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Original Article

GARCINIA COWA ROXB. ETHANOL EXTRACT INHIBITS INFLAMMATION IN LPS-INDUCED RAW 264.7 MACROPHAGES

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ABSTRACT

Objective: The purpose of this study was to examine the effect of *Garcinia cowa* Roxb. Ethanol (EGC) extract in LPS-induced Raw 264.7 macrophages by observing the release of Tumor Necrosis Factor (TNF) and Interleukin-6 (IL-6).

Methods: Using the MTT method, a cell viability assay was performed to observe the cytotoxic effect on Raw 264.7 macrophages. For 24 h, Raw 264.7 macrophages were incubated with various EGC concentrations (100, 50, 10, 1 and 0.1 µg/ml). The medium was taken out after 48 h of incubation, and 100 µl of MTT 0.5 mg/ml was then added. 100 µl DMSO was used to dissolve the crystals and absorbance was measured using a microplate reader. To investigate the activity of EGC to LPS-induced Raw 264.7 macrophages, the ELISA method was used. Supernatant was obtained after treating Raw 264.7 macrophages with complete medium, EGC samples, and LPS (10 g/ml) for 24 h. IL-6 and TNF-α levels were assessed using supernatants with ELISA kit.

Results: Cytotoxic effect of EGC to Raw 264.7 macrophages occurred at a concentration of 100 µg/ml with the cell viability value of 59.5%. At a concentration of 50 µg/ml, no cytotoxic effect occurred and the cell viability value was 105.5%. So, the higher concentration of EGC used for further investigation is 50 µg/ml. It was shown that the production of IL 6 was suppressed by EGC at a concentration of 12.5 µg/ml. The inhibition of TNF-α production was only seen at the concentration of 12.5, 25 and 50 µg/ml; there was an increase of TNF-α production.

Conclusion: It can be concluded that EGC can be developed as a natural immunomodulator that can inhibit inflammation by suppressing IL-6 production to prevent immune system disorders.

Keywords: *Garcinia cowa* Roxb, LPS-induced RAW 264.7 macrophages, Inflammation

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INTRODUCTION

Immunomodulators are compounds which aim to stimulate, suppress and modulate the components of the immune system, both adaptive and innate immune systems. The modulation of the immune system has an important role to prevent immune system-related diseases. Immune system immunomodulation decided host reaction to stimulate or to suppress and to harmonize immune system reaction, and this is the right way to protect host from microorganism infection [1-3]. Immunomodulators from herbs were used to cure infection diseases because they have the potential as an alternative drug [4]. Herbs are the basis for the discovery of new compounds and still hold the interest for the researcher [5].

Known as *asam kandis* in West Sumatra, *Garcinia cowa* Roxb. are small to medium-sized trees that grow everywhere throughout Indonesia, Thailand and Malay Peninsula [6-9]. The fruit has a sour taste and is commonly used as a spice in *Minang Kabau* cuisine [10]. In traditional medicine, India uses the fruit as a treatment for dysentery [11]; the stem bark, sap and roots are utilized as an antipyretic agent [9, 12], while the leaves and fruits are used as a laxative, expectorant, and to enhance blood circulation [9]. It is used in several pharmacological activities as an anticancer [8, 10, 13] and as an anti-inflammatory [9, 13, 14]. It is used in anti-inflammatory activity due to the compounds in genus Garcinia (Guttiferae), they are xanthones, benzophenones, triterpenes, biflavonoids, and benzoquinon [13, 15].

In order to prevent diseases, immune system modulation is crucial. Modulating the immune system plays an important role in disease prevention. Macrophages are important immune cells because they can engulf and kill pathogens directly by the process of phagocytosis. Macrophages cells secrete mediator of inflammation, for example, Nitric Oxide (NO), TNF-α, and IL-6 [2, 16]. In this study, a model of LPS-induced Raw 264.7 macrophages was used to investigate the *in vitro* experiments on the immunomodulatory effect of EGC with parameters TNF-α and IL-6 [17].

MATERIALS AND METHODS

Materials

The main material of this study is the stem bark of *Garcinia cowa* Roxb. Obtained from the forest in Universitas Andalas Padang. The stem bark was washed, sliced, and air-dried. The dried stem bark was ground into powder with a pulverizer.

Raw 264.7 macrophages (ATCC), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penisilin-Streptomisin 2% (v/v), Trypsin-EDTA 0.25% were purchased from Gibco. DimetilSulfoxide (DMSO) was purchased from Vivantis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. Phospate Buffer Saline (PBS) and Lipopolisakarida (LPS) were purchased from *in vitro* gen; Sigma provided ELISA kit for Mouse Tumor Necrosis Factor Alpha and Mouse IL-6.

Methods

Preparation of extracts

1 kg of stem bark powder macerated with ethanol obtained 116 g of ethanol extract.

Cell viability assay

180 µl of Raw 264.7 macrophage suspension was seeded on a 96-well plate, except on blank well and incubated 24 h at 37 °C 5% CO2 for adhesion. The complete medium was used to dissolve the EGC samples and create various gradients; 100, 50, 10, 1 and 0.1 µg/ml at 200 µl medium on well. For 48 h, a series of EGC sample concentrations were applied to adherent raw 264.7 macrophages. To the plates, 100 µl of MTT solution (0.5 mg/ml) was added after the medium had been removed, and they were incubated for 4 h. Each well received 100 µl of DMSO to dissolve the crystals after the solution had been discarded. Microplate reader was used to calculate each well's absorbance at 550 nm.

Assays of IL-6 and TNF-α levels

Raw 264.7 macrophages were seeded on a 24-well plate and incubated for 24 h at 37 °C 5% CO2 for adhesion. EGC samples at the concentration of 12.5; 25; 50 µg/ml were treated into seeded Raw 264.7 macrophages and after 2 h incubation at 37 °C 5% CO2, LPS (10 µg/ml) was added. The amounts of IL-6 and TNF-α generated by Raw 264.7 macrophages were measured in the supernatant after 24-hour incubation. An ELISA kit was applied to identify IL-6 and TNF-α according to the manufacturer's instruction. An optical density standard curve made using the recombinant protein was used to determine the quantity of cytokines and was measured at 450 nm.

Statistical analysis

To analyze the data, SPSS 25 was used and the findings are shown as mean±standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range analysis were used to assess how the experimental groups differed from each other. P<0.05 means that it is statistically significant, while P<0.01 means that it is highly statistically significant.

RESULTS

Effect of ethanol extract on the cell viability of Raw 264.7 macrophages

In fig. 1, it was observed that compared with the control group, EGC at a concentration of 100 µg/ml decreased Raw 264.6 macrophages cell viability to 53.26±2.2 %. The difference was extremely significant (p<0.01) and it means that there was the cytotoxic effect on the Raw 264.7 macrophages. Viability of cell Raw 264.7 macrophages at a concentration 50, 10, 1 and 0.1 µg/ml were 105.46±13.3, 123.08±9.7, 101.69±3.8, and 104.27±13.2 %, respectively. Cell viability>90% means that there was no cytotoxic effect on the Raw 264.7 macrophages [3]. The higher cell viability of Raw 264.7 macrophages at the concentration of 10 µg/ml was 123.08±9.7 %.

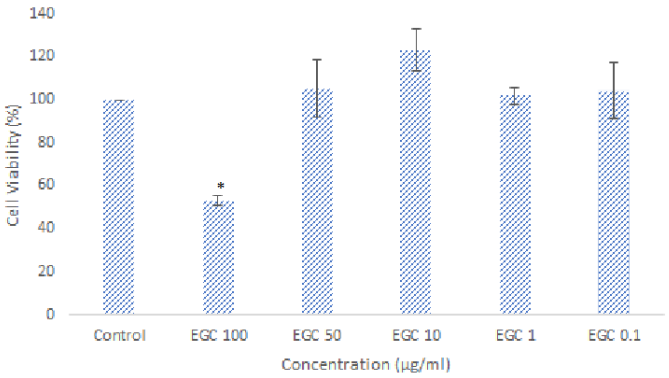

Fig. 1: Effect of EGC on cell viability, *p<0.01 vs control

Effect of ethanol extract on IL-6 and TNF-α generation

Fig. 2 below shows that the release of IL-6 by Raw 264.7 macrophages was promoted by LPS with an extremely significant difference from the control group (p<0.01). Compared with the LPS group, there was a significant decrease on IL-6 level at the concentration of 12.5; 25; 50 µg/ml. The IL-6 concentrations may decrease to 121.76±5.0 pg/ml at EGC 12.5 µg/ml, 82.58±15.9 pg/ml at EGC 25 µg/ml, 107.57±5.7 pg/ml at 50 µg/ml.

As shown in fig. 3, compared with control group, LPS could enhance the production of TNF-α and the difference is extremely significant with the level of TNF-α at 4143.00±25.2 pg/ml. The release of TNF-α slightly decreased to 4116.11±232.6 pg/ml after being treated with EGC with 12.5 µg/ml. The release of TNF-α slightly increased to 4271.03±188.6 pg/ml at EGC with 25 µg/ml and 4970.52±87.7 pg/ml at EGC 50 µg/ml.

DISCUSSION

Effect of ethanol extract on Raw 264.7 macrophages' cell viability

Phagocytic cells are the main defense cells in the immune systems; and one of the most important phagocytic cells are macrophages. Macrophages are capable of engulfing foreign cells and activating immune response [16]. The MTT technique was used to test the cytotoxicity of EGC on Raw 264.7 macrophages and evaluate the cell survival from the treated cells. The results show that at the concentration of 100 µg/ml, there was cytotoxic on Raw 264.7 macrophages and half of the cells died; therefore, the concentration of 100 µg/ml cannot be used for further investigation. Cell viability at concentrations 0.1; 1; 10; 50 µg/ml were above 90%, means the cell was in good growth and no toxic effect on cell viability [3, 18].

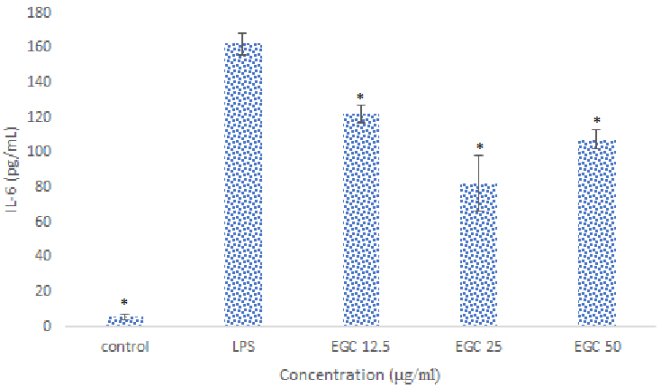

Fig. 2: Effect of EGC on IL-6 level, *p<0.01 vs LPS

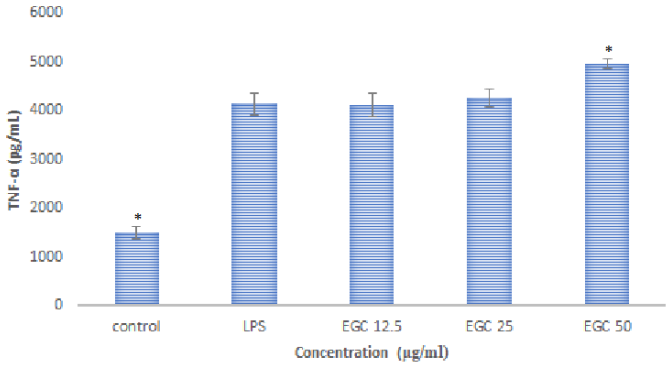

Fig. 3: Effect of EGC on TNF-α level, *p<0.01 vs LPS

Effect of ethanol extract on IL-6 and TNF-α generation

Inflammatory modulators and cytokines modulate the complex process of inflammation. *In vitro* inflammatory, the reaction was tested using macrophages cells that were activated by Toll-like receptor ligands, such as LPS and Interferon-γ which caused secretion of the inflammatory mediator, such as nuclear factor (NF)-κB, proinflammatory cytokines (IL-6, IL-1β, IL-8 and TNF-α) [19]. Immune cells, such as monocytes and macrophages, are activated by LPS [20, 21], thereby increasing the production of IL-6 and TNF-α.

Proinflammatory cytokines like IL-6 are crucial in the inflammatory process because they strengthen the body's defense against antigens and signal the immune system to eliminate foreign substance [22]. TNF-α is the strong proinflammatory cytokines which controls inflammation at the multicellular level and participate in vasodilatation and edema formation [23, 24]. The increased secretion of IL-6 and TNF-α can trigger pathophysiological responses, including tissue damage and loss of tissue function [23, 25].

The results show that LPS (10 µg/ml) was able to induce significant IL-6 production compared to the control group. There was a significant decrease in IL-6 production after being treated with EGC at the concentration of 12.5; 25 and 50 µg/ml. The lowest level of IL-6 occurred at EGC concentration of 25 µg/ml.

TNF-α production increased significantly after being treated with LPS. This shows that LPS induced the production TNF-α as an inflammatory response. The increase was slightly inhibited by EGC treatment at the concentration of 12.5 µg/ml. However, EGC was less potent in inhibit TNF-α production; increasing the concentration of EGC to 25 and 50 µg/ml affects the increase of TNF-α levels. Several theories describe how the active compound prevents and attenuates inflammatory responses. Some mechanisms underlying the anti-inflammatory effect of the natural compound are modulating the assembly of inflammasome, reducing the stability of mRNA, and inhibiting the activation of ERK1/2 and (NF)-κB in macrophages [26]. The further studies still need to confirm the potential mechanism of EGC anti-inflammatory effect.

The results show that EGC has anti-inflammatory activity by suppressing IL-6 levels but not affects TNF-α levels and further study to find out the active compound for this activity can be continued. The compound in stem bark, such as α-mangosteen, Rubraxanthone, and Tetraprenyltoluquinone [14] are likely to be responsible for EGC activity as an anti-inflammatory.

CONCLUSION

The immunomodulatory testing that was performed on Raw 264.7 macrophages indicated that EGC might reduce IL-6 significantly but not affect TNF-α production. Further research is needed to elucidate the compound that is responsible for the activity as an anti-inflammatory and to be developed as a natural immunomodulator to prevent immune system-related disorders.

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AUTHORS CONTRIBUTIONS

Each of the authors named below participated in this project and gave their permission before the manuscript was submitted. Irene Puspa Dewi actually participated in the overall research and wrote the article. In the design of the experiment and the cell experiment, Fatma Sri Wahyuni participated. Yufri Aldi provided literature and edited reference. Dachriyanus participated in project administration and was responsible for editing and reviewing of manuscripts.

CONFLICT OF INTERESTS

Declared none

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