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Original Article

ANTIINFLAMMATORY OF MAIN COMPOUND FORM *PIPER CROCATUM* RUIZ AND PAV INHIBITION OF IL-1B LIPOPOLYSACCHARIDE-INDUCED RAW 264,7 CELLS

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ABSTRACT

Objective: Inflammation is a physiological response to stimuli such as infection and tissue injury that causes an increase in the production of the proinflammatory cytokine IL-1β. Red betel has the potential as an anti-inflammatory drug, the aim study at to determine the anti-inflammatory effect of the main compound of red betel leaves.

Methods: Isolation of the main compound from the ethyl acetate fraction of red betel (*Piper crocatum*) leaves by column chromatography. Cell viability was determined by the MTT method in a concentration range of 1.25; 2.5; 5; 10; 20; 40 ug/ml. The anti-inflammatory effect of the main compound was tested against the inhibition of IL-1β production in LPS-induced RAW 264.7 macrophage cells. IL-1β levels were determined by ELISA.

Results: The viability test of the main compound from *Piper crocatum* leaves showed concentrations that gave viability percentages above 80%, namely 10; 5; 2.5; and 1.25 ug/ml. Concentrations of the main compound *Piper crocatum* leaves very significantly inhibited the production of IL-1β with p<0.001.

Conclusion: The main compound of *Piper crocatum* leaves has the potential to be used as an anti-inflammatory

Keywords: Antiinflamation, *Piper crocatum*, IL-1β, ELISA

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INTRODUCTION

Inflammation is a local tissue reaction to infection or injury and involves more mediators than the acquired immune response. Inflammation can occur locally and systemically and can be acute and chronic, leading to pathological abnormalities [1]. Acute inflammation was caused by releasing various mediators from damaged tissue, mast cells, leukocytes, and complement. Microbes can release endotoxins or exotoxins, both of which promote the release of proinflammatory mediators. Lipopolysaccharide is a component of the cell wall of gram-negative bacteria, a polyclonal activator of the immune system, stimulates the release of various proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, TNF-α, and TNF-β [2]. Under inflammatory conditions, IL-1β is a proinflammatory cytokine involved in pain, inflammation, and autoimmune conditions. IL-1β is considered to be an important mediator in the inflammatory process, for example, exerting response effects that include accelerated growth of target cells (lymphocytes), induces expression of cell surface molecules (ICAM-1 on endothelial cells), and release of secondary mediators such as prostaglandins, from cells, macrophages and IL-2 cytokines by T lymphocytes, TNF-α, IL-6 from fibroblasts [3].

Red betel (*Piper crocatum* Ruiz and Pav) is one of the plants used by the community as traditional medicine. Empirically red betel can treat diabetes mellitus, hyperlipidemia, hyperuricemia, hypertension, cancer, inflammation, and joint pain [4]. Red betel leaf extract shows anti-inflammatory activity because it can produce lower levels of proinflammatory cytokines [5-7]. The secondary metabolite content of red betel leaf is a class of flavonoid compounds, alkaloids, polyphenol/phenolic tannins, steroids, terpenoids, and saponins [8].

Red betel leaves is a plant that has the potential as traditional anti-inflammatory medicine, and there have been many studies on the anti-inflammatory effect of red betel leaf, and no research has been found on the activity of active inflammatory compounds from red betel leaves. IL-1β is a proinflammatory cytokine involved in pain, inflammation, and autoimmune conditions. IL-1β is considered an important mediator in the inflammatory process. It is necessary to research to determine the active compound as an anti-inflammatory by isolating the main compound from red betel leaf and testing for the proinflammatory cytokine IL-1β. The research was conducted *in vitro* using RAW 264.7 cells induced by lipopolysaccharide.

MATERIALS AND METHODS

Sample and plant identification

Piper crocatum were collected 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, West Sumatera, Indonesia, with collection number 106/K-ID/ANDA/II/2020.

Chemicals and reagents

Methanol, n-hexane, ethyl acetate, n-butanol, silica gel (63-200 µm particle size) (Merck), TLC plate, RAW 264.7 cells, DMSO, 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 IL-1β ELISA Kit (BT Lab Cat. No E0192Mo).

Extraction and isolation

The dry powder of *Piper crocatum* leaves (0.6 g) was 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 main compound obtained is 510 mg [9].

Characterization of main compounds

Characterization of the main compounds by organoleptic examination, Thin Layer Chromatography analysis using Silica gel 60

F254 (Merck®), UV spectrum was measured in methanol solvent using UV-Vis Spectrophotometer UV-1900 (Shimadzu®), while the IR spectrum was obtained by FT-IR Spectrophotometer IRAffinity-1S (Shimadzu®), and HPLC spectrum obtained with the HPLC Spectrophotometer Nexera-i LC 2040C Plus (Shimadzu®).

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% CO_2 until the cells were confluent. Cells were then washed and harvested using trypsin-EDTA [10].

Cell viability

A cell viability test was carried out using the MTT test method on RAW 264.7 cells. A total of 1 x 10^4 cells per well were plated in 96 wells and then incubated for 24 h at 37 °C. After 24 h the medium was replaced with a new one and then added test solution main compound with a concentration of 40; 20; 10; 5; 2,5; 1,25 µg/ml and dexamethasone (20; 10; 5; 2.5; 1.25 µg/ml). Incubate for 24 h at 37 °C in a 5% CO_2 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 µl of culture medium and 10 µl of 5 mg/ml MTT to each solution and incubate again for 4 h. The formazan crystals formed were dissolved in 100 µl DMSO. Absorption was measured using a microplate reader at a wavelength of 570 nm [11, 12].

Pro-inflammatory activation of RAW 264,7 cells

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 h at 37 °C. The medium was discarded, and a new medium was added to the cell, and the compound solution was added at a concentration of 10; 5; 2.5 and 1.25 ug/ml, dexamethasone 5; 2.5 and 1.25 ug/ml in the medium and cells without the addition of the test compound as a negative control, then incubated for 2 h. A total of 200 µl of LPS solution with a concentration of 1 µg/ml was added to each well and incubated again for 24 h. The medium was taken and centrifuged at 2000 x g for 20 min at 2-8 °C. The supernatant portion was taken and stored at-80 °C for measurement of IL-1β levels [13].

Measurement of IL-1β levels

Cytokine levels of IL-1β were measured by sandwich ELISA method using ELISA (Bioassay Technology Laboratory). Cytokine levels were measured by the sandwich ELISA method using the BT Lab ELISA kit. Add 50 µl of standard antibody solution to each well plate (except standard solution wells), then add 40 µl of sample to each pre-coated well, and then add 10 µl of IL-1β specific antibody, then add 50 µl of streptavidin-HRP to each sample and standard wells, except control and mixed. Covered and incubated at 37 °C for 60 min. The 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 plate for 10 min at 37 °C in a dark room. Add 50 µl of stop solution to each well. After 10 min, determine the value of optical density (OD) of each well 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 [7, 14].

RESULTS AND DISCUSSION

Organoleptic determination of the main compound

Organoleptic determination aims to determine the identity of the initial introduction to the main compound that can be observed visually.

Table 1: The result of the organoleptic main compound determination

No	Isolate compound identity	Result
1	Shape	Crystal
2	Color	White
3	Smell	No smell
4	Taste	Bitter

TLC examination

The results of the TLC examination, with a mixed mobile phase of ethyl acetate: n-hexane (3: 2), the main compound's retention factor (Rf) value was 0.403. The TLC results show that the main compound is relatively pure.

HPLC examination

The examination of the main compound using HPLC with a mixed mobile phase of acetonitrile: H_20 (70:30) showed a retention time value of 2,564 min with a purity level of 98% (fig. 1).

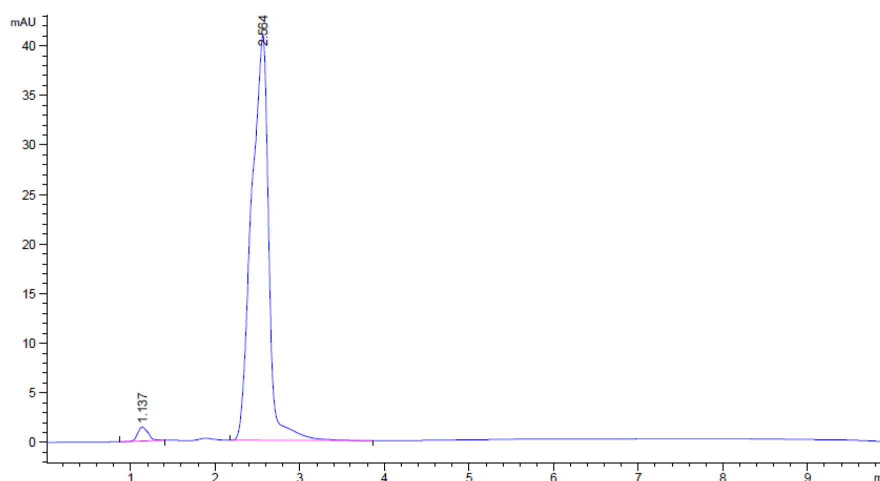

Fig. 1: HPLC Spectrum of the main compound from the ethyl acetate fraction of red betel leaves

Ultra violet spectrophotometric examination

The UV spectrum of the main compound in methanol solvent showed three absorption peaks, namely 205.80; 236.60; and 265.40 nm (fig. 2). The UV spectrum showed that the main compound isolated had several chromophore groups responsible for electron excitation [15].

Infrared spectrum data

From the infrared spectrum, the main compound showed absorption bands at 1588 and 1507 cm^{-1}, indicating the presence of an aromatic C=C group. The absorption band at 1683 cm^{-1} indicates the presence of a C=O group. The absorption band at 3431 cm^{-1} indicates the presence of an OH group. The absorption band at 1375 cm^{-1} indicates the presence of an

aromatic C-H group. The absorption band at 2937 cm^{-1} indicates the presence of C-H alkenes, and the absorption band at 1616 cm^{-1} indicates the presence of C=C alkenes (fig. 3) [15].

Main compound viability

Test of cell viability of RAW 264.7 on the administration of the main compound from red betel leaf using the MTT method. The test results showed a decrease in the number of living cells with an increase in concentration. The concentration of the test substance is: 40; 20; 10; 5; 2.5 and 1.25 µg/ml and dexamethasone as positive control with a concentration of 20; 10; 5 and 2.5 µg/ml. A cell viability test is needed to determine the concentration that is not toxic to RAW 264.7 cells and is used in the test. The concentration chosen is the concentration that gives cell viability above 80% [16].

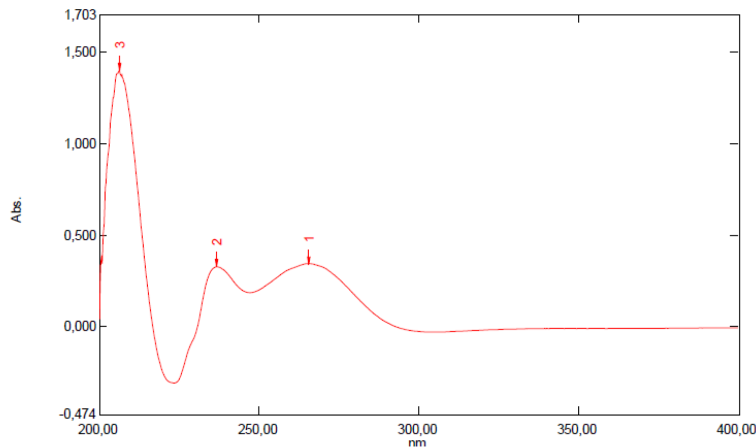

Fig. 2: UV spectrum of the main compound from the ethyl acetate fraction of red betel leaves

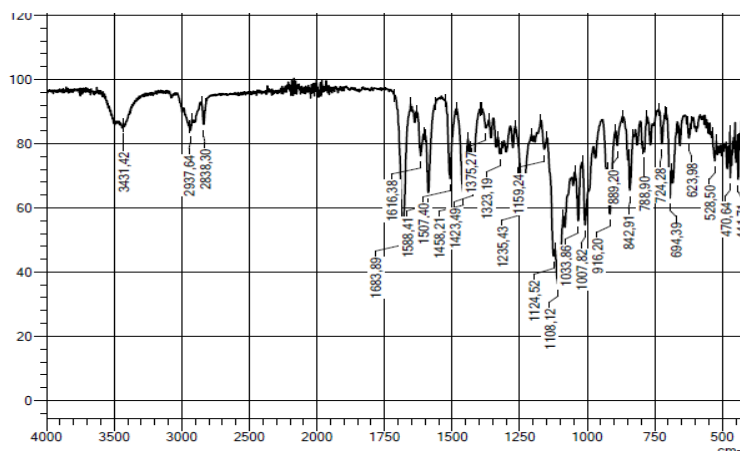

Fig. 3: IR spectrum of the main compound from the ethyl acetate fraction of red betel leaves

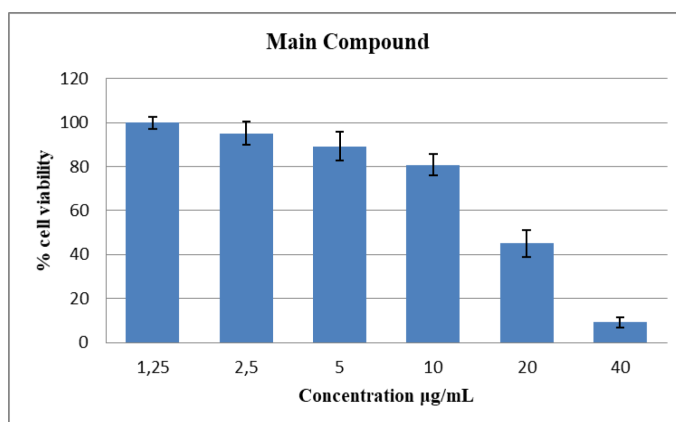

Fig. 4: The average value of the percentage of viabilitas cells from the main compound red betel leaves in RAW 264.7 cells

From the cell viability test results in fig. 1, the concentrations indicating the number of living cells above 80% were concentrations of 10, 5, 2.5, 1.25 µg/ml. This concentration was used to examine the inhibitory effect of IL-1β production on lipopolysaccharide-induced RAW 264.7 cells.

IL-1β levels

The results of the measurement of IL-1β levels from the isolates of the main compound, as well as dexamethasone as a positive control, normal control of cells, and cells induced by LPS. IL-1β is a pro-inflammatory cytokine whose number was increased in RAW 264.7 cells due to the induction of lipopolysaccharide, which is part of the cell wall of gram-negative bacteria. Therefore, administration of the main compound at concentrations of 10; 5; 2.5; and 1.25 μg/ml could very significantly reduce the amount of IL-1β compared to cells that were only induced by LPS with p<0.001.

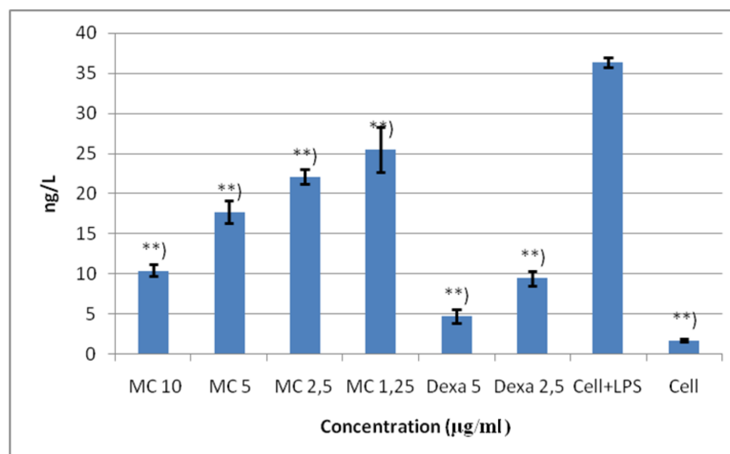

Fig. 5: The average level of IL-1β from the main compound of red betel leaves, dexamethasone in LPS-induced RAW 264.7 cells and normal cells. **)p<0.001 compared to Cell+LPS

CONCLUSION

The main compound of red betel leaf showed non-toxic concentrations of 10, 5, 2.5, and 1.25 µg/ml in 264.7 RAW cells. At concentrations of 10, 5, 2.5, and 1.25 µg/ml, it significantly inhibited the production of IL-1β compared to 264.7 RAW cells induced only by LPS.

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AUTHOR CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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