Stability of shark (*Centrophorus* sp.) liver oil rich in squalene as an immunostimulant

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Abstract. Shark liver oil has been widely produced in Indonesia; however, many shark liver oil products still do not meet the established standards. This research involves the addition of antioxidants to stabilize the oil as an effort to extend its shelf life. The objectives of this study were to determine the immunostimulant activity, assessed the synergistic effect of vitamin E in shark liver oil rich in squalene, and identified the optimum concentration of the antioxidant vitamin E in maintaining its stability. Immunostimulant activity was tested by observing spleenocyte proliferation, phagocytic index, and capacity in the peritoneal cavity cells of mice. The stability of the oil was examined by an accelerated method using an oven, what was called the schaal oven test at 40 °C. The research results indicate that shark liver oil possesses immunostimulant effects; however, with an increasing dosage of fish oil, there is no significant improvement. The presence of vitamin E in cod liver oil exhibited a synergistic effect on spleenocyte proliferation for all dosages, while the phagocytic index and capacity at a dosage of 100 µg/mL showed synergy. However, at a dosage of 50 µg/mL, the phagocytic index and capacity did not exhibit synergy with the immunostimulant. The optimal concentration of the antioxidant vitamin E in maintaining oil stability was found to be 100 ppm.

1 Introduction

Immunomodulators are divided into two categories, immunosuppresants and immunostimulants. Immunosuppressants work by inhibiting the immune response, for example during organ transplantation and autoimmune diseases, while immunostimulants enhance the immune response, such as infections, immune deficiencies (AIDS), and cancer [1]. Immunostimulants are part of immunomodulators defined as substances that capable to activate the immune system to prevent diseases and boost the resistance of viruses and bacteria infections [2]. Immunostimulants have role in cancer prevention by increasing the activity of cytotoxic lymphocytes [3]. According to data from [4], the prevalence of cancer, based on doctor diagnose is higher in the age group above 45 years. The age group 55-64 years occupied the first position, 45-54 years the second position, and 75 years and above the

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third position. The results of a meta-analysis by [5] indicate that unhealthy lifestyles are associated with an increased risk of various cancers, including breast, colorectal, endometrial, esophageal, kidney, liver, lung, rectal, and stomach cancers.

Compound known to have effects on immune system as immunostimulants is squalene [6]. Squalene is known for its anticancer properties [7], cardiovascular benefits [8], and its role as a COVID-19 vaccine adjuvant [6]. The demand for squalene in the pharmaceutical industry is projected to reach 184 million USD by 2025 (9). Squalene defined as an unsaturated polyhydrocarbon compound belonging to triterpenoids, formed by six isoprene units [10]. Raw material sources which contain squalene including plants, animals, and bacteria.

The squalene content reported in some sources, are olive oil 564 mg/100 g; soybean oil 9,9 mg/100 g; rice, wheat germ, grape seed oil 14,1 mg/100 g; and peanuts 27.4 mg/100 g [11]. Biotechnological production techniques of squalene have been developed, with organisms, such as *Saccharomyces cerevisiae*, *Botryococcus braunii*, *Aurantiochytrium* sp., and *Escherichia coli* with lower yields compared to plant sources (5-15 mg/g dry weight) [12]. According to [13], squalene derived from wheat, corn, and grapes is considered not viable due to its very low yield (<1,0%). [14] have patented a method to obtain squalene by producing terpenes from fermented sugar using yeast, but the process needs strict control to ensure good quality. Therefore, squalene produced from shark liver oil is mostly preferred due to its very high yield. The squalene content from shark liver oil is reported to reach 79% of the total oil produced [14].

This research also examines the stability of the product during storage by adding antioxidants to extend its shelf life. Squalene possesses double bonds that render the compound susceptible to oxidation. The stability of oxidized compounds must be maintained through additive intervention by the addition of antioxidant agent, aimed at suppressing the rate of oxidation reactions, thereby enabling squalene to preserve its function optimally. Antioxidants are defined as compounds with the capability of inhibiting oxidation chain reactions [15]. Antioxidants found in food have the major role to control or prevent the oxidation process that leads to a decline in food quality [16]. Vitamin E is a fat-soluble vitamin discovered in 1922 and possesses antioxidant, immunoregulatory, and anti-inflammatory function [17].

Several previous studies related to fish oil stability testing have been conducted; for instance, [18] examined the stability of catfish fish oil with 0,067% vitamin E, and [19] investigated the stability of a combination of sardine and shark liver oil with 0.6% vitamin E. Previous research has reported that the lowest concentration ever tested for stability in fatty products ranges from 50 to 100 ppm [20], and the limit for tocopherol use in fish oil is 6000 ppm [21]. One of the issues encountered with squalene from shark liver oil produced in Indonesia is its low and non-standardized quality due to production without temperature control and inadequate attention to hygiene. Therefore, this study aimed to determine the immunostimulant activity and the synergistic effect of vitamin E in shark liver oil rich in squalene through splenocyte proliferation and macrophage phagocytosis by *in vitro* method and establish the optimum concentration of the antioxidant vitamin E in maintaining the stability of shark liver oil.

2 Materials and methods

2.1 Materials

Shark livers (*Centrophorus* sp.) were purchased from Pelabuhan Ratu, Sukabumi, West Java. All the chemicals used were vitamin E (Blackmores 1000IU), distilled water, 95% alcohol,

phenolphthalein indicator (Merck), KOH (Merck), potassium iodide (KI) (Merck), sodium thiosulfate solution (Na2S2O3) (Merck), starch (Merck), glacial acetic acid (Merck), chloroform (Merck), isooctane (Merck), p-anisidine (Merck), aluminum foil, nylon mesh, MTT Reagent (Sigma-Aldrich), PBS buffer (Sigma-Aldrich), growth medium (RPMI 1640) (Sigma-Aldrich), 3% acetic acid (Merck), ethanol (Merck), ether (Merck), MK and DMSO solvents (Merck), absolute methanol (Merck), Giemsa paint (Merck). Equipment used, including measuring cup, pipette, beaker glass, Erlenmeyer flask (Herma), filter, ruler, titrant, hot plate, oven (DHG-9053A), glass stirring rod, spatula, pan, duran bottle, vial, stirrer (Corning PC-420 D), balance (SF-400 C), UV-VIS spectrophotometer (Agilent 8453), microplate 96 wells, microscope, 40 μm strainer, 15 mL tube, centrifuge, incubator (Faithful WPL-30BE), hemocytometer, latex beads, cover slip. Fatty acid profile testing using a Shimadzu C118047 gas chromatography device.

2.2 Methods

2.2.1 Shark liver oil extraction and addition of antioxidants

The extraction of shark liver oil refers to [27]. Samples were cleaned and then extracted using the dry rendering method at 70 °C for 3 hours in an oven. Every hour, the oil was removed out, filtered, and stored in duran bottles. The antioxidant addition treatment consisted of three levels: 100 ppm (P1), 1000 ppm (P2), and 6000 ppm (P3). The reference used to determine variations in antioxidant concentration was based on previous research and the limits for tocopherol use according to (21). The process of adding antioxidants was carried out by dissolving vitamin E antioxidants in shark liver oil. Subsequently, each treatment was aliquoted into small 12 ml vials for each observation time (day 0, day 1, day 2, day 3, day 4, day 5, and day 6) and repeated three times. Control (without added antioxidants (P0)) was prepared using the same method.

2.2.2 Shark liver oil stability

Oil stability testing was carried out by using accelerated schaal oven test method which refers to (25). Closed bottles were used and stored in oven at 40 ºC. The oil samples were calculated for free fatty acid value (FFA), peroxide value (PV), p-anisidine value (p-AnV), and total oxidation (totox) every 24 hours until the totox had passed the IFOS standard. D1 is the 1st day observation, D2 is the 2nd day observation, D3 is the 3rd day observation, D4 is the 4th day observation, D5 is the 5th day observation, and D6 is the 6th day observation. One day of storage in the oven at 40 ºC when converted is equal to 15 days of storage at room temperature.

2.2.3 Fatty acids profile

Fatty acid profiles were analyzed using gas chromatography refers to [2], is based on the partition of the components of a liquid between mobile phase in the form of a gas and stationary phase in the form of a non-volatile substance. This analysis was carried out through several steps, fat extraction, formation of methyl esters (methylation), injection, and identification of the chromatogram of the analysis results.

2.2.4 Squalene Content Analysis

Analysis of squalene content was carried out semi-quantitatively at Republic of Indonesia Police Forensic Laboratory, Bogor. The instrument used is GC-MS brand Agilent Technology 7890A GC System with a capillary column type Agilent J&W GC Columns HP-5MS with a length of 60 m, a stationary phase thickness of 0,25 µM, and an injector temperature of 150 °C. The injection volume is 1 µL, using an inlet model split 1:10 with a gas flow rate in the column of 1,3 mL/minute.

2.2.5 Fish Oil Oxidation

Analysis including free fatty acids (FFA) referring to [22], peroxide value (PV) referring to [23], p-anisidine value (p-AnV) referring to SNI [24], as well as total oxidation based on the sum of two times the PV value with p-AnV.

2.2.6 Splenocyte Proliferation

The splenocyte proliferation test of balb/c mice using the 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT assay). Separation of single-nucleated blood cells (spleen organ) carried out aseptically. The supernatant fluid was discarded, and the cell suspension was added with 1 mL of lysis buffer solution to lyse the red blood cells, then incubated for 1 minute at room temperature. The cell precipitate was added with 1 mL of growth medium (RPMI-1640) then the number of single-nucleated cells was counted by mixing 10 µL of cell suspension with 90 µL of 3% acetic acid. Cells precipitate of 500 µL each were distributed into 96 microplate wells. Concanavalin A (Con A) solution of 10 µg/mL was added to each well for treatment using Con A. All microplates were incubated at 5% CO_2 at a temperature of 37 °C for 18–20 hours. Fish oil P0, P1, P2, and P3 with a dose of 1,56 each; 3,12; 6,25; 12,50; 25,00; 50,00; and 100,00 µg/mL were added to the wells containing the splenocyte suspension. Each treatment was repeated thrice. All microplates were incubated at 5% CO_2 at 37 °C for 48 hours. Cells were added with 50 µg/well of MTT reagent and incubated for 4 hours at 37 °C with CO_2 5%. The formazan crystals formed were dissolved in 100 µL/well of ethanol, readings were taken at λ 595 nm and numerical data in the form of optical density (OD) was obtained. The viability value (number of living cells) is calculated as follows:

$$\text{Viability} = \frac{\text{OD of cells to which the sample was added}}{\text{Cell control OD - media control OD}} \quad (1)$$

2.2.7 Macrophage Phagocytosis

Mice were fasted for a day, anesthetized with ether until unconscious, then euthanized and dissected in the abdominal cavity. A 10 ml cold RPMI solution was introduced into the peritoneal cavity and gently massaged for 5 minutes. Subsequently, the RPMI fluid in the peritoneal cavity was aspirated and centrifuged at 1200 rpm for 10 minutes. The cell count was determined using a hemocytometer and suspended to a concentration of 2,5 x 10^5 cells/well. Next, macrophage cells were incubated for 24 hours. After that, the test substance was added in a volume of 1 ml. Incubation lasted for 4 hours in a CO_2 incubator at a temperature of 37 °C. Latex beads with a diameter of 3 µm were suspended in PBS to a concentration of $2x10^6$ latex/ml. A 200µl latex bead suspension was added to the wells and incubated for 30 minutes at 37 °C in a 5% CO_2 incubator to allow macrophages to interact

with the latex beads. Coverslips were washed three times with PBS, fixed with absolute methanol for 5 minutes, and air-dried at room temperature. Subsequently, coverslips were stained with giemsa stain until completely submerged for 30 minutes, washed with distilled water, dried, and observed under a microscope. The assessment of phagocytosis ability/activity can be calculated by measuring the Phagocytosis Index (PI) and Phagocytosis Capacity (PC) using the following formulas:

$$\text{Phagocytosis Index (PI)} = \frac{\text{Number of latex phagocytosed}}{\text{Number of active macrophages (100)}} \quad (2)$$

$$\text{Phagocytosis Capacity (PC)} = \frac{\text{Number of macrophages that phagocytosed}}{\text{Number of active macrophages (100)}} \quad (3)$$

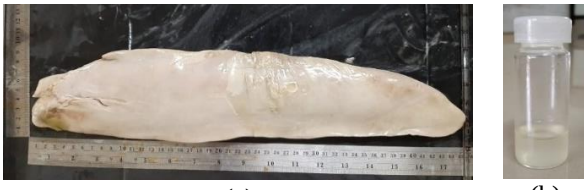
2.3 Data Analysis

Descriptive analysis in this study consists of fatty acid profiles and identification of squalene compounds, and stability during storage. Data obtained from splenocyte viability and macrophage phagocytosis were analyzed using a Randomized Complete Factorial Design with a 95% confidence interval. Data processing was carried out using Microsoft Excel 2019 and SPSS 25.0.

3 Results and discussions

3.1 Evaluation of shark liver oil quality profile

Quality parameters observed were FFA, PV, p-AnV, and totox; then the oil was characterized, including fatty acid profile and squalene content. Oil yield also calculated to determine the ratio of final product and raw material expressed in percent (w/w). The appearance of liver and extracted oil presented in **Fig. 1**.



(a) (b)

Figure 1. Shark liver (a), oil (b).

Length of the shark liver used was 39,02 ± 6,30 cm, width 10,68 ± 1,58 cm, and weight 577,6 ± 188,38 g. Average yield of shark liver oil amounts 40,46 ± 18,55%, with the highest yield was 53,57%. These results are not much different from research conducted by [25], shark liver oil extracted using the dry rendering method at 70 °C resulting 56,96% of yield. Other research shows that yield of extraction results in the liver of *Centrophorus* sp. using a temperature (40, 50, and 60 °C) for 8 hours was 40,4; 46,4; and 49,4%, respectively [26]. Differences in yield results influenced by the levels of fat, protein, water, and others contained in the raw materials [27]. Different extraction methods, temperatures and times will also affect the yield value [28]. [29] stated that the principle of dry rendering extraction aims to damage tissue by coagulating cell wall proteins with the result the oil was extracted.

3.1.1 Quality of shark liver oil

The quality testing of shark liver oil is conducted to determine the suitability of fish oil based on the IFOS standard (2014). The parameters observed include FFA, PV, p-AnV, and total oxidation. The results of the fish oil quality test presented in **Table 1**.

Table 1. Quality of extracted shark liver oil.

Parameters	Shark liver oil	IFOS standard (2014)
FFA (%)	0,14 ± 0,01	≤ 1,5
PV (mEq/kg)	0,60 ± 0,13	≤ 5
p-AnV (mEq/kg)	1,18 ± 0,46	≤ 20
Totox (mEq/kg)	2,37 ± 0,19	≤ 26

Information: FFA: Free Fatty Acids; PV: Peroxide Value, p-AnV: p-Anisidine Value, Totox: Total Oxidation; IFOS: International Fish Oil Standard

Based on **Table 1**, extracted fish oil complies with (30). The obtained results include FFA at 0.14 ± 0.01%, PV at 0.60 ± 0.13 mEq/kg, p-AnV at 1.18 ± 0.46 mEq/kg, and totoks at 2.37 ± 0.19 mEq/kg. This research was not significantly different from the study conducted by [25], which involved the extraction of shark liver oil using the dry rendering method. The obtained values of FFA were 0,6%, PV 2,74 mEq/kg, p-AnV 6,39±0,27 mEq/kg, and total oxidation 7,23±1,34 mEq/kg. Several factors affecting the quality of fish oil are temperature, storage duration, and protective techniques or packaging methods [31].

3.1.2 Fatty acid profile

The fatty acid profile aims to determine the fatty acid content of shark liver oil, including Saturated Fatty Acid (SFA), Mono Unsaturated Fatty Acid (MUFA), and Polyunsaturated Fatty Acid (PUFA). Fatty acid profiles are analyzed using Gas Chromatography (GC). The results of the fatty acid profiles presented in **Table 2**.

Table 2. Fatty acid profile of shark liver oil.

Fatty acids	**Structure**	**Percentages (%)**
Capric acid	C10:0	0,05
Myristic acid	C14:0	0,31
Pentadecanoic acid	C15:0	0,13
Palmitic acid	C16:0	10,54
Heptadecanoic acid	C17:0	3,02
Stearic acid	C18:0	2,75
Arachidic acid	C20:0	0,15
Behenic acid	C22:0	0,13
Lignoceric acid	C24:0	0,02
Total SFA		**17,10**
Palmitoleic acid	C16:1	2,91
Cis-10-heptadecanoic acid	C17:1	0,44
Elaidic acid	C18:1n-9t	0,16
Oleic acid	C18:1n-9c	37,05
Cis-11-eicosenoic acid	C20:1	0,27
Erucic acid methyl ester	C22:1n-9	0,76

Fatty acids	**Structure**	**Percentages (%)**
Nervonic acid	C24:1	1,73
Total MUFA		**43,32**
Linoleic acid	C18:2n-6	0,32
Linolelaidic acid	C18:2n-9	0,07
Linolenic acid	C18:3n-3	5,24
Cis-11,14-eicosadienoic acid	C20:2	0,26
Cis-11,14,17-eicosatrienoic acid methyl ester	C20:3n-3	1,67
Arachidonic acid	C20:4n-6	0,11
Cis-5,8,11,14,17-eicosapentaenoic acid (EPA)	C20:5n-3	0,68
Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA)	C22:6n-3	2,69
Total PUFA		**11,04**
Total fatty acids identified		**71,46**
Unidentified		**28,54**

Based on the **Table 2**, shark liver oil contains 24 types of fatty acids, consisting of 17,10% *Saturated Fatty Acid* (SFA), 43,32% *Monounsaturated Fatty Acid* (MUFA), and 11,04% *Polyunsaturated Fatty Acid* (PUFA). The findings of this study were equal with the research conducted by [19], which stated that fatty acid composition of shark liver oil, from highest to lowest, was MUFA (35,77%), SFA (17,30%), and PUFA (9,30%). The research by [32] suggests that the composition of fatty acids in fish oil can be influenced by sexual maturity, body size, feed type, and water temperature. The dominant MUFA identified was oleic acid (37,05%). Oleic acid, as reported by [33], indicates that subjects consuming a diet high in oleic acid have lower LDL-C concentrations compared to those consuming a diet high in palmitic acid. The dominant PUFA identified was linolenic acid with a content of 5,24%. This aligns with the findings by [19], showed that the dominant PUFA was linolenic acid (6,92%). The amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in this study were 0,68% and 2,69%, respectively. [26] conducted research on shark liver oil extraction and obtained EPA and DHA values ranging from 2,539% - 16,123%. [19] reported EPA and DHA of shark liver oil (*Centrophorus* sp.) were 1,54% and 4,78%, respectively. The fatty acid profile analysis reveals 28,54% unidentified components. This may be due to the limitation of standards used in the fatty acid standards during testing [34]. The unidentified components in the research results are suspected to be squalene compounds because squalene was not classified in the Supelco 37 component FAME mix standard.

3.1.3 Identification of squalene compounds

Identification of squalene compounds is necessary to determine the squalene content in shark liver oil. The analysis is conducted semi-quantitatively by examining the relative percentage of peak area under the curve for each compound. Identification was performed using GC-MS by matching the molecular weight and fragmentation pattern of the tested compounds with the GC-MS library system [35]. The results of organic compounds in the sample using GC-MS apresented in **Fig. 2**.

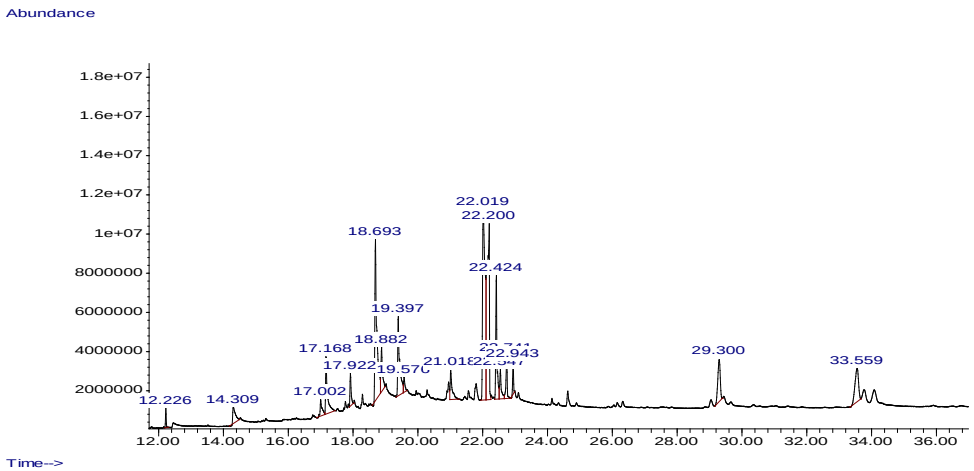


Figure 2. GC-MS chromatogram of shark liver oil.

The chromatogram shows 18 peaks, indicating the presence of 18 organic compounds in the sample, including fatty acids and their derivatives. Other studies have reported that GC-MS analysis of shark liver oil (*Centrophorus* sp.) revealed no fewer than 22 organic compounds [19]. The organic compounds identified in this research, from the first peak to the 18th peak, are presented in **Table 3** below.

Table 3. The compounds in shark fish liver oil from the GC-MS test results.

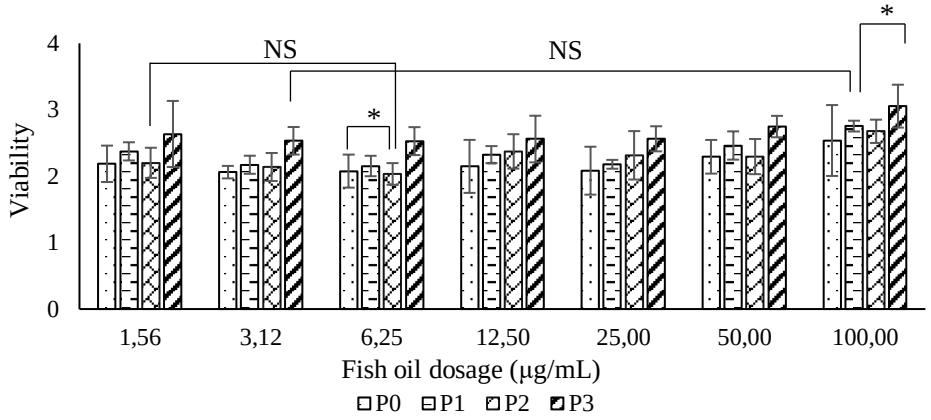
No	Retention time	Compound name	Peak Area (%)	Similarity index (%)
1	12,226	Heptadecane, 2,6-dimethyl-	0,59	93
2	14,309	13-Octadenal, (z)	1,82	62
3	17,002	1-Pentadecyne	1,35	93
4	17,168	17-Pentatriacontene	4,68	81
5	17,922	Glycidyl palmitate	1,61	58
6	18,693	cis-9-Tetradecen-1-ol	13,85	93
7	18,882	Octacosanol	3,03	74
8	19,397	9-Octadecenoic acid (Z)-, oxiranyl methyl ester	6,85	99
9	19,576	9-Octadecenoic acid (Z)-, oxiranyl methyl ester	0,94	87
10	21,018	1-cis-Vaccenoylglycerol	2,57	91
11	22,019	Squalene	21,21	98
12	22,200	Squalene	19,42	99
13	22,424	9-Octadecen-1-ol, (z)-	6,58	95
14	22,547	9-Octadecenoic acid, (E)-	2,12	70
15	22,741	Cholest-3,5-diene		99
16	22,943	3-Trifluoromethylbenzoic acid, 4-hexadecyl ester	1,17	35
17	29,300	9-Tricosene, (Z)-	4,63	83
18	33,559	1,19-Eicosadiene	5,28	60

The most dominant percentage area was squalene, appearing at peaks 11 and 12. Squalene at peak 11 eluted at a retention time of 22,019 minutes, with total area 21,21%, similarity index 98%. Squalene at peak 12 eluted at a retention time of 22,200 minutes, with total area 19,42%, similarity index 99% with the compound in the reference database (NIST.17.L library). The high similarity index indicates a resemblance in the fragmentation pattern present in the NIST.17.L library. A high similarity index suggests that the compound is squalene. The percentage area is determined based on the peak area, representing the quantity of a compound in the tested sample [37]. Fragmentation results show the molecular ion (M+) at 410, derived from $C30H50^{+}$, and a fragment with m/z 395, indicating the loss of CH3. The base peak is found at m/z 69, originating from $C5H9^{+}$, which represents a form of isoprene, a molecule (6 molecules) that constitutes squalene [37].

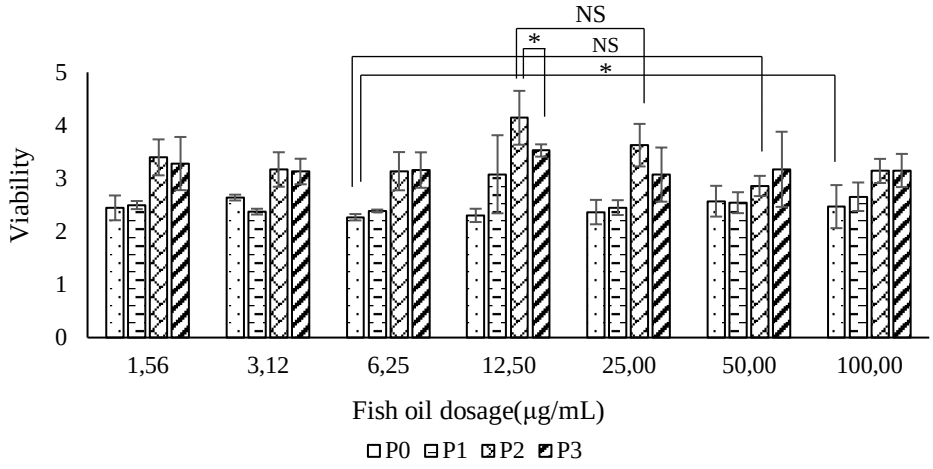
3.2 Immunostimulants

3.2.1 Splenocyte proliferation

Proliferation test aims to determine the immune cell response to cells division. Splenocytes are cells from the spleen containing B cells, cytotoxic T cells, helper T cells (Th), and natural killer (NK) cells, so splenocyte proliferation can depict the proliferation or division of immune cells in the spleen organ [38]. The samples used in the study are labeled P0, P1, P2, and P3. P0 is control (shark liver oil without vitamin E), P1 is shark liver oil with 100 ppm vitamin E, P2 is shark liver oil with 1000 ppm vitamin E, and P3 is shark liver oil with 6000 ppm vitamin E. This test also involves dose variations: 1,56 µg/mL; 3,12 µg/mL; 6,25 µg/mL; 12,50 µg/mL; 25,00 µg/mL; 50,00 µg/mL; and 100,00 µg/mL. Data presented include splenocyte viability for fish oil under two conditions, with and without Concanavalin A (Con A). The splenocyte viability values without and with the addition of Con A presented in **Fig. 3**.



(a)



(b)

Figure 3. Splenocyte viability conditions without the addition of Con A (a) and with the addition of Con A (b) in fish oil with varying dosages and antioxidant additions at different concentrations. 'NS' indicates non-significant treatment, while the symbol '*' indicates significantly different treatment outcomes ($p<0,05$).

Based on the results of ANOVA, the antioxidant concentration factor and fish oil dosage significantly influence cell viability ($p<0,05$). However, the interaction between these two factors does not significantly affect viability values, both with and without the addition of Concanavalin A (Con A) ($p>0,05$). The highest cell viability value without the addition of Con A was observed in P3 at a 100 µg/mL dose (3,05±0.32), which significantly differed from other treatments in post-hoc tests. The highest cell viability with the addition of Con A was observed in P2 with a 12,5 µg/mL dose (4,14±0,51), although it did not significantly differ from the dose of 25 µg/mL (3,63±0,40) in post-hoc tests. Viability values can indicate the level of cell proliferation because proliferating cells increase the mass of living cells. The results of this study show higher viability values when Con A is added at 10 µg/mL compared to without the addition of Con A.

The findings are consistent with the research conducted by [39], which showed that supplementation of ECOMER brand shark liver oil at a dose of 1 g/kg body weight per day in 60-day-old male Wistar rats increased spleen lymphocyte proliferation with the addition of Con A at a 5 µg/mL dose, demonstrating higher and significant values compared to conditions without Con A. According to [40], Con A is mitogenic at doses of 1–10 µg/mL and cytotoxic at doses exceeding 50 µg/mL. The study conducted by [41], mice on 4-5-week-old, weighing 75-80 g, given vitamin E at concentrations of 50, 100, 250, 500, 2500 ppm, also increased lymphocyte proliferation with the addition of Con A. The related results with this study are indicating that an increase of antioxidant concentration, proliferation tends to continue to rise. The results of the proliferation test on shark liver oil splenocytes showed that the sample has an immunostimulant effect, as indicated by cell viability values > 1. The presence of vitamin E in shark liver oil showed a synergistic effect on splenocyte proliferation based on these findings.

3.2.2 Macrophage phagocytosis

Phagocytosis testing aims to determine the macrophage phagocytosis activity, a cell responsible for engulfing and destroying foreign particles [42]. The macrophages used in this study were peritoneal cavity. Peritoneal cavity macrophages are often used because they are easy to isolate, have undergone minimal manipulation, and more abundant than macrophages in other organs. The observation of this index was conducted to understand the phagocytic cell response to antigens [43]. The results of the observation of the phagocytosis index and capacity of shark liver oil at dosage of 50 and 100 µg/mL presented in **Fig. 4**.

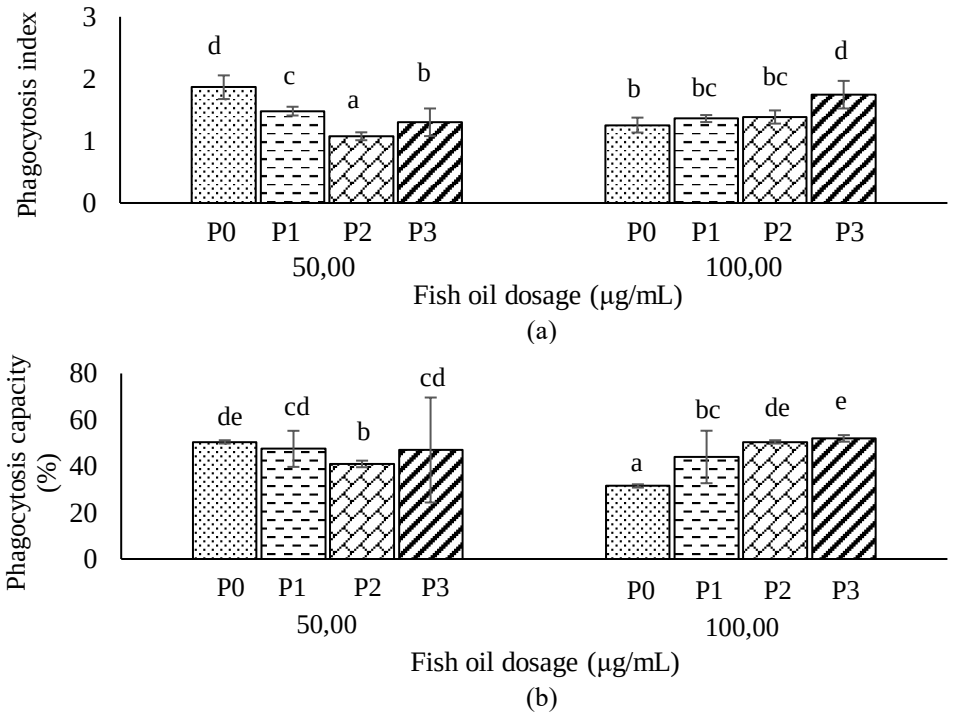


Figure 4. Phagocytosis index (a) and phagocytosis capacity (b) of fish oil at different doses and antioxidant additions at various concentrations. Superscript letters (a, b, c, d, e) indicate significantly different treatment outcomes ($p < 0,05$).

Based on the results by ANOVA analysis, the antioxidant concentration factor and the interaction between the antioxidant concentration factor and fish oil dosage significantly affect the phagocytosis index ($p < 0,05$), while fish oil dosage factor have no significant effect on the phagocytosis index ($p > 0,05$). The highest phagocytosis index value is observed in P0 at 50 µg/mL dose, but it does not significantly differ from P3 at a 100 µg/mL dose. ANOVA analysis results for the phagocytosis capacity parameter showed that the antioxidant concentration factor, fish oil dose, and the interaction between these two factors significantly affect phagocytosis capacity ($p < 0,05$). The highest phagocytosis capacity value is observed in P3 at a 100 µg/mL dose, but it does not significantly differ from P2 at a 100 µg/mL dose and P0 at a 50 µg/mL dose.

The phagocytosis capacity in this study ranges from 31% to 63%, with the research of [44] indicating that the proliferation of peritoneal macrophages in Wistar rats increases when given shark liver oil but does not exceed 100%. [45] analyzed the response of macrophages given squalene; the results showed that squalene is not cytotoxic. Low squalene concentration (1 µM) mediates an anti-inflammatory response despite having a proinflammatory profile,

while higher squalene concentrations (10 and 100 μM) enhance proinflammatory responses. According to (46), compounds with an immunostimulatory factor (IF) <1,0 are not immunostimulatory, IF in 1,0-1,5 are classified as moderately immunostimulatory, and IF >1,5 are classified as strongly immunostimulatory. P0 at a 50 μg/mL dose is classified as a substance with high immunostimulatory activity, while P3 at a 100 μg/mL dose is classified as a substance with moderate immunostimulatory activity. These results indicate that macrophages treated with shark liver oil without addition of antioxidants (P0) at a 50 μg/mL dose exhibit a higher response to antigens compared to other treatments. All treatments are immunostimulatory as the phagocytosis index values are >1. Based on these findings, shark liver oil without the addition of vitamin E shows higher values, especially at a 50 μg/mL dose.

3.3 Stability of fish oil during storage

Fish oil stability test aims to determine the oxidation rate and storage period of the oil [31] based on [30] and [21] standards. Vitamin E antioxidant was added to the fish oil with the expectation to extend its shelf life. The stability measurement method is divided into two categories: real-time and accelerated. Accelerated method involves determining the shelf life of a product by accelerating quality changes using environmental conditions that speed up the degradation reaction [47]. The accelerated method used in this research is the schaal oven test at a temperature of 40 ºC in an oven. According to [48], one day of storage in an oven at 40 ºC is equivalent to 15 days of room temperature storage. The observed parameters including FFA, PV, p-AnV, and totox. Observations of these parameters are conducted on day 1 (D1), day 2 (D2), day 3 (D3), day 4 (D4), day 5 (D5), and day 6 (D6), as elaborated below.

Table 4. FFA Value of Fish Oil During Storage

Sample	FFA (%)					
	D1	D2	D3	D4	D5	D6
P0	0,22 ± 0,05	0,25 ± 0,00	0,40 ± 0,05	0,39 ± 0,09	0,38 ± 0,06	0,42 ± 0,08
P1	0,23 ± 0,04	0,27 ± 0,03	0,32 ± 0,04	0,30 ± 0,04	0,35 ± 0,03	0,38 ± 0,07
P2	0,23 ± 0,04	0,35 ± 0,10	0,44 ± 0,10	0,47 ± 0,10	0,52 ± 0,03	0,53 ± 0,03
P3	0,23 ± 0,04	0,31 ± 0,11	0,51 ± 0,12	0,54 ± 0,06	0,61 ± 0,03	0,61 ± 0,10

Based on **Table 4**, all treatments until day 6 of observation (equivalent to 3 months at room temperature) still meet the [30] standard as the FFA value remain below 1,5%. The increase of FFA from day to day does not show a sharp increase. Research by [18] indicates that catfish oil with added antioxidant vitamin E at 0,67 mg/g resulted in an FFA value of 0,74%, while without antioxidant vitamin E, it yielded an FFA value of 1,02% on day 8 of storage in an oven at 60 ℃. Free fatty acids are formed due to the hydrolysis reaction of triglyceride oil, causing fatty acids to be released from glycerol bonds. Free fatty acids in oil are undesirable because the degradation of free fatty acids produces undesirable odor and taste. Therefore, oil processing aims to minimize the content of free fatty acids [49].

Table 5. PV of fish oil during storage

Sample	PV (mEq/kg)					
	D1	D2	D3	D4	D5	D6
P0	0,90 ± 0,14	1,61 ± 0,14	1,45 ± 0,26	4,44 ± 0,17	6,22 ± 0,29	23,04 ± 0,54
P1	0,73 ± 0,41	1,47 ± 0,42	1,05 ± 0,14	2,98 ± 0,46	5,62 ± 0,13	20,82 ± 0,14
P2	1,21 ± 0,01	2,35 ± 0,28	2,51 ± 0,38	4,96 ± 0,31	5,27 ± 0,33	26,11 ± 0,26
P3	1,31 ± 0,37	3,58 ± 0,68	4,21 ± 0,26	8,72 ± 0,91	12,15 ± 0,27	47,53 ± 0,22

Based on **Table 5**, P0, P1, and P2 have exceeded both [30] and [21] standards by day 5 of storage (equivalent to 2,5 months at room temperature), while P3 has surpassed the standard by day 4 of storage (equivalent to 2 months at room temperature). All fish oil treatments show a sharp increase in observations by day 6. According to [50], FFA affects the oxidation rate of walnut oil products. Even at very low concentrations, such as 0,05%, FFA significantly accelerates lipid oxidation, especially FFA with high degrees of unsaturation. Peroxide compounds are labile compounds formed during the initial oxidation process. These compounds readily react further, forming ketones and aldehydes, causing a rancid odor and taste in the oil and serving as an indication that the oil has deteriorated [51]. According to [52], when α-tocopherol becomes the major homolog in tocopherols, it can have a pro-oxidant effect at high concentrations but turns into an antioxidant as its concentration decreases to its inflection point for certain types of oils. This research indicates that, with the duration of storage, the pv continues to increase.

Table 6. p-AnV of fish oil during storage

Sample	p-AnV (mEq/kg)					
	D1	D2	D3	D4	D5	D6
P0	1,44 ± 0,05	2,05 ± 0,06	2,32 ± 0,01	2,76 ± 0,09	2,84 ± 0,15	3,27 ± 0,06
P1	1,00 ± 0,15	1,83 ± 0,30	2,04 ± 0,04	2,07 ± 0,25	2,00 ± 0,21	2,84 ± 0,08
P2	1,41 ± 0,17	2,01 ± 0,15	2,13 ± 0,08	2,56 ± 0,19	2,25 ± 0,23	3,05 ± 0,14
P3	1,38 ± 0,06	2,40 ± 0,22	2,28 ± 0,16	2,46 ± 0,06	3,42 ± 0,11	4,78 ± 0,21

Based on **Table 6**, all treatments are still within the limits of [30] and [21]. Measurement of the p-AnV was not continued because on the last day of measurement, fish oil had already exceeded its total oxidation limit. According to [53], factors causing the formation of p-AnV compounds include the storage period and natural antioxidants in the fish oil. The p-AnV does not always correlate with the PV, but a low PV can result in a low p-AnV if the process in the fish oil does not allow further degradation [54]. The p-AnV represents the measurement of secondary oxidation products because the decomposition process of hydroperoxides produces aldehydes, ketones, and other polymer compounds, which are secondary oxidation products [55]. The p-AnV in this study tended to be low, and there was no significant increase because, until day 6 of observation, the fish oil had not undergone further degradation.

Table 7. Totox of fish oil during storage

Sam -ple	Totox (mEq/kg)					
	D1	D2	D3	D4	D5	D6
P0	3,24 ± 0,32	5,26 ± 0,27	5,22 ± 0,53	11,63 ± 0,34	15,28 ± 0,64	49,36 ± 1,13
P1	2,46 ± 0,79	4,77 ± 0,86	4,14 ± 0,30	8,03 ± 1,12	13,23 ± 0,37	44,48 ± 0,36
P2	3,83 ± 0,16	6,70 ± 0,71	7,15 ± 0,83	12,48 ± 0,81	12,79 ± 0,51	55,27 ± 0,39
P3	4,00 ± 0,70	9,55 ± 1,37	10,90 ± 0,49	19,90 ± 1,80	27,73 ± 0,53	99,84 ± 0,10

Based on **Table 7**, P0, P1, and P2 have already exceeded the [30] and [21] standards on day 6 of storage (equivalent to 3 months at room temperature), while P3 has exceeded the [30] and [21] standards on day 5 of storage (equivalent to 2,5 months at room temperature). Total oxidation determines all oxidation parameters, including primary and secondary oxidation products [53]. This research shows that as the storage period increases, the peroxide value continues to rise. Total oxidation sharply increased from day 5 to day 6 because free radicals formed more rapidly, accelerating the lipid oxidation rate [56]. 100 ppm antioxidants can better suppress the oxidation rate compared to without antioxidants. However, with the increasing concentration of antioxidants, the oil becomes more oxidized because the main homolog in the vitamin E antioxidant used is α-tocopherol.

4 Conclusion

Although shark liver oil has immunostimulant effects, there is no significant increase in increasing dose of fish oil. The presence of vitamin E in shark liver oil showed a synergistic effect on splenocyte proliferation at all doses and the index and phagocytosis capacity at a 100 µg/mL dose; whereas at a 50 µg/mL dose, the index and phagocytosis capacity are not synergistic with the immunostimulant. The optimal concentration of the vitamin E antioxidant to maintain the stability of shark liver oil was 100 ppm, but with increasing concentrations of vitamin E, the fish oil becomes more oxidized. Results may vary for different types of fatty products.

Thanks to Ministry of Education, Culture, Research, and Technology of Indonesia for the research grants that have been given to the author. The author also would like to express gratitude to IPB University colleagues who were involved in this research.

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