

Effect of Black Sea bream extracts on cytokine production in lipopolysaccharide-induced inflammation

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Abstract: We investigated the effect of black sea bream extracts on changes in pro-inflammatory and anti-inflammatory cytokines induced by lipopolysaccharide (LPS) in murine splenocytes. The levels of pro-inflammatory cytokines [interleukin (IL)-6, tumor necrosis factor- α (TNF- α), IL-12/IL-23(p40) and IL-17A] and anti-inflammatory cytokines [IL-4, IL-10 and interferon- γ (IFN- γ)] were assessed. Incubation of murine splenocytes with acetone+methylene chloride (A+M) and methanol (MeOH) extracts significantly decreased LPS-induced IL-6, IL-12/IL-23(p40) and IL-17A productions after 6 h incubation ($p < 0.05$). The A+M and MeOH extracts significantly increased LPS-induced IL-4 and IFN- γ productions at 48 and 72 h incubation ($p < 0.05$). Treatment with A+M extract resulted in significantly higher IL-10 production in splenocytes after 72 h ($p < 0.05$). Conclusively, black sea bream extracts were shown to be efficient in falling several pro-inflammatory cytokines while rising anti-inflammatory cytokines. Thus our results suggest that black sea bream extracts selectivity modulate immune events.

Introduction

Black sea bream (*Acanthopagrus schlegli*) is an important commercial species of aquaculture market in most countries of Asia (Chang and Yueh, 1990). Southeast Asia markets have enthusiastically welcomed black sea bream because of the deliciousness and nourishing qualities of its meat. Some information has been published concerning the growth (Choi, 1996), maturation and spawning habits (Kwon *et al.*, 2009), sex differentiation (Lee *et al.*, 1994) and adaptation (Yoo *et al.*, 2003) of black sea bream. However, studies on the nutritional value of this species as a food source are still lacking. A previous study has suggested that black sea bream contains the highest percentages of docosahexaenoic acid (DHA, 22:6n-3) compared with other sea breams, such as red sea bream (*Pagrus major*), rock bream (*Oplegnathus fasciatus*), and rudder fish (*Girella punctata*) (Bae and Lim, 2012). Both human and animal studies demonstrated that n-3 polyunsaturated fatty acids (PUFAs) possessed a protective effect against inflammatory properties in macrophage (Rogero and Calder, 2018). Among the PUFAs, DHA is a component of phospholipids in mammalian cell membranes and effective on modulating biological events such as inflammation. It has been known that higher

amounts of interleukin (IL)-1, tumor necrosis factor- α (TNF- α), and IL-6 were associated with the pathogenesis of inflammatory diseases, and marine oil originated n-3 PUFAs inhibited a synthesis of these cytokines in rheumatoid arthritis and amyloidosis (Abdulrazag *et al.*, 2017). Treatment with n-3 PUFAs may influence metabolic effects of cytokines and exert beneficial in delaying the development of these disease states. Therefore, we investigated to determine whether extracts from black sea bream modulate changes in inflammation stimulatory cytokines (IL-6, TNF- α , IL-12/IL-23(p40) and IL-17A) and cytokines against inflammation (IL-4, IL-10, and IFN- γ).

Materials and Methods

Materials and cell culture

6-week-old male C57BL6 mice were purchased from Daehan BioLink (Eumsong, Chungcheongbukdo, South Korea) and were maintained at our thermo-hygrostat facility under conventional conditions of controlled temperature ($23 \pm 1^\circ\text{C}$), relative humidity ($65 \pm 5\%$) and illumination (12-hours light: dark cycle). Lipopolysaccharide (LPS), RPMI 1640, fetal bovine serum (FBS), phosphate buffer saline (PBS) and dimethylsulfoxide (DMSO) were bought from Sigma-Aldrich (St. Louis, MO, USA). Acetone, dichloromethane and methanol were purchased from Duksan Co. (Ansan, Gyeonggido, South Korea). Mouse enzyme-linked immunosorbant assay kits were bought from Biologent (San Diego, CA, USA).

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Extraction and fraction of samples

Live *A. schleglii* was bought in the Jagalchi fish market of Korea on March 2017, respectively. Black sea bream was cleaned and divided on two sides. Sea bream fillet was chopped and homogenized in a homogenizer (HMF-985, Hanil, Seoul, Korea). Homogenized sea bream sample was extracted twice with acetone/methylene chloride (A+M extract, 1:1 volume) and then extracted with methanol (MeOH extract). Each A+M and MeOH extract was concentrated to dryness at 40°C using rotary vacuum evaporator (N-100, EYELA, Japan), and the remainder was set at 4°C until analysis and assay. Both extracts were dissolved in DMSO.

Spleen cell culture supernatants

Cell counts within spleen cell suspensions were subjected to 2×10^6 cells/mL using RPMI 1640 medium with 10% FBS, and the adjusted detached cells were aliquoted into 24-well plates (Costar, Cambridge, MA, USA) at 1 mL/well. The splenocytes were treated with A+M and MeOH samples (1 and 3 µg/mL concentrations) or LPS (2 µg/mL concentrations) alone for 6, 24, 48, 72 h and set at 37°C in a 5% carbon dioxide incubator. As a control, cells were treated with treated with 0.01% DMSO. Cells were incubated for 6, 24, 48, 72 h before collection. The suspensions then centrifuged at 300 x g for 10 min and secondarily centrifuged at 1000 x g for 30 min. The supernatants were kept at -70°C until use in analysis of cytokine expression (Hwang et al., 2004). We measured the expression of IL-6, TNF-α, IL-12/IL-23(p40), IL-17A, IL-4, IL-10 and IFN-γ in samples collected from 6, 24, 48, 72 h treatments.

Measurement of cytokines

Capture antibodies for IL-6, TNF-α, IL-12/IL-23(p40), IL-17A, IL-4, IL-10 and IFN-γ were diluted with 5× concentrated Na-carbonate buffer (pH 9.5), loaded into 96-well plates at 100 µg/well, and set at 4°C overnight. The wells were cleaned with PBS saline with 0.05% Tween 20 (PBST) 4 times, and 200 µL of 5% skimmed milk in PBST was supplemented to each well. The plates were set at room temperature for 1 h. The wells then were cleaned with PBST 4 times again, and 100 µL of the sample (A+M or MeOH extract) was supplemented to each well. The plates were set at room temperature for 2 h. After rinsing 4 times, 100 µL of the detection antibody for the proper cytokine was supplemented to each well, and the plates were set at room temperature for 1 h. The wells were cleaned with buffer 4 times, 100 µL of avidin-horseradish peroxidase was supplemented to each well, and plates were set at room temperature for 30 min. After rinsing 5 times with PBST, 100 µL of substrate fluid containing tetramethylbenzidine was supplemented, and the plates were set at room temperature for 20 min. 100 µL of stop solution was supplemented to break reaction, and the optical density was assessed at 450 nm by ELISA reader (Model 550 microplate reader, Bio-Rad, Richmond, VA, USA) (Choi et al., 2018).

Statistical analysis

Data were represented as mean ± standard deviation. To determine normal distribution, Kolmogorov-Smirnov test was done. Significance of differences observed between the

control and experiment groups using Student's *t* test (STATISTICA package) at $p < 0.05$.

Results

Effect of black sea bream extract on pro-inflammatory cytokine expression

Variations in the amounts of inflammation stimulatory cytokines (IL-6, TNF-α, IL-12/IL-23(p40), IL-17A) were investigated upon treatment of murine splenocyte suspensions with LPS, or by co-administration of LPS and different concentrations of A+M and MeOH extracts from the black sea bream. Treating spleen cells with LPS alone dramatically upregulated the levels of IL-6 with increasing incubation times (Fig. 1). Co-administration of A+M and MeOH extracts (concentration of 3 µg/mL) with LPS significantly reduced the expression of IL-6 after 6 h treatment than LPS alone ($p < 0.05$). The A+M extract at a concentration of 3 µg/mL significantly decreased IL-6 expression 72 h after treatment ($p < 0.05$). Fig. 2 shows that the levels of TNF-α in splenocytes were upregulated by A+M and MeOH extracts from black sea bream, however, there was no significant difference between the control and treatments. The use of LPS as a stimulant, IL-12/IL-23(p40) expression was dramatically increased in a time-dependent manner (Fig. 3). Co-administration of A+M and MeOH extracts (at 1 and 3 µg/mL concentration) with LPS downregulated the IL-12/IL-23(p40) expression after 6 h treatment compared with LPS alone ($p < 0.05$). Co-administration of A+M extract and LPS resulted in significantly lower IL-12/IL-23(p40) expression after 24 h treatment than LPS alone ($p < 0.05$). However, the A+M and MeOH extracts together with LPS significantly upregulated the IL-12/IL-23(p40) expression after 48 and 72 h treatment ($p < 0.05$). Fig. 4 shows that the expression of IL-17A was induced by A+M and MeOH extracts than control. Co-administration of A+M (3 µg/mL concentration) and MeOH extracts (1 µg/mL concentration) with LPS resulted in lower IL-17A expression after 6 h compared to LPS alone ($p < 0.05$).

Effect of black sea bream extract on anti-inflammatory cytokine expression

We investigated changes in the generation of anti-inflammatory cytokines (IL-4, IL-10, and IFN-γ) upon treatment of LPS, A+M and MeOH extracts with splenocyte. Adding of LPS alone with spleen cells promoted level of IL-4 (Fig. 5) respect to the culture times. Co-administration of A+M and MeOH extracts with LPS resulted in significantly higher level of IL-4 expression after 48 and 72 h treatment than LPS alone ($p < 0.05$). In the case of IL-10, treatment with A+M extract (at 1 µg/mL concentration) resulted in significantly higher IL-10 expression after 72 h treatment than LPS alone ($p < 0.05$) (Fig. 6). However, treatment with MeOH extract (at 1 and 3 µg/mL concentration) decreased IL-10 production after 6 h treatment ($p < 0.05$). Fig. 7 shows the expression of IFN-γ induced by A+M and MeOH extracts from black sea bream. There was a similar pattern in IFN-γ expression between A+M and MeOH extracts. Co-administration of A+M and

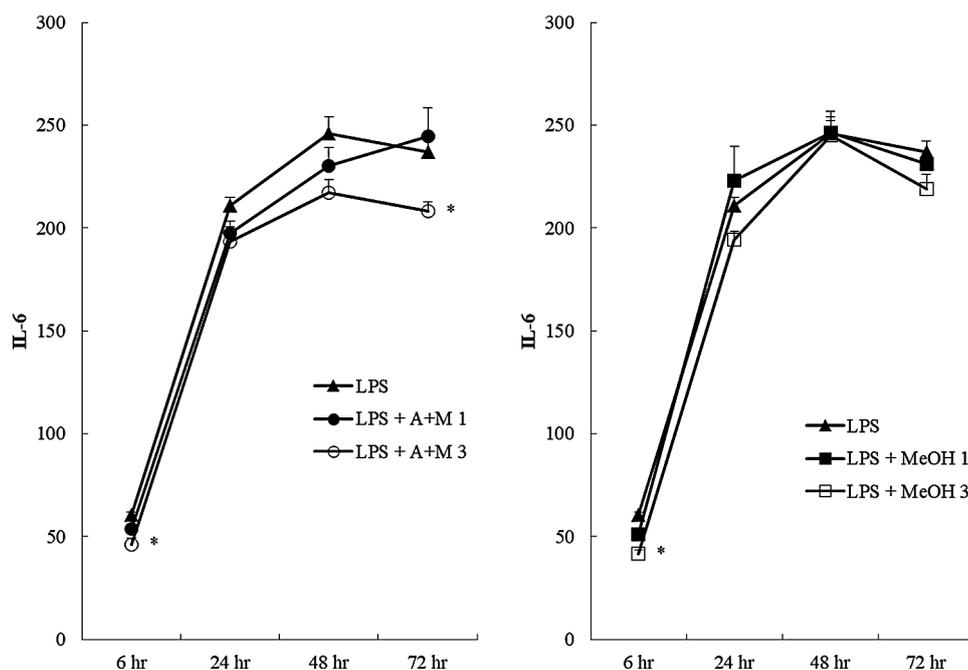


FIGURE 1. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interleukin-6 at different times in mouse spleen cells. The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

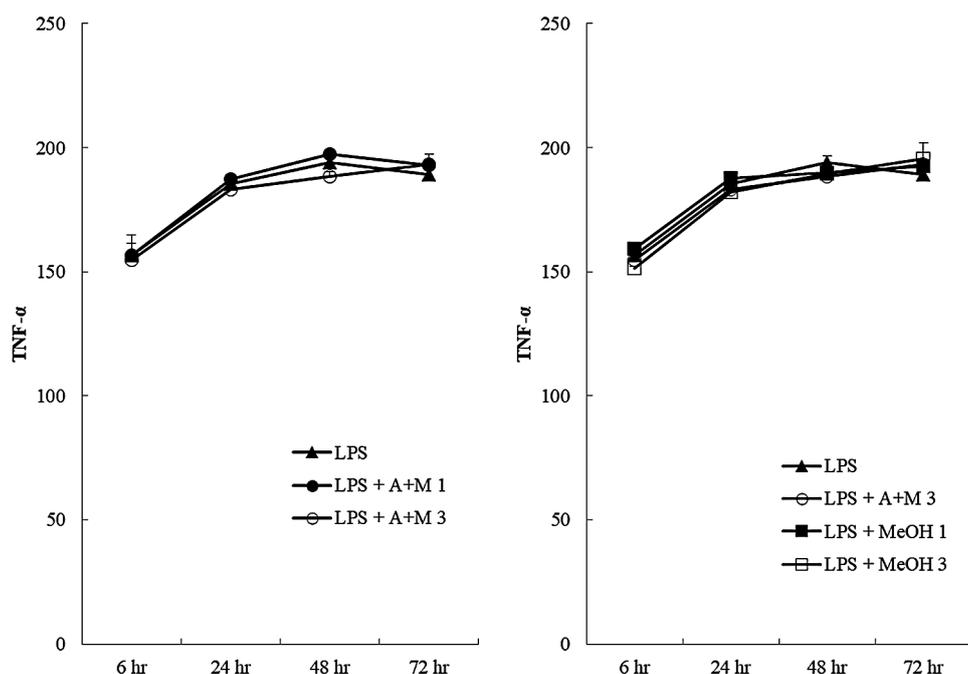


FIGURE 2. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of tumor necrosis factor- α at different times in mouse spleen cells. The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

MeOH extracts with LPS resulted in significantly higher IFN- γ expression after 48 and 72 h treatment than LPS alone ($p < 0.05$).

Discussion

Many evidences suggest that n-3 PUFA enriched marine oils moderate immune responses partially via decrease of T-lymphocyte function by modulating cytokine production. Human and animals studies reported that fish oil administration reduced the amount of the classic inflammation stimulating cytokines TNF- α , IL1 β and IL-6 (Akerele and Cheema, 2018; Hsiao *et al.*, 2018; Chien *et al.*, 2017; Azuma *et al.*, 2018; Laubertova *et al.*, 2017). There is few information of the effects of n-3 PUFA enriched fish oil

on cytokine expression by rodent macrophages. Previous studies have demonstrated that extracts from both tuna (Kim *et al.*, 2013a) and dried mackerel (Kim *et al.*, 2013b) can reduce LPS or Con A-induced TNF- α and IL-6 production. In the present study, it showed that A+M and MeOH extracts from black sea bream reduced the amounts of IL-6, IL-12/IL23(p40) and IL-17A (after 6 h treatment) but the levels of IL-12/IL23(p40) tended to increase after 48 and 72 treatment. As positive control of DHA, the previous study (Choi *et al.*, 2018) suggest that DHA inhibits pro-inflammatory cytokines [IL-2, IL-6 and IL-12/IL-23(p40)] expression on LPS (2 μ g/mL)-induced inflammation. The effective action of n-3 PUFAs on immune cells is still largely unknown. One possible mechanism is that n-3 PUFA from

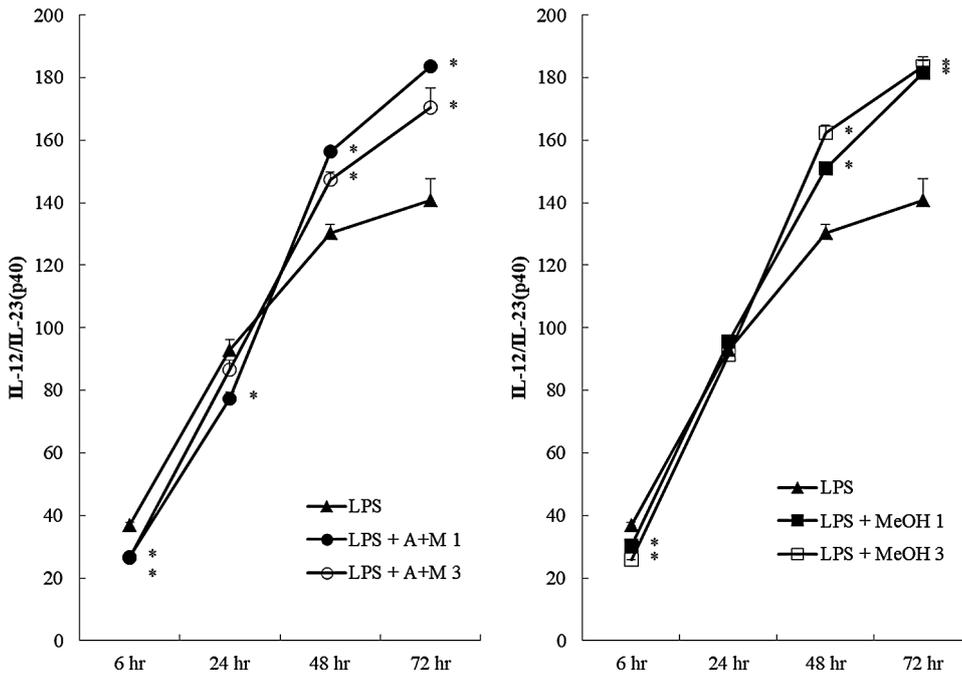


FIGURE 3. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interleukin-12/IL-23(p40) at different times in mouse spleen cells. The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

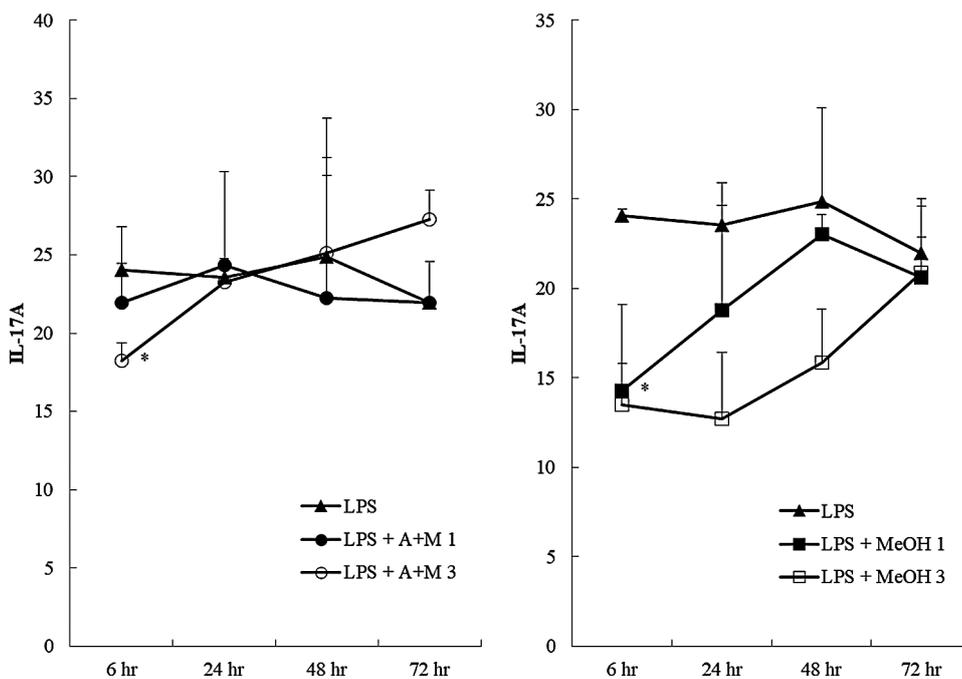


FIGURE 4. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interleukin-17A at different times in mouse spleen cells. The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

fish oils influence arachidonic acid (AA, 20:4n-6) metabolism, which modulate cytokine production. Highly unsaturated eicosapentaenoic acid (EPA, 20:5n-3) and DHA from marine oils can replace AA in membrane phospholipids and reduce generation of inflammation stimulating prostaglandins of the 2 series and leukotrienes of the 4 series (Calder, 2017). Weldon et al. (2007) suggested that n-3 PUFA altered eicosanoid synthesis and reduced pro-inflammatory cytokine production. Bouwens et al. (2009) suggested that dietary fish oil intake decreased the levels of pro-inflammatory genes, nuclear factor κ B (NF- κ B) target genes and genes complicated in eicosanoid synthesis.

Cytokines play important roles in host immune ability, inflammatory reactions, and autoimmune disease (Palomo

et al., 2015). IFN- γ , which is secreted by Th1 cells to activate cell-mediated immunity, suppresses growth of Th2 cells and inhibits the generation of cytokines such as IL-4 and IL-5 (Choi et al., 2012). We observed increased expression of IL-4, IL-10 and IFN- γ upon concomitant exposure to LPS together with A+M and MeOH extracts from black sea bream. There were few studies about effect of black sea bream on inflammation. Sterols and n-3 PUFAs from sea bream were noticed and they are associated with reducing low density lipoprotein cholesterol levels and anti-inflammatory effect (Hamed et al., 2015). Weldon et al. (2007) indicated that n-3 PUFAs (EPA and DHA) exerted anti-inflammatory effects by altering gene expression through direct or indirect actions on cytokine secretion

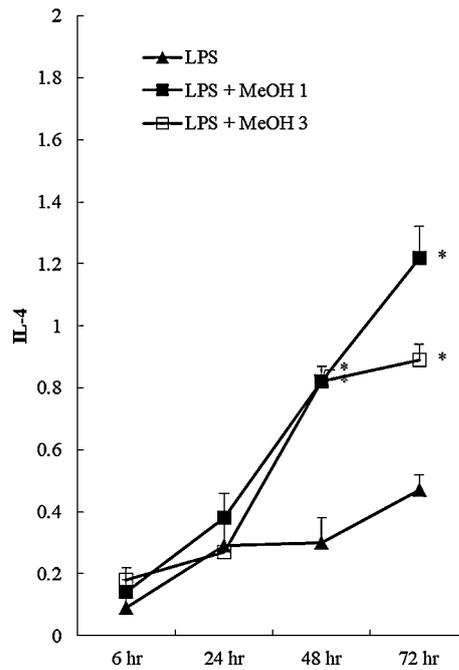
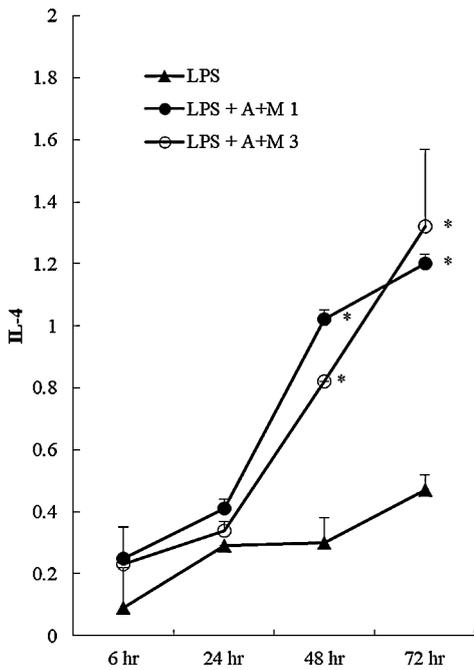


FIGURE 5. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interleukin-4 at different times in mouse spleen cells.

The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

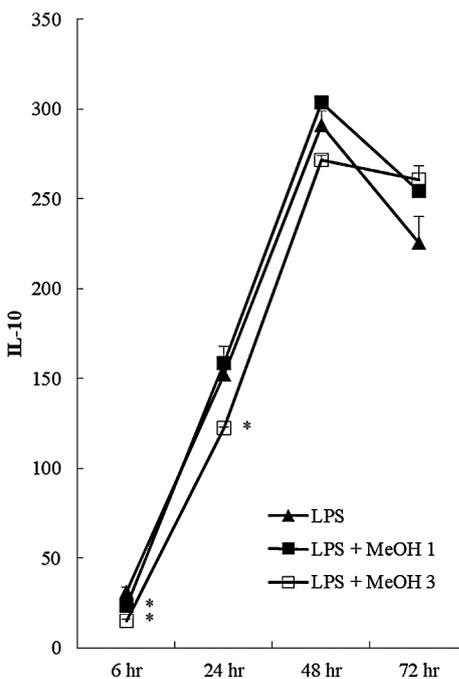
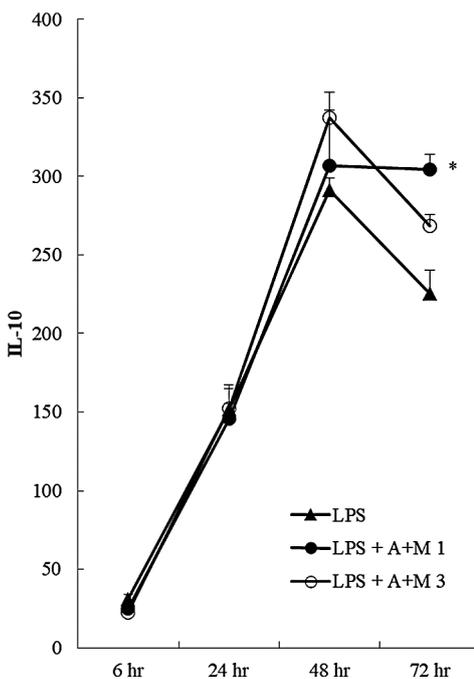


FIGURE 6. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interleukin-10 at different times in mouse spleen cells.

The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

process and inhibiting the stimulation of transcription factors such as NF- κ B. Similar studies supported that n-3 PUFA enriched fish oil inhibited an action of NF- κ B induced by LPS in cultured monocytes (He *et al.*, 2017; Si *et al.*, 2016). Calder (2009) suggested that inflammation inhibitory effect of n-3 PUFAs may enhance peroxisome proliferator activated receptor (PPAR- γ) activity, which activated NF- κ B. Black sea bream is a main commercial species of aquaculture market and a popular fish in Korea as well. Bae and Lim (2012) compared four sea bream species and found black sea bream contained the highest percentages (26% area) of docosahexaenoic acid (DHA, 22:6n-3) among them. Clinical and in vitro studies showed that intake of fish oil including EPA and DHA exerted an anti-inflammatory

effect by decreasing the production of pro-inflammatory cytokines. However, there were few studies evaluating the nutritional and immune effects of black sea bream. Here, our findings indicate that A+M and MeOH extracts, including DHA and EPA, from black sea bream influenced cytokine production.

Conclusion

Here were evaluated the effect of extracts from black sea bream on production of cytokines in splenocyte. There were few studies on relationship between black sea bream and cytokine expression. The A+M and MeOH extracts promoted the generation of protective cytokines (IL-4, IL-10

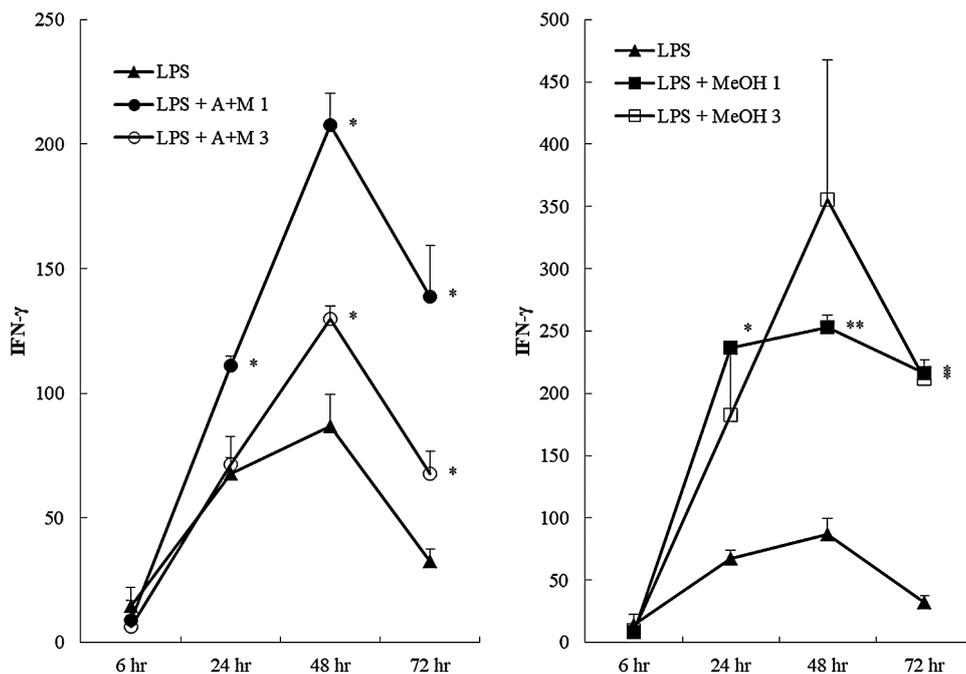


FIGURE 7. Effect of acetone+methylene chloride (A+M) and methanol (MeOH) extracts from black sea bream on the production of interferon- γ at different times in mouse spleen cells. The values were represented as the mean \pm SD and *significantly different between the control and each treatment at $p < 0.05$.

and IFN- γ) against inflammation and reduced the amounts of inflammation-stimulatory cytokines [IL-6, IL-12/IL23(p40) and IL-17A]. Thus steady consumption of black sea seabream is not only intended to provide necessary nutrients and energy for humans but also improve the inflammation related disease.

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Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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