Effect Of Lutein from *Chlorella pyrenoidosa* INK On The Activities And Phagocytic Capacity On Peritoneal Macrophage Cells Of Mice Infected With *Staphylococcus aureus*

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Abstract. *Chlorella pyrenoidosa* is a microalgae that produce lutein compounds that can stimulate an immune response. This study examined the crude lutein extracted from biomass *C. pyrenoidosa* INK as an immunomodulator through observation activity and phagocytic capacity of peritoneal macrophages of mice. This study uses twenty-eight mice were divided into 7 groups each comprising 4 replicates ie Group (I) normal controls, mice were untreated(II) a negative control, mice were induced by *Staphylococcus aureus*; (III) positive control, mice were induced by *S. aureus* and treatment of meniran extract (*Phyllanthus niruri*, stimuno®). The treatment group (IV-VII), mice were induced by *S. aureus* and treatment of crude lutein, respectively: 0.058 mg, 0.117 mg, 0.234 mg and 0.468 mg per 20 g of body weight. Lutein crude was given for 2 weeks and the next day the mice were injected bacterium *S. aureus*. The results showed that crude lutein from *C. pyrenoidosa* INK act as immunomodulators that can stimulate an immune response as the dose increases. Treatment of crude lutein dose of 0.234 mg per 20 g BW of mice showed a proportional response to the positive control treatment (stimuno®). Lutein of *C.pyrenoidosa* had a positive effect on the the activity and capacity of peritoneal macrophage in mice after stimulation with *S. aureus* bacteria.

Keywords: Lutein, *C. pyrenoidosa*, immunomodulatory, macrophage

1. Introduction

The decline of the body's immune system response can lead to the increase of several pathological conditions. Nowadays, treatment using natural ingredients has become an option. The treatment can be done as efforts of keeping and maintaining health, even as efforts of treating a certain disease. The scientific development of how cells interact leads to the development of the manipulation of the communication lines among the cells. The materials that can modulate the body's immune system are known as immunomodulators.

Immunomodulator is a drug or substance that can restore and repair the imbalance in the immune system which function is impaired or suppress its excessive function [1][2]. The function of immunomodulatory is to improve the immune system by restoring impaired immune system function (immunorestore), to improve the function of immune system components by stimulating (immunostimulatory) or by pressing or normalizing abnormal immune reaction (immunosuppressants) [2][3].

Immunostimulator is a way to improve immune system function using immunostimulatory, i.e. a material that stimulates the immune system that coming from inside and outside of the body; thymus hormones, lymphokines, interferons, bacterial, and antioxidant compounds are the examples [1][2]. The immune response of carotenoids was first reported by Bendich and Shapiro [4]. They showed that mice that were fed a carotenoid had a high lymphocyte mitogen induced proliferation and
stimulated phagocytosis, although at a lower level. All these changes indicated increased activity of macrophages when given carotenoids. Lutein is a yellow solid crystalline xanthophyll carotenoid. In addition, lutein improves the antibody response to the vaccine and humoral immune responses. Lutein could increase the antibody response of mice by increasing T-cell antigen. Other studies reported that lutein may improve the immune system. The results of the study Kim et al. (2000) showed that the lutein in dogs increased the number of Th and Tc cells, molecules of the major histocompatibility complex (MHC) class II, resulted the increase of proliferation of peripheral blood mononuclear cell (PBMC) mitogen [5]. The study on dogs given lutein showed stimulation of CD4 + T cell number and the stimulation of IgG production. It suggested that lutein, non-provitamin A carotenoid, had a similar action with β-carotene [6]. Lutein is differentially localized to membrane domains rich in polyunsaturated fatty acids including DHA (docosahexaenoic acid), and therefore is well positioned to block oxidation of these vulnerable lipids [7]. Inhibition of DHA oxidation not only helps to maintain membrane structure and fluidity but also preserves DHA so it remains available for cleavage and conversion into anti-inflammatory molecules [8].

Lutein functions as a powerful antioxidant and as high-energy blue light filtration. It is also an essential nutrient that is required for the prevention of heart disease, stroke, and cancer. Lutein can also be skin protector from UV rays contacts [9]. Lutein is a red, orange, or yellow pigment contained in fruits and vegetables like tomatoes, carrots, and some green vegetables. Lutein is also found in microalgae.

Microalgae are generally green photosynthetic pigment (chlorophyll), brown (fikosantin), turquoise (fikobilin), and red (fikoeritin). Green algae (chlorella) is a single-celled microalgae (unicellular), living in a cluster and efficacious for human health. Chlorella pyrenoidosa is known as the producer of various types of carotenoids, such as lutein, β-carotene, α-carotene, anthaxanthin, neoxanthin, and zeaxanthin [10]. Chlorella pyrenoidosa is a microalgae which is widely consumed by humans and is known to contain compounds lutein as immunostimulation that can stimulate an immune response.

The advantages of lutein production from microalgae : (1). Microalgae are a cheap and effective bioresource that can be used to produce value-added compounds, including chemicals, vitamins, carotenoids, and polysaccharides (2). Microalgae’s growth rate is 5–10 times that of higher plants. (3). Microalgae, which can be cultivated in seawater or brackish water and on non-arable land, do not compete for resources with conventional agriculture. (4) Microalgal biomasses can be harvested all year [11].

Lutein crude is extraction result which has been separated from other compounds, although it has not yet reached the stage of purification. From the previous research results lutein from Chlorella pyrenoidosa INK amounted 0.878 μg per gram wet weight of microalgae cells were obtained. The result of the effectiveness test of antioxidant lutein upon the blood cells of red of mice induced by t-BHP showed that the compounds lutein as the result of microalgae Chlorella pyrenoidosa extraction were able to reduce levels of malondialdehyde (MDA) and were able to increase the superoxide dismutase (SOD) activity and catalase activity [12].

The results of the study conducted by Kim, et al.(2000) reported that administration of lutein dose of 20 mg/kg to dogs was very effective in stimulating an immune response [5]. One of the efforts made by the body’s immune system to defend itself against the entry of antigen, i.e. conducted by destroying antigens through phagocytosis and cells that play a role in phagocytosizing antigen including macrophage cells. Based on the information obtained, this study is focused to determine the effects of lutein crude oil from microalgae Chlorella pyrenoidosa as an immunomodulator through observation activity and macrophage cells phagocytic capacity of peritoneal fluid of mice.

2. Methods
2.1. Cultivation of Chlorella Pyrenoidosa INK.
Chlorella pyrenoidosa INK was isolated from fresh water pond in Cianjur, West Java, Indonesia and it’s cultivated in a one-liter-capacity bottle containing growth medium with 2000 lux fluorescent
lighting. The density of the culture was observed by measuring the OD (Optical Density) using a spectrophotometer until it reached the logarithmic growth phase. Furthermore, culture was transferred into a two-liter-capacity bottle containing Modified Basal Medium (MBM) for the growth of Chlorella with the following composition (2L): 2.5G KNO₃, 2.5g K²HPO₄, 2g MgSO₄, 0.22g CaCl₂, 0.0022g H₃BO₃, 0, 01G Na₂EDTA, 0,0003g MnSO₄.4H₂O, 0,0018g ZnSO₄.7H₂O, 0,00032g CuSO₄.5H₂O, 0,001g Co (NO₃) 2, 2H₂O and 0,001g FeSO₄.7H₂O. Initial OD was 0.4-0.6 [13].

2.2. The weight of Wet Biomass of Chlorella pyrenoidosa INK.
C. pyrenoidosa cultures having reached the stationary phase were harvested. Culture was centrifuged for 10 minutes at a speed of 6000 rpm to separate the cell biomass and liquid media. Furthermore, the biomass obtained was weighed (grams) and was extracted by lutein.

2.3. Lutein extraction.
Wet biomass of microalgae C. pyrenoidosa INK added by n- hexan was sonified at 40,000 Hz, then it was put into a sheaker for 24 hours with 50 ml of n-hexane until was supernatant obtained. The effort was repeated until the supernatant turned into pale yellow. Supernatant was collected and then added with isopropanol and heated at 40 °C while being stirred homogeneously for 60-90 minutes. Then, it was saponified with 50% NaOH and stirred until it became homogeneous with a magnetic stirrer for 2 hours and was left until two layers (the yellow layer and colorless layer) were formed. Yellow layer was taken and then washed with water, stirred until homogeneous for 1 hour at room temperature. After it became homogeneous, it was left for 1—4 hours until two layers (the yellow layer and colorless layer) were formed. Yellow layer was taken and then evaporated using a water bath at a temperature of 40°C, to obtain a crude lutein [14].

2.4. Identification of Crude Lutein by Thin Layer Chromatography.
Lutein crude resulted from C. pyrenoidosa INK extraction and reference standard lutein was weighed (approximately 1 m) and then was dissolved in 1 mL of methanol. Then, spotting on silica gel GF₂₅₄ was conducted. Chromatography thin-layering was done by trying the combinations of three rows eluotropi solvent based solvent thin layer chromatography GF254. The solvent used was a combination of three solvents heksan- chloroform- acetone with a ratio of 6: 2: 2 in a chamber that has been saturated. Observation was done visually in UV light at a wavelength of 254 nm. KLT pattern was observed and its Rf was calculated by comparing it with the pattern of KLT reference standard.

2.5. Determination of drying shrinkage Biomass.
A total of 1 gram of biomass was weighed and then dried at a temperature of 105°C oven, then cooled in excicator and weighed until the weight was constant.

Treatment in mice. A total of 28 seven-week-old male mice were put into seven groups, each group consisted of four mice. The treatment for each group group as follows:

Group I : normal control, mice were given vegetable oil
Group II : negative control, mice were given vegetable oil + 0.5 ml S. aureus bacteria
Group III : positive control, mice were given stimuno® 0.206 mg/20g bw/day + 0.5 mL S. aureus
Group IV : mice were given the extract lutein 0,058 mg/20g bw/day + 0.5 mL S. aureus
Group V : mice were given the extract lutein 0.117 mg/20g bw/day + 0.5 mL S. aureus
Group VI : mice were given the extract lutein 0.234 mg/20g bw/day + 0.5mL S. aureus
Group VII : mice were given the extract lutein 0.468 mg/20g bw/day + 0.5 mL S. aureus

Mice were treated extract for 14 days and on day 15 they were given S. aureus bacteria suspension 0.5 ml except the normal control group.

3. Results and Discussion
Chlorella pyrenoidosa is one type of microalgae that live in fresh water and sea. Chlorella pyrenoidosa INK in this study were grown in medium MBM (Modified basal medium) which was continuously aerated with a light intensity of 2500 lux. The use of aeration as a source of CO2 equalized the distribution of nutrients and prevented sedimentation of the culture. Light administered to algae culture become source of energy in the process of photosynthesis.

Observation of the growth curve during the culturing of microalgae was aimed to determine the phase of growth of Chlorella pyrenoidosa INK and the selection of appropriate harvest time. Making the curve based on the OD (Optical Density) with method turbidimetry was done using spectrophotometer UV-Vis at a wavelength of 680 nm. The initial density (OD) of 0.5. The pattern of cell growth of microalgae Chlorella pyrenoidosa INK consisted of lag phase, logarithmic phase, stationary phase and death phase. The logarithmic phase was characterized by the rising of growth rate to the multiple increase of population density. According Isnansetyo and Kurniastuty (1995), Chlorella sp. can reach the phase within 5—7 days. Dealing with the phase of growth rate decline, the growth rate of algae relatively decreased on days 6—7 and on days 8—10 stationary phase happened, at this phase the increase of the population density was balanced by mortality, so there is no population growth [15].

3.1. Microalgae Biomass Cell C. Pyrenoidosa INK and Lutein Crude.

The growth of C. pyrenoidosa INK microalgae cell density was daily observed using a spectrophotometer at a wavelength of 680 nm. Chlorella pyrenoidosa INK cell culture was harvested at stationary phase to obtain maximum lutein, i.e. on the 8th day after cultivation. The process of extracting lutein from biomass C. pyrenoidosa INK was done using Madhavi method for separating compounds of non-carotenoid pigments and other contaminants. The method was simple, concise, and did not use much solvents. The cell wall of C. pyrenoidosa INK was broken by sonicator in hexane solution to facilitate the withdrawal of carotenoids, especially lutein compounds optimally. Then the digestion process was carried out in a solution of isopropanol; the effort was aimed to remove contaminants such as resin, wax and non carotenoid pigments such as chlorophyll. Furthermore, the saponification process was carried out using NaOH to break ester bonds, because lutein available in nature was in the form of an ester bond. The addition of water was aimed to attract polar compounds, it was to wash contaminants such as salts of fatty acids. The advantage of this method was the hydrolysis of the ester could run efficiently at lower temperatures and shorter time, because oleoresin completely was insoluble in isopropanol at a temperature of around 60º-65ºC[14]. The acquisition of wet biomass and crude lutein from C. pyrenoidosa INK culture were 8,923 ± 1.02 (g per 2L) and 83.0 ± 0.02 (mg per g biomass), respectively. Lutein crude was identified qualitatively using thin layer chromatography test and spectrophotometry.

3.2. Determination of Extract Drying Shrinkage.

The results of the determination of drying shrinkage was 4.428%. This value meets the requirement as required in pharmacopoeia, i.e not more than 10%.

3.3. Analysis of Lutein Using Thin Layer Chromatography (TLC).

Qualitative analysis of lutein using Thin Layer Chromatography, with silica gel GF254 as stationary phase, mobile phase was hexane-chloroform-acetone (6:2:2). The results showed that each of the standard lutein and lutein crude had the value of RF 0.68 and HRF 68. Analysis of thin layer chromatography was carried out using an eluent combination of three hexane-chloroform-acetone with ratio of 6:2:2. Distance spotting reference standards lutein 5.5 cm with eluent creepage distance of 8 cm; the result was equal to the distance at spotting crude lutein obtained, so that both of them had RF value 0.68 and hRF 68. Analysis of lutein in spectrophotometric UV-Vis was carried out through maximum absorption measurement at a wavelength of 400—500 nm with methanol. The spectrum of test samples had the same peak as the peak of standard solution indicated the existence of lutein. Raw Lutein has three peaks in the visible region, i.e. at a wavelength of 468.0 nm, 443.0 nm, and 422.0 nm. The selection of the maximum wavelength in this study was
seen form the highest absorption at the spectrum produced, i.e at 443.0 nm. Meanwhile, crude lutein sample wavelength was at 421.0 nm, 443.0 nm, and 469.0 nm.

3.4. Macrophage Cells Phagocytosis Activity in Mice Peritoneum Fluids.

The observation of the number of phagocytic activity of macrophage cells in each treatment group are listed in Table 1.

| Group | Treatment          | Replicates | Mean | SD  |
|-------|--------------------|------------|------|-----|
|       |                    | 1  | 2   | 3   | 4   |
| G1    | (normal)           | 40 | 39  | 43  | 38  | 40a | 2.16 |
| G2    | (Negative)         | 49 | 48  | 47  | 48  | 48b | 0.82 |
| G3    | (Positive)         | 82 | 80  | 84  | 82  | 82c | 1.63 |
| G4    | (0.058 mg lutein)  | 62 | 64  | 65  | 63  | 63.5c | 1.29 |
| G5    | (0.117 mg lutein)  | 70 | 74  | 73  | 75  | 73d | 2.16 |
| G6    | (0.234 mg lutein)  | 82 | 80  | 80  | 79  | 80.25e | 1.26 |
| G7    | (0.468 mg lutein)  | 88 | 86  | 89  | 89  | 88f | 1.41 |

The value of phagocytic activity is determined based on the number of macrophage cells that conduct phagocytosis (active) in 100 macrophage cells and is expressed in percentage. Table 1 shows that in normal control group, the average number of active macrophages cells (40% ± 2.16) was less than the one in other treatment groups, but not much different from the average number of active macrophages cells in the negative group (48% ± 0.82). In the fifth group the number of cells (73% ± 1.26) was more than the one in normal group, the negative, and the fourth group (63.5% ± 1.29). Meanwhile, the number of the cells in the sixth group (80.25% ± 1.26) was similar to the one in the positive group (82% ± 1.63) and the highest average value was in the seventh group (88% ± 1.41). Phagocytic activity average value of macrophage cells showed increased activity which was in line with the increased dose of lutein *crude* from the microalgae *Chlorella pyrenoidosa* INK, of doses of 0.058 mg/20g BW, 0.117 mg/20g BW, 0.234 mg/20g BW, 0.468 mg/20g BW, even the sixth group is equal to the positive group which was given extracts of *Phyllanthus nirur*.

Testing of immunomodulatory effects of lutein crude from microalgae *Chlorella pyrenoidosa* INK based on activity and capacity of macrophage cell phagocytic. Experiments were performed in vivo in mice given different treatment for each group, i.e. variations of lutein crude *Chlorella pyrenoidosa* dose for 14 days. Observations were done upon differences in mice immune response against the entry of microorganisms and the treatment of *Chlorella pyrenoidosa* lutein dose variation which was different. On day 15 mice from the second group to the seventh group were injected *Staphylococcus aureus* bacteria intra peritoneal (i.p), then, they were left for two hours as a strange microorganisms that can stimulate an immune response in mice. The preparate for peritoneal fluid was prepared and fixed with methanol, and then dyed with Giemsa. Macrophage cells looked purplish red and bacterial cells looked purple-blue. Preparate was viewed under a microscope with a magnification of 1,000 times, to observe the increased activity and capacity macrophage cell phagocytic.

Table 1 shows the number of phagocytic activity of macrophage cells in each treatment group. Value of phagocytic activity was determined based on the number of macrophage cells that conducted phagocytosis (active) process in 100 macrophage cells which were expressed in percentage. Data for phagocytic activity and capacity were obtained after the normality and homogeneity data were tested; and it indicated that the data were normally distributed (p>0.05) and homogeneous (p>0.05), so that data could be analyzed using one way analysis of variance (ANOVA). ANOVA test to see any difference in phagocytic activity and capacity of each treatment group. From the results of the data significance of 0.000 (p<0.05) was obtained, which showed a significant difference between the phagocytic activity and capacity of each treatment group. Statistical analysis was followed by Tukey's test. Phagocytic activities and capacity of macrophage cell of peritoneal fluid in mice in the normal
group, negative group, fourth group, fifth group, and seventh group are significantly different from the other treatment groups. Meanwhile, the positive group and the sixth (lutein crude *Chlorella pyrenoidosa* INK dosed of 0.234 mg/20 g BW) were not significantly different, but were significantly different from other treatment groups. The results of the study showed the influence and the increase of the immune response through increased phagocytic activity and capacity of macrophage cells of peritoneal fluid in mice given *Chlorella pyrenoidosa* INK lutein crude.

3.5. Phagocytic Capacity of Fluid Cells Macrophages in Mice peritoneum.

Figure 1 shows the capacity of macrophage cells that actively phagocytosize pathogenic bacteria *S. aureus* in mice peritoneal fluid. Table 2 shows the phagocytic capacity of macrophage cells in each treatment group. Phagocytic capacity value was number of bacteria phagocytosed by 50 macrophage cells.

![Figure 1. Staphylococcus aureus bacterial phagocytosis by macrophages Cells.](image)

Macrohage cell phagocytic capacity peritoneal fluid of mice (Table 2) shows that in the normal treatment group, the average value of an active macrophage cell capacity (137.75 ± 5.56) was lower than other groups, but not much different from the average value average capacity of macrophage cells active in the negative group (300.75 ± 14.59). In the fifth group, in which the treatment with a dose of 0117 mg lutein per 20 g BW, it was shown that the capacity of active macrophage cells (592 ± 8.45) was higher than the normal group, negative, and the fourth group 4, i.e. the groups with lutein dose of 0.058 mg (485 ± 14.38). Meanwhile, the sixth group, i.e. the group with doses of 0234 mg of lutein (650.5 ± 9.26) was equal with the positive group (644.75 ± 17.46) and the highest average value was highest was found in the seventh group with the doses of 0468 mg lutein that is equal to (690 ± 4.32).

The average value of phagocytic capacity of macrophage cells showed increased activity in line with the increase of lutein crude doses from the microalgae *Chlorella pyrenoidosa* INK, of doses of 0.058 mg/20gBW, 0.117 mg/20g BW, 0.234 mg/20g BW, 0.468 mg/20g BW, even doses in the sixth group is similar to the doses in positive group which is given extracts of *Phyllanthus niruri*.

| Table 2. Macrophage Cells Phagocytosis Capacity of Mice Peritoneum Fluid |
| --- |
| | Macrophage Capacity | Average | SD |
| Treatment Groups | Replicates 2 | 3 | 4 |
| Normal group | 137.75 ± 5.56 |  |  |
| Negative group | 300.75 ± 14.59 |  |  |
| Fifth group | 592 ± 8.45 |  |  |
| Fourth group | 485 ± 14.38 |  |  |
| Sixth group | 650.5 ± 9.26 |  |  |
| Positive group | 644.75 ± 17.46 |  |  |
| Seventh group | 690 ± 4.32 |  |  |
4. Conclusions
The research showed that lutein _crude_ from microalgae _Chlorella pyrenoidosa_ INK had the effect as an immunomodulator to increase phagocytic activity and capacity of peritoneal macrophages of mice along with the increase in dose. Treatment of lutein _crude_ dosed at 0.234 mg/20gBW against mice induced by _S. aureus_ gave results which were similar to the positive control. Meanwhile, the highest activity and capacity was found at the administration of lutein _crude_ dosed at 0.468 mg/20 g BW.

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