Anti-Inflammatory and Th-2 Directed Modulation of Immune Responses by Fresh Water Fish Derived Lipids

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Abstract: Present study analyzesimmunoregulatory potential of lipid extract of a nutritionally important fresh water fish, Catlacatla (FO), in experimental mice. Capillary Gas chromatography-Mass spectrometry technique was used for analysis of components and their quantification. FO (16 mg/kg body weight) caused significant (p<0.05) elevation in lymphocyte proliferation and IL-4 levels upon antigenic and mitogenic stimulation while suppressing the levels of TNF-α, IFN-γ and DTH response. Significant increase (p<0.05) in anti-OVA IgG1 levels, while suppressing anti- OVA IgG2a levels and Nitric oxide induction with no effect on macrophage phagocytic index at highest dose of 16 mg/kg body weight was observed. A significant elevation (p<0.05) in relative lymphoid organ weight and cellularity of spleen and thymus, bone marrow was observed. The present study reveals that FO influences humoral, cell mediated and innate immune responses towards Th2 direction in a dose dependant manner and may be used for the atopic individuals in a serviceable manner.*
Keywords: Cell mediated immune response, Cytokines, Humoral immune response, Immunomodulation and Innate immune response.*

I.   Introduction

Fatty acids make up an important part of the cellular structures apart from being almost omnipresent when it comes to cell signalling, interactions and endocrinological systems. The fatty acid composition of the fish varies with the temperature of the water in which the fish resides as polyunsaturated fatty acids (PUFA) provide tolerance to low temperature, thus, fishes in temperate waters are expected to be high in PUFA [1].*Catlacatla* also known as Indian carp found in rivers and lakes in northern India and is an important aquacultured freshwater species in South Asia. In India it is commonly known as the "Bhakur".

PUFA's have a huge role in immune system modulation as the interfering agents in the synthesis of eicosanoids by inhibiting the oxidation of amino acids and hence limiting their bioavailability [2]. The beneficial properties of PUFA have been well elucidated and documented in case of its impact on lipoproteins and cholesterol where Fish oil has been observed to decrease VLDL and thromboxane levels and increase the HDL_2/HDL_3 ratio [3], making it a suspected tool to avoid stenosis following angioplasty. Fish oils are now characteristically associated with modification of dyslipidemia [4]. The atherogenic properties of dietary fish oil can also be due to its macrophage modified changes in LDL leading to an upsurge in its uptake by these cells [5]. Fish oil treated humans with crohn's disease show reduced platelet responsiveness, relieving the patient of symptoms [6].

Dietary fish oil has also been observed to reduce the hypertension in diabetic patients by balancing the platelet redox reactions [7]. A large amount of experimentation has been made to analyse the anti-cancer activities of n3- PUFA via their effect on prostanoid synthesis through cell signalling, substrate replacement or their impact on gene expression [8]. PUFA have also been observed to show cycloxygenase-2 and NF-κB inhibiting property [9] affecting a number of physiological and medical issues in which these genes are involved. Apart from the conditions discussed above Fish oil has also been observed to be beneficial in rheumatoid arthritis [10] IgA Nephropathy [11], Systemus lupus Erythromatosus[12] and Asthma [13].Advances in the evaluation of role of Fatty acids in the regulation of a number of disorders fuelled a renewed interest in us for evaluating the role of a fresh water fish lipid extract on the innate, humoral and cell mediated branches of immune response.

II.   Materials and Methods

2.1. Chemicals and biologicals

Cytokines; TNF-alpha and Interferon-gamma, Interleukin (IL)-10, IL-4 were purchased from eBioscience (San Diego, CA, U.S.A.). Guinea pig complement, HRP conjugated goat anti-mouse IgG and IgG subtypes, MTT, Ovalbumin, concanavalin A, OPD, FBS, MTT, Penicillin Streptomycin solution, Medium RPMI-1640, HRP were purchased from Sigma-Aldrich Co. (USA). ELISA plates were purchased from Greiner bio-one (Germany). All other chemicals were of analytical grade and procured from the standard commercial sources.

2.2. Extraction of Oil from the Fish (Catlacatla)

The extraction of oil from the fish (Catlacatla) was done as described earlier [14]. The fish were bought fresh from the market and washed after the removal of internal organs. Fish fillet was obtained by cutting the fish lengthwise, cut into small pieces and homogenized (50g) for 2 minutes with a mixture of methanol (100 ml) and chloroform (50 ml) followed by further addition of 50 ml of chloroform. The mixture was blended for 30 seconds and distilled water (50 ml) was added. The homogenate was filtered through a Whatman no.1 filter under vacuum suction. The filtrate was allowed to settle to separate into the organic and aqueous layers. The chloroform layer containing the lipids was taken and water was removed using anhydrous sodium sulphate (3g). The solution was then evaporated to a constant weight in a tared 100 ml round-bottom flask with a rotary evaporator at 40°C.

2.3. Animals and dietary regimen

Male *Swiss Albino* mice, weighing 20-25g, bred and maintained at the Central Animal House facility, JamiaHamdard were used for this study. Mice were housed in room maintained on a 12:12 h light:dark cycle at 23°C and 40–50% relative humidity and had free access to commercial irradiated rodent diet (Aashirwad diets) and acidified water in drilled water bottles. Mice were free of external and internal parasites.
Experimental mice were divided in the four groups containing six mice each. Group I was given standard Rodent diet, Group II, Group III and Group IV were respectively given 4, 8 and 16 mg/kg body weight of FO every day for four weeks.

2.4. Methylation of fatty acids for Characterisation of the oil by Gas chromatography-Mass spectrometry (GC-MS)

The processing of Lipid extract for conversion of Fatty acids to fatty acid methyl esters was carried out by the procedure as described earlier [15]. HCl (35%, w/w) was diluted with methanol to make 8.0% (w/v) HCl. Fish oil sample was placed in a glass test tube and dissolved in 0.20 ml of toluene and 1.50 ml of methanol and 0.30 ml of the 8.0% HCl solution were added respectively. The tube was vortexed and then incubated overnight at 45°C for methylation. After cooling to room temperature, 1 ml of hexane and 1 ml of water was added for extraction of Fatty acid methyl esters (FAMEs). The tube was well mixed by shaking, and then the hexane layer was analyzed by GC-MS directly.

GC-MS analysis was performed using gas chromatography coupled Mass spectromety instrument (Separation was performed on a capillary column SP^{TM}- 2560, 100m ×.25mm ID, and 0. 20µm film). Helium was used as the carrier gas with a flow rate of 20 cm/sec. The temperature of the injector was 260°C; 1.0 µL aliquots were injected and the split ratio of the injector was 100:1. Free fatty acid methyl esters were separated at constant flow with the following temperature program: 140^0C (5 min) to 240°C at 4°C /min. Mass spectra were recorded at 2 scan^{-1} with a scanning range of 40 to 850 m/z.

2.5 Evaluation of cell mediated immune response
2.5.1 Lymphocyte Proliferation (MTT)

The effect of FO on proliferation of lymphocytes was determined by using a colorimetric technique which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [16]. Control and treated groups were assayed in triplicates in three independent experiments. The percentage of proliferation was calculated by the following formula:

$$\text{\% Proliferation} = \frac{[\text{OD sample} - \text{OD control}] \times 100}{\text{OD control}}$$

2.5.2 Cytokine Analysis

The culture supernatants collected during T-cell proliferation were centrifuged at 5000 rpm for 15 min, filtered through a 0.22 µm membrane and quantified for cytokines, interleukin (IL)-4, IL-10, TNF-α and IFN-γ using mouse cytokine ELISA kit (e Bioscience, USA) as per the manufacturer's instructions.

2.5.3 Delayed Type Hypersensitivity (DTH) assay

The DTH response was determined as has been described earlier [17]. Swelling index measurement was performed post- antigen injection with calliper three times and the average was calculated to decrease the experimental error.

2.6 Evaluation of the innate immune response
2.6.1 Estimation of nitric oxide induction (Griess assay)
Peritoneal exudate cells were used to study the macrophage function following a cell viability check by Trypan blue exclusion method. Concentration of nitrite, the stable end product from NO generation by effector macrophages, was determined by the Griess reaction [18]. Nitrite content (μmole/10^6 cells) was quantified by extrapolation from a sodium nitrite standard curve in each experiment.

2.6.2. Phagocytotic response
Phagocytosis by peritoneal macrophages in mice was carried out by intraperitoneal injection of 0.5ml of 5 X 10^6gRBC into each mouse followed by euthanization 1 hr later. The fluid of abdominal cavity was collected to make a smear and incubated at 37 °C for 30 min in a wet box, fixed with 95% ethanol, and then stained by Wright-Giemsa dye. The number of macrophages ingesting gRBC out of a total of at least 100 cells was calculated by direct visual enumeration under the light microscope (Olympus). The phagocytic rate (PR) and phagocytic index (PI) are calculated using the following formula:

PR% $\qquad = \qquad \dfrac{\text{(Number of macrophages ingesting gRBC)} \ \text{X } 100}{\text{Total number of macrophages}}$

PI $\qquad = \qquad \dfrac{\text{Total number of ingested gRBC}}{\text{Number of macrophage ingesting gRBC}}$

2.7. Evaluation of humoral immune response
2.7.1. Plaque forming cell assay (PFC) and Haemagglutinationtiter (HTT)
For assessing the humoral immune function, mice from various groups were immunized with 0.1ml of gRBC suspension comprising 325 X 10^6 cells in the tail vein 24 h. after administrating the last dose of FO. On the fifth day, after the antigen was administrated, PFC and heamagglutination assay were performed [19].

2.7.2. Ovalbumin- specific antibody response
Animals from all the groups were immunized intramuscularly with 50 μg of ovalbumin emulsified in Freund's complete adjuvant after the completion of dosage. Booster dose (25 μg of OVA in Freund's incomplete adjuvant) was given on 21st and 35th day. Blood was obtained from retro-orbitalis vein on 14th, 27th, 42nd and 60th day, and the serum was evaluated for ovalbumin- specific IgG, IgE level and IgG subtype levels and peak titers by ELISA method [19]. The optical density was measured at 492nm in an automatic microplate reader (ECIL, Hyderabad).

2.7.3. Ovalbumin- specific serum anti-isotypic antibody response
Isotyping kit (Sigma, USA) was used to elucidate the type of IgG subclass elicited in the serum samples as collected above.

2.8. Lymphoid-organ weight, cellularity and body weight
Body weight of each mouse was recorded prior to and after the completion of FO dosage. Weight gain and relative organ weight (organ weight/100g of body weight) of kidney, liver, spleen and thymus were determined for each mouse, after their surgical removal and separation of extraneous tissue. A single cell suspension of bone marrow, spleen and thymus was prepared in PBS (Phosphate buffer saline) and the cellularity was counted on a Neubar's chamber.

III. Statistical analysis
Data were statistically analyzed using Dunnett comparison test in GraphPadInStat software to determine the significant differences in the data of various groups. Values of $p < 0.05$ were considered significant. The values are expressed as mean ±S.E.M.

IV. Results
4.1.Gas chromatography-Mass Spectrometry analysis (GC-MS)
The fatty acid methyl esters of FO were characterised by Gas chromatography-Mass spectrometry for the relative quantification of fatty acids in their methyl ester form (fig.1). Relative concentration of Eicosapentaenoic acid methyl ester (PUFA's) was observed to be 22.486 followed by Palmitic acid methyl ester (Saturated fatty acid) at 22.034 and Docosahexaenoic acid methyl ester at a relative concentration of 12.303. The results are shown in table1.

4.2. Effect of FO on Cell mediated Immune Response
4.2.1 Effect of FO on Cytokine response
We next attempted to examine the potential effects of FO on the production of the cytokines (TNF-α, interferon (IFN)-γ, interleukin (IL)-10 and interleukin (IL)-4) following con-A or OVA stimulation. Upon con-A stimulation IFN-γ levels decreased in a dose-dependent manner, with the highest dose of FO (16 mg/kg body weight) showing the maximum suppression (4214.049 pg/ml) when compared to the control group (4847.409 pg/ml) and the similar trend was observed in response to ovalbumin (Figure 2).

Levels of cytokine, interleukin (IL)-4 were observed to increase in a dose dependant manner both in response to mitogen as well as antigen used i.e. Concanavalin A and ovalbumin, reaching the level of significance ($p < 0.05$) at the highest dose, 16 mg/kg body weight.

The observances of Tumour Necrosis Factor-(α) showed that FO had a huge impact on regulation of its secretion in response to con-A and OVA, as its levels decreased in a dose dependant manner in response to con–A with lowest levels (4.098 ng/ml) in 16 mg/kg body weight group in comparison to the control group (6.1814ng/ml). FO also decreased the levels of TNF- α ($p < 0.01$) in response to the antigen given (ovalbumin) from 6.272 ng/ml in control group to 4.173 ng/ml in 16 mg/kg body weight group. Fish oil did not have any significant impact on the levels of interleukin (IL)-10.

4.2.2. Effect of FO on Lymphocyte Proliferative response
The proliferation assay of splenic lymphocytes was performed to study the effect of FO on immune cells (Table 2). FO increased the lymphoproliferative responses to concanavalin A (Con-A) and ovalbumin (OVA). An optimum and significant increase was observed in Con A and ovalbumin stimulated splenocyte proliferation at a dosage of 16 mg/kg body weight ($p < 0.05$). Cells that were grown in absence of mitogen and antigen did not show any significant proliferation.

4.2.3. Effect of FO on delayed type hypersensitivity
FO inhibited the delayed-type hypersensitivity (DTH) response induced by gRBC (as shown in Table 2). The response during the inflammatory phase (48) was reduced in all the groups though, significantly ($p < 0.05$) only in 16 mg/kg body weight group where the response reduced to 9.791% in comparison to 14.671% in control mice.

4.3. Effect of FO on innate immune response
To evaluate the effect of FO on innate immunity, the changes in phagocytic capacity of macrophages and nitric oxide synthesis were measured. Assessment of the functional capacity of macrophages of the peritoneal cavity was evaluated with regard to its ability to ingest gRBC. FO caused an insignificant increase in phagocytotic index of the macrophages (Table 2), but significantly ($p < 0.05$) decreased the production of nitric oxide in a dose dependant manner. Nitrite production decreased from 10.310 $\mu M/10^6$ cells in the control group to 4.078 $\mu M/10^6$ cells at the dose of 16 mg/kg body weight. The viability of peritoneal macrophages was not influenced by FO which was observed to be more than 90% for all groups.

4.4. Effect of FO on humoral immunity
4.4.1. Effect of FO on Antibody secretion (IgG and IgE) to OVA
When the enzyme linked Assay for IgG was conducted on the serum of mice on fourteenth, twenty seventh, forty second and sixth day following the primary antigen loading, it was observed that FO did not have any significant ($p < 0.05$) impact on the overall IgG levels.On 27th and 42nd day following the antigen priming a significant increase ($p < 0.05$) in IgE levels were observed to which eventually decreased to insignificant values by the 60th day (Table 3).

4.4.2. Effect of FO on ova-albumin specific IgG subtype levels
On analysing the impact of FO on IgG subtypes (IgG1, IgG2a, IgG2b and IgG3) it was observed that 16 mg/kg body weight dosage mice showed a significant elevation ($p < 0.05$) in serum IgG1 levels continuously over a sixty day time period. However, 4 and 8 mg/kg body weight dosage groups showed a significant elevation ($p < 0.01$) in IgG1 levels on 27th, 42nd and 60th day post ova-albumin primary challenge.

The levels of IgG2$_a$ in response to FO showed suppression in all groups but, significant reduction ($p < 0.01$) was observed in 16 mg/kg body weight dosage mice. The level of significance decreased post 60 days of priming. The impact of FO on 4 and 8mg/kg body weight dosage mice was not long lasting where the significant suppression was observed only in fourteen and twenty seven day bleeds. Fish oil did not show any significant effect on IgG2$_b$ and IgG3 levels (Table 3).

The effect of FO on Plaque forming cell assay and Haemagglutination titre are asshown in Table 2. All the Groups showed a significant ($p < 0.01$) increase in the number of Plaque forming cells per million

splenocytes and 8 and 16 mg/kg body weight groups showed a significant elevation in anti-gRBChaemagglutination titre when compared to the control.

4.5   Effect of FO on body weight, lymphoid organ weight and relative organ Cellularity
No significant effect was observed on the body weight of mice in all groups in comparison to the control group. Mice fed 16 mg/kg body weight FO showed a significant elevation ($p < 0.05$) in relative organ mass (spleen and thymus) as well as splenic, thymic and bone marrow cellularity. None of the groups showed any significant effect on relative kidney and liver mass. No mortality was observed and none of the animals exhibited any signs of toxicity.

V.   Discussion

The influence of natural compounds on immune cells as immunomodulatory mediators is a highly researched and promising field. In the present study we investigated the effect of Catla fish oil extract supplementation on the immune response of *Swiss Albino mice* at dosage levels of 4, 8 and 16 mg/kg body weight and derived the conclusions after comparing the standard diet fed control mice versus the lipid extract fed ones.

The FO was analysed using Gas chromatography and Mass spectrometry, Relative concentration of Eicosapentaenoic acid methyl ester (PUFA's) was observed to be 22.486 followed by Palmitic acid methyl ester (Saturated fatty acid) and Docosahexaenoic acid methyl ester , these two fatty acids (EPA and DHA) are of nutritional and commercial importance. Presence of good amounts of EPA and DHA in FO makes it a nutritionally important food as the importance of n-3 fatty acids has been well documented earlier [20].

Following the antigen priming with a model antigen, ovalbumin, after the dosage there was no significant impact on serum IgG levels of mice over a 60 day time period. This was followed by the analysis of IgG subtype levels (IgG1, IgG2a, IgG2b and IgG3). A significant elevation and suppression of IgG1and IgG2a levels, respectively, shows that the FO directed the humoral immune response towards Th2 type while suppressing the Th1 type of humoral immune response [21]. There was a short-lived increase in serum IgE levels against ovalbumin in FO fed mice which is in confirmation to the results obtained in earlier studies [22]. In Haemagglutination test and Plaque forming cell assay FO was observed to show significant elevations, this activity can be due to the involvement of fatty acids to modulate the immune system in such a way that agglutinating antibodies like IgM are augmented [23]. The collective inference from above results is that FO may have an impact on the class switching of antibodies

DTH is an indirect marker for cell mediated immunity. It was observed to be suppressed at high concentrations of FO (16mg /kg body weight) in comparison to the control ones. The mechanism behind suppressed DTH may be due to the inability to sensitise the T lymphocytes which carry out the process with the help of IFN-γ [24]. Since, a heightened thickness is not observed in the paw it shows that the signal for the infiltration of the macrophages is weakened or altered.

FO was observed to reduce the levels of IFN-γ in response to concanavalin A as well as ovalbumin in comparison to control. IFN-γ is a potent inflammatory cytokine and is associated with Th1 type of response. The reason may be a possible suppression in the IFN-γ secreting cells as observed by some earlier research [25], where a significant depression in the quantity of IFN-γ secreting cells was reported in response to Fish oil. The results were also found to be consistent with the studies of Wallace et al., 2001 [26]. A significant decrease was also observed in the levels of TNF-α, which is in confirmation to the results obtained from the studies of Xi et al., 1995[27]. Collectively these two cytokines (IFN-γ and TNF-α) make up a major chunk of the inflammatory cytokine machinery. The levels of IL-4 were observed to be elevated and that of IL-10 unaffected. The changes in the cytokine balance give an idea of the subset of T- helper cells activated, here the Th2 immune response seems to be elevated while the Th1 type of immune response (predominantly involved in inflammatory pathways) is suppressed. These results when taken together give an idea that FO has a suppressive influence on the inflammatory response of the immune system, while stimulating its anti-inflammatory wing.

FO was observed to reduce the nitric oxide levels in the splenocyte culture supernatant, lowest levels were observed at a dose of 16 mg/kg body weight. The impact of FO on the levels of nitric oxide can explain the pathway through which its anti-inflammatory action may be mediated, as the triggering of NO synthesis by certain factors like LPS is proceeded by the secretion of a number of inflammatory factors [28]. The Phagocytic potential of the macrophages was not highly altered in response to the lipid extract.

FO at all concentrations showed no significant elevation in the total body weight of the mice. A dosage of 16 mg/kg body weight diet showed significant elevations ($p < 0.05$) in relative splenic and thymic mass. Dosage of 16 and8 mg/kg body weight diet significantly elevated ($p < 0.01$) the cellularity of immune organs (Bone marrow, spleen and thymus), showing the stimulation of bone marrow hematopoietic cells and lymphocytes that home in thymus however, this stimulation may not be permanent.

In summary Catla fish is a good source of polyunsaturated fatty acids. FO, when introduced into the animal diet in high levels were found to increase the relative immune organ mass and cellularity and redistribute the serum antibody levels towards anti-inflammatory side, while suppressing the Th1 response and delayed type hypersensitivity.

VI.   Conclusions

The above results draw the conclusions that fish oil can be used for the treatment of hyper activated or auto immune responses like systemus lupus Erythromatosus, Arthrits, Insulin resistance etc. or their prevention in case of high risk individuals. The pathway of immune suppression by FO needs to be verified by analysing its impact on the expression of inflammatory genes like COX and LOX. Although the proper mechanism of action i.e. receptors on which it acts and the signalling pathways involved need to be searched out as the genetic variations of each individual animal affects the reproducibility and significance of the results greatly.

Acknowledgements

Declaration of Interest
The authors declare no conflict of interest.

Appendices
BHT (of about 0.02 g) was added to FO as an antioxidant after performing GC-MS.

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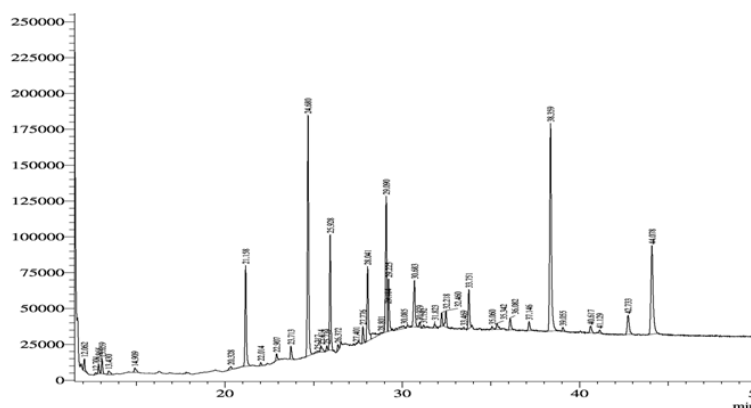
Figures and tables

Figure 1: Gas chromatogram of fatty acid methyl esters (FAMEs) from FO. Lipid extract Fatty acids were converted to fatty acid methyl esters. GC-MS analysis was performed using gas chromatography coupled Mass spectrometry instrument (Separation was performed on a capillary column SP^{TM}- 2560, 100m ×.25mm ID, and 0. 20µm film). Helium was used as the carrier gas with a flow rate of 20 cm/sec. The temperature of the injector was 260°C; 1.0 µL aliquots were injected and the split ratio of the injector was 100:1. Free fatty acid methyl esters were separated at constant flow with the following temperature program: 140^{0}C (5 min) to 240°C at 4°C /min. Scanning range of MS was from 40 m/z to 850 m/z.
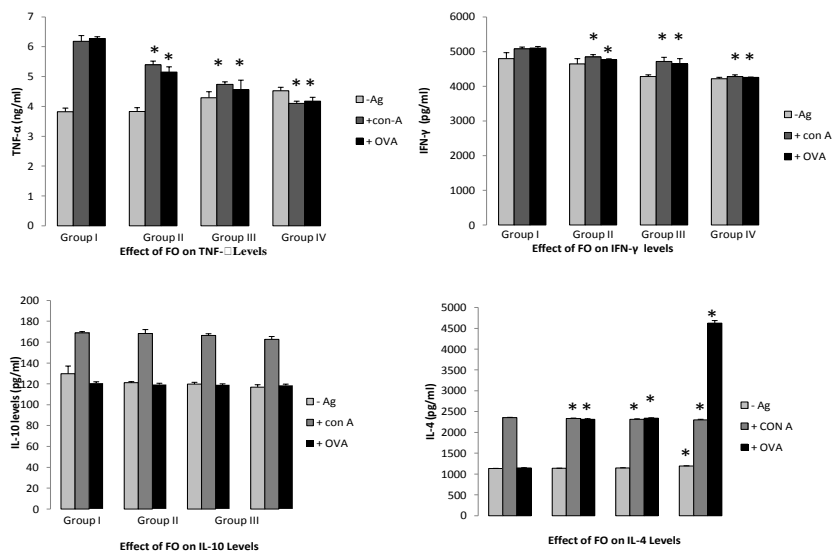

Figure 2: Anti-con A and anti-OVA induced TNF-α, interferon (IFN-γ), IL-10 and IL-4 secretion by total splenocytes from FO fed mice. Splenocytes were unstimulated (- Ag) or stimulated with con-A (2 µg/l) or OVA (5 µg/l) for 72 h. Values are means (n=5), with standard errors represented by vertical bars. * Mean value was significantly different from that of the cells from the control mice (p<0·05).

Table1: Concentration of some important fatty acids in the Catla Fish lipid extract
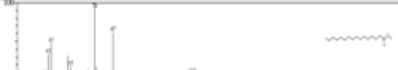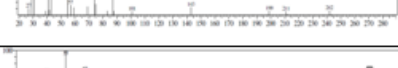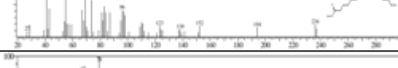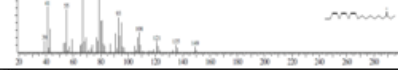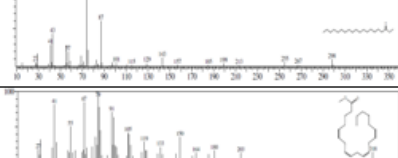
S.NO	Compound	Category	Structure	Relative concentration (%)
1.	Eicosapentaenoic acid methyl ester.	20:5 (n-3)		22.486
2.	Palmitic acid methyl ester	16:0		22.034
3.	Docosahexaenoic acid methyl ester	22:6 (n-3)		12.303
4.	Myristic acid methyl ester	14:0		10.036
5.	Oleic acid methyl ester	18:1 (n-9)		10.130
6.	Linolenic acid methyl ester	18:3 (n-3)		1.354
7.	Stearic acid methyl ester	18:0		2.908
8.	Arachidonic acid methyl ester	20:4 (n-6)		.493

Table 2: Effect of FO on Immune Parameters

Parameters	Groups			
	Group I	Group II	Group III	Group IV
Humoral response Plaque forming cell Assay/ 10^6 splenocytes	460±10	988±3.74[*]	1030±9.79[*]	1260±24.49[*]
Haemagglutination titre **Cell mediated response** Lymphocyte Proliferation: Con-A	1:128	1:384	1:768[*]	1:922[*]
OVA	0.159±0.009	0.167±0.016	0.174±0.013	0.223±0.017[*]
Delayed type Hypersensitivity 48h (%)	0.153±0.002	0.155±0.007	0.160±0.010	0.183±0.002[*]
Innate immune response NO induction	14.671 ± 1.139	13.8776 ±0 .052	10.96 ± 1.473	9.719 ±0.834[*]
Phagocytic index	10.310±0.956 1.04	6.148±1.070[*] 1.07	5.491±0.341[*] 1.08	4.078±0.053[*] 1.1153

Table 3: Effect of FO on anti-OVA IgG, IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) and IgE levels

Second bleed	1.627±0.033	1.606±0.028	1.599±0.039	1.566±0.026
Third bleed	1.672±0.027	1.653±0.013	1.641±0.008	1.633±0.011
Fourth bleed	1.488±0.014	1.450±0.008	1.450±0.019	1.448±0.016
IgG subclass antibody levels				
First bleed				
Anti-OVA IgG1	0.657±0.0002			
Anti-OVA IgG2a	0.248±0.003			
Anti-OVA IgG2b	0.231±0.002	0.657±0.0004	0.662±0.002	0.663±0.003
Anti-OVA IgG3	0.141±0.001	0.210±0.0003*	0.200±0.0003*	0.154±0.001*
		0.231±0.001	0.229±0.0003	0.233±0.002
IgG subclass antibody levels		0.142±0.002	0.142±0.002	0.142±0.003
Second bleed				
Anti-OVA IgG1	0.835±0.001			
Anti-OVA IgG2a	0.320±0.003			
Anti-OVA IgG2b	0.340±0.002	0.856±0.0001	0.857±0.001	0.879±0.006*
Anti-OVA IgG3	0.214±0.002	0.275±0.001*	0.241±0.002*	0.198±0.0005*
		0.335±0.006	0.333±0.012	0.331±0.006
IgG subclass levels		0.215±0.005	0.218±0.004	0.220±0.003
Third bleed				
Anti-OVA IgG1	0.870±0.0002			
Anti-OVA IgG2a	0.393±0.002			
Anti-OVA IgG2b	0.374±0.002	0.877±0.004	0.884±0.001	0.900±0.001*
Anti-OVA IgG3	0.225±0.004	0.389±0.006	0.388±0.001	0.238±0.0016*
		0.370±0.007	0.364±0.007	0.354±0.722
IgG subclass antibody levels		0.228±0.003	0.231±0.004	0.235±0.003
Fourth bleed				
Anti-OVA IgG1	0.7133±0.001			
Anti-OVA IgG2a	0.248±0.0003			
Anti-OVA IgG2b	0.2492±0.009	0.812±0.0008*	0.836±0.001*	0.840±0.0001*
Anti-OVA IgG3	0.1844±0.007	0.247±0.0003	0.246±0.0002	0.246±0.0003*
		0.239±0.009	0.238±0.008	0.234±0.002
IgE Levels		0.185±0.006	0.185±0.008	0.186±0.005
First bleed	0.3668±0.010			
Second bleed	0.393±0.010			
Third bleed	0.425±0.020	0.370±0.012	0.391±0.010	0.408±0.008*
Fourth bleed	0.294±0.010	0.398±0.011	0.438±0.012*	0.438±0.013*
		0.432±0.011	0.506±0.020*	0.518±0.030*
		0.301±0.010	0.307±0.012	0.323±0.012

Table 4: Effect of Fish oil on relative organ weights, cellularity of the lymphoid organs

Parameters	Group I	Group II	Group III	Group IV
Relative organ weights				
Spleen	0.362 ±0 .031	0.376 ±0 .008	0.390 ±0 .003	0.489±0.009*
Thymus	0.0713 ± 0.009	0.1042 ± 0.007	0.160 ± 0.001*	0.294 ±0.046*
Liver	5.590 ± 0.445	6.06 ± 0.040	6.294 ± 0.020	6.616 ± 0.670
Kidney	1.1± 0.024	1.1 ±0 .020	1.1 ± 0.020	1.1 ± 0.024
Cellularity of lymphoid organ				
Spleen	215 ± 13.453	282.6 ± 22.67	446 ± 28.314*	492.2 ± 35.038*
Thymus	108.8 ± 12.559	127.4 ± 12.559	294.8 ± 46.638*	304.4 ± 22.164*
Bone marrow	23.38 ± 1.242	26.616 ± 0.903	47.16 ± 3.522*	57.8 ±3.660*

Legends for Tables

Table 1: Calculated using Gas chromatograpy technique. Fatty acids are in their respective methyl Ester form.

Table 2: Plaque forming cells are represented per million splenocytes. NO induction is expressed as absorbance at 550 nm by un-stimulated and LPS stimulated macrophages. Macrophage phagocytic response is expressed as assessed by phagocytic index. DTH is expressed as footpad thickness (in mm) 48 h after being challenged with gRBC. Lymphocyte proliferation is expressed as absorbance measured at 570 by antigen OVA and mitogen Con-A stimulated spleen cells. Data is represented as mean ± S.E.M (n = 5). *$p < 0.05$ when compared with Control (significantly different).

Table 3: Dietary supplementation with FO increased serum concentrations of anti-OVA IgG1 in OVA sensitized *Swiss albino* mice after immunization with 50 μg of ovalbumin emulsified in Freund's complete adjuvant intramuscularly and booster dose (25 μg of OVA in Freund's incomplete adjuvant) given on 21st and 35th day. Blood was obtained from retro- orbitalis vein on 14th, 27th, 42nd and 60th day and serum separated for measurement of immunoglobulins by ELISA. Data are reported as means ± SEM fir 5 animals/group. *p<0.05 compared to the control group.

Table 4: Group I represents the control group, Group II; 4 mg/kg body weight dosage group, Group III; 8 mg/kg body weight dosage group, Group IV; 16 mg/kg body weight dosage group. Organ weights are expressed as g/100 g body weight. A single cell suspension of Lymphoid organs (bone marrow, thymus and spleen) was prepared in PBS. Cells counting was done using Neubauer chamber. Each value represents mean ±S.E. (n=5) of the relative organ weight in g/100g body weight, the cellularity of the lymphoid organ (1×10^6 cells), bone marrow cellularity (10^6cells/femur), *$p < 0.05$ (Dunnett's test)