IMMUNOMODULATION EFFECT OF Kaempferia galanga ETHANOLIC EXTRACT ON IN VITRO LYMPHOCYTE CELL PROLIFERATION

Efek Imunomodulasi Ekstrak Etanol Kaempferia galanga terhadap Proliferasi Sel Limfosit Secara In Vitro

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ABSTRACT/ABSTRAK

Kaempferia galanga L. is a traditional medicine with antitumor properties, as indicated by its immunomodulatory activities. This study aimed to determine the effect of K. galanga on lymphocyte cell proliferation activity as an indicator of immunomodulatory properties. This study was conducted at the Indonesian Research Center for Veterinary Science (February to April 2018). The immunomodulatory activity of the extract was evaluated with an in vitro splenocyte proliferation assay. The assay was based on cellular enzymatic synthesis to transform the XTT from formazan tetrazolium as an indicator. The K. galanga extract was obtained by 96% ethanol extraction. The test was conducted in an aseptic condition, consisted of five treatment groups with three replications each. Three groups of splenocyte cell culture, each with extract concentration of 2.5 \( \mu g.ml^{-1} \), 25 \( \mu g.ml^{-1} \), and 250 \( \mu g.ml^{-1} \), as well as a positive (Concanavalin A/Con A) and negative (cell only) control. The cell suspension (10x10^4 cells/ml) was distributed on 96-well plates and cultured following the treatment groups. The same five plates were made for five days of observation and retrieved daily by observing an Elisa reader at 450 nm. The extract of K. galanga at 2.5 \( \mu g.ml^{-1} \), 25 \( \mu g.ml^{-1} \), and 250 \( \mu g.ml^{-1} \) significantly (\( P <0.05 \)) promoted splenocyte proliferation compared to control. Therefore, it was expected that K. galanga has a high potential to be used as immunomodulators. Hence, further investigations should be done to clarify the mechanisms of the immunomodulatory effect of K. galanga as an antitumor in vivo.

Kencur (Kaempferia galanga L.) merupakan obat tradisional yang dikenal bermanfaat sebagai antitumor yang diindikasikan dengan aktivitas imunomodulator. Penelitian ini bertujuan untuk mengoAbsent/exists serve sebagaia aktivitas kencur pada sel limfosit sebagai indikator aktivitas imunomodulator. Penelitian dilakukan di Balai Besar Penelitian Veteriner Bogor mulai Februari sampai April 2018. Aktivitas imunomodulator dievaluasi dengan cara menguji kemampuannya dalam meningkatkan proliferasi splenosit in vitro. Metode ini menguji kemampuan enzim dalam sel untuk mentransformasi garam tetrazolium dari XTT menjadi produk berwarna biru yaitu formazan. Ekstrak kencur yang digunakan dalam penelitian ini diekstraksi dengan menggunaan etanol 96%. Uji dilakukan secara aseptis terdiri lima kelompok perlakuan dengan konsentrasi 2.5, 25, dan 250 \( \mu g.ml^{-1} \), serta dua kelompok sebagai kontrol positif (Concanavalin A/Con A) dan negatif (no extract). Uji ini menggunakan 10 x 10^4 cells/ml dalam media RPMI yang dimasukkan ke dalam lempeng sel kultur yang terdiri dari 96 lubang dan dikulturkan selama 5 hari. Setiap hari diambil 1 lempeng untuk diukur serapannya pada panjang gelombang 450 nm. Hasil penelitian menunjukkan adanya peningkatan aktivitas proliferasi sel limfosit pada kelompok yang diberi

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INTRODUCTION

Currently, there is an increasing understanding of the body's immune response in dealing with disease infections and research on the components affecting this immune response. A version of cell communication or interaction enables the development of ways to manipulate these communication lines. The material that can be used to modulate the system of body immune is termed immunomodulation. It is divided into three groups: (1) immunostimulator that has a function to increase the activity of the immune system, (2) immunoregulator that can regulate the immune system and (3) immunosuppression which can inhibit and suppress the action of the immune system (Jantan et al. 2019).

In principle, the immune system's work in dealing with disease infections is highly dependent on the activation of T-helper (Th) CD4+ lymphocytes. These differentiate into two groups, based on the secretion pattern of cytokines, Th1, and Th2 cells, and have a central role in almost all pathogenic microorganisms. Furthermore, Th1 and Th2 cells exhibited mutually inhibiting functions between the opposing subsets, and this mechanism was known as cross-regulation. For instance, IFN (interferon), the main product of Th1 cells, was a potent inhibitor for Th2 cells. Th2 cells produced IL/Interleukin-4 and IL-10, both potent inhibitors of cytokine production by Th1. This mechanism played a central role in understanding the consequences of inadequate response of T lymphocytes to a pathogen and tumor (Delves et al. 2017; Hosseinzade et al. 2019).

An important medicinal plant for Asian people is Kaempferia galanga, in Indonesia, known as kencur. K. galanga is regarded as one of Indonesia's potential medicinal plants with high economic value with increased demand. K. galanga, which belongs to the Zingiberaceae family, contained saponins, flavonoids, polyphenols, polysaccharides, and essential oil (Umar et al. 2011; Shetu et al. 2018). As reported, these bioactive substances have exhibited antioxidant, antitumor, anti-inflammatory, and antimicrobial activities (Gholib 2009; Ali et al. 2017; Yang et al. 2018), which would contribute to the application of curing many diseases. The rhizome has also been widely known in the community as food spices. Furthermore, it has great potential as raw material for traditional and modern medicine and the cosmetics industry (Haryudin & Rostiana 2008). The traditional drink of "beras kencur" is a popular Java beverage made from the K. galanga rhizome and rice. This drink is effectively used to increase immunity, eliminate cold and fatigue, and be applied topically with coconut oil or alcohol to treat foot sprains or tighten leg tendons (Silalahi 2019).

Many in vitro and in vivo studies have shown the anticancer activities of K. galanga extract. K. galanga extract was reported cytotoxic to human cholangiocarcinoma cell lines (CL-6) (Amuamuta et al. 2017) and could effectively protect the thymus and spleen of tumor-bearing mice (Yang et al. 2018). Moreover, K. galangal induced Ehrlich ascites carcinoma (EAC) cell death in a dose-dependent manner (Ali et al. 2017) and showed moderate cytotoxicity in a brine shrimp lethality bioassay compared with vincristine sulfate as the reference compound (Dash et al. 2014). In addition, many studies also showed that K. galanga rhizomes extract contained numerous flavonoids and essential oil, which was revealed to possess antioxidant activity (Ali et al. 2017; Sahoo et al. 2014). Plants that possessed anticancer and antioxidant properties were reported to have immunomodulatory properties (Zhang et al. 2018). However, the precise information on the immunomodulatory properties of K. galanga is still limited.

In understanding the importance of the immune system in fighting pathogens and tumors, research is required to explore the potential of plants as immunomodulators sources. This study aimed to determine the effect of K. galanga on lymphocyte cell proliferation activity as an indicator of immunomodulatory properties. This research was expected to highlight a scientific background for the immunomodulatory activity of K. galanga.

MATERIALS AND METHODS

This study was conducted at the Indonesian Research Center for Veterinary Science (IRCVS/BBLitvet), Bogor, from February to April 2018. The rhizomes of K. galanga were procured...
from the local market and were identified at the Center for Biological Research, Indonesian Institute of Sciences, Bogor.

**Extraction of Kaempferia galanga rhizomes**

For this experiment, 40 g of fresh *K. galanga* rhizome were peeled, thinly cut, mashed, placed in an Erlenmeyer flask containing 80 ml of ethanol 96%. The flask was then covered with aluminum foil and left at room temperature for 24 hours. The result was filtered using Whatman 41 and vaporized using a rotary vacuum evaporator at 40°C to evaporate all ethanol. The extract was then diluted into three concentrations, 2.5 µg.ml⁻¹, 25 µg.ml⁻¹, and 250 µg.ml⁻¹ respectively, then micro-filtered using 0.22 µl diameter-filter (Sujarwadi 1996).

**Preparation of RPMI media (phenol red-free Roswell Park Memorial Institute culture media)**

The lymphocyte cells were cultured in the RPMI 1640 medium. The medium consisted of 10.42 g of RPMI-1640 medium (R8758, Sigma-Aldrich), supplemented with NaHCO₃ 2 g.l⁻¹, 20 mM.ml⁻¹ HEPES organic buffer solution, and antibiotic solutions (0.1% gentamicin sulfate, 100 µl.ml⁻¹ penicillin, 100 µL.ml⁻¹ streptomycins, and 2.5 µl.ml⁻¹ fungizone). This mixture was diluted with distilled water to 1000 ml and filtered with a microfilter (0.22 µm diameter). A 10% fetal bovine serum was added to the medium before the application, and the mixture was then referred to as complete media (Roehm *et al.* 1991).

**Lymphocyte cell isolation**

The lymphocytes used for cell culture were obtained from the spleens of 2-month old female Balb C mice from the Bogor Veterinary Research Institute. The spleen was removed from dead mice, washed with sterile distilled water, and then crushed in a petri dish containing 5 ml of basic media solution. Subsequently, the lymphocyte solution was centrifuged at 1,500 rpm for 10 minutes, and the settled tissue was removed. Next, the basic media solution was added to the cell suspension and centrifuged again at 2,000 RPM for 10 minutes. The precipitated lymphocytes were then diluted with 10 ml of complete media solution. The viability was tested with 0.4% trypan blue and was calculated by a hemocytometer (Roehm *et al.* 1991).

**XTT reagent preparation**

The lymphocyte cell was proliferated in sodium 3-1-phenylamino-carbonyl-3,4-tetrazolium -bis (4-methoxy-6 nitro) benzene-sulfonic acid hydrate (XTT-Menadione reagent). For this process, 1 ml of 10 mg.ml⁻¹ Menadione was dissolved in 9 ml DMSO (Dimethyl sulfoxide), while 100 µl of XTT was measured and mixed with 25 µl of 1 mg.ml⁻¹ XTT (in PBS/Phosphate Buffer Saline solution). The result was seen as a formazan blue product. This conversion occurred only in living cells, and the amount of formazan produced was proportional to the number of proliferating cells (Roehm *et al.* 1991).

**Test for determining lymphocyte cells number and Con A optimum concentration**

A preliminary test was performed to determine the exact number of lymphocytes and the Concanavalin A (Con A) concentration to test lymphocyte cell proliferation activity. This test utilized lymphocyte cell cultures at the concentrations of 1x10⁴ cells/ml, 2x10⁴ cells/ml and 10x10⁴ cells/ml respectively, while the concentrations of Con A used were 10 µg.ml⁻¹, 25 µg.ml⁻¹, 50 µg.ml⁻¹, 75 µg.ml⁻¹ and 100 µg.ml⁻¹ respectively (Table 1). Furthermore, a complete RPMI medium was incubated on cultured cell plates for 48 hours, and the treatment with the highest average number of lymphocytes was used in this study (Roehm *et al.* 1991).

**Lymphocyte cell proliferation activity**

The lymphocyte proliferation activity test was performed in an aseptic condition, consisted of five treatment groups with three replications each. Thus, the treatments were three groups of lymphocyte cell cultures, each with *K. galanga* extract concentrations of 2.5 µg.ml⁻¹, 25 µg.ml⁻¹, and 250 µg.ml⁻¹. Two other groups were a positive control (Con A) and negative control (lymphocyte cell culture only). Each treatment was inserted into a cell culture plate with 96 holes, and the same five plates were observed for five days. It was followed by incubation at 37°C, in 5% CO₂ and 95% humidity. Each hole was then dripped with 40 µl of Menadione XTT solution (Roehm *et al.* 1991). The plates were retrieved daily to determine lymphocyte proliferation activity by observing the absorption (optical density) with an Elisa Reader at 450 nm.

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Statistical analysis

The results were analyzed using Variance Analysis and further tested by Duncan's test at P <0.05 (SPSS Program) if the effect was significant.

RESULT AND DISCUSSION

The optimum condition for splenocyte culture cells

The preliminary test results on the early stages of lymphocyte cell production at different concentrations of lymphocyte cells and Con A concentrations indicated that the lymphocyte proliferated in all treatments (Table 1). The mean value of lymphocyte cell proliferation was expressed as absorption value (optical density at 450 nm). Furthermore, the T lymphocyte proliferation activity in the cells group with Con A (positive control) exhibited a higher response than the group consisting only of lymphocytes (negative control), indicating the lymphocyte cells used in this in vitro test were alive. Lymphocyte cells responded directly through surface receptors to foreign objects or certain mitogenic stimuli, including lectins, one of the glycoproteins of plant origin.

Con A was a type of mitogen that can stimulate T lymphocyte cell populations (Delves et al. 2017). Con A was a well-known T cell mitogen that could activate the immune system, recruit lymphocytes and elicit cytokine production. The result indicated that this method provided an optimum condition for splenocyte culture cells. Therefore, further study was necessary to understand the effect of K. galanga rhizome on the immune system. The complex interactions of the immune system were highly dependent on lymphocyte cells activation (Jantan et al. 2015).

Effect of Kaempferia galanga ethanolic extract on the splenocyte proliferation activity

The increase in lymphocyte cell proliferation activity with K. galanga extract treatments was higher and significantly different (P <0.05) than the control group (Figure 1). Generally, in the treatment groups, proliferation activity peaked on day four and decreased on day five. Delves et al. (2017) also stated that the peak response of normal lymphocytes occurred on the third or fourth day after stimulation. However, the response of impaired lymphocytes was slow or absent.

The highest lymphocyte cell proliferation activity was obtained at a K. galanga extract concentration of 250 μg.ml⁻¹. It was significantly different (P <0.05) compared to the group treated with 2.5 μg.ml⁻¹ and 25 μg.ml⁻¹ (Table 2). The present study showed that the ethanolic extract of K. galanga rhizome significantly stimulated splenocyte proliferation activity. This result indicates the immunomodulatory properties. The proliferation was much higher in the sample treated with K. galanga rhizome than in the splenocyte cells alone. It might be due to the mitogenic effect of K. galanga extract. K. galanga and related species have been the subject of many phytochemical studies, and many biologically active metabolites, such as flavonoids and polyphenols have been identified. Several studies revealed that phytoconstituents in the K. galanga extract had immunomodulatory activities (Nworu et al. 2010; Nani et al. 2015; Pal Jain et al. 2012; Ma et al. 2015).

Table 1. The optimum concentration of Concanavalin A for splenocyte culture cells

| Total cells/Jumlah sel (10⁴) | Con A Concentration/Konsentrasi Con A (μg.ml⁻¹) |
|-----------------------------|-----------------------------------------------|
|                             | 0    | 10  | 25  | 50  | 75  | 100 |
| 10                          | 0.30 | 1.50| 1.70| 1.60| 1.78| 1.47|
| 2                           | 0.29 | 1.17| 1.21| 1.22| 1.37| 1.20|
| 1                           | 0.32 | 1.04| 1.04| 1.20| 1.10| 1.05|

Note: Splenocytes (1x10⁴ cells/ml, 2x10⁴ cells/ml and 10x10⁴ cells/ml) were stimulated by Con A concentration of 10 μg.ml⁻¹, 25 μg.ml⁻¹, 50 μg.ml⁻¹, 75 μg.ml⁻¹ and 100 μg.ml⁻¹ respectively for 48 h. Cell proliferation was measured by XTT assay method.

Keterangan: Splenosit (1x10⁴ sel/ml, 2x10⁴ sel/ml dan 10x10⁴ sel/ml) distimulasi dengan Con A pada konsentrasi 10 μg.ml⁻¹, 25 μg.ml⁻¹, 50 μg.ml⁻¹, 75 μg.ml⁻¹ dan 100 μg.ml⁻¹ selama 48 jam. Proliferasi sel diukur dengan metode XTT.
The lymphocytes cells used in this study were derived from the spleen. The spleen was a peripheral lymphoid organ that played a central role in host defense and was responsible for the differentiation and maturation of immunocompetent T lymphocytes. In addition, T lymphocytes were the main protagonists in orchestrating the antitumor or antimicrobial response, including CD8+T cells and CD4+T cells (Golub et al. 2018). Therefore, it was assumed that increased splenocyte proliferation activation in vitro could represent the proliferation of immune cells in the spleen.

T-lymphocytes represented a fundamental component of the adaptive immune response. The lymphocyte transformation assay was an important tool to measure mitogen-induced lymphocyte proliferation (e.g., Con A) in vitro. Following T-cell receptor (TCR) engagement, one of the early activities in T-cell activation was the phosphorylation of tyrosine kinases and the generation of inositol 1,4,5-triphosphate (IP3), leading to the release and influx of Ca^{2+}, and the rise in cytoplasmic Ca^{2+} concentration. The increase in (Ca^{2+}) I activated via calcineurin induced IL-2 gene expression, consequently stimulating T cells to produce cytokines IL/interleukin 2. These interleukins were produced by Th/helper cell, known as a growth factor for all T lymphocyte cell subpopulations, due to the clonal expansion of T lymphocytes cell (Nani et al. 2015)

The results were generally in line with other studies related to the immunomodulatory properties of K. galanga. (Dash et al. 2018), conducted research to determine the immunomodulatory activity from the essential oil of K. galanga rhizome on T lymphocytes cells from human umbilical CBMCs (cord blood mononuclear cells) by measuring the number of cytokines produced Th1 and Th2 cells. K. galanga extract at a 40 µl.ml^{-1} concentration provided an increased effect of Th1 cells expression with more IFN-gamma secretion than IL-10. IFN was produced mainly by T cells (via the CD4 and CD8 subsets) and NK cells through the stimulation of mitogens or antigens, and IL-2 also induced this
production. The Th1 response pattern in (Dash et al. 2018) study suggested that the essential oil of K. galanga rhizome modulated the immune system by increasing cellular cytotoxicity. It was critical for fighting intracellular pathogens, including viruses, bacteria, parasites, and tumor cells (Kak et al. 2018).

Table 2. The optical density (OD) mean value of lymphocyte activity cell proliferation treated by ethanolic extract of Kaempferia galanga.

| Group/ Kelompok | Mean lymphocyte cell proliferation (OD)/Nilai rataan proliferasi sel limfosit |
|-----------------|--------------------------------------------------------------------------------|
| Control         | 0.659 a                                                                         |
| Con A           | 2.069 c                                                                         |
| Cons 2.5 ppm    | 1.501 b                                                                         |
| Cons 25 ppm     | 1.499 b                                                                         |
| Cons 250 ppm    | 1.629 b                                                                         |

Note: Numbers with the different letters indicated a marked difference following the Duncan test results at P <0.05.

Keterangan: Angka dengan huruf berbeda menunjukkan perbedaan yang nyata hasil uji Duncan pada P<0.05.

The antitumor effect of K. galanga was a consequence of their direct cytostatic or cytotoxic properties, as shown using a different tumor model in vivo or tumor cell lines in vitro. The K. galanga rhizome was rich in bioactive compounds, including flavonoids, phenolic acid, polysaccharides, and essential oil, with ethyl p-methoxy cinnamic as a major phytochemical constituent (Elshamy et al. 2019). These substances might modulate antitumor immunity by enhancing natural killer (NK)-cell activity or the cytotoxicity of macrophages to tumor cells or by increasing certain functions of T lymphocytes (Delves et al. 2017; Poli et al. 2018). Innate and acquired immune responses mediated the immune response to tumor cells. Cells in the innate immune response were responsible for tumor elimination include Natural Killer cells (NK cells) and macrophage cells. Furthermore, cells in the acquired immune response accountable for tumor elimination were cytotoxic T cells (Delves et al. 2017; Poli et al. 2018). T lymphocytes were the main protagonist in orchestrating the antitumor response, including CD8+ T cells and CD4+ T cells (the acquired immune response) (Delves et al. 2017; Hosseinzade et al. 2019).

This work demonstrated that the ethanol extract of K. galanga significantly modulated the proliferation of splenocytes in vitro. It was assumed that the increased proliferation of these cells, mainly T lymphocytes, in the presence of a mitogenic effect of this plant due to increased IL-2 production (Nani et al. 2015). T cells combined strict target specificity and high efficiency for tumor therapy. Antigen-presenting cells (APCs) activated T cells through a two-signal mechanism: (1) the first signal was initiated by T cell receptor (TCR) binding to antigenic peptide presented by major histocompatibility complex (MHC) molecules and (2) the second signal involved costimulatory molecules that interacted with costimulatory receptors on the T cell surface and led to T cell cytokine production and their proliferation (Zhang et al. 2018). (Yang et al. 2018) found that polysaccharides from K. galanga extract could effectively protect the thymus and spleen of tumor-bearing mice from solid tumors and enhance the immunoregulatory ability of CD4+ T cells, the cytotoxic effects of CD8+ T cells, and natural killer (NK) cells in vivo. Polysaccharides have long been recognized as anticancer agents with low toxicity and slight side effects. In addition, plant polysaccharides can function as immunomodulators, which enhance the body’s defense against cancer cells and, if used in conjunction with conventional chemotherapeutic agents, can also help combat the immunosuppression caused by cancer cells (Khan et al. 2019). Other studies showed that polysaccharides from higher plants, such as Borosma sorbilis (Xu et al. 2016), Pavlova viridis (Sun et al. 2016), and Phoma herbarum YS4108 (Chen et al. 2014) could increase and activate the immune cells, i.e., T-lymphocyte cells.

The essential oil, such as ethyl p-methoxy cinnamic in the K. galanga, was thought to be cytotoxic and possess cytostatic, which meant they could inhibit or stop cancer cells growth (Ali et al. 2017; Dash et al. 2014). The in vivo anticancer activity of a p-methoxy cinnamic acid isolate from K. galanga was determined by (Gunasekaran et al. 2019) on 1,2-dimethylhydrazine-induced rat colon carcinogenesis. Their study concluded that the p-methoxy cinnamic acid showed anticancer
properties by increasing the production of tumor necrosis factor (TNF-α) and interleukin (IL)-6. These substances might modulate antitumor immunity by enhancing the cytotoxicity of macrophages to tumor cells. Macrophages cells, which belong to the innate immune system, can rapidly respond to tumor cells. Macrophages could engulf invading-antigens (phagocytosis) and initiate the innate immune response via releasing TNF-α and IL-6. T lymphocytes cells assist the cellular immune response by increasing T-cytotoxic cell populations and activating macrophage cells. (Zhou et al. 2017). The cytotoxic activity of K. galanga was also proven by (Dash et al. 2014) in vivo using the Brine shrimp lethality test. Furthermore, the methanol extract of K. galanga exhibited anticancer activity in Ehrlich ascites carcinoma (EAC) cells induced in mice. It has been proven to significantly reduce the viability of EAC cells and, consequently, inhibit growth by 70.58% at a dose of 10 mg.kg⁻¹. The Brine shrimp lethality bioassay was also used to evaluate the in vitro cytotoxic effect of extract (Ali et al. 2017).

The present study showed that the ethanolic extract of K. galanga rhizome significantly stimulated splenocyte proliferation activity, indicating potential immunomodulatory properties. It might be due to its content of flavonoids, phenolic acid, polysaccharides, and essential oil such as ethyl p-methoxycinnamate. The ability to increase splenocyte proliferation activation in vitro in this work could demonstrate the proliferation of the immune cells in the spleen, which played a central role in host defense and took charge of differentiation and maturation of immunocompetent T lymphocytes, which were the main protagonists in orchestrating the antitumor. There was increasing evidence that many in vitro and in vivo studies of K. galanga extract revealed antitumor activities (Elshamy et al. 2019). Therefore, further investigations should be done to clarify the mechanisms of the immunomodulatory effect of K. galanga as an antitumor in vivo.

CONCLUSION

The extract of K. galanga at 2.5 µg.ml⁻¹, 25 µg.ml⁻¹, and 250 µg.ml⁻¹ significantly (P <0.05) promoted splenocyte proliferation. Thus, K. galanga ethanolic extract had high potential as immunomodulators. Further study is required to understand the mechanism of the immunomodulatory effect of K. galanga rhizome extract in tumor therapy in vivo.

STATEMENT OF CONTRIBUTORSHIP

Dr.drh Ening Wiedosari, M.Sc was a lead author, and drh. Dianita Dwi Sugiantarti, M.Sc was co-author.

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