ORIGINAL ARTICLE

Anti-inflammatory effect *of Alpinia galanga* extract on acute inflammatory cell model of peripheral blood mononuclear cells stimulated with TNF-α

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ABSTRACT

Aim Studies have shown that SARS-Cov-2 has the ability to activate proinflammatory cytokine leading to acute inflammation. During the SARS-Cov-2 infection, an increase of the secretion of production TNF-α in seen in COVID-19 patients along with a decrease in anti-inflammatory cytokine IL-10, and growth factor TGF-β caused cytokine storm and damaged tissues. *Alpinia galanga* extract contains several secondary metabolites with strong antiinflammation and antioxidant effect. The aim of this study was to evaluate the effect of *Alpinia galanga* extract on peripheral blood mononuclear cells (PMBC) acute inflammation cells model stimulated with TNF-α.

Methods *Alpinia galanga* was extracted under maceration methods on ethanol 96%. The PMBC was collected from three healthy humans and isolated using ficol reagent and cultured with the TNF-α 100pg/mL for 72 h. The TNF-α levels were evaluated under ELISA reader. Furthermore, the IL-10 and TGF-β gene expression was analysed using qRT-PCR after 24 h treatment with *Alpinia galanga* extract.

Results *Alpinia galanga* extract has no cytotoxic effect on Vero cells with IC_{50} value of >1000μg/mL. The PBMC acute inflammation cells model stimulated by TNF-α 100pg/mL, after 72 h induction the PBMC cells significantly expressed a high level of TNF-α up to 341±10.87 pg/mL. Furthermore, the treatment of *Alpinia galanga* significantly increased the anti-inflammatory cytokine IL-10 and growth factor TGF-β in dose dependent manner.

Conclusion These findings suggested that *Alpinia galanga* extract has strong antiinflammation activity.

Key words: acute inflammation, *Alpinia galanga* extract, IL-10, PMBC

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Original submission:
16 November 2022;
Revised submission:
10 January 2023;
Accepted:
13 March 2023
doi: 10.17392/1561-23

Med Glas (Zenica) 2023; 20(2):207-213

INTRODUCTION

The novel coronavirus that emerged because of the SARS-CoV-2 virus, known as COVID-19, has become a worldwide epidemic (1). COVID-19 spreads progressively across the continents of Asia, Europe, the Middle East, Africa, and America, covering more than 100 countries (2,3). In Indonesia until April 2022, there were 165,512 cases, with an average of 3,543 new cases per day (4). This endemic virus invites the challenge of quickly finding drugs that match the molecular characteristics of the virus. Previous studies reported that a common feature and presumable cause of death among patients with severe cases of the COVID-19 is the overproduction of pro-inflammatory cytokines arising from excessive immune cell activation (cytokine storm) (5,6). Pro-inflammatory cytokines (interferon-α, interferon-γ, interleukin-1β, IL-2, IL-6, IL-7, IL-10, IL-12, IL-18, IL-33, tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, and CXCL10) came in immoderate amount, responding of cytokine storm by COVID-19 infection (7–9). The inflammation gets out of control inducing COVID-19 deaths (10). It has been shown that cytokine storm is a serious case in COVID-19 (7,11). Efforts to dampen the cytokine storm are still a subject of ongoing studies.

TNF-α is a molecule that actively stimulates the release of several inflammatory cytokines (12). Previous studies reported immune responses of 54 COVID-19 patients and found that TNF-α and IL-6 production by circulating monocytes was sustained(12–14). The increased levels TNF-α could result in the facilitation of viral infection and organ damage. It has been reported that anti-TNF therapy can significantly improve the severe respiratory syncytial virus and influenza in mice, thereby reducing the recruitment of inflammatory cells (15,16). The previous studies reported that IL-10 suppressed inflammation by decreasing TNF-α production. In addition, cytokine synthesis inhibitory factor (CSIF) in human that is also usually called interleukin-10 (IL-10), can suppress Th1 response indirectly leading to inflammation inhibition (17). IL-10 could inhibit the production by dendritic cells and macrophages of many inflammatory molecules such as IL-12, TNF-α, MHC and other costimulatory molecules (18,19). IL-10 also could regulate the B cell survival, proliferation, and antibody production (20). In one hand, IL-10 has the role in remodelling damaged tissues and wound healing (21). However, in the COVID-19 condition expression of IL-10 dramatically decreased leading to acute inflammation and spread damaged tissue. On the other hand, the expression of several growth factors including TGF-β significantly suppresses in acute inflammation of COVID-19 leading to tissues death (18,22–24). Furthermore, anti-TNF-α therapy might accelerate inflammation phase and inhibited tissues damage.

Alpinia galanga is known to have the ability to act as antioxidant and antiinflammation (25–28). Moreover *Alpinia galanga* extract is known to inhibit inflammation and suppress the levels of reactive oxygen species (ROS) (29). 1'-acetoxychavicol acetate (ACA) can inhibit the inflammation *in vitro* and *in vivo* by inhibiting the constitutive, activation of nuclear factor kappa B through suppression of IKKα/β activation (25,28). In addition, our previous study also reported that, ACA has a strong interaction with spike glycoprotein, 3CL protease SARS-Cov-2, and PD-ACE2 of COVID-19 receptor indicating that ACA could inhibit the spreading of SARS-Cov-2 (30). However, the antiinflammation effect of *Alpinia galanga* extract on the PMBC cells that mimic acute inflammation of COVID-19 remains unclear.

The aim of this study was to explore the effect of *Alpinia galanga* extract on the IL-10 and TGF-β gene expression on the PMBC acute inflammation cells model stimulated with TNF- α.

MATERIALS AND METHODS

Material and study design

This post-test only control group study design was conducted in Integrated Laboratory, Universitas Diponegoro, Semarang Indonesia, from August– October 2022. The peripheral blood mononuclear cells (PBMC) were obtained from female healthy subjects. Three healthy subjects were selected with the criteria that the subject was in good health according to the parameters of normal blood sugar levels (<200 mg/dL), normal blood pressure, normal cholesterol levels, normal uric acid levels.

Inclusion criteria were subjects aged 20-30 years, did not have a history of an autoimmune disease as evidenced by a medical history from a doctor, had never been infected with COVID-19 as indicated by a COVID-19 antibody titre of 0, and were not on medication including consumption of the following drugs: antioxidants, vitamins, immunosuppressant, and anti-inflammatories. The PMBCs (1×10^7 cells/flask) were isolated from fresh blood of three subjects as a replication study.

After the isolation of PBMCs from the blood of healthy subjects, the study was continued *in vitro*. It only used fresh blood for PBMC isolation and after that, the PBMCs were cultured *in vitro*. This study consisted of 2 groups, namely the untreated group without TNF-a induction and the TNF-a-induced PBMC group.

All subjects gave a written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Faculty of Medicine Universitas Islam Sultan Agung Semarang (approval number: 301/VIII/2021/Komisi Bioetik).

Methods

Extraction of *Alpinia galanga* extract. *Alpinia galanga* rhizome were collected from Tawangmangu in Central Java Indonesia in May 2021 (Latitude -7.665158 and Longitude 111.129500). They were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried *Alpinia galanga* rhizome was blended to small pieces and sieved with a mesh size of 120 mesh. The 50 g of *Alpinia galanga* was extracted in a maceration apparatus with 500 mL 98% ethanol for 24 h. The filtrate was then evaporated under rotary vacuum evaporator (IKA) and the crude extract was kept in a refrigerator at 4 (31,32).

Cell culture. Vero cells were cultured in high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco, USA) enriched with 10% foetal bovine serum (Gibco, USA), 12.5µg/mL amphotericin B (Gibco, USA), 150µg/mL streptomycin, and 150 IU/mL penicillin (Gibco, USA). The cells were cultured at 37 °C and 5% CO_2.

Cell viability assay. The effect of *Alpinia galanga* extract on Vero normal cell proliferation was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (33). Briefly, $5x10^3$ cells/well were cultured in 96-well plate for 24 h. The treatment with 0-500 **µg**/mL of *Alpinia galanga* extract was applied for 24 h. Untreated cells were considered as negative controls. After the treatment, the medium containing the extracts was replaced by fresh complete medium and 0.5 mg/mL MTT (Sigma-Aldrich, Burlington, MA, United States) was added for 4h; DMSO was added to dilute formazan crystals, and the optical density (OD) of the supernatant was measured at λ595 nm under ELISA reader (Biorad iMarkTM Microplate Reader, Berkeley, United States). The IC_{50} value was calculated through linier regression equation (Y= bX + a). The data were obtained through three replication experiments (31).

PBMC isolation and TNF-α induced-inflammation condition. A total of three females did not receive anti-inflammatory, antioxidant, vitamins, and immunosuppressant therapy or had no previous history of infectious disease, immune disorder and cancer. After obtaining written informed consents, all blood samples were collected. The PBMC was isolated immediately after collecting PBMCs from the healthy subjects using the density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, MO, USA). Human MSCs ($1x10^5$ cells/well) were seeded on corning costar 0.4 µm transwell 12-well plates (Sigma, St. Louis, MO, USA) for 24 h prior to co-culture with PBMCs and cultured in DMEM-LG (Gibco™ Invitrogen, NY, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, 0.25% amphotericin B and 2 mM glutamine, incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The isolated PBMCs from healthy sample were cultured in 12-well plates with the TNF-α 100pg/mL for 72 h in RPMI (Gibco Invitrogen, NY, USA), supplemented with 100 U/mL penicillin–streptomycin and 10% FBS as a treatment group. While the untreated group was a PMBC group without TNF-α induction and only received 100 U/mL penicillin–streptomycin and 10% FBS, isolated PBMCs (1×10^7 cells/flask) from healthy subjects were cultured in a standard medium. After 72 h culture, the PBMCs and supernatant were collected for further analysis.

Determination of IL-10 and TGF-β gene expression. After being induced by TNF-α to mimic inflammation condition, all of PBMCs were treated with *Alpinia galanga* extract on several doses for 24 h. After 24 h treatment, total RNA from PMBC culture was extracted with TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1 g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the IL-10 and TGF-β genes were measured using the respective primers (Table 1). The thermocycler conditions were as follows: initial step at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method).

Table 1. The primer sequence for qRT-PCR

Gene name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
IL-10	TTAAGGGTTA CCTGGGTTGC	TTCACGTGCT CCTTGATGTC
TGF-β	TTGACGTCAC TGGAGTTGTG	CGTTGATGTC CACTTGAAGC
GAPDH	ATCTCGCTCC TGGAAGATG	TCGGAGTGAA CGGATTCG

Statistical analysis

Data were presented as the mean±SD. The statistical significance of differences between the groups was examined using ANOVA with post-hoc Fisher's LSD analysis. The statistical significance of differences between two groups was examined using paired T-test analysis. The $p<0.05$ was considered significant.

RESULTS

The *Alpinia galanga* was successfully extracted with ethanol 96% under maceration methods, the rendement of crude extract was 7.98%. The phytochemical screening of crude ethanol extract of *Alpinia galanga* revealed the presence of some secondary metabolites such as alkaloid, saponin, tannin, and flavonoid.

Isolation of PMBC cells was obtained from the blood of healthy patients as a test taken as much as 5 mL using ficol. The isolated buffy coat was taken and grown on PBMC growth media for 14 days (Figure 1 A-B).

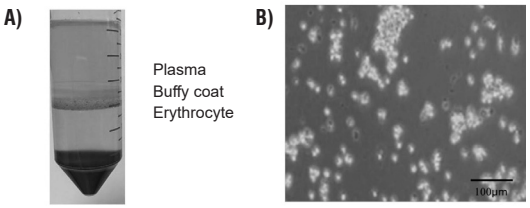

Figure 1. A) Buffy coat isolation results from blood samples; B) Human peripheral blood mononuclear cells (PBMC) cell morphology on day 7 (100x magnification)

Cytotoxic assay was carried out to determine the concentration of *Alpinia galanga* extract which did not cause toxic effects to be used in the further study. Cytotoxic assay was carried out on normal Vero cells, which represent normal cells in humans. The high IC_{50} value of *Alpinia galanga* extract in Vero cells was 1120 ug/mL indicating that *Alpinia galanga* extract had no toxic effect on the normal cells (Figure 2). Furthermore, to evaluate the effect of *Alpinia galanga* extract on the inflammation effect of PMBC with overexpress inflammation factor, TNF- α, several doses of Alpinia galanga extract were used: 1/16, 1/8, ¼, and ½ IC_{50}.

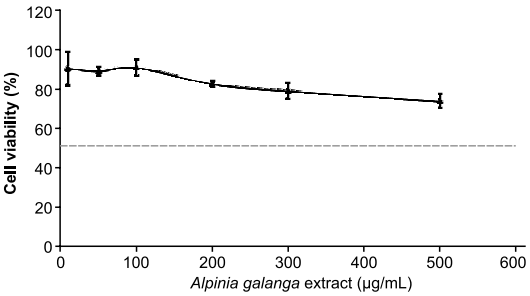

Figure 2. Cytotoxic activity of *Alpinia galanga* extract on Vero cells for 24 h. The data represent the mean±SD of three independent trials with at least 3 replicates

The study found that PBMC cells induced by inflammation had a higher level of TNF-α levels up to 341±10.87 pg/mL compared to untreated cells (34±8.91pg/ml) (Figure 3).

After positive confirmation of overexpression pro-inflammatory cytokine, PBMC cells were treated with *Alpinia galanga* extract at various concentrations for 24 hours. After 24 hours, PMBC cells were harvested and analysed for IL-

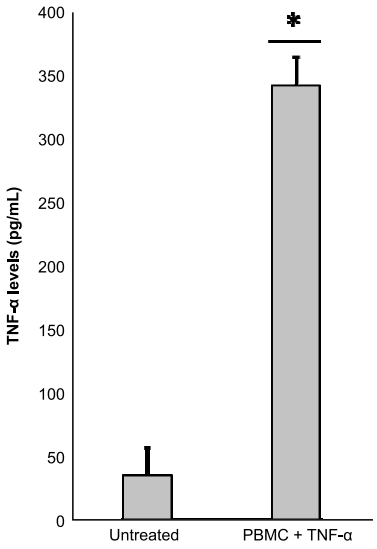

Figure 3. Tumour necrosis factor alpha (TNF)-α levels on human peripheral blood mononuclear cells (PBMC) cells after 72 h induction. The columns represent the mean±SD of three independent trials with at least 3 replicates
*p<0.05

10 and TGF-β gene expression using qRT-PCR. Based on the test, the levels of IL-10 and TGF-β showed that they significantly increased in doses-dependent manner. This result indicated that the secondary metabolite compounds of *Alpinia galanga* extract are proven to suppress inflammation through the induction of anti-inflammatory cytokines and growth factor (Figure 4).

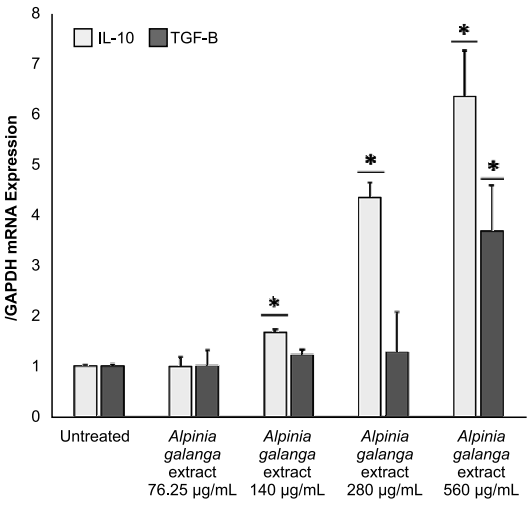

Figure 4. The effect of *Alpinia galanga* extract on IL-10 and TGF-β gene expression on acute inflammation human peripheral blood mononuclear cells (PMBC) cells model. RNA was extracted from the PMBC cells and analysed for mRNA expression by qRT-PCR (n=3 ±SD). Data are presented as fold change in gene expression relative to PMBC untreated group

DISCUSSION

TGF-β and IL-10, potent inhibitory cytokines, have been investigated for their therapeutic potential in inflammatory diseases, including COVID-19 (34,35). Herein, we proposed an inhibitory cytokine synergy with TGF-β and IL-10 that control acute inflammation on the PMBC acute inflammation cells model stimulated with TNF-α by *Alpinia galanga* extract. This study found that *Alpinia galanga* extract significantly increased the IL-10 and TGF-β gene expression. These findings suggest that physiological expression levels of IL-10 might be necessary for the control of inflammation induced by TNF-α. Previous studies reported that ACA, the specific compound of *Alpinia galanga* has direct protein target to MAPK1 and MAPK3 (30). The activation of MAPK pathway correlated with induced expression of anti-inflammatory cytokine IL-10; furthermore, IL-10 inhibits the expression of pro-inflammatory cytokine including IL-6, IL-12, and TNF-α via STAT3 (36,37). In addition, antioxidant effect of *Alpinia galanga* extract has proven inhibited the ROS level (38). High ROS level directly contributes to NF-kB activation pathway leading to activation of pro-inflammatory transcription factors such as IL-6, TNF-α, and INF-γ (39,40). The huge expression of pro-inflammatory cytokine induced acute inflammation and delayed tissues regeneration (41). In addition, this condition also decreased the level of growth factor such as TGF-β. TGF-β is a required signal for Treg differentiation and inhibition of immune imbalance leading to cytokine storm inhibition (42).

In this study, the IL-10 level tends to decrease at untreated PBMC cells (only induced by TNF-α without *Alpinia galanga* treatment). A higher dose of TNF-α causes apoptosis due to acute inflammation (43).

We also found a significant increase of TGF-β gene expression. This finding suggests that *Alpinia galanga* rapidly accelerated inflammation by releasing the IL-10 and TGF-β. TGF-β inhibited inflammatory cytokine-induced iNOS expression in a SMAD3-dependent manner (44,45). Previous studies supported the finding that *Alpinia galanga* extract inhibits inflammation through downregulation of IL-1β in human synovial fibroblast (46). On the other hand, *Alpinia officinarum* ameliorates

oxidative stress by regulated several antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) by nuclear factor erythroid 2-related factor 2 (Nrf2)/protein kinase B (AKT) pathway (47).

In conclusion, *Alpinia galanga* extract suppresses the inflammatory milieu and induced growth factor expression on PMBC acute inflammation cells model stimulated with TNF- α. The higher increase of TGF-β is due to the controlled inflammation by IL-10. These findings suggested that *Alpinia galanga* extract has a strong antiinflammation activity.

ACKNOWLEDGMENTS

The authors express their great appreciation for the financial support received for this work from Universitas Diponegoro RPP 2021.

FUNDING

Financial support was received for this work from Universitas Diponegoro RPP 2021.

TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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