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Supplementation Impact of *Spirulina platensis* Ethanol Extract on Inflammatory Homeostasis Modulation of Rat Spleen at Different Ages

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ABSTRACT

Pro- and anti-inflammatory mediators need to be released in a balanced way to maintain a healthy state as we age. One important regulatory element in the equilibrium of pro- and anti-inflammatory mediators is NF-κB. The purpose of this study was to examine how *S. platensis* affected the control of inflammatory mediators in young, healthy, emerging adults and adults in rats. In this investigation, 200 mg/kg BW of *S. platensis* extract was administered to six groups of male Wistar rats, ages 12, 18, and 24 weeks, along with a control group. In both the treatment and control groups, NF-κB p65 protein expression was lower at 24 weeks than it was at 12 and 18 weeks. TNF-α and COX-2 proteins were lower in the treatment group than in the control group. All age groups in the treatment group had higher levels of IL-10 protein than the control group. The quantity of NF-κB p65 was positively correlated with COX-2 and TNF-α. By raising the concentration of NF-κB p65, the ethanolic extract of *S. platensis* altered a mediator of cellular immunity. A decrease followed this in TNF-α and COX-2 and a rise in IL-10 in the rat spleen at different ages.

1. Introduction

According to a UN report issued in 2019 of the global population, about 9% are 65 years, with a ratio of one in eleven people. This condition is predicted to increase by 16%, with a ratio of one in six people in 2050 (United Nations 2019). Indonesia will enter the elderly period marked by more than 10 percent of the elderly population since 2020. Additionally, projection data indicates that by 2045, the elderly will comprise about one-fifth of Indonesia's population (BPS 2020). It turns out that the quality of life for the aged is not optimal, along with the rise in cases of age-related disorders in the aging global population. Consequently, one of the most significant difficulties in this century is achieving healthy aging. One solution is to perform therapeutic interventions or lifestyle adjustments. (Borgoni *et al.* 2021).

A balanced response between the release of pro- and anti-inflammatory cellular mediators is required to promote a healthy aging process (Van der Geest *et al.* 2014). The dysregulation of the immune response, which results in chronic systemic inflammation, is the primary alteration that occurs during aging. The function of NF-κB, which is essential to the inflammatory response and connected to the control of the innate and adaptive immune systems, is directly tied to this imbalance. NF-κB is present in practically all animal species and is involved in many cellular reactions. Additionally, some factors contributing to worsening or preventing premature aging demonstrate the role of the NF-κB signaling pathway (García-García *et al.* 2021).

The FDA defines functional food as a component of a regular diet of nutrients taken in a food matrix that, through one or more active substances, can enhance health and have physiological effects (Carpio *et al.* 2021). *Spirulina platensis* has a variety of ingredients. Nutrients potentially improving human health can be used as functional food ingredients (Finamore

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et al. 2017). The bioactive compound *S. platensis* is stated to have various biological functions starting from anti-inflammatory, immunomodulation, antioxidant, accelerating wound healing, anti-bacterial, and anti-viral (Wu *et al.* 2016, 2020; Edirisinghe *et al.* 2020).

The immunomodulatory and anti-inflammatory properties of *S. platensis* are mediated via the modulation of multiple important cytokines, including IL-2, IL-4, IL-6, IL-1β, IL-10, and TNF-α. These cytokines are linked to the ability of *S. platensis* to either stimulate or inhibit the transcription factor NF-κB (Mao & Gershwin 2005; Balachandran *et al.* 2006; Liu *et al.* 2017). Reducing NF-κB nuclear translocation inhibits the production of proinflammatory genes, which is how *S. platensis* anti-inflammatory mechanism is exhibited (Chen *et al.* 2012; Hwang *et al.* 2013; Ku *et al.* 2013; Lee *et al.* 2017; Al-Qahtani & Binobead 2019). Phycocyanin from *S. platensis,* as an anti-inflammatory, selectively inhibits cyclooxygenase-2 (COX-2) and affects the negative regulation of NF-κB activity (Reddy *et al.* 2000; Poligone & Baldwin 2001; Shih *et al.* 2009; Khan & Khan 2018).

Supplementation of *S. platensis* has shown its impacts on regulating immune responses in animal conditions and studies on humans in healthy old age. Thus, the purpose of this study is to examine how *S. platensis* administration affects the regulation of inflammatory mediators, such as NF-κB, TNF-α, COX-2, and IL-10, in healthy rats that are emerging adults and young adults. It also compares this group's results with those of the group that received *S. platensis* ethanol extract supplementation.

2. Materials and Methods

2.1. Sample

Thirty male Wistar rats were studied and split into six groups; the study's spleen samples were taken from these animals (Table 1). In this study, only male rats were used to perform the analysis, and the influence of sex was not investigated. The Department of Chemistry at Universitas Indonesia's Faculty of Medicine provided the ethanol extract of *S. platensis*. The *S. platensis* that we used originally comes from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) or Brackish Water Aquaculture Center, at Jepara city, Central Java province, Indonesia. Tests for phytochemistry along with TLC (thin-layer chromatography) were carried out before (Prijanti *et al.* 2022).

Table 1. Group of treatment and steps of *S. platensis* extract administration

Group	Treatment and number of rats
1	Rats in the control group, aged 12 weeks: 5 rats were started at eight weeks and were administered ddH_2O for 29 days
2	Rats used for induction at 12 weeks of age: Five rats, commencing at eight weeks of age, were administered an ethanol extract of *S. platensis* for 29 days
3	Five control rats, each 18 weeks old, were administered ddH_2O for 29 days starting at week 14
4	Rats used for induction at 18 weeks of age: Five rats, commencing at 14 weeks of age, received an ethanol extract of *S. platensis* for 29 day
5	Five rats in all, who were given ddH_2O for 29 days starting at 20 weeks old, served as the control group at 24 weeks
6	Induction rats were administered *S. platensis* ethanol extract at 24 weeks of age. Five rats were administered *S. platensis* ethanol extract for 29 days beginning at 20 weeks of age

Each of the rats in the treatment group received 200 mg/kg body weight of *S. platensis* dissolved in ddH_2O (double-distilled water) as an ethanol extract. During this study, the ethanol-based extract of *S. platensis* was administered orally using a rat gastric probe. Rats were kept in a room with ad libitum feeding, drinking, and changing light and dark every 12 hours. All rats were euthanized on day 30 by intraperitoneal injection of a combination of 0.4 mg/kg BW (Xylazine hydrochloride) and 7.5 mg/kg BW (Ketamine). The University of Indonesia's Faculty of Medicine's Research Ethical Committee has approved every procedure used in this *in vivo* experimental study (No.: KET-223/UN2.F1/ETIK/PPM.00.02/2022).

2.2. Spleen Homogenate Preparation

The two methods listed below were used to create a spleen homogenate for this investigation: (a) Making spleen homogenates for COX-2 and IL-10 analysis: Frozen tissue samples weighing 100 mg were homogenized 1:10 in a cold solution of PBS with fast weighing. The homogenate was centrifuged for 15 minutes at 4°C at 10,000 RPM. The Christian Warburg method was used to determine the amount of protein in the separated supernatant. The sandwich ELISA was used to measure the amounts of COX-2 and IL-10 in the supernatant. The spleen homogenates required for the

measurement of NF-κB p65 and TNF-α were prepared as follows: 50 mg of frozen spleen samples were rapidly weighed and homogenized in 1 ml of RIPA lysis buffer, to which one mM PMSF was added while the mixture was cold. The homogenate was centrifuged at 4°C, 12,000 RPM for 4 min. The total tissue protein concentration on the separated supernatant was measured using the Bradford method. ELISA sandwich methods were used to measure the supernatant's NF-κB p65 and TNF-α concentration (Elabscience 2021; FineTest 2022a, 2022b, 2022c)

2.3. Measurement of the Concentration of NF-κB p65, TNF-α, COX-2 and IL-10

The concentrations of each protein target were determined using the sandwich ELISA technique. In this investigation, TNF-α, COX-2, and IL-10 concentrations were measured using an ELISA kit from Finetest, and the concentration of NF-κB p65 using an ELISA kit from Elabscience (Elabscience 2021; FineTest 2022a, 2022b, 2022c).

2.4. Statistic Analysis

Statistical data analysis will be performed using SPSS software to detect significant variations in the levels of NF-κB p65, TNF-α, COX-2, and IL-10 protein in the spleen of different age groups of rats supplemented with *S. platensis* ethanol extract and those that were not. Using post hoc LSD analysis and the analysis of variance (ANOVA) test, the statistical analysis determined that a significant difference existed if the p-value was less than 0.05. A transformation is applied to the non-normal distribution of data. The data were still not normally distributed and not homogeneous. The Kruskal Wallis test was carried out and continued with the Mann-Whitney test with $p<0.05$, considered significant. Furthermore, Pearson test's statistical analysis for the correlation between the concentration of NF-κB p65 with the expression of TNF-α, COX-2, and IL-10 protein. The concentrations of pro-inflammatory (TNF-α)/anti-inflammatory (IL-10) cytokines were compared by performing ratio analysis. If the ratio ≥1 means pro-inflammatory cytokines are more dominant than anti-inflammatory cytokines.

3. Results

This study analyzed the mediators of cellular immunity by measuring the expression of NF-κB p65, TNF-α, COX-2 and IL-10 in the spleen of rats of various ages who were given the ethanolic extract of *S. platensis* and those who were not given the ethanolic extract of *S. platensis*.

3.1. NF-κB p65 Concentration Analysis

In this study, we can observe a significant difference in the concentration of NF-κB p65 in the group of rats aged 12 and 18 weeks (control and treatment) with rats aged 24 weeks (control and treatment). The measurement of NF-κB p65 protein showed a pattern of increased production in each age group. In the treatment group, there was a higher concentration of NF-κB p65 than in the control group at the same age, although it was not significantly different (Figure 1A). This study also showed an increase in the concentration of NF-κB p65 and the ratio of NF-κB p65 concentration in all age groups in comparison between the treatment group to the control group (Figure 1B).

3.2. TNF-α Concentration Analysis

The concentration of TNF-α in this study showed (Figure 2A) a significant difference in both groups of rats aged 12 and 18 weeks (control and treatment) with rats aged 24 weeks (control and treatment). In addition, the pattern of decreasing TNF-α concentrations and the ratio of TNF-α concentrations (Figure 2B) in the treatment group compared to the control group was presented in each age group.

3.3. COX-2 Concentration Analysis

This study showed a significant difference in COX-2 concentrations between groups of rats aged 12 weeks and 24 weeks (control). In addition, the concentration of COX-2 also showed significant differences in the group of rats treated at 12 weeks of age against groups of 18 and 24 weeks (Figure 3A). Ratio analysis in this study showed a decrease in COX-2 in the treatment rat group compared to the

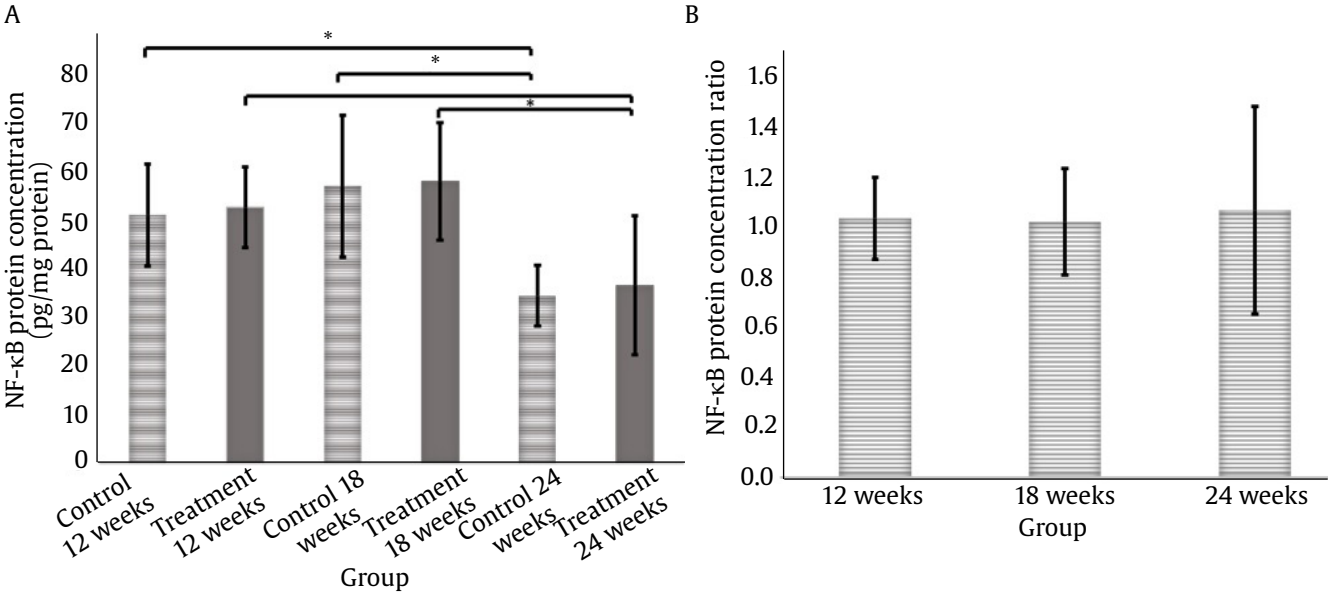

Figure 1. (A) Effect of the administration of *S. platensis* on the concentration of NF-κB p65 in the rat spleen, (B) analysis of the NF-κB p65 ratio of the treatment group to the control group in the rat spleen. Data analysis results with One-way ANOVA were all expressed as mean±SD (n = 5/group). *Showed significant difference ($p<0.05$)

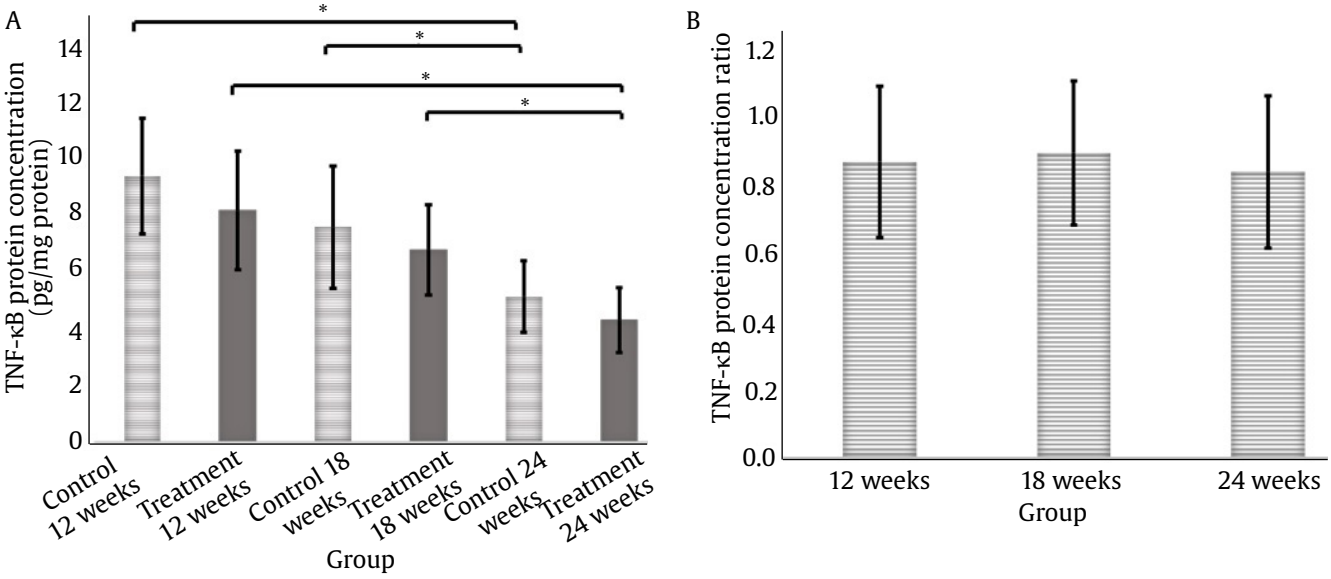

Figure 2. (A) Effect of the administration of *S. platensis* on the concentration of TNF-α in the rat spleen, (b) TNF-α ratio analysis of the treatment group to the group control in the rat spleen. Data analysis results with One-way ANOVA were all expressed as mean±SD (n = 5/group). *Showed significant difference ($p<0.05$)

control rat, although it did not show a significant difference (Figure 3B).

3.4. IL-10 Concentration Analysis

The concentration of IL-10 did not significantly differ between the control groups of 12-, 18-, and 24-week-old rats in this investigation. When comparing rats aged 12 and 18 weeks and rats aged 24 weeks, there was a significant difference in the concentration of these cytokines in the treated rat group, as demonstrated by this study (Figure 4A). Furthermore, the study's ratio analysis revealed that the treatment group's IL-10 concentration was higher than that of the control group. It did not, however, alter noticeably (Figure 4B).

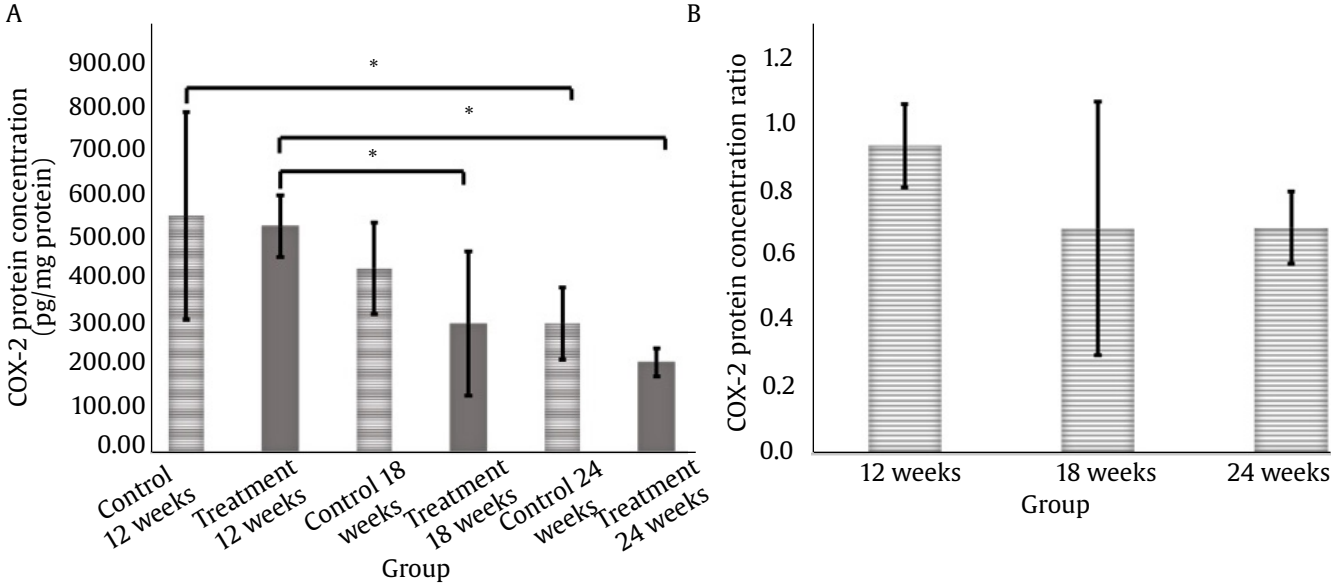

Figure 3. (A) Effect of the administration of *S. platensis* on the concentration of COX-2 in the rat spleen, (B) analysis of the COX-2 concentration ratio of the treatment group to the control group in the rat spleen. Data analysis results with One-way ANOVA were all expressed as mean±SD (n = 5/group). *Showed significant difference ($p<0.05$)

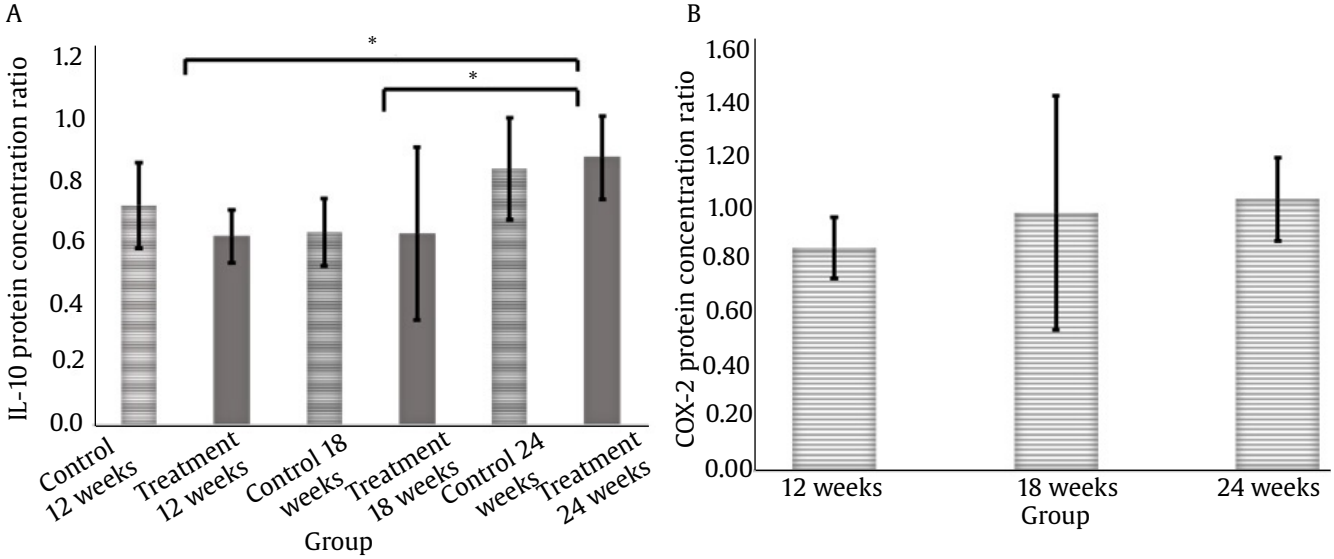

Figure 4. (A) Effect of the administration of *S. platensis* on the concentration of IL-10 in the rat spleen mice, (B) analysis of the IL-10 concentration ratio of the treatment group to the control group in the rat spleen. Data analysis results with One-way ANOVA were all expressed as mean±SD (n=5/group). *Showed significant difference ($p<0.05$)

3.5. NFκB p65 Correlation Analysis with TNF-α, COX-2, and IL-10

Figure 5A displays the correlation coefficient of 0.461 with a significance of 0.005, indicating a significant difference ($p<0.05$) between NFκB p65 and TNF-α. With a coefficient correlation of 0.434 and a significance of 0.016, NFκB p65 and COX-2 likewise displayed significant differences ($p<0.05$) (Figure 5B). In the meantime, Figure 5C correlation analysis between NFκB p65 and IL-10 revealed no significant difference ($p<0.05$), with a correlation coefficient of -0.319 and a value of 0.085.

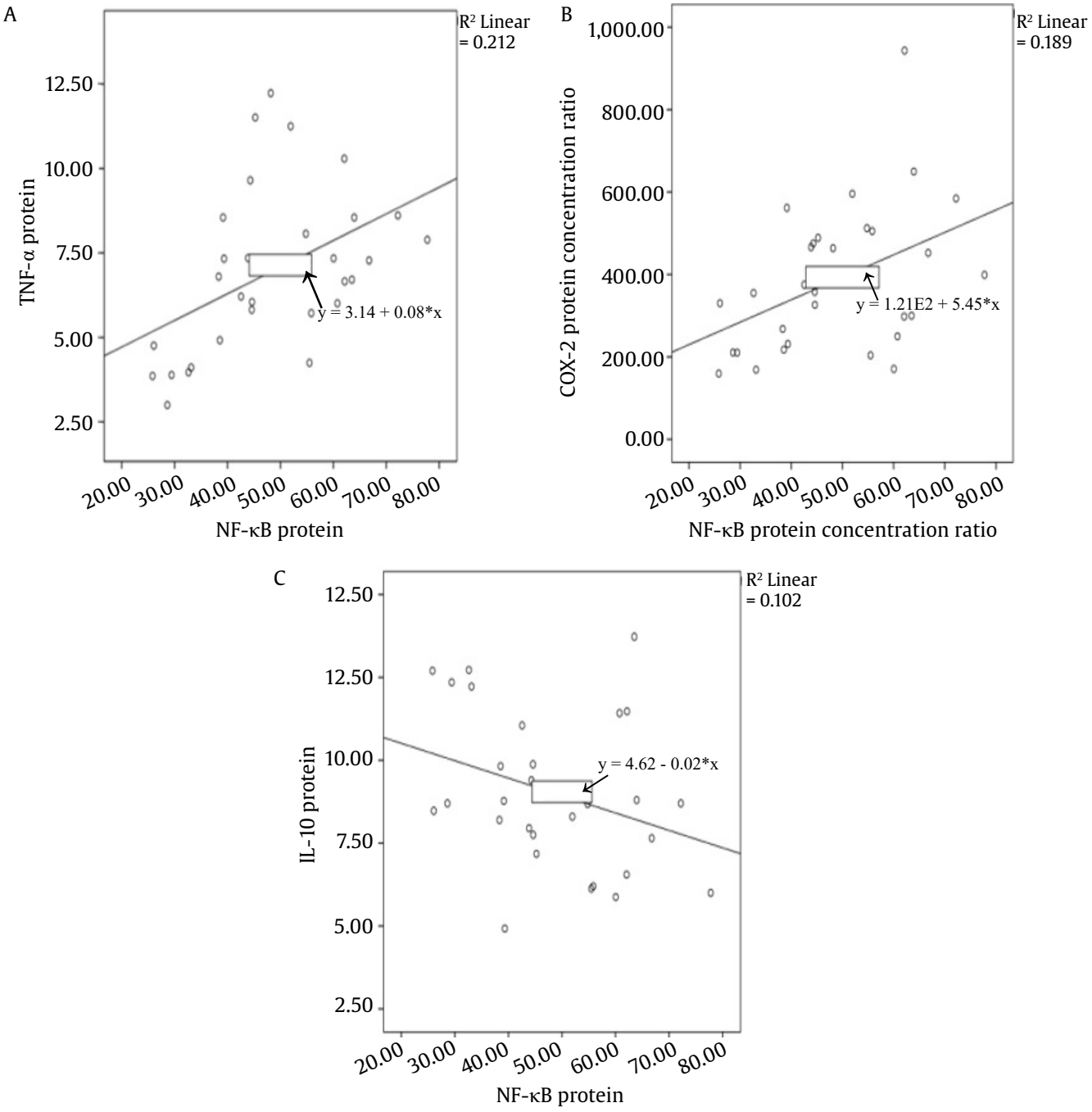

Figure 5. (A) Correlation of NF-κB p65 concentration to TNF-α in the rat spleen, (B) Correlation of NFκB p65 concentration to COX-2 in the rat spleen, (C) Correlation of NFκB p65 concentration to IL-10 in the rat spleen. Results of data analysis using the Pearson test ($p<0.05$, n = 5/group)

3.6. Analysis of the Cytokines TNF-α (Pro-inflammatory) and IL-10 (Anti-inflammatory) Ratios

Figure 6 displays a ratio analysis between the amounts of TNF-α and IL-10. TNF-α to IL-10 ratio is ≥ one at 12 and 18 weeks. On the other hand, at 24 weeks of age, the TNF-α to IL-10 ratio value is less than 1. Ratio analysis revealed an increase in the pro-inflammatory cytokine ratio after 12 and 18 weeks of our investigation. The study's ratio concentration of TNF-α/IL-10 was tested using the Kruskal-Wallis non-parametric statistical test, and the results (Figure 6) indicated that there was no significant difference ($p<0.05$) between the control and treatment groups of rats 12, 18, and 24 weeks aged. At 24 weeks of age, there was a decrease in the ratio of cytokines pro-inflammatory to anti-inflammatory. The rats in the 24-week-old treatment group tend to respond better to the ethanol extract of *S. platensis* than the other treatment groups.

4. Discussion

The blue-green algae *S. platensis* inhabits both freshwater and the ocean. It is a flawless spiral coil under the microscope. The two most prevalent *Spirulina* species found in supplements for humans and animals are *Spirulina platensis* and *Spirulina maxima* (Hwang *et al.* 2011). High-quality protein, carotenoids, C-phycocyanin (CPC), gamma-linolenic fatty acids, vitamins (B1, B2), iron, minerals, and other micro- and macronutrients are all abundant and essential in *S. platensis* (Hwang *et al.* 2011). In several studies, the ethanol extract of *S. platensis* was stated to have a diverse nutritional composition starting from the content of carotenoids, phenols, flavonoids, amino acids, proteins, and carbohydrates (Gabr *et al.* 2020; Martí-Quijal *et al.* 2021; Hidhayati *et al.* 2022). The ethanol extract of *S. platensis* has been reported in multiple investigations to have a varied nutritional composition, beginning with the amount of proteins,

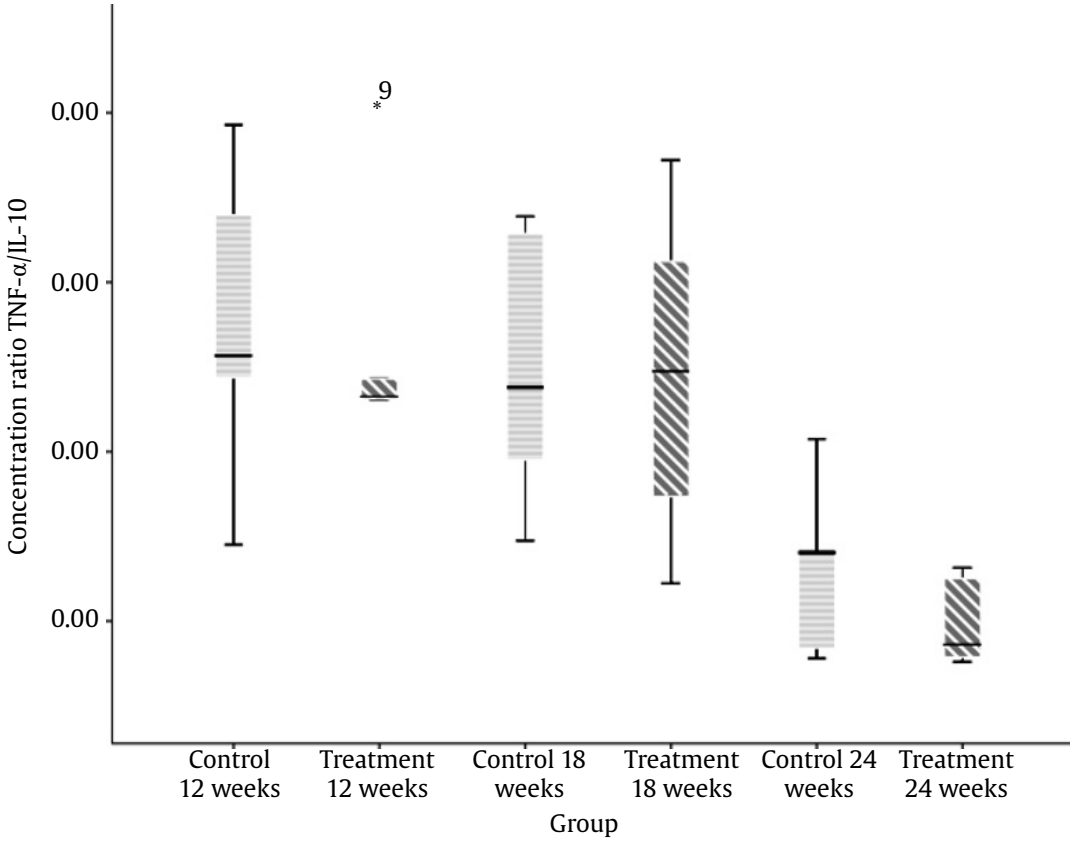

Figure 6. Analysis of the concentration ratio of TNF-α to IL-10 in the rat spleen. Data analysis results with Kruskal-Wallis non-parametric statistical test. There was no significant difference ($p<0.05$; (n = 5/group)

carbs, amino acids, phenols, and flavonoids (Wu *et al.* 2016, 2020).

Health interventions could be implemented from infancy to maturity using a life cycle perspective. One of them is a focused, adequate diet, which has a big impact on keeping older people productive, independent, and healthy in the long run (Infodatin 2016; Calder *et al.* 2017; Sinha *et al.* 2018). Thus, by examining the expression of NF-κB, TNF-α, COX-2, and IL-10, we were able to investigate the impact of supplementing emerging adult and young adult rats with the ethanol-based extract of *S. platensis*.

Rats in this study were supplemented with *S. platensis* ethanol extract from the age of 8, 14, and 20 weeks for 29 days. After termination on day 30 the spleen was used as a sample and analyzed for various parameters. The start of the treatment age range of 8-20 weeks, equivalent to the adolescents-emerging adult stage. Human age at this stage ranges from 12-18 to 18-25 years. At the time of termination for sampling, the age of rats was at the age of 12, 18, and 24 weeks. Rat aged 12 and 18 weeks were in the stage of emerging adulthood and the stage of young adulthood at 24 weeks. Age 12 and 18 weeks in rats, estimated the human age is 18-25 old, and the human age is 25-40 years for rats aged 24 weeks (Ghasemi *et al.* 2021).

In this work, rat spleens of different ages that received an ethanolic extract of S. platensis and those that did not were used to measure the production of NF-κB p65, TNF-α, COX-2, and IL-10, which are mediators of cellular immunity. We can see from this study that at the same age, the treated rat group had higher amounts of NF-κB p65 than the control group. Nevertheless, no statistically significant variation was seen. When examined in human monocytic THP-1 cells, the effect of polysaccharide supplementation from *S. platensis* was found by Pugh *et al.* 2001. This led to an increase in NF-κB activation, which raised the release of the proinflammatory cytokines TNF-α and IL-1β (Pugh *et al.* 2001).

The study by Wu *et al.* 2020 was performed to evaluate the immunostimulatory effect of polysaccharides. NF-κB p65 movement from the cytoplasmic to the nuclei appears to increase in RAW 264.7 cells treated with polysaccharides from *S. platensis* extract and LPS as compared to controls after immunofluorescence staining, as seen using an inverted fluorescence microscope. NF-κB translocation occurs after the degradation of Iκβ-α, which continues with the transcription process (Wu *et al.* 2020). The involvement of TLR4 in RAW 264.7 cells treated with polysaccharide extract and given a TLR4 inhibitor (TAK-242) showed decreased secretion both TNF-α and IL-6. The role of TLR4 as a receptor in mediating the interaction of *Spirulina* polysaccharide extract on macrophages is essential in that decrease. This study showed that the *Spirulina* polysaccharide could stimulate immunity in macrophages by activating the NF-κB signaling pathway induced through TLR4. Giving polysaccharides from *Spirulina* extract shows an increase in NF-κB (Wu *et al.* 2020).

The result of our study is in line with the previous study by Wu *et al.* 2020. Our study showed an increase in NF-κB p65 concentrations and the ratio of NF-κB p65 concentrations in the treatment group compared to controls in all age groups.

TNF-α and IFN-γ levels significantly decreased in the 500 and 1,000 mg/kg treatment group, which equated to the baseline group in Grover *et al.* 2021 study on the effects of *S. platensis* phycocyanin supplementation on mice. In our study, a dose of 200 mg/kg of *S. platensis* ethanol extract administration inhibited TNF-α production. TNF-α modulation appears suppressed in the treated rats in all age groups.

A further investigation conducted in 2019 by Al-Qahtani *et al.* similarly demonstrated that supplementing rats using a 9% aqueous extract of *S. platensis* dramatically decreased the concentrations of inflammatory markers such as TNF-α, IL-6 and also IL-1b after they were exposed to acute hepatotoxicity produced by Wistar (Al-Qahtani & Binobead 2019). Studies analyzed TNF-α secretion and reported no significant differences in age and sex groups in 5 large groups of healthy human subjects starting from age: 21–30, 31–40, 41–50, 51–60, and 61–70 years (Milan-Mattos *et al.* 2019). This seems to align with our study's findings which showed a decrease in TNF-α concentrations at 12 and 18 weeks of age (control and treatment), although there was no significant difference. This is presumably because these two groups are still in the same young adult range (Ghasemi *et al.* 2021).

Although there was no significant difference, this investigation revealed a decline in COX-2 in the treatment group compared to the control rats. The results of this study are in line with *in vitro* studies conducted by Reddy *et al.* 2000 and *in vivo* by Shih *et al.* 2009. Phycocyanobilin and phycocyanin from *S.*

platensis were reported that have anti-inflammatory potential through the inhibition of Cycloocgenase-2 (COX-2) (Reddy *et al.* 2000; Shih *et al.* 2009). Our earlier research verified that the ethanol-based extract of *S. platensis* utilized in this investigation contained phycocyanobilin (Prijanti *et al.* 2022). In our *in silico* study, phycocyanobilin showed its potential as a COX-2 inhibitor. However, its binding energy was not optimal compared to celecoxib and rofecoxib, which are FDA drugs (Iswanti *et al.* 2022).

In this study, rats aged 12–18 weeks and rats aged 24 weeks exhibited a substantial difference in the concentration of the cytokine IL-10 with increasing age. This study's rise in IL-10 cytokine production was consistent with Grover *et al.* 2021 findings, which employed mice given varying doses of the phycocyanin *S. platensis*, ranging from 0 mg/kg to 1,000 mg/kg. Depending on the dosage, the study's healthy mice showed higher levels of IL-10 cytokine expression. At doses between 200 mg/kg and 1,000 mg/kg, it differed significantly from the mice in the control group (Grover *et al.* 2021). Our research demonstrated that taking supplements of *S. platensis* at ages 12, 18, and 24 results in the production of IL-10, an anti-inflammatory. Furthermore, these *in vivo* findings are consistent with an *in silico* investigation wherein the three bioactive substances of *S. platensis* (α-glucan, β-carotene, and phycocyanobilin) all have weak inhibitory effects on the production of IL-10 (Unpublished data).

Correlation analysis showed a positive correlation and significant difference between the concentrations of NF-κB p65, TNF-α, and COX-2. The positive correlation results are consistent with the role of NF-κB, which is essential in expressing various genes, including the pro-inflammatory cytokine gene TNF-α, inflammatory mediator COX-2, adhesion molecules, growth factors, immune receptors, various inflammatory mediators, and several acute phase proteins (Grover *et al.* 2021).

Additionally, studies showed that administering polysaccharides from *S. platensis* boosted NF-κB activation, which in turn increased the levels of the inflammatory cytokines IL-1β and TNF-α (Pugh *et al.* 2001). However, studies differed by Kim *et al.* 2013, who used heptadecane from *S. platensis* in the diet of Sprague Dawley rats and showed anti-inflammatory modulation of NF-κB in rats at 20 months of age. *S. platensis* heptadecane supplementation causes the inhibition of the gene expression regulated by NF-κB. This work demonstrated that the classical NIK/IKK and MAPK pathways, which activate NF-κB, were inhibited, which in turn reduced the production of COX-2 and iNOS (Kim *et al.* 2013). An *in vitro* study by Shih *et al.* 2009 also reported that after lipopolysaccharide induction in RAW 264.7 continued with phycocyanin treatment from *S. platensis* resulted in the suppression of NF-κB activation, which effect a weakening of iNOS expression and the formation of TNF-α, inhibition of the production of leukotriene B4 and COX-2 activity (Shih *et al.* 2009).

Administration of *S. platensis* extracts affected the increasing and decreasing NF-κB as a transcription factor. This is also consistent with changes in TNF-α and COX-2 gene regulation levels controlled by NF-κB. Polysaccharides and phycocyanobilin, the active ingredients of *S. platensis* extract, provide different effects. The concentration and ratio of the NF-κB p65 increased in the treatment group compared to the control shown in this study. It is suspected that the active ingredient polysaccharide in the ethanol extract of *S. platensis* can enhance the levels of NF-κB. Our previous studies have demonstrated the presence of polysaccharides in the ethanol-based extract of *S. platensis* used in this research (Prijanti *et al.* 2022). In addition, *in silico* study that we conducted revealed the potential of polysaccharides of *S. platensis* as an anti-inflammatory through its interaction with NF-κB (Iswanti *et al.* 2022).

Our study showed a negative and insignificant relationship between IL-10 and NF-κB p65. Each protein's functions may align with the negative correlation between IL-10 with NF-κB p65 protein. The immune system's homeostasis is the primary goal of the transcription factor NF-κB's central regulation of the response to inflammation (Christian *et al.* 2016). Only when pro- and anti-inflammatory cytokines function at their peak—in this example, anti-inflammatory IL-10—will inflammation cease and repair occurs (Rea *et al.* 2018). Research by Hobbs *et al.* 2018 provides an overview of how NF-κB p65 can function to suppress the regulation of IL-10 expression directly and indirectly. This suppression mechanism aims to prevent premature anti-inflammatory responses so the repairs or resolution of inflammation can be optimal (Hobbs *et al.* 2018).

Cytokines can control their production through adverse feedback action. Cytokines can also have various effects but, at different times, can give opposite responses (Kubiczkova *et al.* 2012). A study by Hobbs

et al. 2018 also showed how TNF-α stimulates IL-10 expression in macrophages. It is possible only until TNF-α reaches a threshold concentration that IL-10 expression occurs. Other trials support this, whereby active signaling triggered by TNF-α for up to 6 hours is insufficient to trigger an anti-inflammatory response, namely IL-10. To induce optimal expression of IL-10, a threshold concentration of TNF-α is required. Thus, there cannot be an IL-10 (anti-inflammatory) response without a complete pro-inflammatory response from TNF-α (Hobbs *et al.* 2018).

This research has shown that adding *S. platensis* ethanol extract to supplements raised NF-κB p65 concentrations in all age groups. TNF-α concentrations decreased across the board in this study's age categories. The concentration of COX-2 gradually decreased when *S. platensis* extract was added. In this investigation, the concentration of IL-10 grew progressively with age. In the spleen of rats of different ages, the ethanol extract of *S. platensis* modifies cellular immunity by increasing the engagement of NF-κB p65, followed by a decrease of cytokines TNF-α and COX-2 and an increment of IL-10. *S. platensis* supplementation increases the regulation of NFKB, which is the primary regulator of cytokines in the body. This demonstrates how NFKB controls pro-inflammatory and anti-inflammatory mediators' homeostatic activity (Christian *et al.* 2016).

Age-dependent changes in the rodent immunological response are a good representation of the aging human immune system. Numerous advantages of the rat model include the abundance of life-table data, shorter lifespan, ability to control the environment, variety of strains, a relatively low price, and ease of availability (Hazzard 1991; Andreollo *et al.* 2012; Ghasemi *et al.* 2021). As the experimental animals utilized in this study are younger than old age in humans, no signs of aging have yet manifested in them. This study uses the *S. platensis* ethanol extract dose referred to the previous research (Grover *et al.* 2021). However, this dose has not shown optimal NF-κB p65 modulation compared to the treatment and control groups. Therefore, in further research, it is necessary to use experimental animals whose ages describe the elderly in humans. In addition, different doses of *S. platensis* ethanol extract are required in future studies.

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