

# Evaluation of Therapeutic Efficiency of Hilsha Fish Oil on Cardiovascular Disease and Hepatic Disease Marker in Hypercholesterolemic Mice

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## Abstract

Hypercholesterolemia is a clinical situation characterized by the elevated serum cholesterol and associated with the higher risk of cardiovascular disease (CVD), hypertension and stroke. This study aimed to evaluate the therapeutic efficiency of hilsha fish (*Tenualosa ilisha*) oil on diet-induced hypercholesterolemic (HC) albino mice. Mice were divided in three groups consisting each of six mice: control group, HC control group (fed the basal diet containing 1.5% cholesterol and 0.5% cholic acid) and the other group of mice fed the same previous hypercholesterolemic diet supplemented with hilsha fish oil (HFO) 5%. Serum lipid profile (total cholesterol-TC, low density lipoprotein-LDL, high density lipoprotein-HDL, triglyceride-TG and very low density lipoprotein-VLDL) were determined using commercial kits. After treatment with HFO a potential antilipidemic effect was observed as TC, TG, LDL, VLDL showed significant ( $p < 0.001$ ) decrease whereas HDL showed significant increase ( $p < 0.001$ ) compared to the HC control group. The SGPT, SGOT and CRP were also significantly decrease ( $p < 0.001$ ). Therefore HFO might have hepatoprotective activity. Regarding liver tissue extract, the levels of total cholesterol and triglyceride were decreased significantly in treated mice. Gas chromatography (GC)-MS analysis of HFO showed that it contained a high amount of poly unsaturated fatty acids (PUFA) especially EPA and DHA. These Omega-3 fatty acids have an indicative effect to reduce the risk of CVD and other chronic diseases. From the above findings, it can be concluded that HFO has a potential benefit in the treatment of CVD and play a role in its management as well as in reducing the risk of CVD associated hepatic complications.

**Keywords:** Hypercholesterolemia; CVD; Lipid profile; CRP; EPA; DHA

**Abbreviations:** Hilsha Fish Oil (HFO); Hypercholesterolemic (HC); Atherogenic Index (AI); Cardiovascular Disease (CVD); Eicosapentaenoic Acid (EPA); Docosahexaenoic Acid (DHA); Serum Glutamic Pyruvic Transaminase (SGPT); Serum Glutamic Oxaloacetic Transaminase (SGOT); Coronary Heart Disease (CHD)

## Introduction

Hypercholesterolemia, also called dyslipidemia is the presence of high levels of cholesterol in the blood. Hypercholesterolemia has been considered as a major risk factor for coronary heart disease (CHD) and atherosclerosis. Hyperlipidemia, particularly elevated serum cholesterol and Low-Density Lipoprotein (LDL) levels, responsible for the development of atherosclerotic heart disease [1]. Hypercholesterolemia is a major problem to many societies especially the health professionals because of the close relation between cardiovascular diseases (CVD) and lipid abnormalities [2,3]. Dietary factors such as continuous ingestion of high amounts of saturated fats and cholesterol are believed to be directly related to hypercholesterolemia and susceptibility to atherosclerosis [4]. Clinical trials have demonstrated that intensive reduction of plasma low density lipoprotein (LDL) levels could reverse atherosclerosis and decrease the incidence of cardiovascular diseases [5]. It is believed that hypercholesterolemia is correlated to elevated sugar level and hepatic problems.

*Tenualosa ilisha* (Hilsha) belongs to subfamily Alosinae, family Clupeidae, order Clupeiformes, is one of the most important tropical fishes of the Indo-Pacific region. Hilsha fish contain a high amount of protein, minerals, vitamins as well as polyunsaturated fatty acids (PUFAs). Many studies have been conducted on support of various effects of fish oil. It is suggested that fish oil contain long chain polyunsaturated fatty acid [6] more specifically eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA) which have a great benefit for cardiac health [7], controlling blood glucose [8] reduction of arterial disease [9]. Fish oil containing  $\omega$ -3 PUFAs is more effective than the vegetable oil ( $\omega$ -6 PUFAs) in reduction of lipid profile in human [10]. Docosahexaenoic acid ( $\omega$ -6 PUFA) is more effective in lowering serum cholesterol level of experimentally induced hypercholesterolemic rats [11]. Epidemiological studies also show that eating fish or vegetable oil concurrently decreases blood cholesterol and LDL levels and increases HDL, and thus reduces the risk of coronary death [12,13]. Thus fish has medicinal and therapeutic value [14]. Many studies revealed that hilsha fish oil (HFO) can reduce blood glucose and insulin level in diabetes induced rats. Hilsha fish oil can also decrease non-esterified fatty acids (NEFA), platelet aggregation and increase total anti-oxidant status [15].

Previous studies showed that long chain n-3 polyunsaturated fatty acid in fish reduced CHD mortality [16] and cardiovascular risk factors like serum triglyceride (TG) concentration, blood pressure, arrhythmias and inflammation [17]. However there is no

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sufficient evidence of decreasing cholesterol level by consuming HFO in experimental animals. The aim of the present study is to investigate the effect of HFO on serum lipid profile and on hepatic disease marker of experimentally induced HC mice.

## Material and Methods

### Collection of hilsha fish

*Tenualosa ilisha* is essentially a marine water fish but it is abundantly found in the big and small rivers of Bangladesh. 3-5 fishes were collected from Padma River for this study. After collection fishes were cut into small pieces (about 15-20 g of weight) and sun dried at temperature 40-42°C for 6 hours to completely remove moisture from it.

### Extraction of oil

Oil was extracted from the dried fish material with n-hexane by Soxhlet apparatus according to [18]. The extract was evaporated under reduced pressure in a rotary evaporator to obtain the oil.

### GC mass analysis of fatty acids in hilsha fish oil

Fatty acid composition of extracted oil was determined by Gas-liquid chromatography according to [19]. Gas chromatography was conducted with a Gas Chromatograph GC-2025 series with AOC-20i Auto Injector (Shimadzu Co, Japan). GC- 2025 Gas chromatography status include temperature -280°C, pressure-175.4KPa, total flow-165ml/min, purge flow- 3 ml/min, column temperature-270°C. Hilsha fish oil was first saponified to produce the free fatty acid salts. The fatty acid salts then are derivatized to form the fatty acid methyl esters (FAME) according to the American Oil Chemists Society (AOCS). The FAME was extracted with a non-polar solvent (e.g., hexane) for analysis by GC.

Each FAME (Fatty Acid Methyl Ester) in extract was identified by comparing retention times with those of known standard FAME (Lipid Standard Sigma chemical Co, St Louis, MO, USA). The area of fatty acids was measured with GC solution 2011. The results were expressed as relative percentage of fatty acids. The relative percentage of fatty acids was calculated by the formula:

$$\text{Relative percentage of fatty acid} = \frac{\text{Area of fatty acid} \times 100}{\text{Total area of detected fatty acids}}$$

### Experimental animals and treatment

Albino mice weighing ranged (24-26 g) were purchased from the Animal House of International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR), Dhaka, Bangladesh. They were adapted for one week before the experiment. All the animals were kept and maintained under laboratory conditions of temperature (22 ± 72°C), humidity (45.75%) and 12 h day: 12 h night cycle; and were allowed free access to food (standard pellet diet) and water *ad libitum*.

The animals were divided into three groups containing six mice (n=6) in each and divided into following groups:

**Group 1 (G1):** Control mice; fed basal diet according to [20].

**Group 2 (G2):** Hypercholesterolemic mice, fed the basal diet + 1.5% cholesterol + 0.5% cholic acid according to [21]

**Group 3 (G3):** Treated mice; fed hypercholesterolemic (HC) diet supplemented with 5% hilsha fish oil (HFO).

Body weight of mice was measured at the initial and final day of the

experiment of 6 weeks. At the end of experimental period (6 weeks), mice were sacrificed after overnight fasting. Mice were anesthetized with diethyl ether (1.9%) and blood was collected from the heart. The blood samples were left for 15 minutes at room temperature and then centrifuged at 3000 rpm for 20 minutes to separate the serum, then kept in plastic vials at -20°C until analysis. Main organs such as liver, heart and kidney were carefully separated and washed with cold saline and dry on filter paper and the weight were recorded. Liver tissues were quickly dissected, rinsed in ice-chilled normal saline, blotted on filter paper. The tissues were cut into small portions and stored at -20°C until using for measuring cholesterol and triglyceride contents. Ethical guideline was maintained in animal handling during the study and permission was obtained from the concerned department.

### Biochemical Analysis

Serum lipid profile such as triglyceride, total cholesterol, HDL-cholesterol, Very Low Density Lipoprotein were measured using quantification kits (Linear chemicals, Barcelona, Spain) by automatic Bioanalyzer (Hitachi 7180, Hitachi, Tokyo, Japan). Serum LDL was determined according to the Friedewald formula with use of HDL and total cholesterol value. Hepatic enzymes such as SGPT and SGOT as well as CRP were also measured using quantification kits. Atherogenic index (AI) and HTR ratio was calculated by an equations development by Friedewald [22].

Atherogenic index (AI) = (serum total cholesterol -HDL-c)/HDL-c

HTR ratio= HDL-c/TC x100

### Extraction and analysis liver total cholesterol and triglyceride

Estimation of total cholesterol and triglycerides from liver extract was carried out according to [23]. 1 g of liver portion from each animal was homogenized in 10 ml 2-propanol. The liver homogenate was allowed to stand for 48 h at 4°C. The mixture was centrifuged 15 min at 2500 rpm and the supernatant was used for lipid analysis. Total cholesterol and triacylglycerol were quantified enzymatically as described above.

### Results

Fatty acid composition of hilsha fish oil (HFO) was illustrated in Table 1. These data revealed that HFO contain 34.14% total saturated fatty acid (TSFA), 22.81% mono unsaturated (MUFAs) and 15.19% polyunsaturated fatty acids (PUFAs). Among those fatty acids, palmitic acid (C16:0) 25.17%, oleic acid (C18:1) 22.81% and eicosapentaenoic acid (C20:5) 7.01% accounted the highest proportions of fatty acid.

Data in Table 2 showed the changes of lipids profile in different group of mice. These data viewed that in hypercholesterolemic mice (HC) group TC, TG, VLDL and LDL-c concentration increased significantly P<0.001 whereas level of good cholesterol HDL-c significantly decreased compared to healthy control group. Serum TC, TG, VLDL and LDL-c level of HFO treated HC mice decreased by 30.94%, 65.90%, 42.77% and 13.65% respectively whereas HDL-c increased 64.00%.

Data in Table 3 depicted the initial and final body weight as well as body weight gain after the treatment period. These data revealed that there is less increase in body weight in HC mice group. On the other hand by treatment with HFO body weight gain was decreased by 5.59%.

In Table 4, the results was indicated the effect of HFO on AI,

Items	Formula	Hilsha fish oil
Saturated fatty acids (SFA)	C14:1	2.45
	C16:0	25.17
	C16:1	1.65
	C18:0	4.19
	C22:0	0.68
Total saturated fatty acid (TSFA)		34.14
Monounsaturated fatty acid (MUFA)	C18:1	22.81
Omega-6	C18:2	1.43
	C20:4	0.40
Omega-3	C18:3	3.42
	C20:5	7.01
	C22:6	3.33
Total Polyunsaturated fatty acid (PUFA)		15.19

**Table 1:** Relative percentage of fatty acids in hilsha fish (*Tenualosa ilisha*) oil by GC mass.

Groups	TC	HDL	LDL	TG	VLDL
G1 control	5.50 ± 0.08	0.30 ± 0.03	4.53 ± 0.11	1.48 ± 0.12	0.67 ± 0.02
G2 (HC)	9.05 ± 0.38 <sup>a*</sup>	0.17 ± 0.03 <sup>a*</sup>	6.74 ± 0.39 <sup>a*</sup>	3.52 ± 0.21 <sup>a*</sup>	1.59 ± 0.09 <sup>a*</sup>
G3 (HC)+ fish oil	6.25 ± 0.42 <sup>a**</sup>	0.28 ± 0.03 <sup>a**</sup>	5.82 ± 0.47 <sup>a**</sup>	1.20 ± 0.16 <sup>a**</sup>	0.91 ± 0.07 <sup>a**</sup>

Total Cholesterol (TC), High density lipoprotein (HDL), Low density lipoprotein (LDL), Triacylglycerol (TG), Very low density lipoprotein (VLDL). a: P<0.001; \* vs control group, \*\* vs hypercholesterolemic control.

**Table 2:** Effect of hilsha fish oil on the lipid profiles of different group of mice (mmol/L) Mean ± SD.

Groups	IBW	FBW	BWG
G1 control	25.55 ± 0.77	40.06 ± 1.12	14.51 ± 0.86
G2 (HC)	25.40 ± 0.81 <sup>a*</sup>	42.03 ± 1.33 <sup>b*</sup>	16.63 ± 1.02 <sup>b*</sup>
G3 (HC)+ fish oil	25.30 ± 0.76 <sup>a**</sup>	41.00 ± 1.37 <sup>a**</sup>	15.70 ± 0.82 <sup>a**</sup>

Initial body weight (IBW), Final body weight (FBW), Body weight gain (BWG). a: P<0.001; b<0.05, \* vs control group, \*\* vs hypercholesterolemic control.

**Table 3:** Effect of hilsha fish oil on body weight of different group of mice (g) Mean ± SD.

LDL-C/HDL-C Ratio and HTR in hypercholesterolemic mice. Atherogenic index and LDL-C/HDL-C ratio increased markedly while HTR% decreased in the HC group. But by treating HC mice with HFO supplementation AI and LDL-C/HDL-C ratio reduced to 21.32 and 20.78 respectively and HTR% elevated to 4.48.

Lipid level of liver tissue was viewed in Table 5. High cholesterol diet caused significant increase p<0.001 of hepatic cholesterol (50.00%) and triacylglycerol (52.55%) compared to the healthy control group. Administration hypercholesterolemic diet supplemented with HFO reduced hepatic TC and TG by 13.91% and 16.10% respectively.

Effect of HFO on the weight of main organs of HC mice was represented in (Table 6). Results revealed that liver weight was markedly increased in mice fed hypercholesterolemic diet compared to normal control group. Meanwhile, there was no change in the weight of other organs. Supplemented diet of HC mice with 5% HFO reduced liver weight compared to HC group.

There was a significant (P<0.001) increase of SGPT and SGOT level after induction of hypercholesterolemia which was decreased by HFO significantly (P<0.001). SGPT level increased by 70.25% in HC group and decreased by 23.77% with HFO supplementation. Moreover treatment with HFO decreased SGOT level by 30.87%. This result was illustrated in (Figure 1).

C - Reactive protein (CRP) level was also higher in

hypercholesterolemic mice group than the normal control mice which was declined significantly (P<0.001) by 32.35% with HFO supplementation (Figure 2).

## Discussion

Two important groups of PUFA in human nutrition are the omega-6 and omega-3 fatty acids. Many studies have shown EPA and DHA are beneficial to our heart system and have protective effects for different diseases. A study showed that the percentage of polyunsaturated fatty acids (PUFA) in halibut, mackerel, bloater and sprat were 31.9%, 45.4%, 40.8% and 37.0% respectively [24]. These marine fishes also have cholesterol lowering effect. Omega-3 fatty acids have TG-lowering effect that may be mediated by inhibition of the soluble phosphatidate phosphohydrolase and the effect on serum cholesterol may be partly due to inhibition of HMG-CoA reductase which is the rate-limiting enzyme in cholesterol biosynthesis [25]. Omega-3 fatty acids also improve hepatic steatosis in mice and may be used to increase the pool of potential live liver donors that are currently excluded because of the presence of macrovesicular steatosis [26]. Hilsha fish oil effectively reduced serum lipid profile level and this finding was found consistent as reported by others. Dietary marine fish oil reduced blood cholesterol in the experimentally induced hypercholesterolemic rats [27]. Anti cholesterol effects of Hilsha fish oil was also reported in streptozotocin-treated diabetic rats [15]. It has been suggested that the longer lifespan of Japanese and Nordic populations may be partially due to their higher consumption of fish and seafood. A study showed that after 10 months of eating 100g hilsa fish per day, serum total cholesterol level fell from 285.1 to 244.6 mg/dl (14.2% decrease) in the hypercholesterolemic subjects [28]. Induction of hypercholesterolemia significantly increased body weight of experimental mice. This result was supported by a finding which reported that rats fed high cholesterol diet showed significant increase in body weight gain [29]. But feeding diet containing HFO decreases body weight compared with HC mice. This occurs may be due to the catabolism of lipid accumulated in adipose tissue causing a decrease in body weight. HFO also showed significant effect on AI, LDL-C/HDL-C Ratio and HTR%. Reduction in HTR ratio is important

Groups	AI	LDL/HDL Ratio	HTR Ratio
G1 control	17.33 ± 0.87	15.1 ± 1.56	5.45 ± 0.34
G2 (HC)	52.23 ± 2.12 <sup>*</sup>	39.64 ± 2.34 <sup>*</sup>	1.88 ± 0.45 <sup>*</sup>
G3 (HC)+ fish oil	21.32 ± 1.09 <sup>**</sup>	20.78 ± 1.76 <sup>**</sup>	4.48 ± 0.37 <sup>**</sup>

Atherogenic index; \* vs control group, \*\* vs hypercholesterolemic control.

**Table 4:** Levels of AI, LDL/HDL Ratio and HTR of different group of mice Mean ± SD.

Groups	TC (Mean ± SD)	TG (Mean ± SD)
G1 control	2.06 ± 0.25	11.97 ± 0.60
G2 (HC)	3.09 ± 0.53 <sup>b*</sup>	18.26 ± 0.68 <sup>a*</sup>
G3 (HC)+ fish oil	2.66 ± 0.76 <sup>a**</sup>	15.32 ± 0.63 <sup>a**</sup>

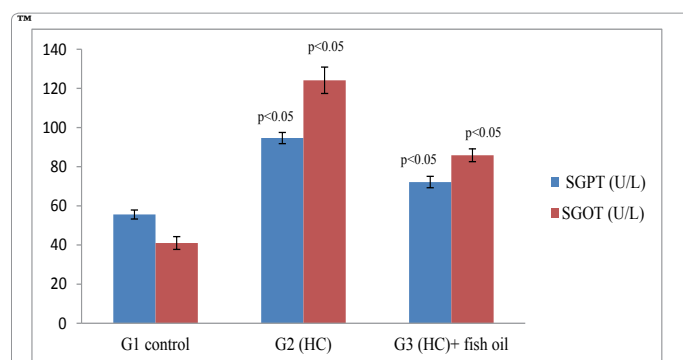
Total cholesterol (TC), Triacylglycerol (TG). a: P<0.001; b<0.05; \* vs control group, \*\* vs hypercholesterolemic control

**Table 5:** Levels of total cholesterol, triglyceride in liver tissue in different group of mice liver (mg/g, wet liver).

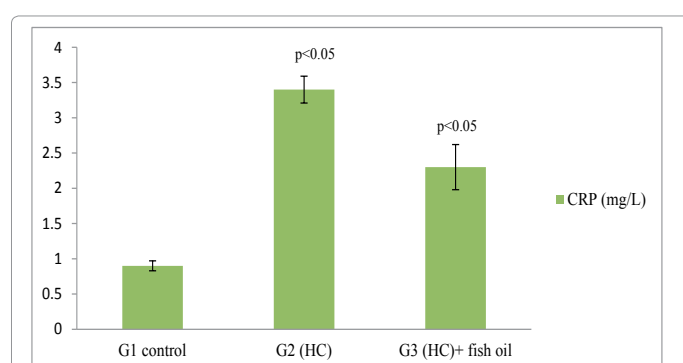
Groups	Liver	Heart	Kidney
G1 control	2.15 ± 0.03	0.18 ± 0.01	0.195 ± 0.01
G2 (HC)	2.61 ± 0.04 <sup>a*</sup>	0.20 ± 0.004 <sup>*</sup>	0.206 ± 0.01 <sup>*</sup>
G3 (HC)+ fish oil	2.03 ± 0.07 <sup>a**</sup>	0.15 ± 0.002 <sup>a**</sup>	0.196 ± 0.01 <sup>**</sup>

a: P<0.001; \* vs control group, \*\* vs hypercholesterolemic control

**Table 6:** Changes of weight of organs different group of mice (g) Mean ± SD.



**Figure 1:** Effect of hilsha fish oil on serum SGPT and SGOT of experimental mice.



**Figure 2:** Effect of hilsha fish oil on serum CRP of experimental mice.

in predicting coronary heart disease in human being, an increase in this ratio is believed to furnish a beneficial effect. This result was supported by another experiment who stated that the increase in HDL-C or HTR ratio is one of the most important criteria of anti-hypercholesterolemic agent [30]. Supplementation of HFO also significantly decreased liver cholesterol and triglyceride level. Animal-derived Hilsha ilisha fish oil is more effective in reducing the serum and liver cholesterol than soybean and palm oil, though both soybean and palm oil are also effective in reducing serum and liver cholesterol [31]. Weight of liver in HC mice increased significantly ( $p < 0.001$ ) which was compensated by treatment with HFO. The increase in hepatic enzymes SGPT and SGOT in hypercholesterolemic mice indicate dysfunctions of liver. On the other hand supplementation of HFO significantly decreased the level of these enzymes indicate effective recovery of the hepatic function by improvement of lipid metabolism or delaying the hepatic disease. C-Reactive Protein (CRP) is a simple cost effective test, which can predict the cardiovascular risk. The addition of CRP-testing to standard lipid screening appears to provide an important method to determine Cardiovascular Disease (CVD) risk factor [32]. Dietary supplementation of HFO declined CRP level significantly and thus reduced the risk of cardiovascular diseases.

## Statistical Analysis

The assays were carried out in triplicate, and the results were expressed as mean values and the standard deviation (SD). Results were analyzed by using Scientific Package of Social Science (SPSS) version 17.0. The descriptive statistic was used to analyze mean, standard deviation where by analytical statistics, one-way ANOVA was used to determine statistical significance ( $p < 0.05$ ) among the groups.

## Conclusion

There is considerable evidence from experimental studies that hilsha fish oil has significant benefit in the management of hypercholesterolemia. This anti lipidemic effect is due to the presence of omega-3 fatty acids that inhibit enzymes in lipid biosynthetic pathway. Hilsha fish oil also reduced SGPT, SGOT and CRP level so it may have hepatoprotective activity. Therefore these results indicate that hilsha fish oil have important implication in the management of hypercholesterolemia as well as cardiovascular and hepatic complications.

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