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Anti-mutagenic property of fish oil from Tamban (*Sardinella lemuru* family *clupeidae*) in female ICR mice

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ABSTRACT

This study aimed to determine the anti-mutagenic property of fish oil from Tamban (*Sardinella lemuru* Family *Clupeidae*) in Mitomycin C induced female ICR mice. Initially, twelve mice were selected for acute oral toxicity testing. The resulted LD50 dose was 2000 mg/kg *b.w*. Twenty-five mice were then randomly divided into five groups: group I positive control; group II-negative control and groups III, IV and V as experimental groups. Group I was given 5 mg/kg *b.w.* of Mitomycin C, group II 5 mL/ kg *b.w.* of normal saline solution, lastly groups III, IV and V received ¼ LD50 (500 mg/kg *b.w*), ½ LD50 (1000 mg/ kg *b.w.*), ¾ LD50 (1500 mg/ kg *b.w.*) of fish oil for seven days, respectively. After the last dose, Mitomycin C was administered intra peritoneal. After 24 hours, bone marrows were obtained and Micronucleus Test was performed. About two thousand erythrocytes were counted for the presence of Micronucleated Polychromatic Erythrocytes (MNPCE) and Polychromatic Erythrocytes (PCE). The results showed that 500 mg per kilogram body weight of Tamban fish oil could exhibit anti-mutagenic property comparable to the negative control. It also possessed the greatest MNPCE inhibition. However, 1000 mg per kilogram body weight and 1500 mg kilogram body weight showed no significant difference with the positive control and significant difference with the negative control. The results proved that the fish oil extract possessed potential anti-mutagenic property against Mitomycin C-induced female ICR mice.

INTRODUCTION

Department of Health identified cancer as one of the top three leading causes of mortality and morbidity in the Philippines, representing 51.8% rate. There are several factors attributed to cancer, one of which is the substance called mutagens. Exposure to these agents causes mutation of the normal cells in the body contributing to the increase incidence of genetic damage [1].

In this world where there is a little chance of survival, prevention offers the foundation in limiting cases of cancer [2]. Studies on the mechanism of anti-mutagenic agents are currently emerging. As described, these compounds are able to counteract the effects of cell damage [3].

Omega-3 fatty acids found in fish oil are important nutrients associated in the treatment of cardiovascular diseases and hypercholesterolemia. Alongside, it is highlighted in its role in the prevention of mutations and cancer. It has been proved that lipidic extract of fish has a significant effect on the mutagenicity of cancer cells [4]. Due to the large utilization of omega-3 fatty acids

as well as the relevant potential for the control of cell mutations in the body, this research effort was aimed to evaluate the possible anti-mutagenicity of Tamban (*Sardinella lemuru Family Clupeidae)* fish oil extract using female ICR mice bone marrow Micronucleated Polychromatic Erythrocytes via OECD-guided Micronucleus Testing.

MATERIALS AND METHODS

Materials

Tamban (*Sardinella lemuru* Family *Clupeidae*) was bought from Navotas Fish Port (Philippines). The samples were then authenticated by the National Museum's Department of Zoology.

Extraction of Fish Oil

About 200 g of Tamban (*Sardinella lemuru* Family *Clupeidae*) were homogenized in a blender for 2 minutes with a mixture of 200 ml methanol and 100 ml chloroform. 100 ml of chloroform was added to the mixture and re-blended for 30 seconds. The homogenate was stirred using a glass rod and filtered through a Whatman no. 1 filter paper on Buchner funnel under vacuum suction. 20 ml of chloroform was used to rinse the remainder. The filtrate was allowed to settle and separate into organic and aqueous layers. The chloroform layer containing the lipids was transferred into a beaker and 3 g of anhydrous sodium sulphate were added to remove the excess water. The mixture was filtered through Whatman no. 1 filter paper. The solution was then evaporated to a constant weight in a 1000 ml round-bottom flask with a rotary evaporation at 50 °C [5].

Fatty Acid Analyses

The fish oil was subjected into organoleptic, stain and miscibility tests. Specific gravity and refractive index were also determined. Chemical tests such as acid value and iodine value were performed. The fish oil was sent to Ateneo De Manila University to conduct Gas Chromatography in order to analyze and characterize the fish oil.

Experimental Animals

Thirty-seven female ICR mice weighing 20-30 g and eight weeks old were procured from Food and Drug Administration (FDA) [6]. They were divided into two groups; random selection of twelve mice for acute oral toxicity test while the remaining were subjected to In Vivo Mammalian Micronucleus Test. The animals were housed in partition cages and acclimatized for one week. All mice had unlimited access to water and food except during the time of administration of fish oil extract. Cages were kept on temperature maintained at a range of about 22°C (±3°C) and a relative humidity range of about 30-70%. All studies performed were approved by the Institutional Animal Care and Use Committee (IACUC).

Determination of Dose Range using Acute Oral Toxicity Test

Twelve (12) female ICR were divided into four groups consisting of three mice per cage. Fasted for three to four hours, each group received four different doses of fish oil extract: 3 mg/kg *b.w.*, 50 mg/kg *b.w.,* 300 mg/kg *b.w.,* and 2000 mg/kg *b.w.* at a single dose via oral gavage. After dosing, animals were observed during the first 30 minutes, periodically during the first 24 hours and daily for seven days. The observation included mortality rate, body weight, physiological and behavioural changes. Median lethal dose was then determined using the OECD chart [7].

Investigation of the Anti-Mutagenic Property

Animals were divided into five groups of five mice each. Group I represented the positive control (Mitomycin C 5 mg/ kg *b.w.*) and group II as the negative control (NSS 5 ml/ kg *b.w.*). The experimental groups III, IV and V were subjected for seven days administration of the fish oil extract. Group III received ¼ LD50 (500 mg/kg *b.w*), group IV ½ LD50 (1000 mg/ kg *b.w.*) and group V ¾ LD50 (1500 mg/ kg *b.w.*) based on the result of acute oral toxicity method [6].

Induction of Micronucleated Polychromatic Erythrocytes (MNPCE)

Parenteral preparation of Mitomycin C was used to induce Micronucleated Polychromatic Erythrocytes (MNPCE) via intra peritoneal 1 hour after the last administration of the fish oil extract.

Extraction of Bone Marrow Cells

The mice were sacrificed after 24 hours of last administration of Mitomycin C via cervical dislocation. Bone marrow cells were collected in a micro centrifuge tube from the femur of the mouse. The bone end was punctured with needle and was flushed with 1 ml fetal bovine serum. Collected bone marrow cells were centrifuged at 3000 rpm for 5 minutes.

Staining and Analysis of Bone Marrow Smear

Two smears per mouse were made from experimental tube using a rubber bulb and a Pasteur pipette. Slides were fixed with methanol for 5 minutes and a combination of May-Grunwald and Wright-Giemsa stain was used. Slides were analyzed under Binocular Olympus CX41 microscope and two thousand erythrocytes were counted and screened to determine the number of Micronucleated Polychromatic Erythrocytes (MNPCE) and Polychromatic Erythrocytes (PCE) [6].

Statistical Analysis

The results were expressed as mean ± S.D. Statistical analyses were carried out by using Analysis of Variance (ANOVA) followed by Tukey HSD for Normal Data, Kruskal-Wallis Test and Mann-Whitney Test for Non-Normal Data. P value <0.05 was considered as statistically significant [8].

RESULTS

Acute toxicity studies

Acute oral toxicity test was conducted prior to the Micronucleus Test in order to find the minimum threshold concentration of the fish oil extract that could cause mortality.

All test animals were subjected to gross necropsy. The external examination showed healthy mice with good conformation, clean and coat skin. Gross necropsy of liver, kidney, lungs, spleen, heart, intestines, and other major organs showed normal results. However, mild pallor was observed on some mice with kidneys as the most prominent organ. Pale lungs and liver were also observed on some mice. Since *Sardinella lemuru* produced no mortality on any of the mice group, the concentration of 2000 mg/kg was used as the basis for LD50. Therefore, one-fourth LD50 (500 mg/kg), one-half LD50 (1000 mg/kg) and three-fourth LD50 (1500 mg/kg) were selected in this study respectively [7].

Anti-Mutagenic Activity of Tamban (Sardinella lemuru Fam. Clupeidae in Female ICR Mice

Results in figure 1 show the positive control group (MMC 5 mg/kg *b.w.)* yielded the highest number of MNPCE; on the other hand, negative control (NSS 5 ml/kg *b.w.*) obtained the least. It implies that Mitomycin C caused significant chromosomal aberration. The ratio of the occurrence of MNPCE was lesser in groups treated with the fish oil extract (Groups III, IV and V) than with the positive control treated with Mitomycin C. Among the experimental groups, the one treated with 500 mg/kg *b.w.* (Group III) yielded the lowest MNPCE ratio suggesting its effectiveness in suppressing mutations.

Statistical analyses shown in Table 1 interpreted that among the doses administered only group III with 500 mg/kg *b.w.* fish oil has no significant difference with the negative control and near significant difference at $p< 0.1$ with the positive control in terms of MNPCE. Groups IV and V with 1000 mg/kg *b.w.* and 1500 mg/kg *b.w.* fish oil respectively show significant difference with the negative and no significant difference with the positive control.

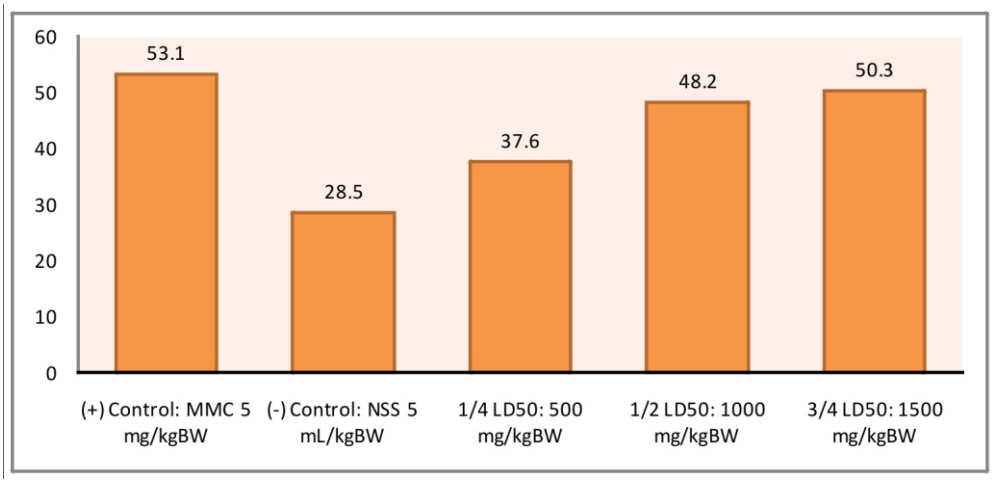

Fig 1. : Average Micronucleated Polychromatic Erythrocytes (MNPCE) per Test Group

Figure 2 represents a graphical presentation of the number of Polychromatic Erythrocytes (PCE), with group V of 1500 mg/kg *b.w.* having the highest value, while negative control having the lowest. The presence of PCE is expected to mature within two days and as the dose is increased the number of PCE also increases even beyond the positive control.

The percentage inhibition of the experimental groups in terms of Micronucleated Polychromatic Erythrocytes is presented in figure 3. MNPCE was used to determine the capability of fish oil to inhibit mutagenesis as compared to the negative control. Group III treated with 500 mg/kg *b.w.* of fish oil extract recorded the highest inhibition of 29.19%. Group IV (1000 mg/kg *b.w.*) and V (1500 mg/kg *b.w.*) yielded 9.23% and 5.27% respectively.

Histopathological observation

The histopathological observations basically support the results obtained from the data analysis. The red arrow which indicated the Polychromatic Erythrocytes (PCE) is also called reticulocytes. These are immature red blood cells found both in the bone marrow and in the blood stream. Once the PCEs mature, it will become Normochromatic Erythrocytes (NCEs, shown in blue arrow) or the mature red blood cells. DNA damage caused by the inducer like Mitomycin C causes mutation in the bone marrow.

When this damage is not repaired there will be replication errors thus mutation occurs. The altered DNA will not be incorporated in the nucleus. The left genetic material will make its

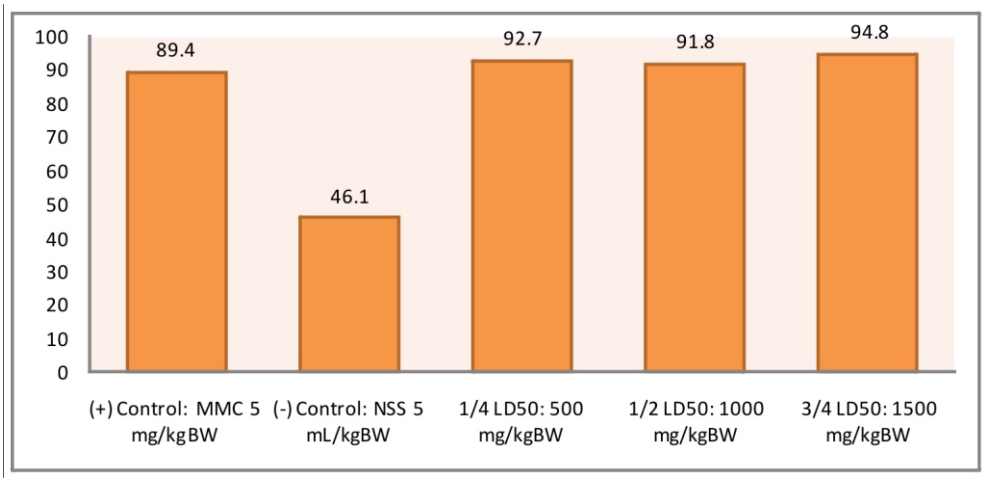

Fig 2. : Average Polychromatic Erythrocytes per Test Group

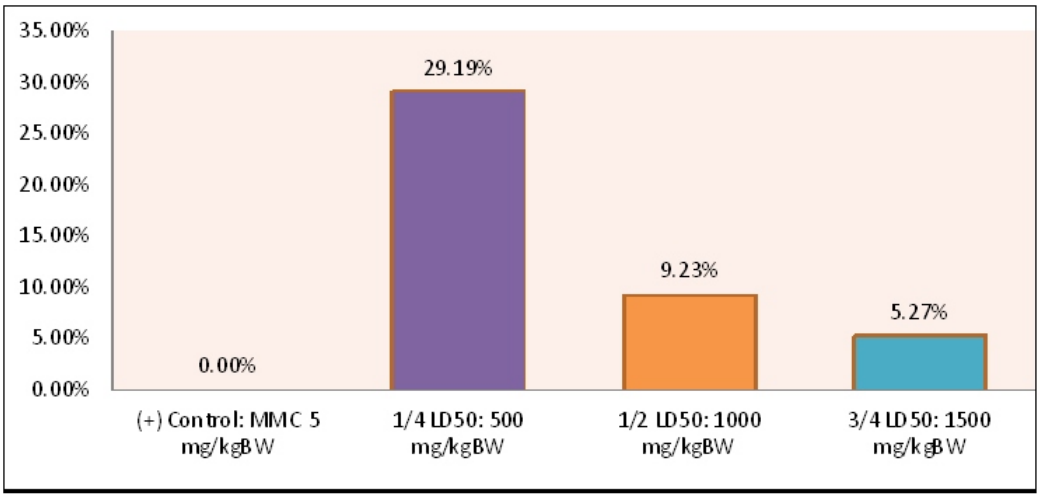

Fig 3. : Percentage Inhibition of Micronucleated Polychromatic Erythrocytes Frequency

Table 1. : Comparison of MNPCE in Treated Group vs Control Groups

Groups	vs (+) Control	P Value*	vs (-) Control	P Value**
3 (1/4 LD50) 500mg/ kgBW	0.095	Near Significant difference at p<0.1	0.151	No significant difference
4 (1/2 LD50) 1000mg/ kgBW	0.841	No significant difference	0.032	Significant difference
5 (3/4 LD50) 1500mg/ kgBW	0.421	No significant difference	0.008	Significant difference

n=5 mice per group
*p<0.1
**p<0.1

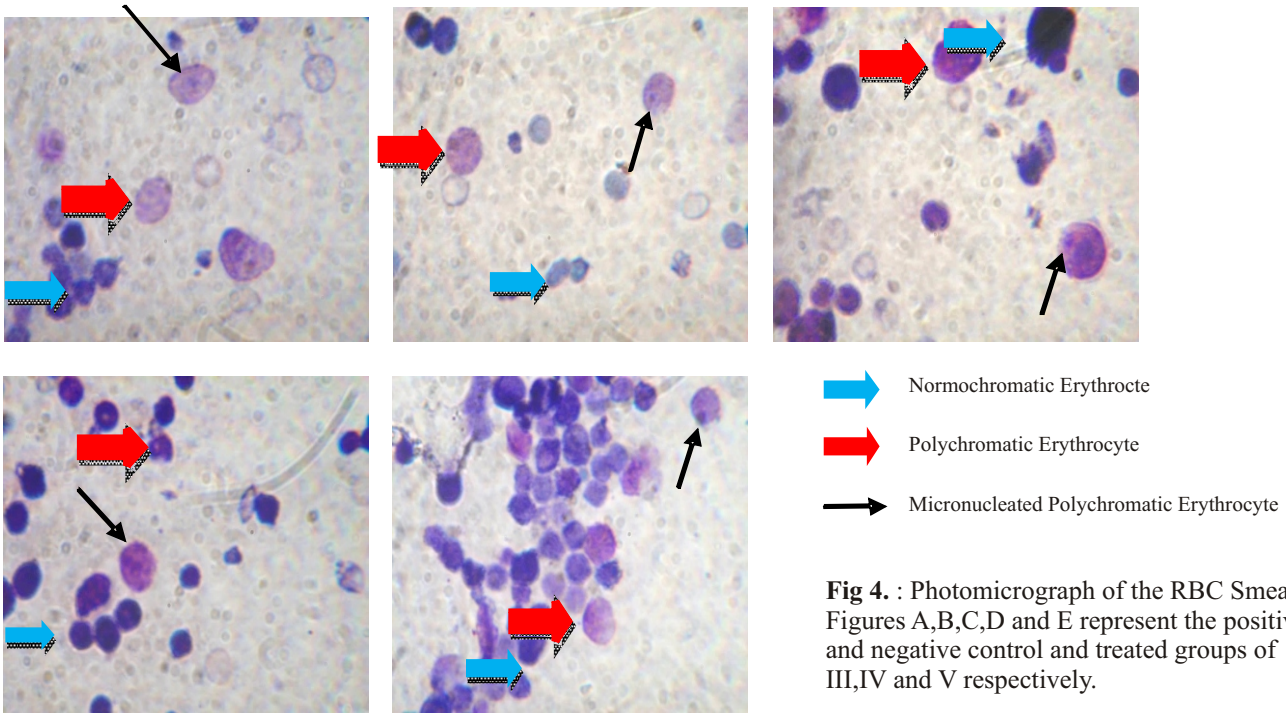

Fig 4. : Photomicrograph of the RBC Smear. Figures A,B,C,D and E represent the positive and negative control and treated groups of III,IV and V respectively.

own nucleus but only fragments of abnormal chromosomes, thus a micronucleus is formed. This is now the Micronucleated Polychromatic Erythrocytes (MNPCE) which is represented by the black arrow (Fig.4)

DISCUSSION

Dietary fish oil had long been associated in the prevention of cardiovascular diseases and in the regulation of cholesterol levels in the body. The inquisitive minds of researchers all over the world have prompted them to further study the benefits of fish oil, including its usefulness in the prevention and treatment of cancer [9].

In this study the Tamban *(Sardinella lemuru Family Clupeidae*) fish oil exhibited anti-mutagenic effects against Mitomycin C specifically at dose 500 mg where the highest percentage inhibition and lowest MNPCE ratio is recorded. For doses 1000 mg and 1500 mg, some studies have suggested that at higher doses fish oil can actually contribute to induction of cancer. Although it is unknown why high levels of fish oil increases the possibility of cancer, the protective effect of Tamban *(Sardinella lemuru Family Clupeidae)* can be attributed due to some potential mechanisms of action in which inhibition of prostaglandin, as the most relevant. Prostaglandin synthase, the enzyme responsible for the production of prostaglandin is the specific target of fish oil. Through its inhibition, stimulation of tumor growth by the said compound is also suppressed [10].

CONCLUSION

Based on the data obtained, the researchers therefore concluded that the fish oil, extracted from Tamban *(Sardinella lemuru* Fam. *Clupeidae)* has a potential anti-mutagenic property at the lowest dose of 500 mg. This activity may be attributed to the fatty acids contained in the Tamban fish oil.

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