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Original Article

In vitro Immunomodulatory Effect from Edible Green Seaweed of *Caulerpa lentillifera* Extracts on Nitric Oxide Production and Phagocytosis Activity of RAW 264.7 Murine Macrophage Cells

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ABSTRACT

Background: *Caulerpa lentillifera* is edible macroalgae which has several biological properties. This marine plant also contains beneficial secondary metabolites that can be used for treatment and prevent several deficiencies. **Objectives:** This research was conducted to evaluate immunomodulatory effect on nitric oxide production and phagocytosis of RAW 264.7 murine macrophage cells from edible green seaweed of *C. lentillifera* extract. **Methods:** Extraction was applied to obtained ethanol extract, water extract and insoluble fraction. Alamar blue inclusion method was used to determine cytotoxicity and proliferation of RAW 264.7 after treated with sample. Griess assay using colourimetric commercial kits was selected to evaluate NO production and phagocytosis effect of samples was determined using phagocytic kit. **Results:** *C. lentillifera* extracts did not show any cytotoxic effect against RAW 264.7 macrophage. One hundred µg/mL of water extract increased the production of nitrite oxide. This extract revealed suspected-giant cells formation as a results of phagocytosis activity. The extract of *C. lentillifera* also enhanced phagocytosis activity of murine macrophage against *E. coli*. *C. lentillifera* extract enhanced activity of RAW 264.7 murine macrophage by increasing NO production and phagocytosis activity. This extract also caused suspected-giants' cells as indicator of phagocytosis effect. **Conclusion:** This result provides an insight that the potency of *C. lentillifera* extract as immunostimulant.

Key words: *Caulerpa lentillifera*, Immunomodulatory, Macrophage, Nitric oxide, Phagocytosis.

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INTRODUCTION

The immune system provides homeostasis mechanism within the body in healthy host. Immunomodulator is a substance that can modify immune functions either up or down its activity.[1] Professional phagocytes including macrophage play a fundamental role in defensive mechanism against pathogens. In response to invading bacteria, phagocyte cell will migrate towards the site of infection followed by engulfment of foreign materials through phagocytosis activity.[2,3] Macrophage also enables to produce various chemical intermediates such as nitric oxide (NO) and hydrogen peroxide (H_2O_2), as well as inflammatory mediators that can eliminate bacteria or cause of infection in the host.[4] The use of immunomodulator is important to gain defense response of the host and it has become a promising therapeutic strategy for the treatment of various disorders, including immunodeficiency. Moreover, immunomodulator can also prevent the host from several ailments due to malfunction of the immune system.[5] Thus, it has been proposed that modulation phagocyte cell by enhancing its activity could be used a basis for the development of immunostimulant agents.

The natural product is a potential source of new drugs. Nowadays, the application of marine seaweed has become prominent for discovering and development of new therapeutic agents.[6] Macroalgae which contains bioactive compounds has been evaluated regarding its potency as a functional ingredient for human health.[7] *Caulerpa lentillifera* is edible seaweed that has been cultivated in Indonesia (Takalar, South Sulawesi). Our previous study showed that *C. lentillifera* contained high protein (14.4%), dietary fibre (8.5%), carbohydrate (32.95%) and low lipid (0.84%).[8] It has been reported that this plant exhibits several pharmacological properties, such as immunomodulator, anti-microbial, antidiabetic, anti-fungal and anti-cancer.[9-12] *C. lentillifera* also possessed strong anti-oxidant activity due to their phenolic compound.[13,14] Some bioactive compounds have also been isolated from this plant, such as clionasterol, 1,4 α-glucan dan 1,3-β-glucan.[13,12] β-glucans showed potential for treating several diseases and recognized as potent immunological stimulators in humans.[15] Therefore, the study was undertaken to evaluate immunomodulatory activity towards macrophage cell from edible green seaweed of *C. lentillifera* extracts and compared with 1,3-β-glucan.

MATERIALS AND METHODS

Plant materials

Seaweed (*Caulerpa lentillifera*) were collected from Takalar, Makassar, South Sulawesi, Indonesia (cultivating seaweed). The material was identified at Research Center for Oceanography, LIPI. The plant material was hot air oven-dried at 50°C and ground to a powder (>80 mesh).

Extraction

The powdered of seaweed was soaked with ethanol, then filtrated. The ethanol extract (CL-EE) was obtained by removing the organic solvent under reduce pressure. Meanwhile, the residue was then re-extracted with water for 3 × 6 h, at 80°C, followed by addition of ethanol with ratio 1:2 to produce water extract (CL-W) and insoluble fraction (CL-In).

Cell culture

Murine macrophage RAW 264.7 was cultured in complete medium containing RPMI-1640 media supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin-streptomycin in 25 cm^2 T-Flask (GIBCO, USA). The cells were incubated in humidified atmosphere with 5% CO_2 at 37°C.

Cell proliferation assay

Cell proliferation was quantified using colorimetric assay. In brief, macrophage RAW 264.7 cell (1 × 10^4 cells/well) was platted into 96-wells plate. Afterward, various concentration of samples (10, 25, 50 and 100 µg/mL) were added each well and incubated for 24 h at 37°C under 5% CO_2. After incubation, 50 µL of Alamarblue™ solutions was added into each well and incubated for 4 h. Next, 150 µL of an absolute DMSO was added after aspiration of media. The cell proliferation was measured using a microplate reader at 560/590 nm (Varioskan Flash, Thermo Scientific). A percentage relative to the proliferation of control cells was obtained.

Measurements of nitric oxide (NO) production

The amount of nitrite, an oxidized product, in the cell culture supernatants were measured in order to calculate the amount of NO. The assay was performed using colourimetric commercial kits (Griess Reagent G2930-Promega, USA). LPS was used as a positive control. Briefly, RAW 264.7 cells were seeded at a density of 5× 10^3 cells/well in 96-well cell culture plates. Cell suspensions were then treated with various concentrations of each sample (10, 25, 50 and 100 µg/mL) or 500 ng/mL LPS (Sigma-Aldrich Co., USA). The treated cell suspension was then incubated for 24 h at 37°C under 5% CO_2. Cell culture supernatant was mixed with Griess reagent (1:1) in a new 96-well plate. Reading was taken at 520 nm using a microplate reader. Nitrite concentrations were determined.

Phagocytosis assay

A modified protocol phagocytic kit (Cayman-500290, USA) was used to determine phagocytosis activity. In brief, RAW 264.7 cells were seeded in 96-well cell culture plates (5× 10^3 cells/well) were treated with various concentrations of each sample (10, 25, 50 and 100 µg/mL) or 500 ng/mL LPS (positive control) (Sigma-Aldrich Co., USA) and then incubated for 24 h at 37°C under 5% CO_2. Next, 2 µl/mL latex bead-FITC was added to each well and incubated for 3 h, washed with buffer solution, quenched with 10 µl *trypan blue quenching solution* for 1 min and washed again with buffer solution. The absorbance of phagocytic cells was measured using a microplate reader at 485/535 nm (Varioskan Flash, Thermo Scientific).

Statistical analysis

Statistical analysis was performed using Student's *t* test (Excel 2013 software; Microsoft, Redmond, WA). *P* values less than 0.05 were considered significant.

RESULTS

Extraction

The extraction of *C. lentillifera* using various solvents and methods produced per cent (%) yield of ethanol extract (CL-EE), water extract (CL-WE) and insoluble fraction (CL-In) were 12, 8 and 6% respectively.

Measurements of nitric oxide (NO) production

The effect of the extract in NO secretion was determined by measuring the nitrite content in the culture medium. As shown in Table 1, the various concentration of extracts and LPS significantly ($p<0.05$) increased nitrite level compared to the untreated cell and only high concentration of β glucan enabled to elevate nitrite level significantly ($p<0.05$) on cells.

Phagocytosis assay

The phagocytosis activity of treated RAW 264.7 cells using phagocytosis assay. The results showed that some of RAW 264.7 cells with LPS, β glucan and seaweed extracts treatment performed suspected-giant cells formation as a results of phagocytosis activity. As shown in Figure 1, *C. lentillifera* extracts and β- glucan ($p<0.05$) showed phagocytosis activity compared to untreated cells ($p<0.05$).

DISCUSSION

Beta (β) glucan, known as an active component in seaweed, was selected as a marker. It has been known that *C. lentillifera* has several medicinal properties including modulation of immune cells. In this study, RAW 264.7 cell line was selected to assess immunomodulatory effect of *C. lentillifera* extracts. In order to know the cytotoxic effect of this extract against macrophage, viability test was performed by using alamar blue assay.[16] It showed that percent (%) viability all test was greater than 80% after 24 h incubation. These results indicated in all tested concentration were non-toxic and it can be used for further experiments.

Macrophage plays an important role to modulate the immune system. This cell enables to kill invading pathogens through phagocytosis activity. In response to infection, macrophage also releases some inflammatory mediators, cytokines as well as free radical.[17] Nitrite oxide (NO) is one of the important indicators for inflammation. Increasing of

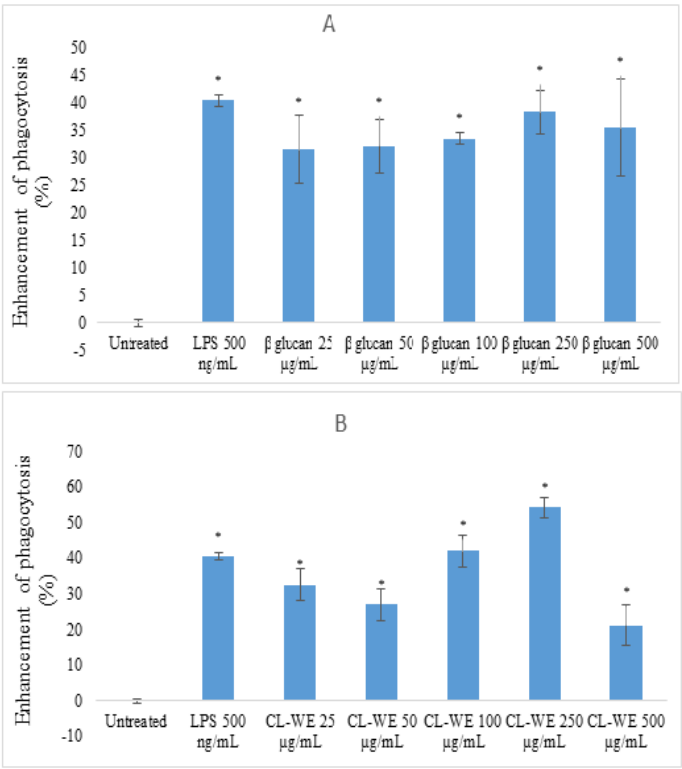

Figure 1: The effect of samples; A. β glucan, B. CL-WE (*Caulerpa lentillifera* Water Extract) on phagocytic activity of RAW 264.7 murine macrophage cells. (mean ± SEM, n=3). **P* value < 0.05 (student *t*-test) was considered to indicate significance vs untreated cells.

NO production in macrophages will stimulate immune cells activity in the host. Therefore, in this study, macrophage was selected as *in vitro* cell model. Lipopolysaccharide (LPS) is used in this research is an endotoxin found in the outer membrane of Gram-negative bacteria.[18] It can trigger activation of immune cells, due to its function, LPS was selected as a positive control in regard of immuno-stimulant. The effect of the extract in NO secretion was determined by measuring the nitrite content in the culture medium. As shown in Table 1, the various concentration of extracts and LPS significantly ($p<0.05$) increased nitrite level compared to the untreated cell and only high concentration of β glucan enabled to elevate nitrite level significantly ($p<0.05$) on cells. This finding exhibited that *C. lentillifera* extract in all tested concentrations and β glucan induce production of NO on RAW 264.7 cells. This result was in aggreement with previous study which reported that polysaccharides from *C. lentillifera* possessed immunostimulatory activity by increasing the secretion of NO and inflammatory cytokines.[19] In addition, polysaccharides derived from this algae also enabled to stimulate NO production on murine macrophage.[20]

Phagocytosis is a fundamental function of immune cells to eliminate foreign materials including pathogens.[21] In order to know correlation between NO secretion and phagocytosis, then we also observed the phagocytosis activity of treated RAW 264.7 cells using phagocytosis assay. The results showed that some of RAW 264.7 cells with LPS, β glucan and seaweed extracts treatment performed suspected-giant cells formation as a results of phagocytosis activity. As shown in Figure 1, *C. lentillifera* extracts and β- glucan ($p<0.05$) showed phagocytosis activity compared to untreated cells ($p<0.05$). However, dependent concentration response was not found in this study. Amongst all of tested sample, 100 µg/mL of CL-WE and β-glucan (250 and 500 µg/mL) produced phagocytosis activity which were similar to LPS as positive control. Overall, it can be seen that CL-WE enable to enhance phagocytosis activity as one indicator of immuno-stimulant. This result was in agreement with previous studies. It has been reported that polysaccharides isolated from *C. lentillifera* promoted proliferation of macrophage. This compound also enhanced phagocytosis activity of immune cells.[20] Another study also reported that polysaccharides derived from seaweed was able to modulate macrophage function.[22] It has been known that seaweed and its active compound enabled to enhance immune system in response of invading pathogens. Polysaccharide is a major active compound derived from seaweed which has possessed immunomodulatory activity. Sulfated polysaccharides derived from *Laminaria ochroleuca*, *Porphyra umbilicalis* and *Gelidium corneum* were able to increase the proliferation of murine macrophage.[23] Recently study showed that *C. lentillifera* contain sulfated polysaccharide.[24] Furthermore, sulfated polysaccharides from *Caulerpa cupressoides* Var. *Flabellata* exhibited strong activity in enhancing activity of innate immune cells. This compound increased the secretion of proinflammatory cytokines including IL-6 and TNF-α, as well as intracellular reactive oxygen species.[25] This finding proposed that seaweed, an edible plant as a source of functional food which provide many beneficial effects for health including immunomodulator.

Table 1: The effect of samples on NO production of RAW 264.7 murine macrophage cells (mean ± SEM, *n*=3).

Sample	Concentration	Concentration of nitrite (µM)
Untreated	-	1.3 ± 0.1
LPS	500 ng/mL	2.9 ± 0.1*
β glucan	25 µg/mL	1.5 ± 0.2
	50 µg/mL	1.4 ± 0.1
	100 µg/L	1.9 ± 0.1*
	250 µg/L	1.9 ± 0.2*
	500 µg/L	2.7 ± 0.5*
CL-WE	25 µg/mL	1.7 ± 0.1*
	50 µg/mL	1.6 ± 0.1*
	100 µg/mL	1.9 ± 0.2*
	250 µg/mL	2.5 ± 0.3*
	500 µg/mL	2.7 ± 0.3*

*P value <0.05 (student t-test) was considered to indicate significance vs untreated cells.

CL-WE (*Caulerpa lentillifera* Water Extract)

CONCLUSION

In conclusion, *C. lentillifera* extract derived from cultivation contained various nutrients, as well as active components, can enhance the immune response. This extract increased the NO secretion on murine macrophage and also enabled to raise phagocytosis activity of phagocyte cell. This finding indicates that *C. lentillifera* extract has potential as immunomodulatory agents.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

NO: Nitric oxide; **LPS:** Lipopolysaccharide; **IL:** Interleukin.

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