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Original Article

CURCUMA AERUGINOSA ROXB. EXTRACT INHIBITS THE PRODUCTION OF PROINFLAMMATORY CYTOKINES ON RAW 264.7 MACROPHAGES

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ABSTRACT

Objective: The study explores the potential of *Curcuma aeruginosa* Roxb. extract for anti-inflammatory properties.

Methods: *Curcuma aeruginosa* Roxb. simplicia was macerated with distilled ethanol. *In vitro* testing was done on Raw 264.7 macrophages to fulfill this aim by observing Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6 production and phagocytosis activity. The production of IL-6 and TNF-α were determined using the ELISA method while phagocytosis activity using the neutral red uptake method.

Results: The results showed that *Curcuma aeruginosa* Roxb. extract inhibited production of TNF-α and IL-6 and phagocytic activity and on Raw 264.7 macrophages.

Conclusion: The results demonstrated that *Curcuma aeruginosa* Roxb. extract could be developed as an anti-inflammatory, which can be improved as a novel pharmaceutical approach for treating inflammation-related illness.

Keywords: Anti-inflammatory, *Curcuma aeruginosa* Roxb., Immune response, LPS, Raw 264.7 macrophages

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INTRODUCTION

Inflammation is a biological response that maintains homeostasis in the body as a defense mechanism against various infections and injuries. Several cellular and molecular processes commonly occur during an inflammatory reaction. A prolonged and uncontrollable inflammatory response can disturb the regular balance between cellular and molecular responses [1] and lead to the severity of numerous diseases, for example, rheumatoid arthritis, asthma, type 2 diabetes, neurogenic disorders, and cancer [2-4].

The primary goal of anti-inflammatory medications is to reduce production of the proinflammatory mediator TNF-α and IL-6 and the inflammatory mediator prostaglandin E2 (PGE2) [5, 6]. Currently, NSAIDs are still widely used as an anti-inflammatory. Still, adverse effects such as gastrointestinal disturbances, kidney damage, increased cardiovascular risk, and liver disorders can occur if used in the long term [7]. Medicinal plants derived from nature still have opportunities to be developed as anti-inflammatories, which have lower side effects and better effectiveness [8, 9].

A typical murine macrophage cell line for studies on immunomodulation is known as Raw 264.7 macrophages [10]. Macrophages are differentiated blood monocyte cells and include cells of innate immunity, found mainly in tissues throughout the body. These cells are crucial to the inflammatory response [11]. Lipopolysaccharide is the most abundant element of the cell wall of gram-negative bacteria that can stimulate macrophage cells to produce inflammatory mediators and proinflammatory cytokines such as TNF-α, IL-6 and propagation of numerous immune responses [12, 13]. This cell is often used as a model in inflammation research [5, 14].

Thailand, Northern Australia, Papua New Guinea, Indonesia, and Malaysia are among the countries that use the ethnomedicinal herb *Curcuma aeruginosa* Roxb. Common names for *Curcuma aeruginosa* Roxb. include Temu Ireng in Indonesia and Pink and Blue Ginger in English [15], waan-maha-mek in Thailand, and Temu Hitam in Malaysia [16]. Traditional uses of *Curcuma aeruginosa* Roxb. include treating gastrointestinal disorders and acting as an antibacterial and anti-inflammatory agent in Indonesia [15]. However, scientific evidence for the bioactivity of this rhizome as an anti-inflammatory is still limited. This research explores the potential of *Curcuma aeruginosa* Roxb. extract for anti-inflammatory properties.

MATERIALS AND METHODS

Materials

Raw 264.7 macrophages was provided by European Collection of Authenticated Cell Culture (ECACC), England; Trypsin-EDTA, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Pen-Strep 2% (v/v) were provided by Gibco, New Zealand; Dimetil Sulfoxide (DMSO) was provided by Vivantis, Malaysia; Neutral Red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ELISA kit for mouse TNF-α and IL-6 were provided by Sigma, Japan; Lipopolisakarida (LPS) and Phosphate Buffer Saline (PBS) were provided by Invitrogen, USA.

Methods

Preparation of extracts

Curcuma aeruginosa Roxb. rhizomes were obtained from Teluk Kabung, Bungus, West Sumatra, Indonesia. Dr. Nurainas, Herbarium Universitas Andalas, Indonesia, identified the collected samples (No: 479/K-ID/ANDA/X/2022). The collected specimens were cleaned and air-dried. The samples were made into powder by grinding. The samples were macerated with distilled ethanol for two days and filtered. The maceration process was repeated twice. All liquid extract was evaporated using a rotary evaporator (Buchi, Switzerland) to obtain sticky extract.

Cell viability assay

After being cultured in DMEM supplemented with 10% FBS and Pen-Strep 2% for 24 h, the cells (10^3 cells/well) were given samples of *Curcuma aeruginosa* Roxb. extract with concentrations of 0.1, 1, 10, and 100 µg/ml for 48 h. After the medium was discarded, the cells were given 100 µl of MTT solution for 4 h. 100 µl of DMSO was added after removing the MTT solution. A microplate reader (Biorad, California) measured the solution's absorbance at 550 nm [17, 18].

Assays of IL-6 and TNF-α levels

After cultured in DMEM supplemented with 10% FBS and Pen-Strep

2%, the cells (10^4 cells/well) were given *Curcuma aeruginosa* Roxb. extract samples with 12.5, 25, and 50 µg/ml concentrations and LPS (10 µg/ml). After being incubated for 24 h, the levels of IL-6 and TNF-α on the supernatant were determined using an ELISA kit [19].

Phagocytosis assays

After cultured in DMEM supplemented with 10% FBS and Pen-Strep 2%, the cells (10^4 cells/well) were given *Curcuma aeruginosa* Roxb. extract samples with 12.5, 25, and 50 µg/ml concentrations and LPS (10 µg/ml). After being incubated for 24 h, the cells received 100 µl of neutral red liquid after two PBS washes. The solution was discarded, and after the cells were washed, ethanol and glacial acetic acid (1:1) were added and left for one hour. A microplate reader measured the solution's absorbance at 540 nm [19, 20].

RESULTS

Cell viability assay of *Curcuma aeruginosa* Roxb. extract on Raw 264.7 macrophage

Fig. 1 demonstrates the viability of cells after *Curcuma aeruginosa* Roxb. extract treatment at concentrations between 1–50 µg/ml was more than 90%, meaning *Curcuma aeruginosa* Roxb. extract at this concentration is not toxic to the cells. However, the cells viability after treatment of *Curcuma aeruginosa* Roxb. at a concentration of 100 µg/ml was less than 90%, which means *Curcuma aeruginosa* Roxb. extract at 100 µg/ml was cytotoxic, so it was not used for further testing.

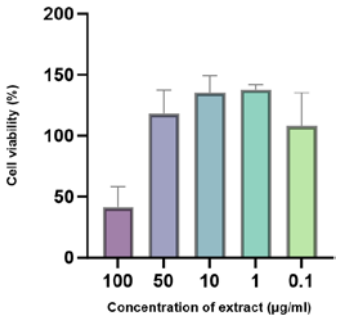

Fig. 1: Cell viability of Raw 264.7 macrophages. Data are expressed as the means±SD (n = 3). The error bar represents the standard deviation

Effect of *Curcuma aeruginosa* roxb. extract on secretion of IL-6 and TNF-α

Fig. 2 demonstrated that after being treated with LPS, there was a significant ($p<0.01$) increase in IL-6 secretion up to 177.75±0 pg/ml. After treatment of *Curcuma aeruginosa* Roxb extract. at a concentration of 12.5, 25, and 50 µg/ml, there was a significant decrease in IL-6 secretion compared to LPS ($p<0.01$) up to 105.04±12.2, 70.9±0.47, and 57.48±5.3 pg/ml.

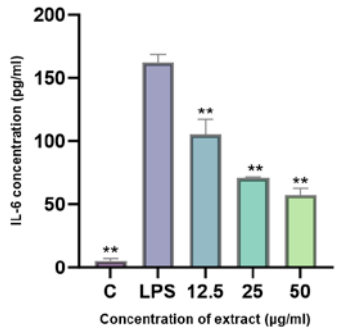

Fig. 2: Effect of *Curcuma aeruginosa* Roxb. extract on IL-6 secretion. Data are expressed as the means±SD (n = 3). The error bar represents the standard deviation. The difference between the treated and control groups was assessed for statistical significance through the application of one-way ANOVA, followed by post hoc duncan analysis. ** p<0.01 vs LPS

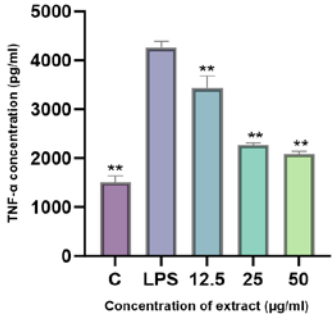

Fig. 3: Effect of *Curcuma aeruginosa* Roxb. extract on TNF-α secretion. Data are expressed as the means±SD (n = 3). The error bar represents the standard deviation. The difference between the treated and control groups was assessed for statistical significance through the application of one-way ANOVA, followed by post hoc duncan analysis. ** p<0.01 vs LPS

Fig. 3 shows that TNF-α secretion caused by LPS induction was significantly restrained ($p<0.01$) by the *Curcuma aeruginosa* Roxb. extract administration. After being induced with LPS, the cells increased TNF-α secretion by up to 4259.40±130.65 pg/ml. After being given *Curcuma aeruginosa* extract at concentrations of 12.5, 25, and 50 µg/ml, TNF-α secretion decreased to 3437.27±243.77, 2271.70±41.80, and 2084.42±60.00 pg/ml.

Effect of *Curcuma aeruginosa* Roxb. extract on secretion of phagocytic activity

Fig. 4 shows that after treatment with LPS, there was a significant ($p<0.01$) increase in phagocytosis index compared to control cells. After being given *Curcuma aeruginosa* Roxb. extract, the phagocytosis index was significantly ($p<0.05$) reduced compared to the LPS group.

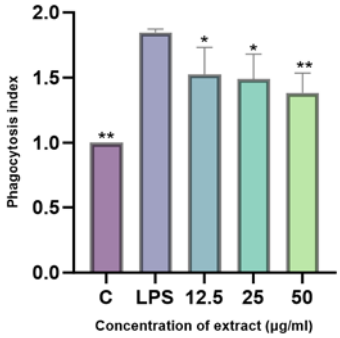

Fig. 4: Effect of *Curcuma aeruginosa* Roxb. extract on secretion of Phagocytic activity. Data are expressed as the means±SD (n = 3). The error bar represents the standard deviation. The difference between the treated and control groups was assessed for statistical significance through the application of one-way ANOVA, followed by post hoc Duncan analysis. *p<0.05, **p<0.01 vs LPS

DISCUSSION

Cell viability assay of *Curcuma aeruginosa* roxb. extract on raw 264.7 macrophage

The cell viability of *Curcuma aeruginosa* Roxb. extract was determined using the MTT method. *Curcuma aeruginosa* Roxb. extract is not toxic to the cells up to a maximum concentration of 50 µg/ml. In contrast, *Curcuma aeruginosa* Roxb. extract could promote cell proliferation. However, at a 100 µg/ml concentration, *Curcuma aeruginosa* Roxb. extract was toxic to the cells and was not used in future studies.

Curcuma aeruginosa roxb. extract inhibits the secretion of IL-6 and TNF-α

Raw 264.7 macrophages is a cell line widely used in immunomodulatory research and can be stimulated by LPS [21]. When stimulated by LPS, Raw 264.7 macrophages secrete pro-inflammatory cytokines, namely TNF-α and IL-6, and secrete inflammatory factors, namely nitric oxide (NO) and Prostaglandins E2 (PGE2) [22]. TNF-α and IL-6 stimulate the acute phase of the immune system. They are the first cytokines released to respond to pathogens and affect multiple organs, such as increasing the release of corticotropic hormone and inducing fever. As an inducer of inflammatory reactions, excessive amounts of TNF-α and IL-6 can react pathologically to diseases, including cancer, inflammatory bowel disease, psoriasis, rheumatoid arthritis, asthma, and other auto-immune diseases [23].

To determine the anti-inflammatory activity of *Curcuma aeruginosa* Roxb. extract, the levels of IL-6 and TNF-α produced by LPS-induced Raw 264.7 macrophages were measured using the ELISA method [24]. Fig. 2 and 3 show that *Curcuma aeruginosa* Roxb. extract inhibited IL-6 production by 67.7% and inhibited TNF-α production by 51.1% compared to LPS. It means that *Curcuma aeruginosa* Roxb. extract has strong anti-inflammatory properties. TNF-α is a potent inducer of IL-1, IL-2, and IL-6 [25]. Inhibiting the secretion of TNF-α by endotoxin-induced macrophage cells will also inhibit the secretion of IL-6. The inhibition of TNF-α and IL-6 production increases with the rising concentration of the given extract.

The anti-inflammatory properties of Curcuma species are attributed to the symmetric structure and position of substituents, as well as the number of methoxy groups. α-and β-unsaturated carbonyl groups and electron-withdrawing constituents also increase their reactivity [26]. One of the main ingredients of the Curcuma species, namely curcumin, is reported to have activity as a potent immunomodulator, antioxidant, anti-inflammatory, and antitumor. Sesquiterpenoids isolated from *Curcuma aeruginosa* Roxb., such as curcumenol, isocurcumenol, zedoarol, isofuranidiene, furanodiene, zedoarondiol, zedoalactone A, and zedoalactone B are thought to be the compounds responsible for its anti-inflammatory activity [16].

Curcuma aeruginosa roxb. extract inhibits the phagocytosis activity

In the body's immune system, macrophages are differentiated from blood monocytes and play an essential role [27]. Macrophages are phagocytic cells that are found in numerous tissues [28]. These cells are the first to recognize foreign objects that enter the body and become active. These activated macrophages will initiate an immune response and kill foreign bodies directly by phagocytosis [27, 28]. As shown in fig. 4, the phagocytosis index decreased significantly after being treated with *Curcuma aeruginosa* Roxb. extract. It means *Curcuma aeruginosa* Roxb. extract inhibits the ability of Raw 264.7 macrophages to phagocytose foreign objects and reduce the inflammatory response.

In this study, we found that *Curcuma aeruginosa* Roxb. have the anti-inflammatory properties that inhibited the secretion of IL-6, TNF-α and phagocytosis ability of LPS-induced Raw 264.7 macrophages. Previous study mentioned that based on NO reduction in Raw 264.7 macrophages stimulated by LPS, *Curcuma aeruginosa* Roxb. exhibits potency as an anti-inflammatory drug [30]. These findings will add information regarding the anti-inflammatory properties of *Curcuma aeruginosa* Roxb. and could be a starting point for developing new anti-inflammatory drugs. However, a limitation of this study is that the mechanisms underlying its pharmacological characteristics and chemical components still need to be fully understood.

CONCLUSION

Curcuma aeruginosa Roxb. extract inhibited the production of IL-6, TNF-α, and phagocytosis ability of LPS-induced Raw 264.7 macrophages. This research suggests that *Curcuma aeruginosa* Roxb. extract can potentially prevent and suppress inflammatory disease. These finding could be a starting point for developing new anti-inflammatory drugs.

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AUTHORS CONTRIBUTIONS

Irene Puspa Dewi: Investigation, Project administration, Writing–original draft. Dachriyanus: Conceptualization, Project administration, Supervision. Fatma Sri Wahyuni and Nor Hadiani Ismail: Conceptualization, Supervision. Yufri Aldi: Supervision. Dira Hefni, Meri Susanti and Suryati Syafri: Investigation.

CONFLICT OF INTERESTS

Declared none

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