Stimulating Activity on Human Lymphocytes *in vitro* of Nori like Product (*Geluring)* Made from *Gelidium* sp. and *Ulva lactuca* Seaweeds

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***Abstract*— Seaweed has been reported to contain bioactive compounds that have an immunomodulatory activity, such as stimulating activity on human lymphocytes. Nori like the product from the mixture of *Gelidium* sp. and *Ulva lactuca* seaweed in this research named "*geluring*" may improve the immune system. This research is aimed to determine the stimulating activity of *geluring* by observing proliferation activity and interleukin-2 (IL-2) production of human lymphocyte by in vitro method. Two types of *geluring* were prepared, that were P1 (unseasoned *geluring*) and P2 (seasoned *geluring)* according to the commercial nori process with some modification. *Gelurings* were extracted with water and the extracts were added into lymphocyte cultures with various concentrations. The results showed that extracts of P1 and P2 *gelurings* could stimulate lymphocyte proliferation and IL-2 production significantly ($P<0.05$) compared with the stimulations demonstrated by the cultures stimulated with PHA, RPMI, and extracts of unprocessed dried *Ulva lactuca* (UP0) but not significantly as compare to cultures added with unprocessed dried *Gelidium* sp.(GP0). Moreover, P2 *geluring* showed stimulation of lymphocyte proliferation and IL-2 production higher than P1 *geluring* and those of the control cultures. There was a positive correlation of proliferative activity with IL-2 production of the lymphocytes. The stimulation of lymphocyte proliferation and IL-2 production by P1 and P2 *gelurings* was significantly influenced by the concentration of the extracts. The concentration of 2.66 mg/ml culture showed the highest proliferation and IL-2 production of the cells. Base on this research, it can be concluded that *geluring* products made from the mixture of *Gelidium* sp. and *Ulva lactuca* stimulate the activity of lymphocytes, which indicates the potential to be used as a health food to improve the immune system.**

***Keywords*—immune system; interleukin-2; lymphocytes proliferation; *geluring*; seaweed.**

I. INTRODUCTION

Seaweed has been studied to contain bioactive compounds that have biological activities to improve health [1]–[5]. One of the important biological activities of seaweed is the immunomodulatory activity, which is the ability of a substance or chemical compound in modulating the function and activity of the immune system. The immune system is a complex biological system, which happens through various mechanisms to guard and defend the body from attacking microorganisms. The existence of a well-functioning and good immune system activity could suppress a number of diseases and preserve the health [6],[7].

Bioactive components of seaweed such as phenolic, flavonoid, carotenoid, sulfates polysaccharide have the potency of immunomodulation through various mechanism [8]–[11]. One of the bioactive compound mechanisms in increasing the immune system is through the stimulation of lymphocyte proliferation activity [12],[13]. Lymphocyte cell stimulation process will stimulate various biochemical reactions within T cell, one of them is the production of several types of important cytokines needed for the immune

response including interleukin-2 (IL-2). This cytokine has an important role in stimulating lymphocyte cells to proliferate, regulating the signals during the immune response, and increasing differentiation of T and B lymphocyte cells [14],[15].

Research on lymphocyte cell stimulation activity by seaweed extracts or compounds isolated from seaweed have been done [16]–[19], however lymphocyte stimulation activity by processed food products made from seaweeds, especially dry sheet products or nori like product has not been conducted. *Geluring* is a processed seaweed product in form of dry thin sheet made from the mixture of dried *Gelidium* sp. and *Ulva lactuca*, adapted from nori production in Japan or Korea that are made from *Porphyra* seaweed. *Geluring* has been studied to contain a bioactive compound such as phenolic and flavonoid [2]. *Geluring* has the potential to be used as immunomodulation, particularly to increase the activity of lymphocyte stimulation due to the presence of bioactive compounds reported to have immunomodulation capacity. *Geluring* processing that involves heating process may reduce several bioactive components in the product, which could affect the biological activities, including lymphocyte cell stimulation *activity.Cox* et al.[20] and Cervantes-paz et al.[21] stated that the processing of food involving heat, can reduce bioactive components in food.

This research is aimed to determine the stimulating activity of *geluring* by observing proliferation activity and interleukin-2 (IL-2) production of human lymphocytes by in vitro method. Proliferation activity of lymphocyte and production of IL-2 in vitro are affected by the concentration of extracts, therefor it is important to study the optimum concentration of *geluring* extracts. The result of this research shall be an initial information to demonstrate the potential of *geluring* as health food product to be used for improving the immune system.

II. Material and Method

A. Geluring Processing

Geluring product was processed by Erniati et al [2] methods.

B. Extraction of Geluring Products

Extraction of UP0, GP0, P1 and P2 were done by maceration process using water. The powdered of *geluring* products and unprocessed dried *Ulva lactuca* (UP0) and dried *Gelidium sp.* (GP0) weighing 1.25 g each were added with 25 ml aquadest, shaken for 8 hours, macerated for 24 hours and centrifuged at 500 g for 30 minutes. All supernatant was filtered with a 0.22 μm sterile membrane for use in the lymphocyte proliferation analysis and IL-2 assay.

C. Preparation of Peripheral Blood Lymphocytes

This study has received ethical approval from the Research Ethics Committee of our Institution. Lymphocytes were isolated from 40 ml peripheral blood of a healthy volunteer who signed the informed consent forms. The blood was centrifuged at 300 g for 10 min to obtain buffy coat layer containing lymphocyte cells. The buffy coat was passed in the *ficoll-hypaque* (Sigma) and centrifuged at 900 g for 30 min. The cell pellets were harvested washed with PBS and added with 9 ml RPMI (Sigma). Cell viability was calculated using 10 µl cell suspension using 3% acetic acid with hemocytometer. The cell viability should be 95% living. The lymphocyte suspension was then added with FBS (Sigma) 10% and Penicillin (Sigma) 100 U/ml, Streptomycin (Sigma) 100 µg/ml [22].

D. Lymphocyte Proliferation activity assay

Suspension of lymphocytes cells ($1x10^6$ cells/ml), 80 µL, were cultured in 96 well microplate and incubated at 5% CO2, 37^{o}C and RH 90% for 24 h. After 24 hours, 20 µL of each *geluring* extract was added to the wells with concentrations in the cultures of 1.33 mg/ml (K1), 2.66 mg/ml (K2), 3.99 mg/ml (K3) and 5.32 mg/ml (K4) and then reincubated for 48 hours. In other wells, lymphocytes were added with GP0 and UP0 extracts at concentration of culture 2.66 mg/ml, and mitogen PHA (10 µg/ml, Sigma) as a positive control, and RPMI media as a negative control. Four hours before the incubation period, the cultures were added with 10 µl MTT (3-[4,5-dimetilthiazol-2yl]-2,5-difenil tetrazolium bromide, Sigma) at concentration 5 mg/ml. After the incubation, all cultures were added with 100 µl ethanol (p.a). Absorbance value was read using microplate reader at λ 595 nm [12]. The activity of stimulating was expressed as the proliferation activity (% proliferation).

$$\% \text{ proliferation} = \frac{Absorbance\ of\ sampel}{Absorbance\ of\ control} x\,100\% \quad (1)$$

E. Interleukin-2 (IL-2) of lymphocyte cell assay

The suspension of lymphocyte cell (10^6 cells/ml) was introduced into 96 well microplate and cultured for 24 hours as in the proliferative activity assay. After 48 hours cultured, the cultures were centrifuged for 10 minutes at 300 g. The supernatant obtained was used to analyze the IL-2 production of lymphocyte cells with the procedures of IL-2 ELISA KIT (*Elabscience*) [23].

F. Statistical Analysis

The design of experiment used was completely randomized design. The obtained data was statistically analyzed using One Way Analysis of Variance, then followed with Duncan test on 5% level of significance. Data processing was done using SPSS program.

III. Result and Discussion

A. A. Lymphocyte Proliferation Activity

The result of this research shows that the extracts of *geluring* (P1 and P2) could increase proliferation activity of human lymphocytes *in vitro*. The lymphocyte proliferation (% proliferation) of the extracts were significantly different ($P < 0.05$) with the cultures added with PHA mitogen and dried *Ulva lactuca* leaf extract (Fig.1).

In Fig. 1, it can be seen that the highest lymphocyte proliferation activities occurred in culture added with P2 *geluring* (seasoned *geluring*) extract. The addition of spices such as garlic and pepper 0.1% in *geluring* P2 may contribute to increase the lymphocyte proliferation activity. This is because garlic and pepper contain bioactive

components that can stimulate lymphocyte to proliferate. Garlic extract was reported to stimulate lymphocyte proliferation activity in in vitro methods [24]. Bioactive components in pepper was also reported to be capable to increase lymphocyte proliferation activity. Reference [25] stated that piperine compound extracted from pepper could increase mouse lymphocyte proliferation under maximum dosage of 1 µg/ml using MTT method.

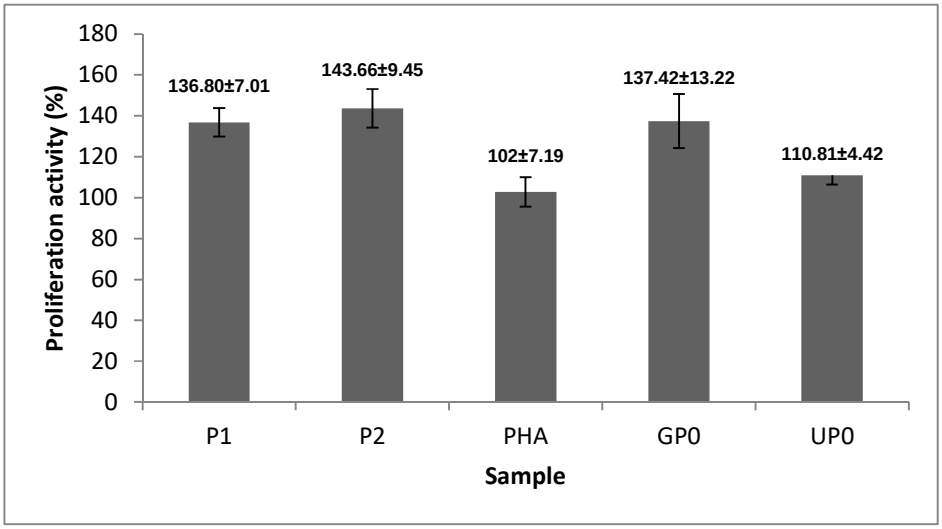

Fig. 1 Lymphocyte proliferation activity by *geluring* extract (P1= Unseasoned *geluring*, P2= Seasoned *geluring)* and dried *Gelidium* sp. (GP0) and *Ulva lactuca* (UP0) extract.

Lymphocyte cell proliferation activity by *geluring* product is closely related with bioactive components in the seaweed. *Geluring* product contains a number of bioactive components that has been studied to be able to stimulate lymphocyte proliferation activity [2]. *Ulva lactuca* contains bioactive components such as phenolic compound [26], [1], flavonoid [27], chlorophyll, carotenoid [28], alkaloid and terpenoid [29].

One of the possible mechanisms of lymphocyte activation by seaweed *geluring* product is allegedly based on the binding of phenolic or flavonoid compounds acting as mitogen on the surface of T cell and B cell [30]-[31]. The binding of mitogen on T cell receptor surface can activate G-protein, which subsequently produces phospholipase C enzyme. This enzyme can hydrolyze phosphatidyl inositol bisphosphate (PIP2) into reactive products, such as diacyl glycerol (DAG) and inositol trisphosphate (IP3). The reaction stimulates the release of Ca2+ into cytoplasm so that the concentration of Ca2+ increases, which has an important role in stimulating the work of kinase C and 5-lipoxygenase protein enzymes. The kinase C protein stimulates the production of interleukin−2 (IL-2) that activates B cell and T cell so that they proliferate [32]-[33]. Lymphocyte proliferation activities by *geluring* products were higher than the unprocessed *Gelidium* sp. and *U. lactuca*. It may be that not all native bioactive compounds or flavonoids in unprocessed *Gelidium* sp. and *U.lactuca* stimulate the lymphocyte cells, and possibly even some may suppress the cell proliferation. Lyu and Park [34] reported that catechin and epicatechin flavonoids increased lymphocyte proliferation, but curcumin was reported to inhibit the cell proliferation [35]. *Geluring* process involving pulping and drying may reduce some bioactive components in the raw material which inhibits lymphocyte proliferation. The activity of lymphocyte proliferation is also highly dependent on the suitability of bindings between ligand and binding sites of lymphocyte receptor [19]. The phenolic or flavonoid components of P1 and P2 *gelurings* may perfectly bind on the binding site of T cell receptor, resulting in higher lymphocyte proliferation activity.

Lymphocyte cell is a highly sensitive cell type, therefore in vitro test of bioactive compounds using lymphocyte cells may also function as a test for compound cytotoxicity [36]. Based on this research, it can be concluded that P1 and P2 *geluring* products can increase lymphocyte proliferation and at the same time is not toxic to the cells.

B. Production of Interleukin-2 (IL-2) by Lymphocyte cells

The results of this research showed that extracts of the *geluring* products could increase IL-2 production of lymphocyte cells higher than those of the control. Production of IL-2 by lymphocyte cells added with P2 *geluring* extract was the significantly higher ($P < 0.05$) than that of RPMI added but not significantly different ($P < 0.05$) with the cultures added with PHA mitogen, *geluring* P1 and dried *Gelidium* sp. (GP0) and *Ulva lactuca* (UP0) extracts (Fig. 2). The addition of garlic and pepper to P2 *geluring* may also increase the IL-2 production of lymphocyte cells.

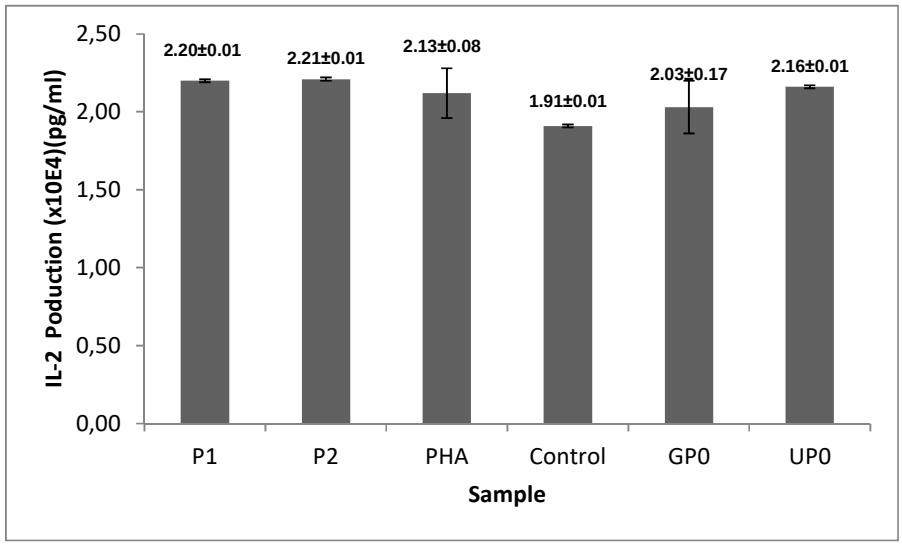

Fig. 2 Production of IL-2 by lymphocyte cells added with *geluring* extracts (P1= unseasoned *geluring*, P2= seasoned *geluring* and dried *Gelidium sp* (GP0) and *Ulva lactuca* (UP0) extracts.

Production of IL-2 lymphocyte cells by *geluring* extracts were correlated with proliferative activity (Fig. 3). In the immune system, IL-2 has several functions, including stimulates lymphocyte proliferation, regulates signals during immune response and increases differentiation of T and B cells [14],[15]. Stimulation of lymphocyte cells by inducers such as phenolic or flavonoid components promotes various biochemical reactions in the T cells producing more IL-2 necessary for further cell proliferation [15]. High lymphocyte proliferation by the cells added with *geluring* extracts is associated with the increase production of IL-2. This research result is similar to the report of Ahn et al [23] stating that *Ecklonia cava* seaweed enzymatic extract was also able to stimulate lymphocyte proliferation and increase IL-2 production.

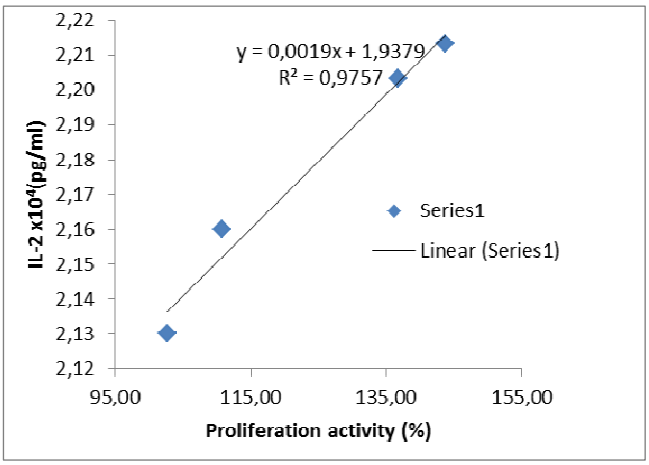

Fig. 3 The correlation of proliferation activity with IL-2 production of lymphocyte cells cultured with the addition of *geluring* extracts and controls.

C. *The Effect of Geluring Extract Concentrations on Proliferation Activity and IL-2 Production of Lymphocyte Cell*

The proliferation of activity and IL-2 production of lymphocyte cells induced by *geluring* extracts (P1 and P2) was affected by the level of consumption. The level of consumption was based on the average consumption of seaweed by Asian people which is about 8 grams/day [37]. In the cell culture, the concentrations were calculated to include dilution in 6 liters human blood when the dry seaweed is consumed. Thus, the concentration used were K1 = 1.33 mg/ml, K2 = 2K1 (2.66 mg/ml), K3 = 3K1 (3.99 mg/ml) and K4 = 4K1 (5.32 mg/ml). K2 concentration in the culture is assumed to be equivalent to the concentration of *geluring* compounds in the blood resulting from the intake of twice the average seaweed consumption by Asians.

The results showed that the difference of concentrations (K1, K2, K3, K4) of P1 and P2 *geluring* extracts have significantly affected (P<0,05) the proliferative activity (Fig. 4) and IL-2 production (Fig. 5) of the lymphocyte cells. The concentration of K2 (2.66 mg/ml) resulted in the higher proliferation activity and IL-2 production compared to the other concentrations. Increasing the extract concentrations of K3 and K4 decreased lymphocytes proliferative activity and production of IL-2.

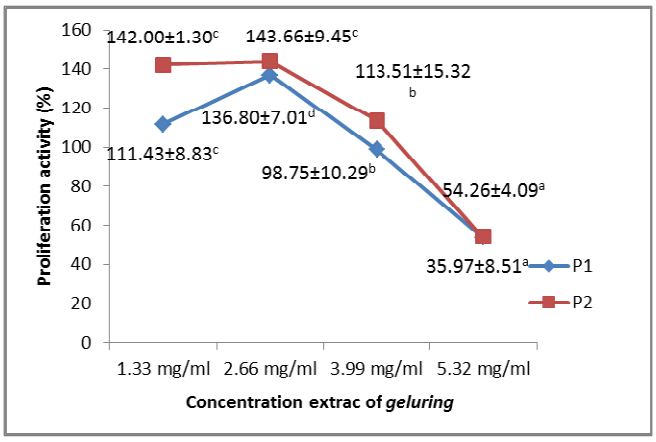

Fig 4. The effect of concentrations of *geluring* extracts (P1 and P2) on the lymphocyte proliferation activity

The stimulating activity of lymphocytes by inducer compounds such as phenolics or flavonoids in *geluring* products is depending on their binding conformity to the

receptor binding sites of lymphocyte cells. At the low concentrations, the inducer compounds might not bind perfectly on the surface of the cell receptors, whereas at high concentrations there may be saturation of the receptor on the cell surface resulting in a low stimulatory effect, low proliferation activity and IL-2 production. As seen in Figure 4, both proliferation activity and IL-2 production were the highest at K2 concentration. References [19]; [38]; [39] suggested that lymphocyte stimulation activity was affected by the amount of stimulator attached to the surface receptor and compatibility with the binding sites of the lymphocyte cells.

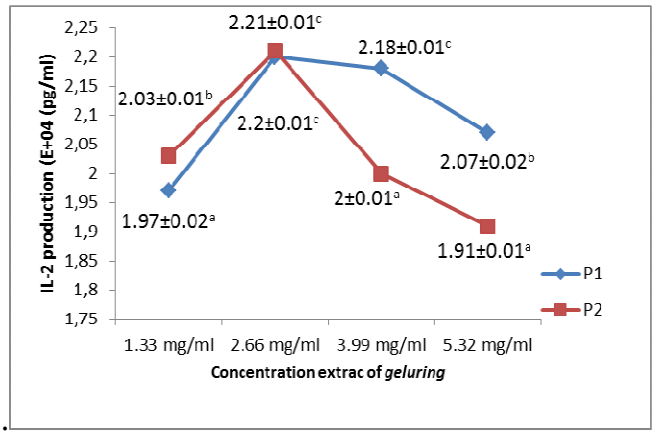

Fig 5. The effect of concentrations of *geluring* extracts (P1 and P2) on the IL-2 production.

IV. Conclusion

Geluring product from dried *Gelidium* sp. and *Ulva lactuca* seaweed could increase the proliferation activity and interleukin-2 (IL-2) production of human lymphocyte cells by *in vitro* methods. The seasoned *geluring* product (P2) has higher proliferation activity and IL-2 production. The stimulation of lymphocyte proliferation and IL-2 production by P1 and P2 *geluring* extracts were influenced by concentration of the extracts. The highest proliferation activity and IL-2 production was obtained at K2 (2.66 mg / mL). The activity of lymphocyte proliferation in this study was positively correlated with IL-2 production. Further in vivo research is necessary to confirm the potential of *geluring* products as immunomodulators.

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