblasts, mast cells, T cells, and natural killer cells. TNF- α can also trigger fever through stimulation of PGE2 synthesis in vascular endothelial cells and the hypothalamus. The continued presence of TNF- α has been reported to be responsible for a group of chronic inflammatory diseases such as parasite infections, cancer, and rheumatoid arthritis (17).

Overall data indicated that selligueain A was proven to reduce NO, iNOS, and TNF- α levels of RAW264.7

macrophage cells induced by LPS. These findings may be related to the anti-inflammatory mechanism of selligueain A, which was previously reported (4).

Conclusion

Selligueain A of the *P. feei* roots had anti-inflammatory activity through the inhibition of the production of inflammatory mediators, NO, iNOS, and TNF- α , the highest activity of which was shown by the concentration of 150 μ M. These results increase scientific proof of the pharmacological activity of the *P. feei* roots.

Authors' contributions

DWS made research designs, conducted all research experiments, compiled and processed data and wrote the scripts. TT assisted in conducting in vitro experiments and biomarker analysis. M supervised the extraction and isolation processes. AS directed the development of research designs and organized all stages of the experiment.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The experimental protocols were approved by the Research Ethics Committee of Faculty of Medicine, Universitas Padjadjaran, Indonesia, with the ethical clearance number of 325/UNG.KEP/EC/2019.

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