

Biochemical compounds and sub-chronic toxicity test of *Chlorella* sp. and *Spirulina* sp. isolated from Glagah Coastal Water

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Abstract

Microalgae are microscopic photosynthetic organisms found in marine and freshwater environments. This organism is a eukaryotic group of polyphyletic and very diverse. This study investigated the biochemical composition and sub-chronic test of *Chlorella* Sp. and *Spirulina* Sp. isolated from Glagah coastal water (Yogyakarta, Indonesia) as a preclinical study to create potential nutritional supplement for human health. Microalgae culture was analyzed for biochemical composition using several methods: AAS for metal analysis (Pb, Cu, Fe, Mg, Zn, and Hg), GC-MS and UV spectrophotometer for fatty acid profiles analysis (linolenic acid, eicosatrienoic acid, arachidonic acid, linoleic acid, oleic acid, eicosenoic acid, EPA, DHA, omega 6 and 9), Alkaloids, Steroids, Tanin and Saponin. The next step is to do a sub-chronic test of microalgae biomass using experimental animals. The result shows that *Spirulina* sp. and *Chlorella* sp. contain 16 and 10 different biochemical compounds respectively. Furthermore, organic nutritional properties in both of microalgae are relatively high with a rich biochemical profile of Fe and nutritious poly-unsaturated fats (such as linolenic acid, arachidonic acid, omega 6, and 9). Regarding sub-chronic toxicity test, it was clear that *Spirulina* and *Chlorella* did not affect the blood profile. These findings suggest that both of Glagah strain microalgae are considered to have great potential as a multi-nutritional human health supplement.

Keywords: Biochemical composition, blood profile, *Chlorella* Sp., Glagah Coastal, *Spirulina* Sp.

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Introduction

Currently, there has been commercialization of *Spirulina* and *Chlorella* as functional food to improve human and animal health (Hutadilok-Towatana et al., 2010). *Spirulina* sp. and *Chlorella* sp., are microscopic, unicellular, and photosynthetic aquatic algae. It has been used for hundreds of years as a food source for humans and animals. Due to excellent nutritional profile, as a rich source of nutrients and biologically active substances, such as protein, amino acids, polyunsaturated fatty acids, microelements, vitamins, antioxidants, and carotenoids.

Spirulina belongs to the cyanobacterium group with the characteristic multicellular trichomes in the cylindrical shape. These microalgae have high protein content and are rich in vitamins, minerals, and other components that are beneficial to health such as essential fatty acids and antioxidant pigments such as carotenoids, chlorophyll, and fikocyanin (Hutadilok-Towatana et al., 2010). In addition, another microalgae that has a high protein content is *Chlorella*. This algae in addition to providing the majority of amino acids, also contains many vitamins and minerals. *Chlorella* also contains *Chlorella* Growth Factor that can strengthen immunity and prevent or destroy cancer wounds (Tang and Suter, 2011), while *Spirulina* is widely used as a supplement to animal feed. Based on previous research, the addition of 10% or 20% *Spirulina* decreased the rate of chicken growth. Addition of 50-100

g/kg of *Spirulina* in feed ratio will keep the growth rate while the excess level of 200 g/kg will decrease the growth rate of chicken (Holman and Malau-Aduli, 2013). Another research resulted the addition of *Spirulina* on the best poultry diet ranged from 0.5% - 12.5%. Based on the research of Cheong et al. (2015) recommended addition of 4% *Spirulina* on quail feed resulted in good performance, weight of karakas, and meat quality.

Thus, it is necessary to conduct research to determine biochemical compounds of *Spirulina* and *Chlorella* before it was tested by sub-chronic toxicity of to test animals especially to know its effect on hematology profile. The final purpose of this research is to create nutraceutical products microalgae to create potential nutritional supplement for human health. Before, in the first phase, nutrient content of microalgae has been obtained by HPLC (High Performance Liquid Chromatography), AAS (Atomic Absorption Spectroscopy) and GC-MS (Gas Chromatography-Mass Spectrometry) methods. Furthermore, nutraceutical formulation has been obtained preclinically concerning the sub-chronic toxicity of *Spirulina* and *Chlorella* microalgae through animal model in particular to determine its effect on the hematologic profile.

Method

Ethical Clearance

This research method has been approved by ethical commission for preclinical research in The Integrated Research and Testing Laboratory Universitas Gadjah Mada through The Certificate of Ethical Clearance No: 00044/04/LPPT/VIII/2016, date 01 August 2016.

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Preparation of Microalgae

Microalgae was cultured at Biotechnology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia.

Analysis Microalgae Nutrient Content

Microalgae are cultivated using a medium F/2 + silica in liquid form which is taken 1 ml of liquid silica solution with a concentration of 30 mg/L. The dry weight of samples is taken on days 0, 7, 14 and 21 as much as 2 mL for a scale laboratory. After 21 days the microalgae was harvested and used to be tested on experimental animals and nutrian analysis and chemical profile. Microalgae biochemical and heavy metal profile were analyzed by AAS; GC-MS and spectrophotometer for fatty acid and other compounds. Biochemical profile was analysis by GC-MS at Chemistry Department Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. A hundred gram of microalgae were dried in temperature 45 °C until the weight is constant, 64.45 g of peeled single black garlic was crushed with blender and followed by maceration using ethanol 70% for 24 h, repeated three times. The filtrate was collected and was evaporated in 45 °C, the crude ethanol extract was collected. For the n-hexane fraction, 10 g of ethanol extract was dissolved in 100 mL ethanol and partitioned with 100 mL n-hexane. The upper suspension layer was filtered and evaporated at 45 °C, the ethanol extract and n-hexane fraction were kept at 4 °C before use. The ethanol extract and n-hexane fraction from microalgae were analyzed using GCMS-QP2010S (Shimadzu Co., Japan) instrument with AGILENT HP 5MS capillary column of 0.25 µm thickness, 0.25 mm diameter insert and 30 m long. The analysis was performed by injecting 1 µL sample (ethanol extract and n-hexane fraction of microalgae). Helium gas (He) is used as a carrier gas with a flow rate of 3 mL/min. The ionization electron system with 70 eV ionization energy is used for GC-MS detection. The initial 50 °C column temperature held for 5 min was then gradually increased to 280 °C with an increase of 5 °C per minute. Then when the temperature reached 280 °C held for 19 min. The obtained spectra were then compared to the database in WILEY libraries (Hoboken, 2012).

Animal and Experimental Design

Fifteen male Wistar rats were purchased from The Integrated Research and Testing Laboratory Universitas Gadjah Mada. The animals were divided into three groups consisting of five animals per group. Group I were placebo control, group II and III are Treatment Groups, were treated with dry biomass of *Spirulina* 5000 mg/kgWB and *Chlorella* 5000 mg/kgWB orally, respectively.

Sub-Chronic Toxicity Test

Sub-chronic Toxicity Test conducted by giving microalgae orally every morning and evening for 90 days. The physiological conditions of the test animals were observed in the day of 0, 30, 60 and 90 by studying the hematology profiles such as red blood cells, white blood cells, and platelets (PLT). Red blood cells include the

total number of red blood cells (RBC), hemoglobin (HGB), and hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC). White blood cells include the total number of white blood cells (WBC), the percentage of lymphocytes (LYM) and the percentage of neutrophils (NEUT). Platelets include the total number of PLT, platelecrit (PCT), and Platelet Distribution Width (PDW). At the end of the study, the rats were anesthetized using ketamine for blood collection. Then, the mice were mutated by eksanguinasi (Muñoz-Antoli et al., 2015).

Data Analysis

The haematological data were tabulated and analyzed statistically using Two-Way ANOVA with 95% confidence level. If there is any difference, then proceed with Post hoc LSD. If the data obtained are not homogeneous, then it is analyzed by using One-Way ANOVA by using software program (software) SPSS 20.

Results

Nutritional and Chemical Profile of *Spirulina* sp.

Analysis on the content of volatile compounds on *Spirulina* sp. and *Chlorella* sp. were done using GC-MS method. Based on figure 1, the chromatogram of *Spirulina* sp. was obtained 26 peak and successfully identify 19 compounds while *Chlorella* sp was obtained 11 peak and successfully identify 10 compounds. Tabel 1 shown the biochemical profile of *Spirulina* sp. The volatile compounds of *Spirulina* sp. were successfully identified various classes of biochemical compounds including cycloalkanes, benzenes, alkanes, terpenes, monoterpenes, alcohol, fatty acids, carotenoids, benzene and fatty alcohol groups, whereas in the fraction of n-Hexane have been identified 26 compounds, originating from various groups between other classes of alkanes, fatty acids and organochloride. *Chlorella*'s compounds came from various classes of compounds including phenyl propanoids, terpenes, sesquiterpenes, fatty acids and alkanes.

The results of GC-MS analysis as shown in table 1, indicate the abundance of biomolecules as shown by their peaks, including cyclooctanone, toluene, ethylbenzene, alpha-pinene, eucalyptol, tridecane, tetradecane, eicosane, hexadecane, octadecane, octadecanoic acid, heneicosane, methyl 9,9-dideutero-octadecanoate and 2h-1,4-benzodiazepin-2-one, 7 chloro-1,3-dihydro-5-phenyl-.

Nutritional and Chemical Profile of *Chlorella* sp.

The biochemical compounds of *Chlorella* sp. were eugenol, azulene, hexadecanoic acid, methyl 9,9-dideutero-octadecanoate, 1,2-benzenedicarboxylic acid, santalol, tridecane, hexatriacontane and dodecanoic acid as shown in table 2. Eugenol can be used in perfumes, flavorings, and essential oils. It is also used as a local antiseptic and anaesthetic. The dominant compound that is found in *Chlorella* is fatty acid such as hexadecanoic acid, methyl 9,9-dideutero-octadecanoate, 1,2-benzenedicarboxylic acid and dodecanoic acid.

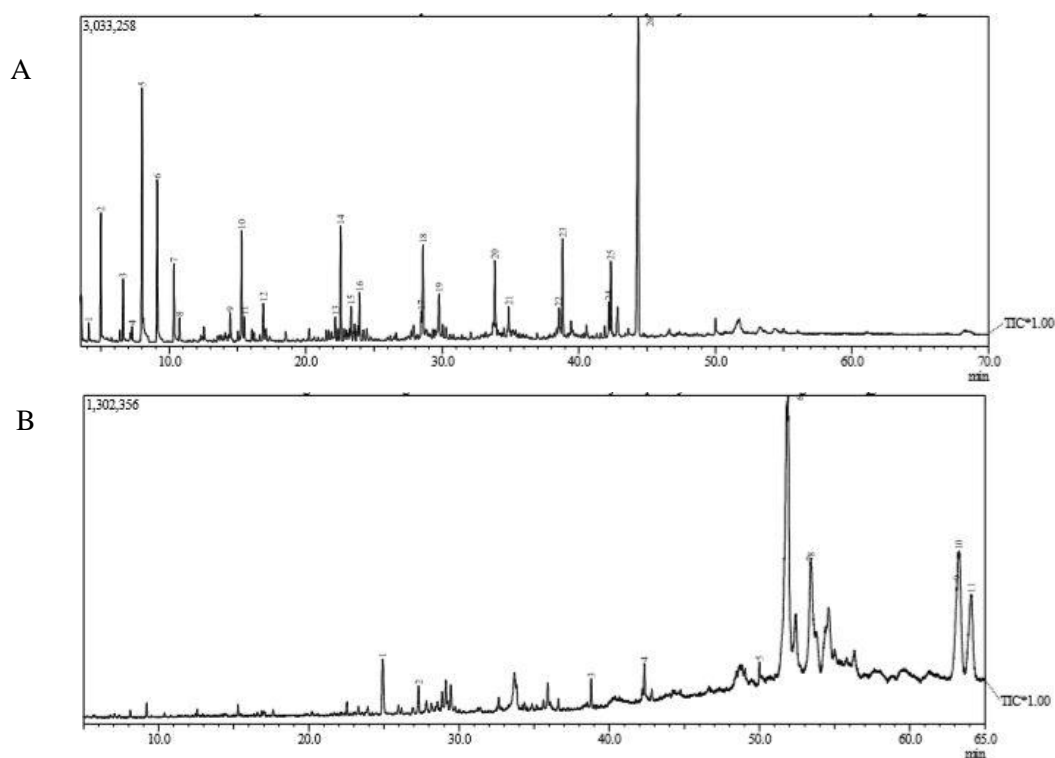


Figure 1. Chromatogram of *Spirulina* sp. (a) and *Chlorella* sp. (b)

Table 1. Chemical compounds of *Spirulina* Sp.

No	Structure	Name	Class
1	C ₈ H ₁₄ O	Cyclooctanone (CAS)	Cycloalkane
2	C ₇ H ₈	Toluene	Benzene
3	C ₉ H ₂₀	Hexane,2,3,4-Trimethyl-	Alkane
4	C ₉ H ₁₈	2,4-Dimethyl-L-Heptene	Alkene
5	C ₈ H ₁₀	Ethylbenzene	Benzene
6	C ₈ H ₈	Bicyclo[4,2,0]octa-1,3,5-Triene(CAS)BYCYCLO(4,2,0)OCTA-1,3,5-TRIENE, Benzenocyclobutane	Benzene
7	C ₉ H ₁₂	Benzenocyclobutane, 1,2-dihydro-Cardene,1,2Dihydrobenzocyclobutene	Benzene
8	C ₁₀ H ₁₆	Benzene,(1-methylethyl)-Alpha,-Pinene	Terpene
9	C ₁₀ H ₁₈ O	Eucalyptol	Monoterpenes
10	C ₁₃ H ₂₈	Tridecane	Alkane
11	C ₁₃ H ₂₈	Tridecane	Alkane
12	C ₁₃ H ₂₈	Tridecane (CAS) n-tridecane	Alkane
13	C ₁₄ H ₃₀	Tetradecane(CAS) n-Tetradecane	Alkane
14	C ₂₀ H ₄₂	Isotetradecane	Alkane
15	C ₁₂ H ₂₆ O	Eicosane n-Eicosane	Alkane
16	C ₁₆ H ₃₄	1-octanol, 2-butyl-Hexadecane	Alcohol
17	C ₂₀ H ₄₂	Hexadecane	Alkane
18	C ₁₈ H ₃₈	Eicosane n-Eicosane	Alkane
19	C ₂₀ H ₄₂	Octadecane	Alkane
20	C ₁₈ H ₃₈	Eicosane n-Eicosane	Alkane
21	C ₁₈ H ₃₈	Octadecane	Alkane
22	C ₂₁ H ₄₄	Octadecane	Alkane
23	C ₁₉ H ₃₈ O ₂	Heneicosane n-Heneicosane	Alkane
24	C ₁₉ H ₃₄ O	Octadecanoid acid, methyl ester	Fatty acid
25	C ₁₉ H ₃₆ D ₂ O ₂	8,11-octadecanoid acid, methyl ester	Fatty acid
26	C ₁₅ H ₁₁ ClN ₂ O	Methyl 9,9-Dideutero-Octadecanoate	Fatty acid
		2H-1,4-Benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-	Organochloride

Table 2. Chemical compounds of *Chlorella* Sp.

No	Structure	Name	Class
1	C12H12O2	Eugenol	Phenylpropanoid
2	C15H24	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, 1S-(1alpha., 4.alpha., 7.alpha.)-	Terpene
3	C17H34O2	Hexadecanoic acid, methyl ester	Fatty acid
4	C19H36D2O2	Methyl 9,9-dideutero-octadecanoate	Fatty acid
5	C24H38O2	1,2-Benzenedicarboxylic acid, diisooctyl ester	Fatty acid
6	C15H24O	Alpha,-Santalol 2-Penten-1-ol, 5,-(2.3dimethyltricyl [2.2.1.0(2.6)hept-3-yl)-2-methyl-, stereoisomer (CAS) 2-Penten-1-ol, 5,-(2.3dimethyltricyl [2.2.1.0(2.6)hept-3-yl)-2-methyl-,[R-(Z)]-(CAS)S	Sesquiterpene
7	C15H24O	Santalol	Sesquiterpene
8	C15H24O	Santalol	Sesquiterpene
9	C19H40	Tridecane, 7-hexyl-7-Hexyltridecane	Alkane
10	C36H74	Hexatriacontane	Alkane
11	C39H74O6	Dodecanoic acid, 1,2,3-propanetriyl ester (CAS), Glyceril tridodecanoate, Trilaurin, Laurin, tri-Glycerol trilaurate, Glyceril trilaurate, lauric acid triglyceride, Lauric acid triglycerin ester	Fatty acid

Discussion

Octadecanoic acid is anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematocide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, antipsychotic and insectifuge (Dandekar et al., 2015). Eicosane derivative is a good antioxidant (Linton et al., 2013). Alkanes compounds are the major volatile compounds in *Spirulina* sp which generally have anti-inflammatory, antiarthritic, antidiabetic, antiulcer, hypolipidemic, antiatherosclerotic, anti-HIV and cytotoxic activities (Safayhi and Sailer, 1997).

In addition, Eucalyptol contained in *Spirulina* is the main compound of essential oils of many plant. As an isolated compound, eucalyptol is known for its mucolytic and spasmolytic action in the respiratory tract, with proven clinical properties. It has also shown therapeutic benefits in inflammatory airway diseases, such as asthma and chronic obstructive pulmonary disease (Juergens, 2014). At low exposure levels, Alpha-Pinene generally is a bronchodilator in humans, and is highly bioavailable with 60% human pulmonary uptake with rapid metabolism or redistribution. Alpha-Pinene is an anti-inflammatory via PGE1 (Russo, 2011), and seems to be an antimicrobial (Nissen et al., 2010). It exhibits activity as an acetylcholinesterase inhibitor, aiding memory and acts as a positive modulator of GABAA receptors at the benzodiazepine binding site (Yang et al., 2016).

Hexadecanoic acid is antioxidant, hypocholesterolemic, nematocide, pesticide, and lubricant, antiandrogenic, as flavoring agents, hemolytic and as 5-

alpha reductase inhibitor. (Rajeswari et al., 2013). Based on research that has been done, dodecanoid acid or lauric acid (LA) has been associated to certain health-promoting benefits of coconut oil intake, including the improvement of the quality of life in breast cancer patients during chemotherapy. As it concerns the potential to hamper tumor growth, LA was shown to elicit inhibitory effects only in colon cancer cells (Lappano et al., 2017).

Eugenol also can be combined with zinc oxide to form zinc oxide eugenol which has restorative and prosthodontic applications in dentistry. For example, zinc oxide eugenol is used for root canal sealing. Moreover, it detected a substance of azulene. It functions as a skin conditioning agent in cosmetic formulations, including hair dyes. Azulene is reported to be used in a wide range of cosmetic formulations, but these reported uses are likely to be uses of guaiazulene, a chemically related colorant, because there are currently no suppliers of Azulene to the cosmetics industry. The anti-inflammatory action of Azulene has been demonstrated in several animal studies. Effects at the cellular level are reported to include inhibition of respiration and growth, but no effect on ciliary activity or membrane permeability (Andersen, 1999). *Chlorella* also contains santalol that generally used as anticancer. A research by Bommaredy et al. (2012) showed that santalol able to against the development of skin cancer and human prostate cancer both in vitro and in vivo. Santalol induced apoptotic cell death and activation of caspase-3.

From the table 3, it is clear that both of *Chlorella* and *Spirulina* contain five different heavy metals such as Cu,

Cd, Fe, Mg and Zn. The levels of all types of metals measured in chlorella are higher than Spirulina, except Cd and Fe. The large gap is detected in Fe where Spirulina consist almost double than Chlorella. The high number of Fe reveals that both of that microalgae have potency as therapy agent of anemia. Similarly, Zn is one of the most important trace elements in the organism, with three major biological roles, as catalyst, structural, and regulatory ion (Chasapis et al., 2012).

Spirulina and Chlorella also contain omega 3, omega 6 and omega 9 that is play an important roles in the body

system. The result shows that Spirulina contains methals and biochemical profile, while Chlorella with the exception of EPA and DHA. A high number of omega 3, 6 and 9 depicts that Spirulina has a potency to be a multi-nutritional human health supplement. The steroid consisting in Spirulina and Chlorella is a potential compound to make as supplement because it is a basic to form hormones and it can impact a number of things from growth to sexual development (Fountain et al., 2008).

Table 3. Biochemical Profile of *Chlorella* sp. And *Spirulina* sp.

No	Test Parameters	Unit	<i>Chlorella</i> sp.	<i>Spirulina</i> sp.
1	Cu (Copper)	mg/kg	< 0.01	< 0.01
2	Cd (Cadmium)	mg/kg	< 0.01	< 0.01
3	Fe	mg/kg	741.51	1323.9
4	Mg (Magnesium)	% b/b	1.75	0.73
5	Zn (Zinc)	mg/kg	46.27	44.62
6	OMEGA 3	% relative	17.29	6.26
7	Lenolenic Acid	% relative	< 0.1	0.24
	Eicosatrienic Acid	% relative	< 0.1	0.93
	Arachidonic Acid	% relative	< 0.1	< 0.1
	EPA	% relative	5.59	2.54
	DHA	% relative	11.7	2.54
	OMEGA 6	% relative	< 0.1	8.08
8	Lenoleat Acid	% relative	< 0.1	7.15
	Eicosadienic Acid	% relative	< 0.1	0.93
	OMEGA 9	% relative	< 0.1	3.63
9	Oleic Acid	% relative	< 0.1	2.81
	Eicosenic Acid	% relative	< 0.1	0.82
	Alcaloid Total	% b/b	0.04	0.45
10	Steroid (Sitosterol)	mg/kg	< 68	< 68
11	Tanin Total (Tannic Acid)	% b/b	0.16	7.85
12	Saponin	% b/b	0.86	1.36
13	Caroten Total	% b/b	0.04	0.1

Sub-chronic test based on hematology profile

In order to know the sub-chronic toxicity of Spirulina and Chlorella, the hematology profile test of white rat (*Rattus norvegicus*) was conducted. The blood profile of the analysis results is shown in tables 4, 5, 6 and 7.

Table 4 shows that total erythrocytes, hemoglobin levels, and hematocrit in the control group tended to increase with the duration of treatment. Although the statistical test analysis is not significant. In contrast, erythrocyte levels, hemoglobin, and total hematocrit from the Spirulina and Chlorella treatment group were decreased with increasing treatment time. Compared to the control group, there was no significant increase in erythrocyte profile during the time of the experiment. It shows that treatment of Spirulina and Chlorella have no toxic impacts based on the erythrocyte profile.

From table 5, it is known that in the control there is a decrease in MCV and MCH values, and an increase in the MCHC value. Likewise in Spirulina treatment. In Chlorella treatment, the values of MCV, MCH, and MCHC show no significant level increase.

The second hematological parameter is leukocytes or white blood cells. These leukocytes play a role to keep the body from the antigen. From table 6, it can be seen that the control number increases on day 30 and then decreases until day 90. In group II that Spirulina treatment, the total leukocyte was relatively constant and slightly decreased on day 90. In Chlorella treatment

(group III), the increase of total leukocyte was noted on the day 30, then decreased until day 90. At the first glance, it is noticeable that there are no significant different between control and treatment groups in the leukocyte profile, indicating that Spirulina and Chlorella have no negative impact on the animal model.

The third hematological parameter is platelet profile. Platelets play a role in blood clotting when injuries occur. Table 7 gives information about profile of platelet. In control, there was an increase in total platelet and plateletrit in the first month then decreased in the second and third months. Similarly, it occurs in the treatment of Spirulina with minimum increase and decrease while Chlorella treatment decreased the total platelet and plateletkrit. For PDW values, the control group decreased whereas Chlorella treatment has increased the value of PDW and Spirulina witnessed a fluctuation.

Overall, the main facts that stand out from this sub-chronic toxicity test was that there are no significant effects on blood profile due to the treatment of Spirulina and Chlorella. It was indicating that both of microalgae have a positive influence and do not have a toxicity effect in the body of experimental animals.

Based on the theory, consumption of Spirulina and Chlorella microalgae can improve body immunity (Tang and Suter, 2011). Previous studies have suggested that Spirulina microalgae has a significant therapeutic effect

for animals and humans (Watanuki et al., 2006). Spirulina is thought to act as an immunomodulator especially related to the non-specific immune system (Watanuki *et al.*, 2006; Abdel-Tawwab and Ahmad, 2009). While Chlorella contains chlorella growth factor that plays a role

in strengthening the immune system and prevent or destroy cancer (Tang and Suter, 2011). Other references suggest that some micronutrients contained in Spirulina and Chlorella such as selenium may help the immune system (Litman et al., 2005).

Table 4. Profile of erythrocyte

Group/Day		I	II	III
RBC ($10^6/\mu\text{L}$)	0	6.47 ± 1.06	7.32 ± 0.07	7.58 ± 0.23
	30	6.75 ± 0.60	6.78 ± 0.42	7.39 ± 1.05
	60	7.72 ± 0.80	6.64 ± 0.68	7.79 ± 0.69
	90	7.05 ± 0.07	6.96 ± 0.22	6.83 ± 0.31
Hemoglobin (g/dL)	0	11.7 ± 2.4	14.3 ± 0.2	14.5 ± 0.3
	30	11.4 ± 1.2	$13.4 \pm 0.6^*$	$14.6 \pm 1.4^*$
	60	13.9 ± 0.2	12.3 ± 1.3	14.8 ± 1.2
	90	13.3 ± 0.6	12.5 ± 0.9	13.1 ± 0.9
Hematocrit (%)	0	38.4 ± 7.7	41.6 ± 0.5	43.0 ± 1.1
	30	34.9 ± 2.6	38.0 ± 2.8	43.0 ± 4.8
	60	41.2 ± 1.6	35.1 ± 3.7	43.8 ± 4.1
	90	39.6 ± 1.3	37.0 ± 1.7	38.8 ± 2.2

*significantly different by Duncan's multiple range test ($p < 0.05$)

Table 5. Index of erythrocyte

Group		I	II	III
MCV (fL)	0	59.1 ± 4.1	56.8 ± 0.6	56.8 ± 1.6
	30	52.1 ± 8.0	56.1 ± 2.9	58.4 ± 1.6
	60	53.6 ± 3.7	52.9 ± 0.3	56.2 ± 0.2
	90	56.2 ± 2.3	53.2 ± 2.9	56.8 ± 0.7
MCH (pg)	0	18.3 ± 3.6	19.5 ± 0.2	19.2 ± 0.5
	30	17.0 ± 3.3	19.9 ± 1.3	19.9 ± 1.0
	60	18.1 ± 1.8	18.5 ± 0.8	19.0 ± 0.2
	90	18.8 ± 1.0	18.0 ± 1.6	19.2 ± 0.6
MCHC (g/dL)	0	30.9 ± 5.1	34.3 ± 0.2	33.7 ± 0.1
	30	32.6 ± 1.3	35.4 ± 1.5	34.1 ± 0.9
	60	33.8 ± 1.3	35.0 ± 1.3	33.8 ± 0.5
	90	33.5 ± 0.6	33.9 ± 1.2	33.8 ± 1.0

*significantly different by Duncan's multiple range test ($p < 0.05$)

Table 6. Profile of lymphocyte, WBC and Neutrophyl

Group		I	II	III
WBC ($10^3/\mu\text{L}$)	0	5.4 ± 2.4	7.7 ± 0.9	7.9 ± 0.6
	30	10.7 ± 4.4	7.6 ± 3.1	9.0 ± 2.9
	60	6.3 ± 1.5	7.7 ± 2.5	8.4 ± 2.7
	90	6.3 ± 1.0	6.6 ± 2.3	4.8 ± 3.8
Lymphocyte (%)	0	70.0 ± 11.3	71.4 ± 6.6	70.2 ± 3.2
	30	72.8 ± 13.7	64.1 ± 8.0	74.0 ± 4.3
	60	72.3 ± 5.8	56.3 ± 11.7	69.5 ± 6.0
	90	70.0 ± 5.5	51.0 ± 21.0	58.5 ± 4.6
Neutrophyl (%)	0	0	0	0
	30	16.5 ± 0.5	$28.3 \pm 0.3^*$	$19.5 \pm 0.7^*$
	60	23.6 ± 3.5	$37.8 \pm 0.1^*$	23.9 ± 6.2
	90	21.0 ± 5.2	23 ± 0.6	$37.7 \pm 2.8^*$

*significantly different by Duncan's multiple range test ($p < 0.05$)

Table 7. Profile of platelet

Group		I	II	III
PLT ($10^3/\mu\text{L}$)	0	523 ± 540	1003 ± 478	1259 ± 235
	30	1258 ± 278	1158 ± 340	1138 ± 463
	60	1003 ± 129	908 ± 400	976 ± 288
	90	1023 ± 103	972 ± 530	1022 ± 197
Platelecyt (%)	0	0.35 ± 0.36	0.65 ± 0.28	0.83 ± 0.15
	30	0.83 ± 0.20	0.79 ± 0.16	0.75 ± 0.28
	60	0.62 ± 0.07	0.58 ± 0.28	0.67 ± 0.12
	90	0.65 ± 0.06	0.66 ± 0.35	0.70 ± 0.09
PDW (fL)	0	8.4 ± 1.2	7.4 ± 0.8	7.3 ± 0.1
	30	7.6 ± 0.2	8.0 ± 1.5	7.5 ± 0.7
	60	7.0 ± 0.3	7.1 ± 0.7	8.2 ± 1.5
	90	7.2 ± 0.2	8.3 ± 1.8	7.9 ± 0.6

*significantly different by Duncan's multiple range test ($p < 0.05$)

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