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The Effect of Black Soybean Tempe Extract on the Increase of Proliferation Stimulation Index (PTI), Protein Tyrosine Kinase Enzyme Activity and Proliferating Cell Nuclear Antigen of Human Lymphocytes

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Abstract: Phytochemical components of tempe could interact with receptors on the surface of lymphocytes, which then increase the activity of the enzyme of protein tyrosine kinase and DNA polymerase. The aim of this study was to determine the effectiveness of black soybean tempe extract on the increase of proliferation stimulation index, protein tyrosine kinase enzyme activity, and proliferating cell nuclear antigen of human lymphocytes, *in vitro*. A number of 15 ml blood samples were drawn from a total of five respondents. The lymphocytes were then separated from these blood samples and later cultured. The black soybean tempe extract was added in varied concentrations of 0, 25, 50, 100, and 200 μg/ml in the culturing process and used as a positive control in cultures with added PHA. Lymphocyte cultures were incubated for 72 hours and after that their PSI, PTK enzyme activity and PCNA were analyzed. The results showed that the extract of black soybean tempe added to cultured human lymphocytes significantly affected the lymphocytes PSI, PTK enzyme activity and PCNA, where the increased level of black soybean tempe extract added into the lymphocyte culture tended to raise the values of PSI, PTK enzyme activity and PCNA. The addition of black soybean tempe extract to cultured human lymphocytes affected the lymphocytes PSI, PTK, and PCNA.

Keywords: Black soybean tempe, PSI, PTK, PCNA

1. Introduction

Tempe is made of soybean fermented by *Rhizopus spp.* Such *as R.oligosporus, R.oryzae,* and *R.stolonifer,* with characteristics of white color products, compact texture and distinctive flavor of mushroom and soy mixture. The fermentation process makes tempe have several advantages compared to non-fermented soybean. The advantages include the composition of nutrients, the digestibility of protein, and the content of essential amino acid, anti-nutritional substances, antitrypsin, and phytic acid, which are lower than the non-fermented soybean.

Tempe contains antioxidant components such as isoflavones, vitamin E and β-carotene. Antioxidant compounds (isoflavones) in soybean may also contribute to gene expression [1]. The activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are significantly increased by genistein [1]. Phytoestrogen in certain condition acts as an antioxidant and protects DNA from oxidative damage. They also found that human lymphocytes incubated with genistein *in vitro* are resistant to damage by H_2O_2 [2].

Black soybean tempe has the potential functional properties because the black soybeans contain phenolic content, tannins, anthocyanins and isoflavones with antioxidant activity higher than

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yellow soybeans [3]. Black soybean tempe has higher antioxidant activity, while its flavor is preferred over yellow soybean tempe. Groups of rats consuming feed containing black soybean tempe flour for one month showed higher T cell proliferation stimulation index than the control group [4]. Yet, the consumption of black soybean tempe did not affect B cell proliferation stimulation index and the activity of secretory IgA antibody production [5].

Consuming isoflavones contained in soy food could modulate cytokine production[6]. The role of components of black soybean tempe on the proliferation of lymphocytes is to stimulate the formation of lymphokines, particularly interleukin 1 (IL-1) and interleukin 2 (IL-2). IL-1 is produced by macrophages to increase the growth and differentiation of lymphocytes. It also plays a role in stimulating the non-specific expression of various receptors on the cell surface antigen indirectly increasing the specific immune response. Besides, IL-1 stimulates the production of lymphokines, including IL-2, B-cell growth factor, gamma interferon, and chemotactic factors. IL-2 is produced mainly by Th cells playing a role to increase proliferation of T cells, B cells, NK cells and activity of macrophages [7].

Mice treated with soybean diet experienced proliferation response (T cells and B cells) higher than those treated with casein diet. There are three possible groups of components in tempe, which could improve the immune system of the body [8]. The first group of elements in tempe such as vitamin E, β-carotene, folic acid, pyridoxine, riboflavin and vitamin B_{12} [9] and some amino acids such as lysine, methionine, tryptophan, threonine, and leucine [10] could improve the performance of immune cells. The second group contains components that increase the activity of antioxidant enzymes (Cu, Zn and Fe), which cause the ability to inhibit the oxidation reaction and the performance of the body cells including lymphocytes increase [1]. The third group consisted of the phytochemical components in soybean, which could interact with receptors on the surface of lymphocytes, which then increase the activity of the enzyme of protein tyrosine kinase (PTK) and DNA polymerase. Genistein, an isoflavone found in soybean and soybean products, is able to bind to estrogen receptors [11]. Isoflavone also showed estrogenicity effects, as it can bind with estrogen receptors and induce specific products of genes that respond to estrogen [8].

Consumption of black soybean tempe affects the activity of macrophage and increases the level of IL-1 on feed containing black soybean tempe instead of casein by 50 and 75 percent, respectively. The increased activity of macrophages positively correlated with the amount of IL-1, with a correlation coefficient value of 0.9. Consumption of black soybean tempe affected the T cell proliferation stimulation index and level of IL-2. Increased T cell proliferation stimulation index is positively correlated to the amount of IL -1, with a correlation coefficient of 0.8. The increased activity of macrophages, proliferation of T cells, level of IL-1, and level of IL-2 showed an increase of the immune system. Hence, it could be concluded that eating tempe could increase the immune system, particularly the cellular immune response [12].

When consumed by rats, feed containing black soybean tempe could increase level of IL-1 produced by macrophages and could increase level of IL-2 produced by Th, so that the T cells of the rats proliferate. ConsumPSIon of black soybean tempe by a person could enhance his or her immune system by increasing the T cells proliferation and lymphocyte resistance to hydrogen peroxide [13]. However, a research explaining how the black soybean tempe component could interact with the T cell receptor in human has not been done. The aim of this research was to determine the effectiveness of the black soybean tempe extract on the increase of PSI, PTK enzyme activity and PCNA of human lymphocytes, *in vitro*.

2. Materials and Method

2.1 Materials

Materials used in this research were black soybean of mallika variety, blood of human adults (age 20-25 years), 70% ethanol, Phitohemaglutinine (PHA, Sigma), antibody of PCNA (BIOSS), Histostain reagent kit (Biocare Medical), Protein Tyrosine Kinase reagent kit (Qayee-Bio), RPMI 1640 (Sigma), Histopaque (Sigma), and common chemicals for lymphocyte proliferation analysis.

2.2 Procedure

Five blood samples with a volume of 15 ml of each were drawn from a total of five respondents. The lymphocyte cells were separated from the blood samples and then cultured. In the process of culture preparation, the soybean Tempe extract was added as much as 0, 25, 50, 100, and 200 µg/ ml successively as a positive control in the PHA added culture. The obtained lymphocyte cultures were incubated for 72 hours and then subjected to analyses of PSI [13], PTK enzyme activity (according to the manual kit) and PCNA (according to the manual kit).

2.3 Processing of Black Soybean Tempe

The technique of tempe preparation adopted in this research was the common way of tempe making and hull separation in Indonesia, where 10% of the hull was remixed with the cotyledon. Black soybeans were cleaned to remove dirt and contaminant objects, washed with water, soaked for 30 minutes, and then boiled with water for 30 minutes. Boiled soybeans were de-hulled by hand and after that 10% of the hull was put back with the cotyledon, soaked for 36 hours, thoroughly drained, and then steamed for 1 hour. The steamed de-hulled soybeans were allowed to cool for 30 minutes prior to inoculation with inoculum. The used inoculum has a ratio of starter to soybeans of 2:1000. The inoculated soybeans were packed in plastic bags having tiny holes and then incubated at room temperature (25-27° C) for 36 h. The resulted tempe was then dried at temperature of 40-45° C for 24 hours and grounded into tempe powder (60 mesh).

2.4 Antioxidant Extraction of Tempe Powder with Ethanol

Successive antioxidant extractions were conducted on black soybean tempe powder according to the method of Xu and Chang (2007). A number of 100 g of tempe powder was put in Erlenmeyer flasks, mixed with 200 ml of ethanol 70% and allowed to settle for 24 hours. The extracts obtained from the extractions and the remaining residue were separated through filtration. The residue was mixed again with 100 ml of 70% ethanol and then re-filtrated. Both filtrates were combined and the solvent was evaporated to obtain dry extract (freeze-drying).

2.5 Isolation of lymphocytes

All blood samples drawn from a total of 5 patients were centrifuged at 514 x *g* for 10 minutes to obtain plasma and lymphocyte layers. After centrifugation, the heavier parts of blood, the red blood cells, will be at the bottom of centrifugation tubes. A buffycoat layer containing lymphocyte cells appeared between the layers of red blood cells and blood plasma. The plasma liquid was isolated from the mixture, and then stored at a temperature of 20° C. The buffycoat layer was separated using Pasteur pipette, then the isolated liquid was put into 5 ml of RPMI-1640 medium. The separation of lymphocyte cells from the mixture was conducted based on density differences using Histopaque. Buffycoat part was mixed with synthetic RPMI-1640 medium, which was then placed on top of 10 ml Histopaque in a 50-ml centrifuge tube. Centrifugation of the buffycoat mixture was carried out at 1430 x *g* for 30 minutes. Centrifugation resulted in the formation of a ring of white layer in centrifuge tube, which could be seen between the synthetic medium RPMI-1640 phase and the Histopaque. The white layer was carefully isolated using a Pasteur pipette and then mixed with 10 ml of synthetic medium RPMI-1640. Washing process was done through centrifugation of lymphocyte suspension, which was mixed with synthetic media at 228 x *g* for 10 minutes. These washing steps were repeated twice. The live and dead cells were calculated with hemocytometer by using tryphan blue.

2.6 Lymphocyte proliferation

First, lymphocyte culture count was carried out on each blood sample of five respondents. After that, 2 x 10^{6}/ml lymphocyte from each respondent's sample was introduced into standard media. The obtained cell suspension from each respondent's sample was divided and put into 12 wells (requiring 96 well plate in total) 100 µl of each. After that, 2.5 µl PHA mitogen was added to each well. To prepare cultures for lymphocyte proliferation, 100 µl solution of black soybean tempe extract in varied concentrations of 0, 25, 50, 100, and 200 µg/ml respectively was added into 5 wells of each respondent's samples. Into each of these 5 wells, which have been filled with cell suspension, 20 µl AB serum was then added, so that each of them had 200 µl total volume. Control sample of this experiment only contained lymphocytes and standard media. All of the lymphocyte culture

preparation steps were carried out in Duplo for each respondent. Later, all cells in wells were cultured in an incubator with 5% CO_2 humidity 95% at 37 ^{o}C. After incubation for 72 hours, cultures of lymphocytes were subjected to PSI, PTK enzyme activity and PCNA analyses.

2.7 Experiment Design

The completely random experimental design was used in this research with tempe extracts as independent variable (added in varied concentrations of 0, 25, 50, 100, and 200 µg/ ml, respectively). The dependent variables of this study were PSI of T cells, the enzyme activity of PTK and the number of PCNA of human lymphocytes. For every different treatment, samples were read 3 times. The obtained data were presented in charts, and statistically analyzed with single factor ANOVA method.

3. Result and Discussion

3.1 Lymphocyte Proliferation

The extraction of black soybean tempe was conducted using a method previously reported by Xu and Chang (2007), which could produce high antioxidant activity by incorporating 70 % ethanol as solvent. The solvent was separated from the obtained extracts by freeze-dryer resulting dry powder. An aliquot of the resulted dry powder was re-dissolved in RPMI fluid. The obtained tempe extract liquid was then added to lymphocyte culture in various amount to evaluate its immunomodulatory activity (mitogenic effect). Phitohemaglutinine (PHA), which is specific for T-cell mitogen (Snow, 1991), was used as a comparison to observe the mitogenic effects of tempe extracts.

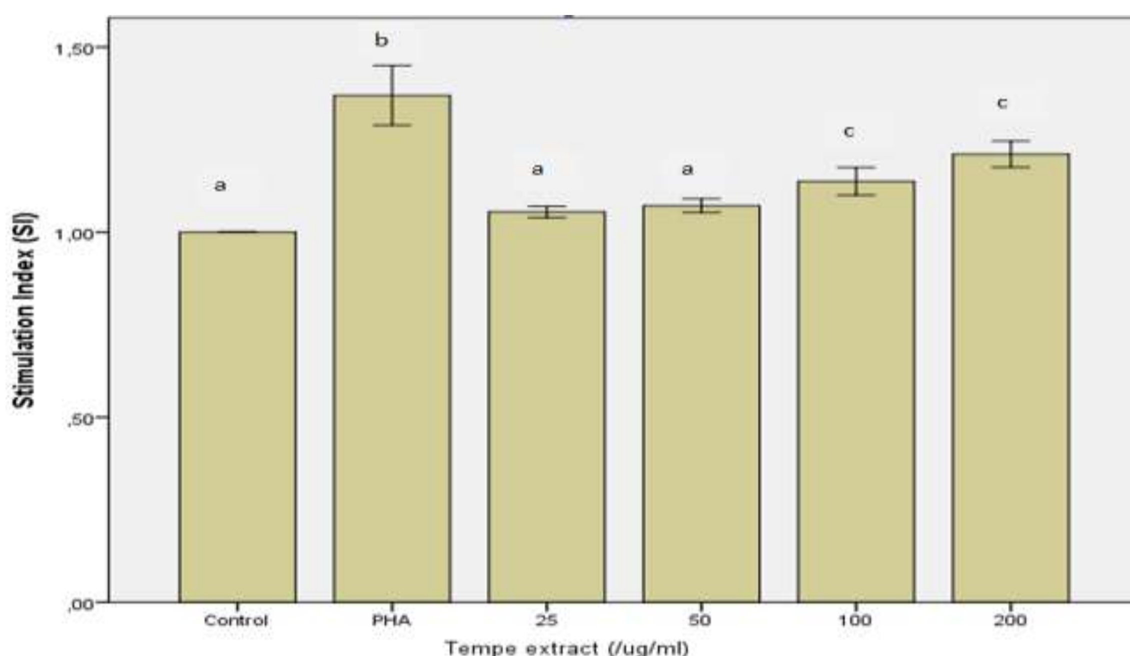

Figure 1. Graph of effect of the added mitogen (PHA = phitohemaglutinine, ET0 = without the addition of mitogen; ET25 = with the addition of 25 µg/ml tempe extract; ET50 = with the addition of 50 µg/ml tempe extract; ET100 = with the addition of 100 µg/ml tempe extract; ET200 = with the addition of 200 µg/ml tempe extract) on cultured lymphocytes in terms of proliferation stimulation index. Note: Letters on top of bars indicate significance difference ($p \leq 0.05$)

Figure 1 is a graph displaying the effect of the addition of tempe extract to lymphocyte cell culture, particularly on its proliferation stimulation index. The average proliferation stimulation index of lymphocyte cells in cultures with added PHA of 2.5 µl and tempe extract in varied concentrations of 0, 25, 50, 100, and 200 µg/ml were 1, 1.37, 1.06, 1.07, 1.14 and 1.21, respectively. The obtained data showed that PHA response was higher than tempe extract ones. Lymphocyte cultures without

added mitogen used as controls had lowest the proliferation stimulation index. The higher concentration of tempe extract (25 to 200 µg/ml) added to the cultures resulted in the higher lymphocyte responses.

Results of statistical analysis using single factor ANOVA showed the significant effect of mitogen on the lymphocyte cultures in terms of their proliferation stimulation index ($p \leq 0.05$). There was significantly increase of proliferation stimulation index ($p \leq 0.05$) due to the addition of 100 µg/ml tempe extract. Interestingly, the addition of 25 and 50 µg/ml tempe extract to samples of lymphocyte cultures could not give significantly different proliferation stimulation index when compared to control sample. This means that the tempe extract could interact with lymphocytes and could have a mitogenic effect on lymphocyte proliferation at concentration of 100 and 200 µg/ml, but its mitogenic effect was lower than PHA's.

Black soybean tempe extract contains genistein and daidzein of 6.06 ± 0.54 and 11.39 ± 0.55 mg/g, where both components were classified in isoflavone. The isoflavone was one of the isoflavone genistein found in soy and soy products, which was able to bind to estrogen receptors [11]. Isoflavone shows estrogenesity effect, could bind to estrogen receptors and induces specific products of genes that respond to estrogen [8]. Genistein could boost Tc and NK cell activity [6]. The presence of components in soy products interacted with T cells making them activated to proliferate [8].

Isoflavone might decrease proliferation and yet increase apoptosis of breast cancer cells *in vivo* [14]. Several other researchers also stated that daidzein and genistein could reduce the risk of breast cancer through a mechanism where the second component of isoflavones could inhibit cancer cell proliferation [15-17]. These reports were in contrary to those by Zhao and_Wang as well as the other reports obtained by researchers using lymphocytes to evaluate the effect of isoflavone on cell proliferation [6,8].

From the discussion, it could be concluded the effects of isoflavone on cell proliferation are different between normal cells (lymphocytes) and abnormal cells (cancer cells). Until now, researchers have not found the explanation of such difference. Therefore, further research is needed to explain the differences in the interaction between isoflavone with normal and abnormal cell.

3.2 Enzyme activity of Protein Tyrosine Kinase (PTK)

PTK enzyme is an enzyme playing an important role in the mechanism of intracellular mitogenic signaling. There is evidence showing that growth factor receptors such as dermal growth factor receptor (DGFR), nerve growth factor receptor (NGFR) and stem cell factor receptor (SCFR) relate to enzyme activity of PTK [18]. PTK enzyme plays a role in T-cell proliferation in response to the stimulation of the T cell receptor (TCR).

Figure 2 shows a graph of effect of addition of the tempe extract to lymphocyte cell culture, particularly on the activity of PTK enzyme of the lymphocyte cells. The average enzyme activity of PTK of lymphocyte cells in cultures with added PHA of 2.5 µl and tempe extract in varied concentrations of 0, 25, 50, 100 and 200 µg/ml were 417.42, 653.55, 439.03, 476.45, 571.61 and 626.13 ng/ml, respectively. According to the data, the PTK enzyme activity of lymphocyte cell cultures with added PHA gave the highest value of all including those with added tempe extract. Culture of lymphocytes without added mitogen (control) had the lowest PTK enzyme activity. The higher concentration of tempe extract (25 to 200 pg/ml) added to the lymphocytes, the higher PTK enzyme activity of the lymphocytes.

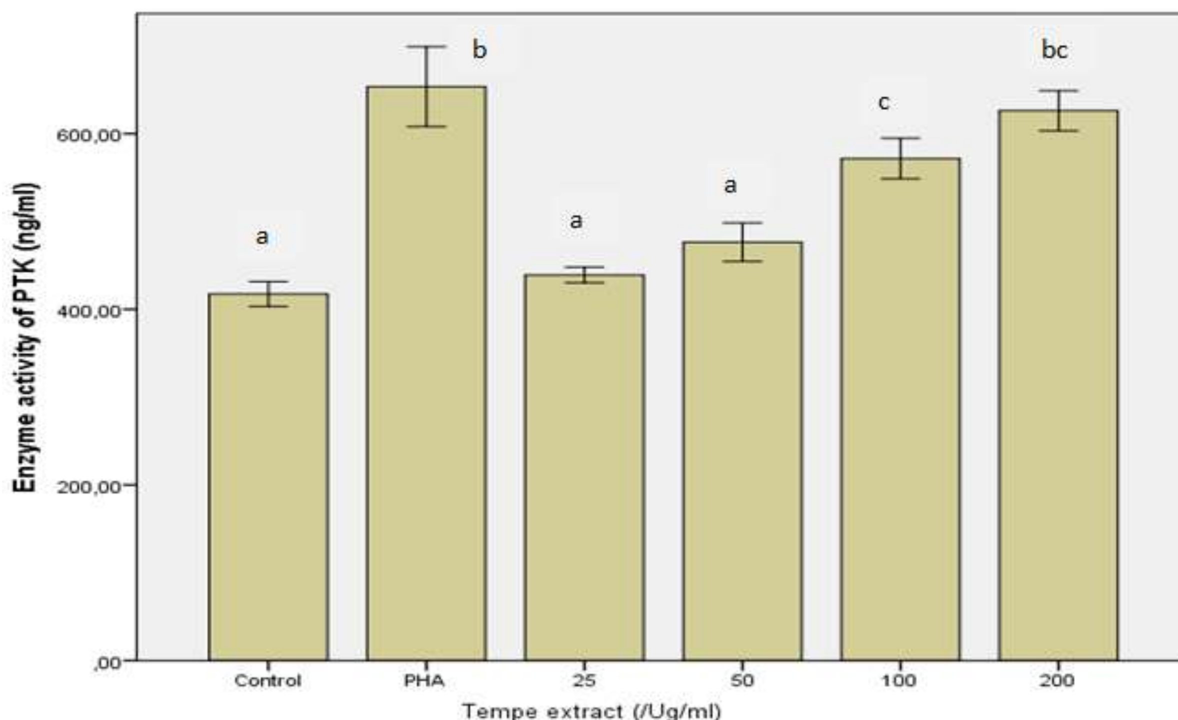

Figure 2. Graph of effect of the added mitogen (PHA = fitohemaglutinin, ET0 = without the addition of mitogen; with the addition of 25 µg/ml tempe extract; ET50 = with the addition of 50 µg/ml tempe extract; ET100 = with the addition of 100 µg/ml tempe extract; ET200 = with the addition of 200 µg/ml tempe extract) on cultured lymphocytes in terms of PTK enzyme activity.

Letters on top of bars indicate significant difference ($p \leq 0.05$)

Results of statistical analysis using single factor ANOVA showed no effect of mitogen on lymphocyte culture in terms of PTK enzyme activity ($p \leq 0.05$). The increase of PTK enzyme activity by the addition of 25 and 50 µg/ml tempe extract was not significantly different, when compared to that of the control sample. Effect of tempe extract on PTK enzyme activity began to manifest at the concentration of 100 µg/ml ($p \leq 0.05$). Tempe extract at the concentration of 100 and 200 µg/ml added to the lymphocyte culture increased the PTK enzyme activity but it was not significant. When compared, PTK enzyme activity of lymphocyte cell culture with added PHA solution of 2.5 µl and those with added 0, 25, 50, and 100 ug/ ml tempe extract were significantly different ($p \leq 0.05$). However, the addition of 200 µg/ml tempe extract could not give significantly different PTK enzyme activity. In other words, the addition of 200 µg/ml tempe extract and the addition of PHA gave the same effect on lymphocytes in terms of the enzyme activity of PTK.

The data demonstrate that the components contained in tempe extract (isoflavones) could interact with the TCR, which is the case binding to the receptor increasing the enzyme activity of PTK.TCR is an antigen receptor on the surface of T cells containing ligand-binding subunit and chain related to signal transduction [19][20]. Immunoreceptor stimulation occurs when interacting with antigens that result in protein tyrosine phosphorylation reaction speed increases, so that the cells undergo activation [19]. PTK enzyme becomes more active after stimulation of the TCR, and this enzyme also participates encourage the production of cytokine (IL-2) [21]. IL-2 is cytokine produced Th which induces T cell proliferation [7].

Based on the mentioned explanation, it could be said that components (isoflavones) of black soybean tempe extract could interact with T cells through binding ligand inducing Th to produce IL-2. Nurrahman and Nurhidajah (2014) reported that the cultures of lymphocyte of rats consuming black soybean tempe show effects in a proliferation stimulation index of the lymphocytes and the production of IL-2.

3.3 Proliferating Cell Nuclear Antigen (PCNA)

Proliferating Cell Nuclear Antigen (PCNA) is a cofactor of DNA polymerase δ synthesized in the G1 to S phases of the cell cycle. Expression of PCNA is a sign of the activity of the DNA polymerase and DNA replication enzymes, which increase during cell proliferation [22]. Determination of cell proliferation could be done through the interaction of monoclonal antibody immunohistochemistry with PCNA [7].

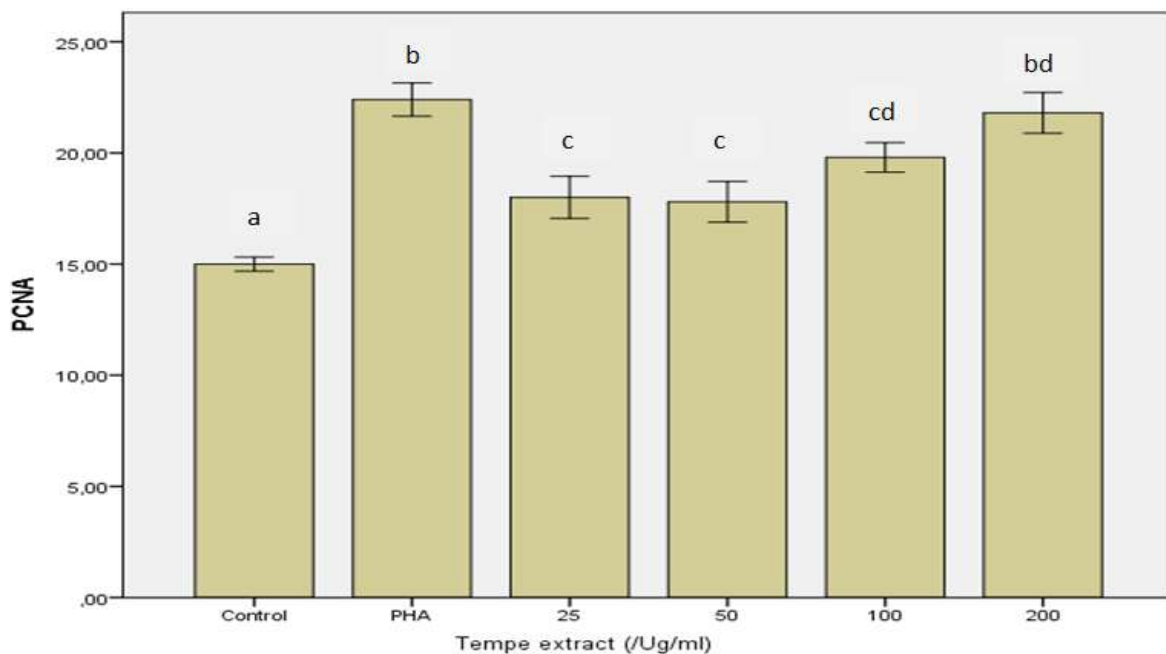

Figure 3. Graph of effect of the added mitogen (PHA = fitohemaglutinin , ET0 = without the addition of mitogen; ET25 = with the addition of 25 μg/ml tempe extract; ET50 = with the addition of 50 μg/ml tempe extract; ET100 = with the addition of 100 μg/ml tempe extract; ET200 = with the addition of 200 μg/ml tempe extract) on cultured lymphocytes in terms of PCNA.

Letters on top of bars indicate a significant difference ($p \leq 0.05$)

Figure 3 shows a graph explaining the effect of the addition of tempe extract to cultured lymphocytes in terms of PCNA. The average number of PCNA of lymphocytes in cultures with PHA and tempe extract in various concentrations of 0, 25, 50, 100, and 200 μg/ml, respectively, were 22.4, 15, 18, 17.8, 19.8, and 21.8. Data on Figure 3 showed that the number of PCNA in culture of lymphocytes with added PHA was the highest of all. The cultures of lymphocytes, which were without the added mitogen (control) had lowest number of PCNA. When higher concentration of tempe extract (from 25 to 100 μg/ml) added to these cultures, higher number of PCNA was observed.

Results of statistical analysis using single factor ANOVA showed significant effect of mitogen to the PCNA of lymphocyte cultures with $p \leq 0.05$. It increased the number of actual PCNA ($p \leq 0.05$) in addition to the increased number by tempe extract, PHA, and control. Effect of soybean extract on real number of PCNA started to appear at concentration of 25 μg/ml ($p \leq 0.05$) as compared to the control. Tempe extract at the concentration of 25, 50, and 100 μg/ml added to lymphocyte cultures did not show significantly different number of PCNA. Tempe extract with concentrations of 50 and 200 μg/ml added to the lymphocyte cultures showed significantly different number of PCNA ($p \leq 0.05$). When compared to PHA, the lymphocyte cultures plus soybean extract of 200 μg/ml also did not show significant difference. Thus, it could be said that the black soybean tempe extract of 200 μg/ml with PHA gave the same effect on the number of PCNA. Although the number of PCNA in cultured lymphocytes with added extract of tempe of 200 μg/ml was lower than that with added

extract of tempe of 100 µg/ml, it could be said that the 100 and 200 µg/ml black soybean tempe extract added into cultured lymphocytes gave the same impact on the number of PCNA.

The results showed that the addition of tempe extract to lymphocytes culture could increase the number of lymphocytes' PCNA. Number of PCNA is an expression of the activity of DNA polymerase enzyme. Increasing the number of PCNA shows an increasing activity of the DNA polymerase activity enzyme in cells associated with DNA replication [23].

When evaluated, it appeared that data of PTK enzyme activity and data of the increased number of PCNA due to the addition of PHA and tempe extract (0 to 200 µg / ml) had the same pattern. Similar pattern was also shown in the data of proliferation stimulation index by MTT method. Increased concentrations of tempe extract added to the lymphocyte cultures tended to increase the proliferation stimulation index, the PTK enzyme activity and the number of PCNA. It could be concluded that an increase of PTK enzyme activity characterizes the proliferation of lymphocytes. PTK enzyme activity increases the activity of DNA polymerase enzyme (indicated by an increasing in the number of PCNA), resulting DNA replication. DNA replication is an early stage of cell division process, so that the cells could undergo proliferation.

4. Conclusion

The addition of black soybean tempe extract to cultured human lymphocytes affected the lymphocytes proliferation stimulation index (PSI), protein tyrosine kinase (PTK) and proliferating cell nuclear antigen (PCNA). Furthermore, the increased concentrations of the tempe extract added to lymphocyte culture tended to increase the lymphocytes PSI value, PTK enzyme activity of and PCNA number.

5. Acknowledgments

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