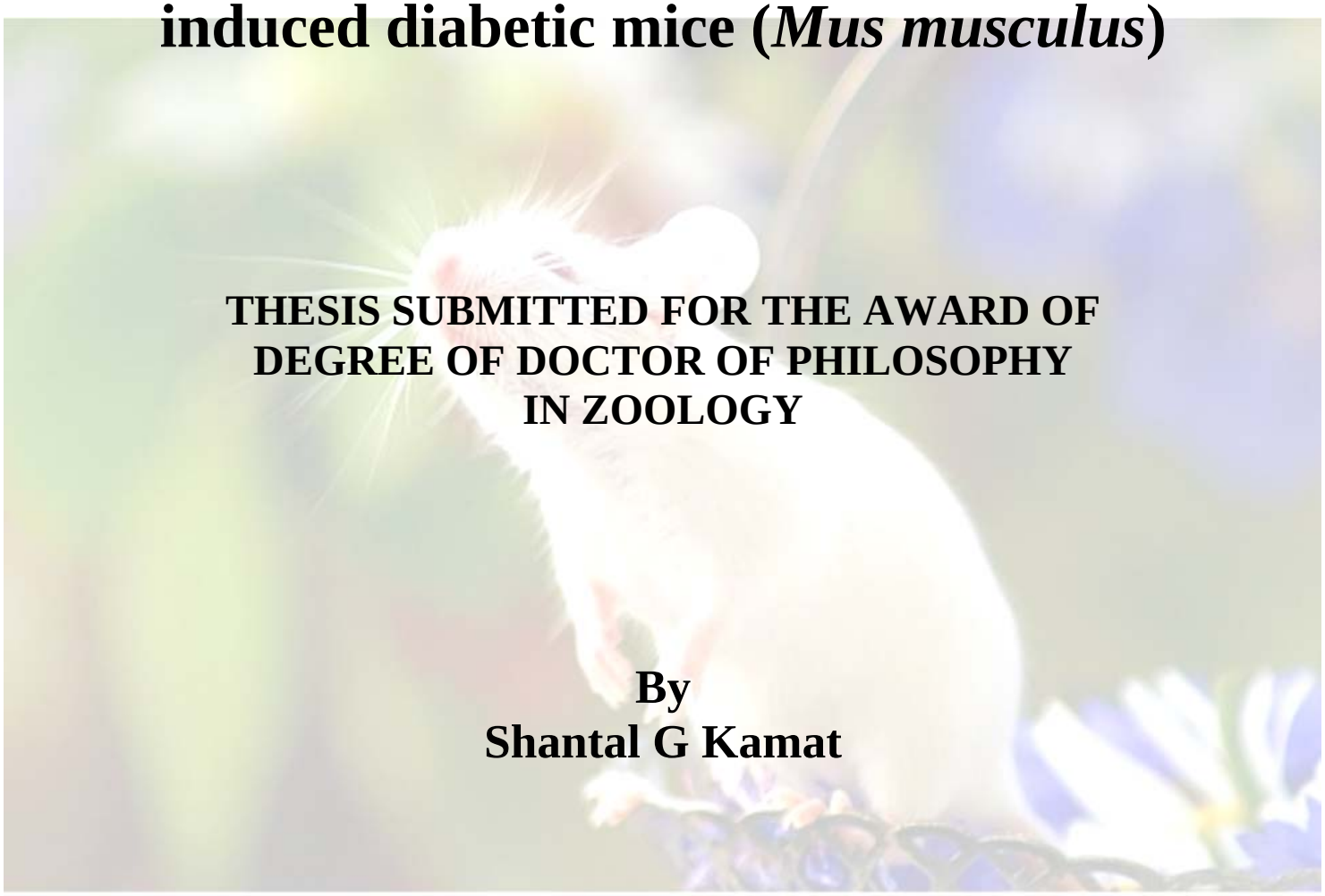
Effect of dietary fish oil in alloxan induced diabetic mice (*Mus musculus*)



THESIS SUBMITTED FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY

By
Shantal G Kamat

Research guide
Prof R Roy

GOA UNIVERSITY
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- - Certificate - -

This is to certify that **Ms. Shantal Ganapati Kamat** has worked on the thesis entitled “Effect of dietary fish oil in alloxan induced diabetic mice (*Mus musculus*)” under my supervision and guidance.

This thesis, being submitted to Goa University, Goa, for the award of the degree of Doctor of Philosophy in Zoology, is an original record of the candidate herself and no part of the work has been submitted to any other University or Institute for the award of any degree or diploma. The thesis presented is worthy of consideration for the award of the Ph.D. degree.

Date:

Ramaballav Roy

Department of Zoology

Declaration

I declare that the thesis entitled

“Effect of dietary fish oil in alloxan induce diabetic mice (*Mus musculus*)”

is my original contribution and that the same has not been submitted

on any previous occasion for any other degree or diploma

of this university or any other university / institute.

To the best of my knowledge, the present study is the

first comprehensive study of its kind from the area mentioned.

The literature related to the problem investigated has been cited.

Due acknowledgements have been made wherever

facilities and suggestions have been availed of.

Shantal Ganapati Kamat

Place: Goa University

Date:

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1. Introduction:

Diabetes mellitus (DM) is a multifactorial disease, triggered by different factors characterized by a chronic high level of blood glucose related to disturbances in the metabolism of carbohydrate, fat, and protein resulting from defects in insulin secretion, insulin action, or both (Azevedo and Alla, 2008). Deficiency of pancreatic hormone insulin results in failure to metabolize sugar or adequacy of another pancreatic hormone glucagon result in the increased liver glucose production. Inhibition of glycogen synthesis, and or stimulation of both glycogenolysis and gluconeogenesis lead to hyperglycemia. Due to irregularity in these hormones the sugar level increases and gets accumulated in the blood and leads to advanced glycosylated product formation and in the advanced stage it also affects the lipid metabolism (Singh *et al*., 2014). Even in 200 A.D. the diabetes was present in humans as described by a Greek physician Arateus, who gave the disease its name. Although, it has been present for so many years, still it was not considered as a threat until the 20th century.

The recent report of the International Diabetes Federation (IDF) and the World Health Organization (WHO) attributed 3.2 million deaths a year is due to high risk of cardiovascular complications associated with diabetes (Grundy *et al*., 1999). The hyperglycemia is assumed to be the reason for the vascular complications recognized in diabetes. Several mechanisms, like alterations in the metabolism of lipid (Bayens, 1991), the antioxidant defense mechanism and production of reactive oxygen species imbalance (Matough *et al*., 2011), changes

related to the inflammatory pathway (Donath *et al*., 2003) which play an important role in increased oxidative stress in diabetic patients.

1.1 Classification of Diabetes:

Diabetes is a complex, heterogeneous disease with the diverse etiological mechanism; therefore any given classification is arbitrary but yet not useless. There are two major classes of diabetes mellitus viz. diabetes type 1 and diabetes type 2. Gestational diabetes and other forms related to endocrine disorder, pancreatic disease, drugs and genetic mutation make up the small percentage of all the cases (Inzucchi *et al*., 2010). Three main forms of diabetes mellitus were recognized by the WHO are Type 1, Type 2 and Gestational diabetes, which show similar signs, symptoms and consequences, but different causes and population distribution.

1.1.1 Type 1 Diabetes mellitus:

The diabetes type 1 results from autoimmune destruction of pancreatic β-cells, resulting in inadequate insulin production, which possibly trigger in genetically susceptible people by an exposure to environmental factors. The β-cell damage which can take over months or years until the insulin concentration is no longer adequate to control plasma glucose (Atkinson and Eisenbarth, 2001). Type 1 DM has mostly progressed in childhood or adolescence yet it can also develop in adults (Chiang *et al*., 2014). The interactions between susceptibility genes, auto-antigens and environmental factors involved in pathogenesis of the autoimmune β-cell destruction are incompletely understood. It is also postulated that early diet plays a role in the development of diabetes type1. In 90% of patients with type 1 DM the major histocompatibility complex (MHC), are included in the susceptible gene list (Akerblom *et al*., 2005; Raha *et al*., 2009).

1.1.2 Type 2 Diabetes mellitus:

The diabetes type 2 results from insulin resistance, leading to an inability to inhibit production of glucose ensuing in hyperglycemia (Sollu *et al*., 2010). A high intake of dietary sugars chiefly leads to surplus demands on the production of insulin, which over time leads to insulin resistance. Receptor cells that have developed resistance to insulin are incapable to eliminate glucose from the blood, which leads to higher blood glucose and larger demands on insulin production. In the early stage of disease often insulin concentration is very high, levels may fall later with the development of the disease, further worsening hyperglycemia. Generally type 2 DM progresses in adults and develop more common with age, up to one third of adults greater than age 65 has impaired glucose tolerance. Due to increased visceral and abdominal fat accumulation and decreased muscle mass glucose levels take longer to return to normal (Kesavadev *et al*., 2003). The cause of progress of diabetes type 2 is a combination of lifestyle and environmental factors. It also has a stronger connection to family history and heredity than type 1 DM.

1.1.3 Gestational Diabetes:

In the period of pregnancy the 2-4% of women gets affected by gestational diabetes mellitus (GDM) and is manifested as a heterogeneous group of disorders. The genetic determinants are suggested to have a susceptibility to GDM (Martin *et al*., 1985) although the precise genetic basis for GDM has yet to be interpreted. Both resistance to insulin and reduced secretion of insulin are associated with the metabolic pathogenesis of GDM in addition to risk factors such as obesity, family history, pregnancy and age of mother (Ryan *et al*., 1995).

1.2 The factors causing diabetes:

The particular cause of diabetes is not clear, however, it is assumed that some infection (mainly viral), environmental factors or stress, genetic factors and lifestyle elicits diabetes (American Diabetes Association, 2004, Pruthi *et al*., 2014).

1.2.1 The genetic factors:

The human leukocyte antigen (HLA) the highly polymorphic major genetic region indeed has been recognized as the strong linkage and influencing risk for type 1 diabetes. The super locus encodes a cell-surface antigen-presenting proteins related to immune system function in humans. There is strong evidence suggesting that either, HLA-DR4/HLA-DQ8 or (HLA) -DR3/HLA-DQ2 haplotypes of human leukocyte antigen are related to type 1 diabetes (Raffel *et al*., 2008). Moreover, to the HLA, the relationship of cytotoxic T-lymphocyte related antigen 4 (CTLA-4) loci on chromosome 2 has specified that CD4+ T cells play a vital role (Paust *et al*., 2004) in regulation of inflammatory response which was first studied in a small cohort of Italian multiplex families (Nistico *et al*., 1996). Since the CTLA4 plays vital role in the regulation of the immune response, it shows strong link in association with autoimmune diseases (Marron *et al*., 1997; Ueda *et al*., 2003). Studies have also shown that mutations in genes involved in glucose regulations may cause of diabetes type 2. Some of the genes associated are insulin and glucose secretion controlling gene TCF7L2, urea receptor gene ABCC8, glucose transporter (GLUT2), glucagon receptor gene GCGR (McEntyre, 2004).

In humans the other gene, which play a vital role in the progression of diabetes is an insulin gene (INS) positioned on chromosome 11p15.5 and comprises of 3 exons and 2 introns. The increasing number of proofs suggests other tissues/cells can actively transcribe Ins producing low concentration of insulin transcription/pro-insulin of which has been typically thought to be merely limited to pancreatic β-cells (Pugliese and Micelli, 2002; Pugliese, 2005). Despite some insulin production in extra-pancreatic tissues, most of the cells in the body do not transcribe INS, and only β-cells have the necessary machinery for storing and secreting insulin in response to glucose stimulation. The transcription of INS is stimulated by glucose through its multiple effects on transcription activation complex associated proteins, including transcription factors that are vital for the secretion of insulin as well as for the growth and differentiation of the pancreas and the β-cells (Pugliese and Micelli, 2002). Mutations of the PDX-1 gene can have effects on the progression pancreatic damage and INS transcription (Stoffers *et al*., 1997; Stoffers *et al*., 1998) and related to a severe impairment of sensitivity of beta-cell towards glucose and an obvious increment in peripheral tissue sensitivity to insulin in vivo (Clocquet *et al*., 2000, Nanclares *et al*., 2003).

1.2.2 The environmental factors and lifestyle:

Many studies have shown that the concordance rate for the progress of type 1 diabetes among monozygotic twins is not higher than 50% (Knip *et al*., 2005), favoring the hypothesis that other factors like some environmental factors are involved in the development of diabetes. The part of environmental variables in actuating or quickening autoimmune disease is ineffectively comprehended when they happen in hereditarily susceptible people.

There is a link between development of diabetes type 1 and some environmental aspects such as foods, viruses, and toxins (Oikarinen *et al*., 2007). The enteric viruses which attack the intestinal tract are among under investigation. *Coxsackie* viruses are a group of enteric viruses specifically noteworthy. An association between *Coxsackie* viruses and diabetes type 1 has been suspected for more than 40 years. They infect million individuals every year, and are transmitted in outbreak style by respiratory secretions – salivation, sputum and nasal bodily fluid or by the fecal oral way (Schulte et *al.*, 2010). Studies expose, that contaminations of enterovirus might start and quicken beta cell harm years before a clinical indication of diabetes type 1 (Boucher and Notkins, 1973; Yoon *et al*., 1980).

The risk of developing type 2 diabetes is not only increased due to obesity but it also complexes, health risks and obscures management of diabetes type 2. Recent epidemic studies in the U.S. reported that obesity and diabetes type 2 are a consequence of extensive environmental changes disturbing energy stability and its regulation (Nichols, 2015). Obesity has a tendency to to run in families, and families tend to have related eating and exercise practices. Simple sugars and low in fiber and vital nutrient rich diet are more likely to lead to type 2 diabetes. The environmental changes range from acquaintance to pollutants disrupting endocrine to reduced sleep period of physical dormancy to extra caloric intake (Ershow, 2009).

1.3 Blood glucose homeostasis:

The steadiness of blood glucose is achieved through well-balanced hepatic glucose release, transport and peripheral glucose disposal maintained by excellently tuned, synchronized network of metabolic, signalling, regulatory pathway. Within narrow physiological range, the complex balance of dietary intake helps to maintain blood glucose level, de novo glycogen storage, synthesis, release and insulin dependent and independent glucose acceptance of tissue (Baum *et al*., 2006). The entrance of glucose into the circulation is influenced by the rate of assimilation of carbohydrates (Kloover and Mooney, 2004; Postic and Girard, 2008).

The glucose is used by the liver as fuel and also it has the capability to store it as glycogen and synthesize it from non-carbohydrate precursor. The glucose that is taken up by a cell in cytosol may be oxidized to pyruvate (Levinthal and Tavill, 1999). The electrons produced for this course are transported to the mitochondria to form energy. In the mitochondria, the acetyl CoA produced by oxidizing pyruvate through the glycolytic pathway undergoes complete oxidation through the tricarboxylic acid cycle and inner membrane electron transport system generates nearly 36 moles high energy phosphate from each molecule of glucose (Szablewski, 2011).

Even though main fuel for most organs is the free fatty acids, under physiological conditions the obligate metabolic fuel for the brain is glucose. The reason is may be due to small circulating concentrations of other possible alternative substrate (e.g. ketone bodies) or due to limitations of alternative

transport across the blood brain barriers (Siesjo, 1988). The brain is dependent on a continuous supply of glucose from plasma because it cannot store glycogen and also it cannot synthesize glucose. Glucose transport becomes rate limiting for brain to utilize glucose if the plasma glucose concentration is below normal levels. The cerebral function gets impaired if the concentration of plasma glucose decreases below 55mg/dl, while more severe and prolonged hypoglycemia results in seizures, permanent damage to the brain and death (Jarrett and Keen, 1976; Tominaga *et al*., 1999). Further, even mildly higher plasma glucose concentrations, in patients with decreased glucose tolerance increases the risk for cardiovascular morbidity (Sharyyef and Gerich, 2010; Howarth *et al*., 2012).

1.3.1 Role of pancreas in glucose metabolism:

The pancreas is one of the major organs of the body, beside with the liver. It has both endocrine and exocrine functions. It carries two functions, the production of enzymes in the digestive system in the exocrine tissue and creates hormones as part of the endocrine system (Hellman *et al*., 2007). Several hormones regulate the carbohydrate metabolism, particularly which are produced by pancreatic cells of islets of Langerhans. Insulin and glucagon produced by β cell and α cell respectively, have an important contribution on glucose metabolism. D cells and F and D1 cells produce hormone somatostanin and pancreatic polypeptides having a modulating effect on the secretion of insulin and glucagon. The carbohydrate metabolism is also affected by some other hormones like epinephrine, thyroid hormones, glucocorticoids, etc. (Kim and Egan, 2008).

The large nutrient molecules were reduced by gastric juice in stomach by breaking down the large quantity of food and the nutrients released were absorbed into the bloodstream by the action of the intestine. The large nutrient molecules are cut down to smaller molecules by enzymes secreted by the pancreas, these molecules through the walls of the intestines can be absorbed into the bloodstream (Boundless, 2015).

1.3.2 Insulin, Glucagon and their metabolic action:

Insulin is a protein chain or a peptide hormone consisting 51 amino acid molecules. Like the receptor for other protein hormones, the receptor for insulin is entrenched in the plasma membrane. The hydrolysis of dietary carbohydrates such as starch and sucrose within the small intestine liberate the glucose. Elevation of the concentration of glucose in the blood results in the release of insulin, which stimulate glucose uptake, use and storage (Figure A). Depending upon the target tissue the effects of insulin on glucose metabolism vary (Wilcox, 2005).

The entry of glucose into muscle, adipose and several other tissues is facilitated by Insulin. The cells can uptake glucose by facilitated diffusion mechanism through a family of hexose transporters. Through the action of insulin in many tissues the major transporter used for the uptake of glucose (called GLUT 4) is made available in the plasma membrane. When the insulin concentration is low the GLUT4 transporters present in cytoplasmic vesicles inadequate for transporting glucose (Kido *et al*., 2001) and are recycled back into the cytoplasm. But there are some tissues like the brain and liver use transporter that is not

insulin-dependent (like GLUT 2) for importing glucose and do not require insulin for efficient uptake of glucose (Levy *et al*., 2005; Guyton, 2006).

The storage of glucose in the form of glycogen in the liver is also stimulated by insulin. The hepatocytes takes up the large fraction of glucose absorbed from the small intestine and converts it into polymer glycogen for storage. Insulin has several effects in the liver, which stimulates glycogen synthesis (Bowen, 2009). The activity of glucose-6-phosphatase is inhibited by the action of insulin it also phosphorylates glucose and traps it within the cell by activating the enzyme hexokinase. The net result is when the supply of glucose is abundant insulin conveys messages to liver to store as much of as glucose possible for later use (Alan, 2006; Burtis *et al*., 2006). The glucagon presence in lack of insulin stimulates the glycogen breakdown (Burks and White, 2001). The extra glucose taken up by hepatocytes is lead into the pathways involved in the synthesis of fatty acids and is exported from the liver as lipoproteins, when the liver is saturated with glycogen (Postic and Girard, 2008). In the circulation the lipoproteins are shredded apart, providing free fatty acids for use of other tissues, which use them to produce triglyceride (Bowen, 2009).

Glucagon is having the inverse impact of insulin. As specified previously brain can't use substitute vitality sources like fatty acids to any substantial extent hence is certainly dependent on glucose as fuel. When the level of glucose in the blood start falls below normal glucagon commands to find and pump extra glucose to the brain. Within the liver two essential metabolic pathways (Figure A) leading the organ to distribute glucose in the body are controlled by glucagon

(Bowen, 2009). The breakdown of glycogen stored in the liver is stimulated by glucagon. When blood glucose levels fall down glucagon is secreted and acts on hepatocytes to trigger the enzymes that depolymerizes glycogen and releases glucose. Glucagon also helps to activate hepatic gluconeogenesis in which the amino acids (non-hexose substrates) are converted to glucose, which provides another source of glucose in the blood (Jiang and Zhang, 2003). When the glucose concentration of blood falls, the lipase enzyme activated by glucagon, which in turn hydrolyzes the stored triacylglycerols to discharge glycerol's and free fatty acids in the blood. These free fatty acids are carried by serum albumin to the tissues and liver absorbs the glycerol for gluconeogenesis (Miles, 2003).

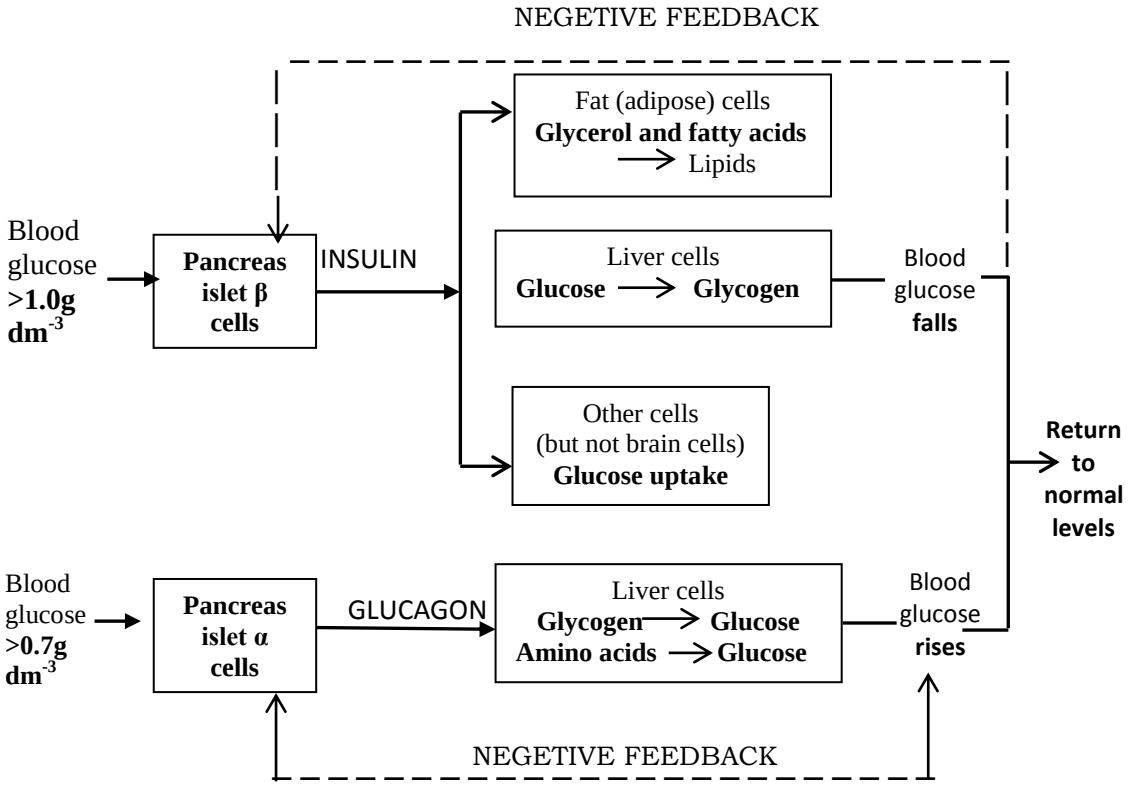

Figure A: The homeostatic control of blood glucose level. (http://www.saburchill.com/IBiology/chapter02/005.html)

1.4 Oxidative Stress, Lipid Peroxidation and Diabetic Complications:

The complications associated with diabetes type 1 leads to increasing disability and shortened life expectancy and are main threat to the health and life of patients. Although the hyperglycemia is recognized as a risk factor for progression of diabetic complications by the Diabetes Control and Complication Trial (DCCT), there is no agreement concerning the pathogenic relation among hyperglycemia and complications (Brownlee, 1992; Aronson, 2008). There are numbers of equally acceptable reasons for the origin of complications. Although there are improved treatments available, metabolic and hemodynamic abnormalities associated complications of prolonged diabetes often leads to increasing disability and shortened life expectancy (Hostetter, 1992; Moore *et al*., 2009). Years of poorly controlled hyperglycemia lead to major types of diabetes related multiple, primarily microvascular (affect small vessels) and macrovascular (affect large vessels) complications or both and oxidative stress plays a pivotal role (Bayens, 1991).

In diabetes over generation of reactive oxygen species (ROS) because of glucose auto-oxidation, metabolism and the progress of advanced glycosylation end products, stimulates toxic impacts. This leads to unsuccessful scavenging of ROS and disturbances in the regular redox state that damage all constituents of the cell, including proteins, lipids and DNA resulting in tissue impairment and damage (Shradha and Sisodia, 2010). Mainly the oxidative stress and years of inefficiently controlled hyperglycemia plays a vital part in the progress of diabetes complications that affect small vessels which damages tissues like eye, kidney (microvascular) and large vessels (macrovascular) causing cerebrovascular

disease, coronary heart disease, peripheral arterial disease or both (Bayens, 1991). Endogenous ROS in mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes are produced through a variety of mechanisms, including auto oxidation of numerous compounds (Moldovan and Moldovan, 2004). The two most pervasive ROS that can significantly influence the lipids are chiefly hydroxyl radical (HO·) and hydroperoxyl ($HO\cdot_2$). The prolonged production of superoxide causes the initiation of 5 noteworthy pathways (Ayala *et al*., 2014) included in the pathogenesis of intricacies: (1) polyol pathway flux, (2) increased arrangement of AGEs (advanced glycation finished items), (3) elevated articulation of the receptor for AGEs and its actuating ligands, (4) activation of protein kinase C isoforms, and (5) over activity of the hexosamine pathway. A few lines of proof demonstrate that each of the 5 mechanisms triggered by upstream occasion of ROS overproduction by mitochondria (Giacco and Brownlee, 2010). A cell produces around 50 hydroxyl radicals every second, which can be neutralized or un-specifically attack biomolecules (Halliwell and Gutreridge, 1984; Ayala *et al*., 2014) situated less than couple nanometres from its origin of generation which can cause oxidative damage and participate in cellular diseases such as neurodegeneration (Venero *et al*., 2003; Castellani *et al*., 2004), cardiovascular disease (Lipinski and Pretorius, 2012), diabetes (Arora *et al*., 2013) and cancer (Dizdaroglu and Jaruga, 2012; Kanno *et al*., 2012).

1.5 Role of PUFA in health:

Dietary lipid helps to keep up well-being and plays an essential role in physiological developments. Members of poly unsaturated fatty acid (PUFA) cluster are directly vital substances and these are accepted to be intricate in tissue

lipid and are at current assuming increase prominence in biochemistry (Glick, 2009). The alpha-linolenic acid, the precursor molecule of omega-3 PUFAs, and linoleic acid, the precursor of omega-6 PUFAs are the two essential fatty acids (EFAs) in human nutrition (Simopoulous, 1999) and PUFAs belong to omega-3 or omega-6 families cannot be interconverted. Both linoleic and alpha linolenic acid can be extended to various long chain (C-20, C-22) polyunsaturated fatty acids through elongation and desaturation processes. Therefore, humans must obtain these essential fatty acids from dietary sources (Baur, 1994; Berghe *et al*., 2003). While the natural distribution of omega-6 fatty acids is cosmopolitan, the distribution of omega-3 fatty acids is restricted in terrestrial and freshwater ecosystems but abundant in marine ecosystem. These long chain omega-3 and omega-6 PUFAs produce distinct types of prostaglandins and thromboxanes through lipoxygenase and cyclooxygenase pathways, each of which has very different effects in the body and act in antagonistic manner. These eicosanoids act as potent regulator of vital body functions and play role in immune system and inflammatory responses (Perkins, 2007; Ricciotti and FitzGerald, 2012).

The distinct types of prostaglandins and thromboxanes (collectively known as eicosanoids) were produced by arachidonic acid (AA, 20:4, ω-6), dihomo γ-linolenic acid (DGLA, 20:3, ω-6) and eicosapentaenoic acid (EPA, 20:5, ω-3) along with other long chain omega-3 and omega-6 fatty acids through lipoxygenase, cyclooxygenase and epoxygenase pathways (Figure B). Each of these eicosanoids like hydroperoxides, prostaglandins, lipoxins, leukotienes and epoxy fatty acids and other bio reactive molecules has very diverse effects in the body and act in antagonistic way (Baur, 1994; Berghe *et al*., 2003). As a result

enzymes involved in the production of these eicosanoids have become the target for the development of anti-inflammatory drugs (Aparoy *et al*., 2012). Eicosanoids act as potent regulator of vital body functions and play role in immune system and inflammatory responses (Perkins, 2007; Ricciotti and FiltzGerald, 2012).

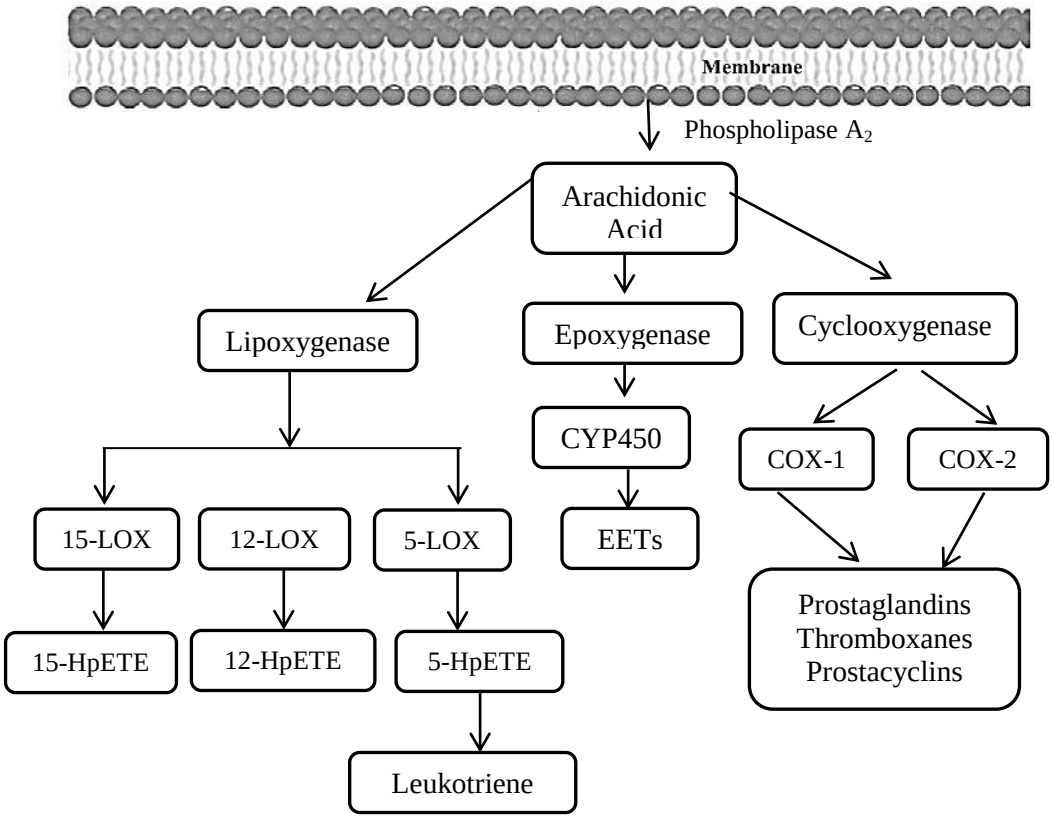

Figure B: Overview of Oxygenation of Arachidonic Acid via COX, LOX and EPOX pathway (Aproy *et al*., 2012). COX- cyclooxygenase, LOX- lipoxygenase, EPOX- epoxygenase, HpETE- hydroperoxyeicosatetraenoic, CYP450- cytochrome 450, EETs- epoxyeicosatrienoic acids

Studies have shown that the both n-6 and n-3 fatty acids have an ameliorating effect on rheumatoid arthritis (Simopoulos, 2002a). It is also reported that fish oil helps to clinically improve tender joint notches and morning stiffness (Guesens *et al*., 1994). It is reported that for a period of 24 weeks the γ-

linolenic acid daily supplementation reduces the number of swollen joints (Leventhal *et al*., 1993).

The studies have confirmed that the risk of progress of some types of cancer, particularly prostate, breast and colorectal cancer was modulated by consumption of dietary PUFA (Willet, 1994). It is reported that the growth of tumor can be reversed or inhibited by essential fatty acids and their metabolites (Bougnoux, 1999). Moreover, during the past decades, among Japanese women the increased intake of vegetable oils rich in n-6 fatty acids and decreased consumption of fish has been reason for increased breast cancer rates (Lands *et al*., 1990).

The studies related with consumption of omega-3 PUFAs have shown to improve the well-being of cancer, cardiac diseases and few mental illnesses (Riediger *et al.,* 2009). The membrane fluidity which is dependent on level, composition and the percentage of PUFA and level of membrane cholesterol, regulate the proper functioning and development of various tissues like retina and brain (Simopoulos, 1991). PUFA also plays a significant role in newborn development and growth (Broadhurst *et al.*, 2002; Lauritzen *et al*., 2004). It also helps for the intellectual growth of the brain (Matorras *et al*., 1999). PUFA, mainly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) existing in marine sources, have been found to have therapeutic effects against numerous oxidative stress related complication (Simopoulos, 2002b; Ruxton *et al.*, 2004; Zamaria, 2004; Siriwardhana *et al*., 2012).

Therefore, it is hypothesized that the omega 3 long chain polyunsaturated fatty acids might be helpful in the prevention of all the diseases caused due to oxidative stress that leads excess generation of free radicals and lipid peroxidation.

1.6 Review of literature:

The much clearer understanding of the association among diabetes, insulin deficit, and lipid/ lipoprotein metabolism are provided by the study of plasma lipoproteins in patients with diabetes, which gets altered due to defects in insulin action and hyperglycemia (Goldberg, 2001).

The chronic inflammation due to the infiltrate of inflammatory cells like macrophages, lymphocytes, etc. (Kotanko *et al*., 2006). In diabetes also contribute to the cardiovascular disease (Packard and Libby, 2008) and diabetic kidney disease (Hou *et al*., 2004) and are largely responsible for tissue destruction. The presence of lipid deposits has been associated with diabetic kidney disease (Wang *et al*. 2005) and the increased nerve lipid peroxidation results in diabetic neuropathy (Nickander *et al.* 1996).

Given the inflammatory basis of atherosclerosis, it suggests that type 1 diabetes may accelerate atherosclerosis (Kanter *et al*., 2008). Cholesterol carried by lipoproteins through the blood goes to the artery wall and builds up in huge quantities, resulting in, inflammation, tissue damage and fibro-proliferative scarring. The heart attack and stroke are caused by the blood clots due to failure of tissue in the inner part of the artery wall (Guyton, 2006).

Alterations in lipids related to increased cardiovascular risk are also associated with central obesity in diabetes type 1 (as well as type 2 DM). Individuals with type 1 diabetes are as much at risk for hypercholesterolemia as the non-diabetic population (Donaghue *et al*., 2009). The risk of individual developing cardiovascular disease (CVD) is relatively high in diabetic patient and is the primary cause of death in people with either both type of diabetes. The association between the diabetes and the possibility of atherosclerotic plaque formation are not completely clear; the association between the two is profound. The utmost factor of health care expenditures in people with diabetes accounts for CVD (Flower, 2008).

The patients with diabetes show the abnormal lipid profile mainly because the deficiency of insulin, which regulates the steps involved in lipid metabolism (Rivellese *et al*., 2004). In the severe form of diabetes disturbance in the fat metabolism is also frequently observed, which is marked in two ways, by the appearance of the acetone bodies in the urine and due to the tendency to accumulate in the blood as fat and other derivatives of the fatty acids, such as phospholipids and also cholesterol (Bloomgarden, 2003). The long chain PUFA deficiency has been observed in diabetes (Tikhonenko *et al*., 2010).

There are lots of studies has been carried out regarding the beneficial effect of fish oil on disease and in maintaining health. There is a large body of data that strongly suggests that the highly unsaturated omega-3 fatty acids (EPA

and DHA), possibly other minor fatty acids, are multipotent compounds which are the active molecules of fish oil (Nestel, 2000).

Studies carried out to check the effect of fish oil supplementation on D-galactosamine induced hepatitis (Pujari and Roy, 2012) and alcoholic liver damage (Surliker and Roy, 2011) showed that PUFAs present in fish oil shows a hepatoprotective effect, reduces the inflammatory response due to damage and diminishes the intoxication.

Last two decades of research mainly focused on the potentiality of fish oils to counter atherosclerotic vascular diseases which has been supported by a progressively lengthy list of purposes, certainly associated with lipid metabolism but others facilitated through non-lipid mechanisms (Nestel, 2000). The eicosapentaenoic and docosahexaenoic acid fatty acids supplementation has been proven to have positive physiological effects on cardiovascular system, insulin resistance and lipid metabolism (Devarshi *et al*., 2013) and exhibits depletion of low density lipoprotein and rise of the high-density lipoprotein cholesterol (HDL-C) in vitro (Foulon *et al*., 1999) and prevents lipid peroxidation (Montrol *et al*., 2000) and increase concentration of glutathione reductase, activity of glutathione peroxidase involved in oxidative defence (Iraz *et al*., 2003).

In diabetes ineffective scavenging of reactive oxygen species is led by alteration in the endogenous free radical scavenging defense, resulting in oxidative damage and tissue injury (Shradha and Sisodia, 2010). In vitro studies of endothelial cells revealed that the glucose-mediated inhibition of nitrous oxide

production is decreased by eicosapentaenoic acid (Okuda *et al*., 1997). Supplementation of dietary omega-3 fatty acid also increases activity of superoxide dismutase by reducing free radical generation (Erdogan *et al*., 2004). Fish oil elevates arachidonic acid (omega-6) in cell membrane phospholipids, which results in a reduced free radical production (Hussein *et al*., 2014).

The polyunsaturated fatty acids content increase in the cell membrane helps to improve the insulin receptor quantity and binding and action of insulin, while saturated fatty acids decrease the same (Field *et al*., 1990). The decreased insulin sensitivity is associated with the reduced content of long chain PUFAs, in specificarachidonic acid, and the total percentage of C20-C-22 polyunsaturates (Das, 2002).

Numerous studies have been also demonstrated the hypo-triglyceridemic effects of dietary fish oils in controlling cardiovascular disease (Harris, 1989; Nestel, 1990). In addition, reports suggest that consumption of dietary fish oil may lower blood pressure in hypertensive patients, decreases blood viscosity, and reduces platelet aggregation showing anti-atherogenic properties (Knapp and FitzGerald, 1989). Fish oil rich with omega-3 PUFAs particularly EPA and DHA are associated with prevention of inflammation (Calder, 2006) and several metabolic diseases (Gillies *et al*., 2012).

The experimental animal models are one of the best strategies used to comprehend the pathophysiology of any disease (Rees and Alcolado, 2005) and to develop the drugs for its treatment (Chatzigeorgiou *et al*., 2009). In the prior few

years of diabetes mellitus studies and testing anti-diabetic mediators that include manipulations like chemical, surgical and genetically, several animal models have been developed (Etuk, 2010). A survey of the literature reveals that rats and the mice are the prime animals for diabetes study. The animal models seem indispensable for elucidating the aspects of diabetes. Several drugs are used for the induction of diabetes, but the cytotoxic alloxan and streptozotocin glucose analogues are known to cause permanent diabetes and are the most prominent diabetogenic chemical agents (Lenzen, 2010). Insulin producing beta cells found in the pancreas were destroyed by alloxan and reduction product, dialuric acid, which undergo dismutation to hydrogen peroxide (H_2O_2) and more extremely reactive hydroxyl radicals formed by alloxan selectively by establishing a redox cycle with the formation of superoxide radicals (Rohilla and Ali 2012). Thus, it has been noted that alloxan injection induce disease like an insulin-dependent type 1 diabetes and all the morphological characteristics of beta cell damage are distinctive for a necrotic cell death (Lenzen, 2008).

1.7 Rationale and Objectives of the study:

DM is perhaps the world's fastest growing metabolic disease and one of the major causes of illness and death (Grundy *et al*., 1999). Scientific proof suggests that with aggressive treatment with exercise, diet and blood glucose control the morbidity and mortality associated with diabetes can be reduced. Regardless of all the improvement in diabetic therapy, recent research suggest that the commonly prescribed insulin therapy for type 1 diabetes and drugs for type 2 diabetes may delay the process but can't cure it, it is not helpful in preventing morbidity and death due to microvascular and macrovascular complications

associated with diabetes. The literature survey reveals the increasing need of safe, effective therapies for diabetes, which is a major challenge (Nyenwe *et al*., 2011).

In diabetic patients several mechanisms, like alterations in lipid metabolism (Bayens, 1991), imbalance between production of ROS and the antioxidant defense mechanism (Matough *et al*., 2011), changes in the inflammatory pathway (Donath *et al*., 2003) play an important role in increased oxidative stress. As insulin plays a central role in the regulation of lipid metabolism, in poorly controlled type 1-diabetes, ketoacidosis, hypertriglyceridemia and reduced HDL concentrations commonly occurs (Goldberg, 2001). The dyslipidaemia associated with diabetes type 1 is likely a major risk factor for the accelerated macro-vascular diseases like atherosclerosis and coronary heart disease seen in diabetic patients (Goldberg, 2001; Guy *et al*., 2009). Therefore, regulating the lipid metabolism through the dietary supplementation or drug might be helpful to enhance the insulin secretion and thus ameliorate the diabetes and its related complications.

Over the past 10–15 years of research have demonstrated the health benefits associated with consumption of omega-3 PUFA rich fish oil and it has been the utmost effective way of omega-3 intake (Lavie *et al*., 2009; Devarshi *et al*., 2013). It has also been confirmed by various studies that dietary supplementation with fish oils has hypo-triglyceridemic effects (Jacobson, 2008; Saraswathi *et al*., 2009). In addition, consumption of fish oil has been reported to have hypo-triglyceridemic effects, in hypertensive patients it decreases the blood pressure, reduce blood viscosity, and decrease platelet aggregation, signifying that

dietary fish oil consumption may have anti-atherogenic properties (Caterrina and Zampolli, 2006). The omega-3 enriched fish oils are associated with the infant development (Lauritzen *et al*., 2004) and prevention of several metabolic disorder (Gillies *et al*., 2012) including cancer (Li and Hu, 2009), alcoholic liver disease (Surliker and Roy, 2011), hepatitis (Pujari and Roy 2012), mental diseases (Peet *et al*., 1996; Timonen *et al*., 2004), to attenuate inflammation (Calder, 2006), oxidative stress (An *et al*., 2009) and useful for treatment of cardiovascular disease (Garman *et al*., 2009).

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Goa being a coastal state, high intake of marine fish rich with omega 3 PUFA is common in the state, still diabetes on the rise in the state (according to report Times of India, 14 Nov 2013). However, often there are a controversy, conflicts and lacunae on consumption of which fish offers better protective effect. The effects of fish oil consumption are unclear in diabetes and there is no clear conclusion about the net benefits of administering omega-3 PUFA to diabetic mice (Tsuduki *et al*., 2011). Hence, the present work was designed to test the **hypothesis that the dietary fish oil rich with EPA and DHA might also help in reversing the metabolic changes and prevent the tissue damages due to prolonged diabetes**. To achieve this proposed research work objective were as followed:

1. To find out the effect of fish oil on the diabetes induced mice.
2. To find out changes in lipid profile and lipid metabolism of different tissues of mice (both diabetic and fish oil treated diabetic mice).
3. To study molecular mechanisms of fish oil by measuring the expression of some genes in diabetic mice

2 Materials and Methods:

2.1 Collection of materials:

2.1.1 Chemicals and Reagents: The chemicals and reagents used in this study were analytical grade. Chemicals like organic solvents (chloroform, methanol, benzene, ether, etc.), acids (sulfuric acid, hydrochloric acid, acetic acid etc.) and salts (potassium chloride, sodium chloride, sodium hydroxide, copper sulphate, sodium sulphate, potassium hydroxide etc.) were purchased from M/s., Sd-fine Chemicals or M/s., Hi-media. All the Standard chemicals like glycine, urea, albumin, tocopherol, ascorbic acid, adenosine triphosphate, cholesterol, tripalmitin, fatty acid methyl esters, etc. as well as the chemicals required for enzymatic analysis were obtained from M/s., Sigma Aldrich Chemicals USA. The Trizol reagent required for RNA extraction was procured from M/s., Life Technologies Corporation, Singapore, the one-step reverse transcriptase-PCR kit was purchased from M/s., Hi-media and the primers required for RNA expression study were obtained from M/s., Qiagen India.

2.1.2 Glass wares and Lab wares: General laboratory glass wares like pipette, beaker, conical flask, funnels, test tubes, measuring cylinder were purchased from M/s., Borosil and other plastic lab wares like plastic storage bottles, micro vials, centrifuge tubes and pipette stands etc. were purchased from M/s., Tarsons.

2.1.3 Instruments: The precise instruments used for the study are TLC visualizing iodine chamber, Rotary evaporator, Shimadzu UV-visible spectrophotometer (UV 2450), nanodrop- spectrophotometer (ND 1000), PCR etc. were available in the department. GC-MS study was carried out in National

Institute of Oceanography, Goa. For the visualization of gel bands Gel-doc study was carried out in department of Botany, Goa University.

2.2 Biological Materials:

2.2.1 Collection and maintenance of experimental animals: From Sri Venkateshwara Enterprises, Bangalore, India the mice *Mus musculus* were acquired. The concern party has the licensed for the supply of live animals for laboratory uses from CPCSEA (No. 237). The mice were housed in an air condition room (22±2 °C) in the animal house of Department of Zoology, Goa University. All the experiments with mice were carried out as per the guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India". The prior permission for work was obtained from the Institutional Animal Ethics Committee (GU/Zoo/2013-14/03 dated 14/5/2013). "The animals were housed in polypropylene cages (42cm x 25cm x 15cm, each cage contained 6 mice) with stainless steel lid, having a provision of food and water". Animals were provided with paddy husk bedding, which was regularly changed to maintain hygiene. Commercial feed (Mfd. by Goldmohur Food & Feed Ltd., Mumbai, India) was used to feed the animals. This feed was procured from Sri Venkateshwara Enterprises, Bangalore, India. The mice were pellet fed with water ad libitum every day.

2.2.2 Collection of fishes and extraction of fish oil: The fishes (*Sardinella longiceps*/ *Clarias batrachus*/ *Rastrelliger kanagurta*) were collected from the market during pre-spawning / post spawning period (June-October) of the fishes when the lipid content of the fish were maximum. In the clean beaker 250g

minced flesh of fishes was taken to that 250ml of hexane (AR grade) was added and mixed thoroughly, covered with aluminum foil and kept in the sonicator bath (Elma transonic T460). Sonication was carried out for one hour with a stop of 5 minutes after every 15 minute. After each sonication using a clean glass rod the tissue was mixed well with the solvent. The extract was filtered out and stored. The residual flesh was mixed again with fresh 100ml of hexane and re sonicated for one hour. The two extracts thus obtained were pooled together and the solvent was completely evaporated, leaving no trace of its odor using vacuum evaporator. The extracted oil was stored in an amber color bottle. To prevent auto oxidation of the oil butylated hydroxyl toluene was added (1µmole /per liter).

2.3 Experimental Design:

2.3.1 Induction of diabetes*:* The Alloxan induced model of diabetes was used in the current study and is the most popularly used diabetic models that is capable of inducing type I diabetes mellitus in 95% of experimental animals with 1-2% mortality. Administration of alloxan to experimental animals, particularly destroys the beta cells, thus inducing hyperglycemia and produces a disease that is similar to diabetes type 1 (Rohilla and Ali, 2012).

After the initial acclimatization in the laboratory conditions for a period of two weeks, two months old male Swiss albino mice of uniform weight (23-30g) were selected for the present study. Animals are divided into two groups with 24 mice in each group; Group I: Control (C) is maintained as a non-diabetic group (with 20µl of intra-peritoneal saline injection) whereas the Group II : Diabetic (D) received intra-peritoneal injection of alloxan in 20µl of saline at the dose of

100mg/ kg body weight (Chougule *et al*., 2007). Diabetes was verified after 2 days by obtaining the blood samples from the tail tip vein of all experimental animals to determine the fasting blood glucose using glucometer (Accu check Go). The mice having blood sugar level higher than 250mg/dl were selected as diabetic and considered for further experiment. Uslu *et al*. (2009) developed experimental diabetic mice by injecting 150 mg/kg alloxan and repeated the injection 3 times with 48 hour intervals. It was observed that animals with this dose the mortality rate of the mice were more than 5%. Whereas at lower dose of 100mg/kg the mortality rate restricted within 2%, but the hyperglycemic condition was stable up to 6 days and then reverted back to normal within a couple of days. Therefore, hyperglycemic condition in the diabetic groups was maintained by repetitive injection of alloxan after every 5^{th} day for a period of one month with mortality less than 2%.

2.3.2 Dietary treatment with fish oil: Both the group I and II were again divided into four sub groups. Mice of one subgroup from both Control and Diabetic groups were maintained with normal pellet feed and labeled as NC and ND respectively, and other three subgroups from Control and Diabetic groups were fed with the same pellet feed, but freshly blended with 10% *Sardinella, Clarias* and *Rastrelliger* fish oils were labeled as SC, CC, RC and SD, CD, RD respectively. The 10% fish oil supplementation was selected for the study based on previous observation by Pujari *et al.* (2010). It may be noted that the commercial pellet feed used in the present work contains 5% lipid. The mice were fed with regular/ experimental diet for 30 days and were starved for an overnight before sacrificing. The fatty acid profiles and lipid profiles of the extracted fish oils are mentioned in Table A and Table B.

2.3.4 Work plan: To find the effect of supplementation of fish oil on diabetes induced mice biochemical parameters (free sugar, total carbohydrate, free amino acids, total protein, triglyceride and free fatty acids), antioxidants parameters (Vitamin C, Vitamin E, reduced glutathione, activities of superoxide dismutaseand catalase enzymes), lipid peroxidation parameter (Thiobarbituric acid reactive substance and activity of gamma glutammyl transpeptidase) in various tissues (serum, liver, kidney, heart and pancreas) were evaluated.

Table A: Lipid composition of fish oil extracted from *Sardinella longiceps, Rastrelliger kanagurta and Clarias batrachus* (µmole/ml of oil). Mean values of four replicate and their standard error were tabulated.

Lipid Parameters	***Sardinella longiceps***	***Rastrelliger kanagurta***	***Clarias batrachus***
Cholesterol	2.76±0.085	1.56±0.042	3.11±0.093
Phospholipid	47.8±1.04	21.05±0.44	20.76±0.39
Glycolipid	5.8±0.081	1.63±0.060	1.93±0.11
Triglyceride	39.8±0.48	21.1±0.41	23.1±0.28
Free fatty acid	69.1±0.41	67.03±0.41	67.6±0.64

Table B: Fatty acid composition (relative percentage) of oil extracted from *Sardinella longiceps*, *Rastrelliger kanagurta* and *Clarias batrachus.* Mean values of four replicate and their standard error were tabulated.

Type of Fatty acid	***Sardinella longiceps***	***Rastrelliger kanagurta***	***Clarias batrachus***
C14:0	8.66	10.92	11.54
C16:0	10.86	10.96	15.76
C16:1	7.08	8.07	8.03
C16:3	2.52	2.14	2.42
C18:0	6.81	6.73	7.27
C18:1	9.53	9.4	10.17
C18:2 n6	2.79	1.22	6.94
C20:0	0.21	1.26	-
C20:1	0.63	0.64	1.49
C20:3 n6	1.73	2.2	2.67
C20:4 n6	9.54	9.13	5.77
C20:5 n3	12.89	12.81	9.64
C22:5 n3	6.60	8.72	5.83
C22:6 n3	20.15	15.8	12.47
Total saturated	46.3	50.12	56.68
Total Unsaturated	53.7	49.88	43.32
Total n3	39.64	37.33	27.94
Total n6	14.06	12.55	15.38

Along with these, activities of alanine transaminase, aspartate transaminase, alkaline phosphatase, lactate dehydrogenase and acid phosphatase in these tissues have been measured. Besides, the concentrations of glycosylated hemoglobin, insulin in serum were also assessed. Histological evaluation of liver, kidney, heart and pancreas was carried out using light microscopy.

To find out the importance of lipid and lipid metabolism in diabetic mice the changes in serum lipoprotein along with lipid profiles and fatty acid profiles of liver, kidney, heart and pancreas tissues were evaluated. To understand the changes in the lipid metabolism due to induction of diabetes and ameliorative effect of intake of fish oil on diabetes the activities of HMG CoA reductase, HMG CoA synthase and Glucose-6-phosphate-dehydrogense were measured. Besides, the fluidity of phospho-liposomes prepared from the phospholipid of liver, kidney, heart and pancreas were recorded.

To understand the molecular mechanism of induction of diabetes and its vitiating effects on vital organs and ameliorative effects of fish oil we studied the expression of certain mRNA by one-step reverse transcriptase PCR. The gene expression of Ins1 and Ins 2 (Insulin), Glu (Glucagon) in the pancreas; GLUT2, GLUT4 (Glucose transporter), COX1, COX-2 (Cyclooxygenase or Prostaglandin-endoperoxide synthase) and SREBP1 (Sterol regulatory element-binding protein-1) in the liver; TGFβ (Transforming growth factor β) in the kidney; PPARα (Peroxisome proliferator-activated receptor alpha) in the heart were checked. The expression of some inflammatory genes like TNFα (Tumor necrosis factor alpha), IL1α (Interleukin-1 alpha) in liver, kidney and heart tissues were also monitored.

2.4 Histology (Light microscopy studies):

After opening the general viscera, liver, kidney, heart and pancreas were perfused thoroughly with phosphate buffer saline pH7.0. PBS was injected through the hepatic portal vein, left ventricle of the heart and renal artery in the respective tissue and the wash was drained by cutting the post cava vein, right atrium and renal vein respectively. The tissues were cut into small pieces

thickness of 1x1 mm and for 24 hour fixed in 10% formalin. After overnight, placing under running tap water, for dehydration tissues were transferred in 50% alcohol. Then passed through a series of ascending grades of alcohols, keeping it for 1 hour in each grade i.e.70 %, 80% and 90%, again passed in 100% alcohol with 3 changes, for each changes kept for 1 hour. The tissues were then placed in each jar of xylene for a total 45 minutes (3 changes, 15 minutes for each jar). Then the tissues were transferred to molten paraffin maintained for 1 hour each at 68˚C (3 changes). Using molds it was then made into blocks allowed to set overnight and transferred to a refrigerator (4˚C) for cutting. After 1 day, the blocks with good sections for the slide preparation were selected and sectioned (10μm) using a microtome. The slides were smeared with egg albumin and allowed to dry overnight. The slides were passed through 2 changes of xylene, and descending grades of alcohol (100%, 90%, 80%, 70%, 50%, 5 minutes in each) and then with distilled water and stained using hematoxylin after standardizing stain for 5 minutes. "It was strengthened with acid alcohol and then stained with eosin after washing with water, passed through ascending grades of alcohol, xylene and then mounted using DPX mountant (Arora and Prakash, 1998)". The slides were viewed under microscope (Olympus BX 41) for pathological changes.

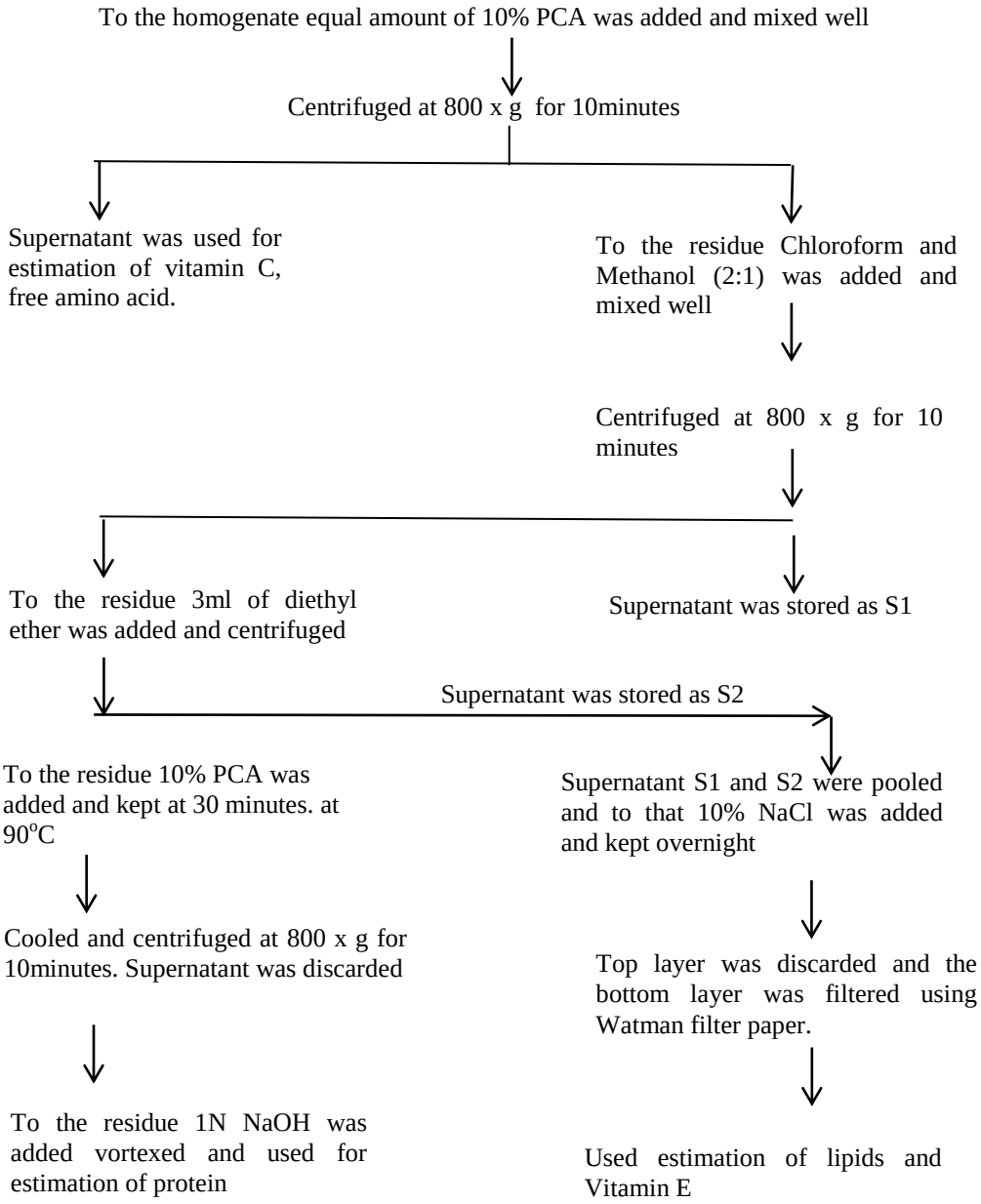
2.5 Biochemical estimation:

Mice, *Mus musculus* were sacrificed by dislocation of cervical. The blood was collected by moderate suction by the cardiac puncture. Before transferring blood into the appendorf tubes the needle was detached to avoid hemolysis (Chawla, 1999) for the collection of serum. The blood was allowed to clot completely at room temperature for half an hour for the collection of serum, then

the appendorf tubes were centrifuged at 4ºC for 800 x g for 10 minutes, serum thus separated was stored in (-) 20ºC for further biochemical analysis.

The general viscera were opened and various tissues like liver, kidney, heart and pancreas were collected. With phosphate buffer saline (PBS, pH 7.0) all these tissues were washed and 10% homogenate was prepared using PBS and divided into two parts. The one part of the homogenate was deproteinized by adding equal amounts of 0.3N barium hydroxide and 5% zinc sulfate, the mixture was vortexed and centrifuged for 10 minutes at 800 x g and the supernatant was stored for estimation of urea, total carbohydrates and free sugars (Roy *et al*., 1991). Similarly serum samples were also deproteinized by adding equal amounts of 0.3N barium hydroxide and 5% zinc sulfate for quantification of previously mentioned parameters. From the other part of the homogenate various biomolecules like free amino acid, protein, vitamin C, vitamin E, and lipid were extracted (Roy *et al*., 1991). The flow chart 1 represented precise outline of the extraction. For further quantification the extracts were stored in (-) 20ºC.

Flow chart 1: Extraction of various biomolecules from the tissue



2.5.1 Total carbohydrate**:** "*With the conc. H_2SO_4 carbohydrates are desiccated to procedure furfural, which condenses with anthrone to form a blue colored compound, intensity of which can be measured at 620nm".*

"1ml of deproteinized aliquot was made up to 1.0ml with distilled water and to that 4ml anthrone reagent (0.2gm/100ml of H_2SO_4) was added, in boiling water bath incubated for 10 minutes. The absorbance of the color formed was measured using suitable blank at 620nm (Carroll *et al*., 1956). The total carbohydrate content was measured with the help of total carbohydrate standard curve (100µg of glucose/ml)".

2.5.2 Free sugars: *The cuprous oxide is formed when the sugars are heated with alkaline copper reagent, which with arsenomolybdate reagent gives a blue colored complex, the intensity of which can be measured at 540 nm.*

1.0ml alkaline copper reagent was added to the 1.0ml deproteinized sample and incubated in the boiling water bath for 20 minutes. To the cooled mixture 1ml of arsenomolybdate color reagent was added and diluted with 7ml distilled water. The intensity of the color was read at 540nm against a suitable blank (Nelson, 1944). The concentration of free sugar was calculated using glucose standard curve, using 200µg/ml glucose as a standard solution.

2.5.3 Total protein: *Copper from alkaline copper reagent reacts with protein to form a protein complex. The tungstic acid from Folin Cio-Calteau reagent reacts with the amino acids from the complex, to form a blue color. The intensity of which can be measured at 690nm and directly proportional to the amount of tyrosine and tryptophan present.*

For protein estimation, 0.1ml of the tissue extract or 20µl of serum was diluted up to 0.5ml using distilled water. To this, 5ml of Lowry's reagent was added and at room temperature incubated for 15 minutes. 0.5ml of (1:2 dilutions)

Folin Cio-Calteau (commercially available) reagent was then added and the mixture was further incubated for a period of 30 minutes. The intensity of the blue colored complex was measured against a suitable blank at 690nm (Lowry *et al*., 1951). Quantification of the protein content of the sample was done with the help of a standard curve of bovine serum albumin (250µg/ml in 1N NaOH).

2.5.4 Free amino acid: *With free alpha amino group (NH_2-C-COOH) ninhydrine reacts and produces a purple color product. The intensity of the color depends upon the amount of amino acids present, which can be measured at 575nm.*

0.1ml of tissue extract or 20µl of serum was diluted with distilled water to make a final volume 1ml, to that 2ml of freshly prepared ninhydrine reagent was added and in boiling water bath kept for 10minutes and then cooled. By adding 50% ethyl alcohol the volume of the mixture was made up to 5.2ml and for 5 minutes was kept at room temperature. The intensity of color developed was measured at 575nm using proper blank. The quantification of amino acid (Moore and Stein, 1948) in the sample was done with the help of standard curve of glycine (100µg/ml in 5% PCA).

2.5.5 Triglycerides: "*The alkali hydrolization of triglycerides in the presence of iodate and arsenite ions forms glycerol which reacts with the chromotropic acid reagent to produce a brown colored compound, and intensity of the color can be measured at 570nm*".

"For complete solvent evaporation 0.05ml of lipid extract was kept in hot water bath. The lipid sample thus obtained was then hydrolyzed by adding 0.5 ml of 0.4% ethanolic sodium hydroxide at 60°C for half an hour and then to the mixture 0.5ml of 0.25N sulphuric acid was added. It was kept in hot water bath for 10 minutes to that with a gap of 10 minutes 0.1ml of 0.05M sodium per iodate and

0.1 ml of 0.5M sodium arsenate were added. The mixture was allowed to stand for additional 10 minutes. To that 5ml chromotropic acid reagent was added and kept in the boiling water bath for 30 minutes. The triglycerides were measured using suitable blank at 570nm (Kates, 1986). Quantification of triglycerides was done using standard curve of tripalmitin (3.2mg/ml in benzene)".

2.5.6 Free fatty acids: "*Free fatty acids react with copper reagent to form copper salt of the fatty acid which further forms a pale yellow coloured compound by reacting with diethyl dithiocarbamate of the colour reagent. The absorbance of which can be measured at 440nm*".

"For complete solvent vaporization 0.1ml extract of liquid was kept in hot water bath, to that 6ml of chloroform was added and mixed well and later 3ml of copper reagent was added. Two layers were formed. The upper layer was discarded carefully using a micropipette. To the lower layer 0.5ml of color reagent (0.1% sodium diethyl dithiocarbamate in n-butanol) was added. The intensity of the color developed was measured at 440nm (Anstall and Trujillo, 1965). Quantification of free fatty acid content was done with the help of standard curve of palmitic acid (1mg/6ml of benzene)".

2.5.7 Total cholesterol: "*Cholesterol reacts with ferric chloride reagent in acidic medium to form a brownish green compound and the intensity of which can be measured at 550nm*".

"0.05ml of lipid extract was dried by keeping in a hot water bath. To that, 3.0ml of glacial acetic acid and 2.0ml of ferric chloride reagent were added and incubated for 5 minutes at room temperature. The intensity of the color was measured at 550nm against a suitable blank (Kates, 1986). The amount of

cholesterol was quantified with the help of a standard curve of cholesterol (500µg/ml in benzene)".

2.5.8 Phospholipids: "*The charring at 300°C with an alcoholic magnesium nitrate solution converts the organic phosphate present in the phospholipids to inorganic phosphate. The ammonium phosphomolybdate formed by reaction of inorganic phosphate with ammonium molybdate is reduced by ascorbic acid to produce a blue colored complex. The intensity of the color can be measured at 820nm. Therefore, the amount of inorganic phosphate present in the sample is equal to the amount of phospholipids*".

"0.2ml of 10% alcoholic magnesium nitrite was added to 0.1ml of lipid extract, and charred at 300˚C till a complete white powder was formed with no trace of yellow color. The powder was dissolved in 1.5ml of 0.5N hydrochloric acid and to that 3.5ml of freshly prepared ammonium molybdate reagent was added and was kept in a water bath for 30 minutes at 60°C. The intensity of the color developed was measured against a suitable blank at 820nm (Kates, 1986). With the help of a standard curve of ATP (4µmole of ATP/ ml which equivalent to 12umole of phosphate /ml or 12µmole of phospholipid/ ml) the quantification of phospholipids was done".

2.5.9 Vitamin E: "*The ferric ions were reduced by tocopherol to ferrous ions which react with 2, 2'-dipyridine and produces red colored complex. The intensity depends on the concentration of tocopherols can be measured at 520nm*".

"1.5ml ethanol and 2.0ml of hexane were added to 0.5ml of tissue lipid extract or 20µl serum and centrifuged. The supernatant was evaporated at 80°C to dryness, to that 0.2ml of each 2, 2'-dipyridyl solution (0.5% in ethanol) and ferric chloride (0.2% in ethanol) were added. For 5 minutes the mixture was kept in the dark and then 4ml of butanol was added. The color developed was read against a suitable blank at 520nm (Baker *et al*., 1980). With the help of standard curve of

tocopherol (0.2µmole /ml in benzene) the concentration of vitamin E in the samples was quantified".

2.5.10 Vitamin C: "*In the presence of aniline and bromine ascorbic acid forms a complex with dinitrophenyl hydrazine to give a colored complex, intensity of which can be measured at 540nm*".

"With distilled H_2O the 0.1 ml of the tissue extract or 20µl of serum was made up to 2ml. To that 0.1ml of bromine water was added and then exactly after 10 seconds, 0.1ml of 3% aniline and 0.5ml of 2% Dinitro phenylhydrazine (in 9N H_2SO_4) were added and the samples were incubated in the boiling water bath for 5 minutes, then kept in the ice bucket and 5ml of 70% sulfuric acid was added. The intensity was read against a suitable blank at 540nm (Roe and Kuether, 1943). The vitamin C content present in the samples was quantified by using a standard curve of ascorbic acid (100µg/ml)".

2.5.11 Thiobarbituric acid reactive substances (TBARS): "*Malondialdehyde forms a compound with thiobarbituric acid and produces a colored complex. The absorbance of the color can be measured spectrophotometrically at 535 nm. Trichloro acetic acid precipitates out the other lipoproteins and evades from interfering in the reaction. Only water soluble malondialdehyde reacts with thiobarbituric acid and produces a colored complex*".

"The tissue was homogenized in 0.1M Tris HCl buffer pH 7.5 to prepare a 2% homogenate. To 0.1ml of tissue homogenate or 10µl serum 2ml of TBA-TCA-HCl reagent was added and for 15 minutes kept in boiling water bath, then cooled and centrifuged at 3000rpm at room temperature for 10 minutes. The absorbance of the supernatant collected was measured against a suitable blank at 535nm (Niehaus and Samuelsson, 1968). With the help of standard curve of

malondialdehyde (10ηmole/ml) thiobarbituric acid reactive substance concentration was estimated".

2.5.12 Reduced glutathione (GSH): "*GSH reacts with 5, 5'- dithiobis, 2-nitrobenzoic acid to yield a yellow colored compound. The intensity of the color can be measured spectrophotometrically at 412nm*".

"Tissue was homogenized with 5% TCA to prepare a 2% homogenate and by centrifugation at 300rpm for 5 minutes. 2ml of 5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent was added to the 1.0 ml of diluted tissue extract or serum to make the final volume 3.0ml (Moron *et al*., 1979). At 412nm absorbance was read using a suitable blank. With the help of a standard curve of reduced glutathione (0.2µmole/ml in 5% TCA) reduced glutathione content of the samples was quantified".

2.6 Estimation of serum Glycosylated hemoglobin and Insulin:

Serum glycosylated hemoglobin and insulin levels in mice were assessed by enzymatic assay and chemiluminesence ELISA procedure respectively. The samples were given to the Ashwini Pathology laboratory, Goa. The concentration of glycosylated hemoglobin and insulin were measured using the diagnostic kit manufactured by Crystal Chem, USA and ALPCO Mouse Insulin ELISA kit, USA respectively.

2.6.1 Determination of serum glycosylated hemoglobin: In each well of microplate 112µl of Reagent CC1a and 48µl of Reagent CC1b was added and mixed well by repeated pipetting. To the each well 25µl lysate of sample, calibrator, or control was added and mixed well by repeated pipetting. The microplate was placed in an incubator (37ºC) for 5 minutes. Absorbance was

measured using a microplate reader at A700 and noted as 0 second readings. To the microplate again 70µl of Reagent CC2 was added and mixed well by repeated pipetting. The increase in absorbance was measured after 3 minutes and noted as 180 second readings. Using linear graph paper, the HbA1c calibration curve was constructed. Mouse HbA1c concentrations in the samples were inserted using mean absorbance values and the calibration curve for each sample. The HbA1c concentration is expressed directly as %HbA1c.

2.6.2 Determination of serum Insulin: The 96-well microplate coated with a monoclonal antibody specific for insulin was used. 10µl of each standard, control, and sample were pipetted out into their respective wells. 75µl of working strength conjugate was added into each well. Microplate was covered with a plate sealer and incubated for 2 hours at room temperature, shaking at 700-900 rpm on a microplate shaker. The contents of the wells were decanted and the microplate was washed 6 times with 350µL of working strength wash buffer per well using a microplate washer. Alternatively, the wells were filled with working strength wash buffer using a wash bottle equipped with a wash nozzle or manual washer. The wells were soaked for 1 minute. The microplate was inverted to discard the liquid and firmly taped the inverted microplate on absorbent paper towels. The wash and soak procedure was repeated 2 more times, for a total of 3 washes. After the final wash, any residual wash buffer and bubbles from the wells was removed by inverting and firmly tapping the microplate on absorbent paper towels. 100µl of working chemiluminescent substrate was pipetted into each well. In a microplate reader the microplate was placed capable of reading the luminosity of the wells. The microplate was analyzed at 1 minute after the addition of the chemiluminescent substrate for not more than 20 minutes. The plate was read

using a 1 second integration time. The concentration of insulin is expressed as ng/ml of serum.

Sensitivity and Precision:

The sensitivity of the assay was 0.06 ng/ml. The percentage coefficient of variation (CV%) within run precisions (intra assay) was recorded between 2.8-4.9%. Similarly, the CV % between the run precision (inter-assay) was recorded between 2.6-4.3%. The % cross reactivity of the antisera was recorded <0.01 for Human C-peptide, Mouse C-peptide1and 2, Rat C-peptide1and 2, Human IGF1 and 2, Mouse IGF1 and 2.

2.7 Lipid and fatty acid profiling:

From a crude tissue lipid extract (extracted from tissues as mentioned in flow chart1) using chloroform, acetone and methanol solvents for column chromatography neutral lipid, glycolipid and phospholipid were fractioned respectively. Further each fraction was separated by thin layer chromatography (TLC) into different fractions using different solvent systems. Hexane: diethyl ether: acetic acid (80:20:1.5, v/v) for further fractionation of neutral lipid; chloroform: methanol: acetone: acetic acid (40:25:3:7, v/v) for fractionation of glycolipid and chloroform: methanol: water (65:25:4, v/v) for fractionation of phospholipid were used.

On the pre coated TLC Silica gel 60 plates (20x20cm), obtained from M/s., Merck, Germany 50µl of each lipid fraction was spotted, using a Hamilton syringe. Till the complete run the plate was put in the solvent chamber containing specific solvent mixture depending upon the fraction to be separated (Kates,

1986). The dried plate was placed in an iodine chamber and the spots were viewed. The spots were identified against authenticated standards run along with the sample.

"Each one of these spots / fractions were scraped and analytically quantified. All acylglycerides, namely triglycerides, diglycerides and monoglycerides were estimated by chromotropic acid reagent. Free cholesterol and esterified cholesterol were assessed by ferric chloride reagent. Free fatty acids were assayed by sodium dithiocarbamate reagent. All glycolipid and phospholipids spots were estimated by anthrone reagent and ammonium molybdate reagent respectively (Pujari, 2010)".

Fatty acid profiling: Total lipid was subjected to saponification by adding 0.7 ml of 10 *N* KOH in water and 5.3ml of methanol. The tube was incubated in a 55°C water bath for 90 minutes with every 20 minutes vigorous handshaking for 5 seconds to properly permeate, dissolve, and hydrolyse the sample. After cooling below room temperature, 0.58 ml of 24N H_2SO_4 (in water) was added and mixed by inversion and precipitated K_2SO_4 was incubated again at 55°C in water bath for 90 minutes with every 20 min handshaking for 5 seconds. After FAME synthesis, the tube was cooled under cold tap water. To that 3ml of hexane was added and the tube was vortex-mixed for 5 minutes. The tube was centrifuged for 5 minutes in a tabletop centrifuge and the hexane layer with the FAME, was stored in a GC vial (O'Fallon *et al*., 2007) analyzed using "GC-MS equipped with FID detector and using a fused silica capillary column 30m x 25 mm1D x 0.2µm thickness. The column was initially maintained at 150°C for 5 minutes, increased by 12°C/minutes to 160°C and next by 3°C/minutes to 220°C

and there it was kept isothermal for 10 minutes. Injector and detector ports were maintained at 220 °C and carrier gas (nitrogen) pressure was maintained at 18psi (Pujari, 2010)".

2.8 Serum lipid profiles:

The serum lipid profiles were measured by using the diagnostic kit manufactured by Crest Biosystems, Coral Clinical System, Goa, following the method of Trinder, (1969).

2.8.1 Serum triglyceride: "*The lipoprotein lipase hydrolyzes triglyceride to glycerol and free fatty acids. The glycerol formed in the presence of glycerol kinase with ATP forms glycerol 3 phosphate the enzyme glycerol phosphate oxidase, oxidizes glycerol 3 phosphate to form hydrogen peroxide, which further reacts with 4-aminoantipyrine and phenolic compound by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. The intensity of which is directly proportional to the amount of triglycerides present in the sample*".

The test solution (marked as T) contained 1ml of working reagent and 0.01ml of serum sample. The standard solution (marked as S) contained 1ml working reagent and 0.01ml of triglyceride standard (conc. 200mg/dl). The solutions were mixed properly and for 5 minutes incubated at 37˚C. The standard and test sample absorbance was measured against a suitable blank at 505nm within 60 minutes.

Triglyceride content present in the sample was calculated using the formula: Triglycerides in mg/dl = (Abs T/ Abs S) * 200

2.8.2 Serum cholesterol: *Free cholesterol is formed due to hydrolysis of esterified cholesterol by cholesterol esterase. Oxidation of free cholesterol forms hydrogen peroxide which further reacts with 4-amino antipyrine and phenol peroxidase acts as catalyst and forms quinoneimine dye complex (red colored). The intensity of the red color is directly proportional to the amount of cholesterol present in the sample.*

"The test solution (marked as T) contained 0.01ml of serum sample and 1ml of working reagent. The standard solution (marked as S) contained 0.01ml of cholesterol standard (200mg/dl) and 1ml working reagent. The solutions were mixed properly and incubated for 5 minutes at 37˚C. The absorbance of samples (standard and test) was measured against a suitable blank at 505nm".

Cholesterol content present in the sample was calculated by using the formula: Cholesterol in mg/dl = (Abs T/ Abs S) * 200

2.8.3 Serum HDL cholesterol: "*Polyethyleneglycol content in the precipitating reagent when reacts with serum, precipitates all the LDL and VLDL. In the supernatant the HDL remains and is assayed as a sample for cholesterol using the cholesterol reagent*".

"To the 0.1ml of serum sample 0.1ml of precipitating reagent was added. The contents were mixed well and incubated at room temperature for 5 minutes. The samples were then centrifuged at 800 x g for 10 minutes to obtain a clear supernatant. The clear supernatant was assayed for cholesterol as mentioned above using HDL standard (25mg/dl). Using the formula the concentration of HDL cholesterol was calculated:

HDL Cholesterol in mg/dl= (Abs T/ Abs S) * 25*2

(where, 2 is a dilution factor due to the de-proteinization step)"

2.8.4 Serum LDL cholesterol and VLDL cholesterol:

From the data obtained of serum triglyceride, cholesterol and HDL cholesterol, "serum LDL- and VLDL- cholesterol were calculated using the following formula:

VLDL cholesterol = TG /5

LDL cholesterol= total cholesterol- [HDL-cholesterol+ VLDL-cholesterol]"

2.9 Estimation of Membrane fluidity:

100µg of phospholipid sample and 1µg of 1mM 1,6-diphenyl-1,3,5-hexatriene (i.e. 100:1 v/v ratio) was taken in a cryo-vial and dried completely under nitrogen gas. The vial was rehydrated with 2ml of 0.05 M HEPES buffer (pH 7.4), then vortexed and sonicated for 1 minute at 37^0C. The above step repeated for 2 times and filtered using 200um membrane syringe filter to obtain Small Unilaminar Vesicles (SUV) of same diameter phosphor-liposome. Fluorescence polarization of DPH was monitored using Shimadzu spectroflurometer (RF 5301 PC) equipped with a polarizer. Excitation and emission wavelength were set 360nm and 440nm respectively to 5nm slit width each. Fluorescence intensities were recorded by keeping the excitation polarizer at 0^o with emission analyzer at 0^o (LH) and 90^o (LV) and again excitation polarization at 90^o with emission analyzer at 0^o (VH) and 90^o (VV) with time programmer from 0 minutes to 30 minutes with an interval of 2 minutes at 37°C. Using the formula by Kleinfeld *et al*. (1981) fluorescence anisotropy value was calculated.

P= [VV-(LV * VH/LH)] / [VV+ (LV *VH/LH)]

r=2P/ (3-P)

LH= Excitation polarization at 0° with emission analyzer at 0°

LV= Excitation polarization at 0° with emission analyzer at 90°

VV= Excitation polarization at 90° with emission analyzer at 90°

VH= Excitation polarization at 90° with emission analyzer at 0°

VH/LH= Gaussius factor i.e. sensitivity of the detection system for vertically and horizontally polarized light.

2.10 Enzyme activity analysis

2.10.1 Alanine amino transferase [EC 2.6.1.2]: " *α-Ketoglutaric acid reacts with alanine and undergoes transamination reaction, in which α-ketoglutaric acid receives an amino group of alanine. As a result α-ketoglutaric acid becomes glutamic acid and alanine becomes pyruvate. Pyruvate reduces 2, 4-dinitrophenyl hydrazine to dinitrophenyl hydrazone. In alkaline medium, hydrazone produces a colored complex. The absorbance of which can be measured at 540nm*".

0.5ml of ALT substrate was incubated at 37°C and to that 0.1 ml of 5% tissue homogenate (in 0.01M phosphate buffer, pH 7.0) or diluted serum 0.5ml was added and for 15minute incubated to assess the activity enzyme. A suitable enzyme blank was also prepared by taking 0.5ml of substrate and 0.1ml of phosphate buffer. "By adding of 0.5ml dinitrophenyl hydrazine reagent (DNPH) the reaction was stopped and was mixed thoroughly, for 20 minutes kept at room temperature (25°C ± 5°C). To this 5ml of 0.4N NaOH was added to develop the color. The intensity of color developed was measured at 540nm using the suitable enzyme blank (Reitman and Frankel, 1957). With the help of a standard curve of pyruvate (220µg/ml) the product formed (pyruvate) during this reaction time was quantified. The enzyme activity was expressed as µg of pyruvate formed / minute reaction / mg of enzyme protein. Protein content in the enzyme was estimated as described earlier (2.5.3)".

2.10.2 Aspartate amino transferase [EC 2.6.1.1]: "*α-Ketoglutaric acid reacts with aspartate and undergoes transamination reaction by receiveing amino group from aspartate α-ketoglutaric acid becomes α-ketoglutaric acid becomes glutamic acid and alanine becomes oxaloacetic acid. Oxaloacetic acid reduces 2, 4-dinitrophenyl hydrazine to dinitrophenyl hydrazine which produces a colored*

complex in alkaline medium. The intensity of the color can be measured at 540nm".

"The activity of aspartate amino transferase was assayed using AST substrate by following the same method mentioned above (Section 2.5.1) (Reitman and Frankel, 1957). The activity of the enzyme was quantified with the help of a standard curve of sodium oxaloacetate (220µg/ml)".

2.10.3 Lactate dehydrogenase [EC 1.1.1.27]: "*LDH converts Lactate to pyruvate, generating NADH from NAD. Pyruvate reacts with dinitrophenyl hydrazine (DNPH), to form dinitrophenyl hydrazone. The formation of dinitrophenyl hydrazone gives a measure of LDH concentration and can be measured after alkaline dilution at 440nm".*

1.0ml of buffered substrate was incubated at 37°C for 5 minutes to that 0.2ml of NAD^+ solution and 0.02ml of 5% tissue homogenate in 0.2M glycine buffer, pH 7.0 or serum was added. "The mixture was incubated at 37°C for exactly 15 minutes. To that 1.0ml of DNPH reagent was added and the mixture was kept at room temperature (25±5°C) for 15 minutes. To the mixture 10ml of 0.4N NaOH was added. Mixed thoroughly and absorbance was measured at 400 nm against suitable blank (King, 1959). Enzyme activity was quantified with the help of a standard curve of pyruvate. Protein content in the enzyme was estimated as described earlier (Section 2.5.3). LDH activity was expressed as IU/mg protein".

2.10.4 Alkaline phosphatase [EC 2.6.1.2]: "*The enzyme in the acid medium splits off the colorless phosphate group from the substrate to form p-nitrophenol. In alkaline conditions this is converted to p-nitrophenoxide ions, which shows yellow color. The intensity of the color developed is directly proportional to the enzyme present in the sample and can be measured at 405nm".*

"To assay the enzyme activity, to the pre incubated (37°C) mixture of 2.7 ml of 0.2M glycine buffer (pH 7.0), 0.2 ml of substrate (freshly prepared) 0.1ml of 5% tissue homogenate (prepared in glycine buffer) or diluted serum was added. Simultaneously to prepare an enzyme blank 2.7ml buffer, 0.2ml substrate and 0.1ml of buffer were used. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 5ml of 0.25N NaOH. The intensity of the products of this reaction (p-nitrophenol) was measured at 405nm (King and Armstrong, 1934). Using the p-nitrophenol standard curve (250µg/ml) the amount of the p-nitrophenol released by the action of alkaline phosphatase was quantified. Protein content in the enzyme was estimated as described earlier (Section 2.5.3). The enzyme activity was expressed as (IU/mg protein)".

2.10.5 Acid phosphatase [EC 3.1.3.2]: "*The enzyme acts on p-nitrophenyl phosphatase in citrate buffer (pH4.9) to cleave the phosphate from p-nitophenol phosphate. The liberated nitrophenol produces yellow colored complex under alkaline conditions which can be measured at 405 nm. Intensity of nitrophenol is measured after diluting it in 0.1N sodium hydroxide*".

To the 1.0ml of working p-nitrophenol phosphate solution (substrate) 0.2ml of tartarate solution (1.5g of tartaric acid was dissolved in 0.02M citrate buffer, pH4.9) was added and incubated at 37°C for 5 minutes. "To that 0.2ml of 5% tissue homogenate in citrate buffer or diluted serum was added, mixed and incubated at 37°C for 30 minutes. To that 4.0ml 0.1 N NaOH was added, mixed thoroughly and after 5 minutes read O.D at 405nm against suitable blank (Andersch and Szczypinski, 1947). The standard was prepared using 30mM/dl nitrophenol standard (4.173mg/dl). The enzyme activity was expressed as (IU/mg protein)".

2.10.6 Catalase [EC 1. 11. 1. 6]: "*The decomposition of hydrogen peroxide into molecular oxygen and water is catalyzed by catalase. The remaining H_2O_2 in the samples, which was not decomposed by the enzyme, reacts with dichromate to give a blue precipitate of perchromic acid. The precipitate which unstable is then decomposed by heating to give a green colored stable compound. The intensity of the green color can be measured at 620nm*".

In tubes, 1.5ml of phosphate buffer (0.01M, pH 7.0) and 0.4ml of substrate (0.2M H_2O_2) were taken and incubated for 5 minutes at 37°C. In one tube 0.1ml phosphate buffer was added to prepare enzyme blank and in remaining sets 0.1ml of 10% tissue homogenate (prepared in phosphate buffer) or diluted serum was added to assay the enzyme activity. "This reaction mixture was incubated for 15 minutes at 37°C and then stopped by adding 2.0ml of dichromate acetic acid reagent. The test tubes were kept for 10 minutes in a boiling water bath, mixed well and the intensity of the color was measured against reference blank at 620nm (Sinha, 1972). Protein content in the enzyme was measured as described earlier (Section 2.5.3). With the help of a reference curve of hydrogen peroxide (2µmole/ ml) the enzyme activity was quantified and expressed as µmoles of H_2O_2 consumed / min/mg protein".

2.10.7 Superoxide dismutase [EC 1.15.1.1]: "*The dismutation of the superoxide (O_2^-) into hydrogen peroxide and molecular oxygen was catalyzed by superoxide dismutase (SOD). Illumination of riboflavin in existence of oxygen and electron donors like methionine or EDTA generates a flux of superoxide anions. The reduction of Nitroblue tetrazolium chloride by superoxide anions produces a blue formazan compound the intensity of which can be measured at 560nm*".

"To diluted serum or 0.1ml tissue homogenate (5% homogenate was prepared in ice cold 10mM of potassium phosphate buffer, pH 7.0, and it was brought down to 2.5% with 50mM potassium phosphate buffer, pH 7.0) then centrifuged. The clear supernatant was used for the assay) 2.9ml of freshly

prepared SOD substrate was added. Enzyme blank was prepared by adding 0.1 ml 50mM potassium phosphate buffer, pH 7.0 instead of samples. Illumination was carried out in the aluminium foil lined box fitted with 15V fluorescent lamp for exactly 10 minutes. The reaction was stopped by switching off the source of illumination. The intensity of the color was read at 560nm (Beauchamp and Fridovich, 1971). Enzyme activity was quantified with the help of a standard curve of SOD (400µg/ml). Protein content in the enzyme was estimated as described earlier (Section 2.5.3). SOD activity was expressed as IU/mg protein".

2.10.8 Gamma glutamyl transpeptidase [EC 2.3.2.2]: "*Gamma-glutamyl transpeptidase (GGT) is primarily a liver enzyme which catalyzes the separation of the gamma-glutamyl unit of glutathione (GSH) and gamma-glutamyl related compounds. Gamma-glutamyl transpeptidase acts upon L-γ glutamyl p-nitroanilide. The glycyl glycine acts as an acceptor for the glutamyl moiety. The sodium nitrite in an acidic medium diazotises p-nitroaniline cleavage product to form a purple colored product intensity of which can be measured at 570 nm*".

"To 1.7ml buffer mixture (120 mM Tris- HCl, 4µM Sodium nitrite, 12mM $MgCl_2$, 90mM glycyl glycine, pH 7.0), 0.2ml GGT substrate (48mM of L-γ glutamyl p-nitroanilide dissolved in 150mM hydrochloric acid) was added and warmed for 5 minutes at 37°C. To this 0.1 ml 5% tissue homogenate (prepared in the above mentioned buffer) or diluted serum was added. To prepare enzyme blank, instead of sample 0.1ml of glycyl glycine buffer was added. After incubation at 37°C for 15 minutes, by addition 2ml of glacial acetic acid the reaction was stopped. The intensity of the color was read against a reference blank at 570nm (Fiala *et al*., 1972). Using L-γ glutamyl p-nitroanilide reference curve (10µg/ml) the enzyme activity was quantified. Protein content in the enzyme was estimated as described earlier (Section 2.5.3). The enzyme activity was expressed as IU/mg protein".

2.10.9 3-Hydroxy-3-Methylglutaryl-CoA Reductase [EC1.1.1.34]: "*HMGR (3-hydroxy-3-methylglutaryl-CoA reductase) is a transmembrane glycoprotein placed on the endoplasmic reticulum. The four-electron reduction of HMG-CoA to coenzyme A (CoA) to produce mevalonate is catalyzed by the enzyme. The assay is established on a spectrophotometric quantity of the decrease in absorbance, which signifies the oxidation of NADPH by the catalytic subunit of HMGR in the existence of the substrate HMG-CoA. The intensity of reduction of four-electrons of HMG CoA is equivalent to the production of mevalonate per unit time*".

The enzyme activity was measured with UV-visible spectrophotometer capable of exact measurement at 339nm with thermostat cuvette holder as described by Siedel (1983). In a cuvette the reaction was developed, contained 0.9ml reagent mixture, 0.02ml serum sample or 0.05ml 1% tissue homogenate whose final volume was made up to 1ml with distilled H_2O. The mixture was stirred and incubated in thermostat cuvette holder for 5minutes at 37°C to obtain a persistent base line rate at 339nm (ΔA1). Then 0.02ml of HMG-CoA was added. The decrease in intensity was monitored over a period of 5 minutes after addition of HMG-CoA (ΔA2). The catalytic concentration of the enzyme in the sample (µmole of HMG-CoA reduced per minute and liter) is calculated from the decrease in absorbance according to formula: "Enzyme catalytic unit IU= [79.4 * ΔA]/ [v *Δ t]. The final result was expressed in IU/mg of protein. Where, ΔA=ΔA1-ΔA2, 79.4 is excitation coefficient for NADPH, v is the sample volume in ml and t is the time of reaction in minutes".

2.10.10 3-Hydroxy-3-Methylglutaryl-CoA Synthase [EC 2.3.3.10]: "*By activating the methyl group of an acetylated cysteine 3-hydroxy-3-methylglutaryl synthase catalyzes the formation of a carbon–carbon bond. On acetylation of the enzyme by the substrate acetyl-CoA it forms coenzyme A (CoASH), leads to condensation with acetoacetyl-CoA and produces the HMG-CoA product*".

The reaction was developed in cuvette and the enzyme activity was measured with UV-visible spectrophotometer capable of exact measurement at 300 nm with thermostat cuvette holder as described by Miziorko (1985). The standard assay mixture contained 0.5ml of 0.2M Tris-HCl buffer (pH8.2) containing 0.2mmole EDTA, 0.05ml of 1mM acetoactyl CoA (pH 4.5) and 0.03ml serum or 0.08 ml 1% tissue homogenate in Tris-Hcl buffer whose final volume was adjusted to 1ml. For 5 min at 37°C the enzyme is incubated in cuvette the base line absorbance (ΔA1) at 300nm is obtained earlier to initialization of the reaction by the addition of 0.02ml of 10mM acetyl CoA (pH 4.5). The decrease in the absorbance (ΔA2) was monitored over a period of 5 minutes after addition of acetyl CoA. "The catalytic activity was calculated by the following formula: Enzyme catalytic unit IU=ΔA1-ΔA2/ Δ t (Where, t is the time of reaction in minutes). The final result was expressed in IU/mg of protein".

2.10.11 Glucose-6-phosphate dehydrogenase [EC 1.1.1.49]: "*Oxidation of glucose-6-phosphate utilizing $NADP^+$ and/or NAD^+ is catalyzed by regulatory enzyme glucose-6-phosphate dehydrogenase (G6PD). The enzyme possesses the dehydrogenase, which is unique with dual coenzyme specificity. Under conditions that are ideal for the particular coenzyme, the assay ratio observed for catalytic activity NAD/NADP is equal to 1.8. The velocity of reaction is determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD or NADP*".

In a cuvette 2.52ml of 100mM Tris HCl buffer (pH 9), 80µl of 100mM D-glucose-6-phosphate, 100 µl 20mM NADP and 200 µl 600mM $MgCl_2$ were taken and mixed well, monitored at 340nm until constant gassing spectrophotometer. To that cuvette 100µl of diluted serum or 1% tissue homogenate in 5mM glycine buffer was added. Immediately mixed by inversion and absorbance at A340nm (Anderson and Nordlie., 1968) was noted for about 5min for test and blank.

Instead of serum or tissue homogenate using distilled H_2O the blank was prepared. The enzyme activity is measured by using an equation:

"(A340nm/min test - A340nm/min blank)*V*df / 6.22*v

Where, V -total volume of the assay mixture (3 ml), df- dilution factor (for serum 5 and for tissue homogenate is 1), 6.22- milimolar extinction coefficient of NADP at 340nm, v- volume of sample (0.1ml). Finally the result was expressed U/mg protein"

2.11 Gene expression study:

2.11.1 RNA extraction from tissues: The RNA was extracted from the tissues following the method by Chomczynski and Sacchi (1987). Tissue was homogenized with TRIzol reagent (Ambion, life technologies, 1ml/ 50-100mg tissue) incubated in at room temperature for 5 minutes. To that 200µl chloroform per 1ml TRIzol reagent was added, mixed thoroughly by vigorous handshaking for 15 second and incubated at room temperature for 2-3 minutes. At 12,000 × g at 4°C for 15 minutes the homogenate was centrifuged. By angling the tube at 45° the aqueous phase of the sample is removed and pipetting the solution out into a new tube and to that 0.5ml of 100% isopropanol, per 1 ml of TRIzol® Reagent was added and incubated for 10 minutes at room temperature. At 12,000 × g the mixture was centrifuged for 10 minutes at 4°C. Supernatant was removed and with 1ml of 75% ethanol the pellet was washed. The sample was briefly vortexed, and then at 7500 × g for 5 minutes at 4°C centrifuged. Wash was discarded and RNA pellet was vacuum or air dry for 5–10 minutes. The RNA pellets were passed in RNase-free water or 0.5% SDS solution (20–50µl) up and down several times through a pipette tip for re-suspension. Incubated in a water bath at 55–60°C

for 10–15 minutes. The quality of isolated RNA was determined by using a Nanodrop spectrophotometer which provides an absorbance ratio at A260/A280 and concentration of RNA in the sample. The sample having an absorbance ratio >1.8 is used for further downstream application, or stored at –70°C.

2.11.2 cDNA synthesis: cDNA was synthesized using HiScript One Step reverse transcriptase-PCR cDNA synthesis kit (HIMEDIA).The amount of total RNA taken ranges from 200-500ng. The reaction mixture was prepared in the PCR tube as mentioned in the kit and placed in thermal cycler as per the program mentioned below so that cDNA synthesis was followed immediately automatically for PCR amplification.

A. cDNA synthesis and Pre-denaturation: 1-cycle of: 45-60°C for 30 minutes, 94°C for 2 minutes. **B.** PCR amplification: 40 Cycles of: Denaturation, 94°C for 15 seconds, Anneal, 55-60°C for 30 seconds, Extend, 68°C for 1 minute. **C.** Final extension: 1 Cycle of 68°C for 5 minutes.

mRNA expressions of Ins1, Ins2, Gcg, TGFβ, GLUT2, GLUT4, PPARα, TNF-α, IL-1α, SREBP1, COX1 and COX2 were studied as per conditions mentioned above. The β actin gene was used as the housekeeping gene to study the mRNA expression. The reference nucleotide sequences (RefSeq) for the specific genes were obtained from the National Center for Biotechnology Information (NCBI) database. The primers (Table C) used for present study were designed using Primer 3 software default parameters.

Table C: The sequences of the primers used and their temperature

Gene	Primers	Base pairs	Temperature
β-actin	LP5'TCTAGGCACCAAGGTGTG3' RP 5'TCATGAGGTAGTCCGTCAGG3'	460bp	56°C
IL1α	LP 5'GCTCGTCAGGCAGAAGTTTG3' RP 5' CCCAAGTGAATAGACTCCCGA3'	458bp	59°C
TNFα	LP 5'GGATTATGGCTCAGGGTCCA3' RP 5' GCTCAGCTCCGTTTTCACAG3'	455bp	58°C
TGFβ1	LP 5'AGGGCTACCATGCCAACTTC3' RP 5' CCACGTAGTAGACGATGGGC3'	168bp	53°C
Ins1	LP 5'AACCACCAGCCCTAAGTGAT3' RP 5'CCCACACACCAGGTAGAGAG3'	200bp	55°C
Ins2	LP5' CCTCAGGGACTTGAGGTAGGA3' RP5'TTCATTGCAGAGGGGTAGGC3'	460bp	56°C
Gcg	LP 5'AATGAAGACAAACGCCACTCA3' RP5'TCCCAAGTGACTGGCACGA3'	456bp	59°C
GLUT2	LP 5'ATCTTCACGGCTGTCTCTGT3' RP 5'ACCTGGCCCAATCTCAAAGA3'	198bp	58°C
GLUT4	LP5'TGGGAGGGAGCCTTTGGTAT3' RP5'GAATCTAGAGGCCTCGGTGC3'	320bp	57°C
COX1	LP 5'CACCAAGACCTGCCCCTATG3' RP 5'AATGCCAAGCCACGAAAACC3'	175bp	55°C
COX2	LP 5' GCCCTCTACAGTGACATCGA3' RP 5' GCCCTCTACAGTGACATCGA3'	170bp	55°C
SREBP1	LP 5'CCCACCTCAAACCTGGATCT3' RP 5' TATGGTAGACAACAGCCGCA3'	175bp	59°C
PPARα	LP5' AGACAAAGAGGCAGAGGTCC3' RP 5' CGATCAGCATCCCGTCTTTG3'	206bp	58°C

2.12 Statistical Analysis:

All the statistical calculations were done using online software and Statistical Package for the Social Sciences, Version 21 (IBM SPSS Statistic) and XLSTAT. All the recorded observation was tabulated in the form of mean of six samples and their standard errors by using the following formula:

"Standard Error = Standard deviation÷ √ n,

Standard deviation= $\sqrt{[(X-X')^2 \div (n-1)]}$

where, X = The arithmetic mean

X′ = the individual observation

n= number of observations

Student "t" test: Comparison test of the obtained data for each sample group (control, diabetic and treated) was calculated by using common student 't' test formula:

$$t = (X_1-X_2) \div \sqrt{[(SE1)^2+ (SE2)^2]}$$

where, X_1, X_2 = Mean of the two data which are under comparison.

SE1 and SE2 = Standard Errors of the two respective mean values.

Degree of freedom = (n1+ n2) - 2

At the probability level 0.05-0.0001 the tabulated-'t' values are used to tally the calculated "t" values (at respective degree of freedom, in our case it was 10). If the calculated value was higher than the tabulated value at the probability level 0.05 then the difference was considered significant".

Analysis of Variance Test (ANOVA): "The comparison of the different parameters between control and all the treated groups was also statistically verified by calculating 'F' value of ANOVA. This was calculated by calculating the variation between the columns and their sum of square on one hand and on another hand by calculating variation within the column and their sum of squares and finally 'F' value was calculated by calculating the values of both the variance that means (the ratio of the variance between the column and the variance within the column) at their respective degree of freedom. The calculated 'F' value was tallied from the statistical table (probability table) to find whether the variation

was significant or non-significant. The ANOVA has been performed using the Statistical Package for the Social Sciences, Version 21 (IBM SPSS Statistic)".

Tukey's Post Hoc test: " Tukey's HSD test is a post-hoc test, meaning that after an analysis of variance (ANOVA) test to maintain integrity Tukey's HSD (honest significant difference) is performed which is single-step multiple comparison process. It can be used to check raw data or in conjunction with an ANOVA (Post-hoc analysis) to find significantly different means from each other. The formula for Tukey's HSD: $M_1 - M_2 \div \sqrt{MS_n(\frac{1}{n})}$

M= treatment/ group mean, n= number per treatment/ group"

Appendix:

- **Alkaline copper reagent:**

  Solution1: a) 12.0g anhydrous sodium carbonate and 6.0g sodium potassium tartarate were dissolved in 125ml of distilled water.

  b) 2.0g of copper sulphate was dissolved in 25ml distilled water.

  Both the solutions (a) and (b) were mixed, 8.0g of sodium bicarbonate was added to it by stirring to prepare solution (1).

  Solution 2: 90.0g of anhydrous sodium sulphate was dissolved in 250ml of distilled water. Boiled to expel air and then cooled to room temperature to prepare solution (2).

  Now both the solutions (1) and (2) were mixed and the volume was made up to 500ml with distilled water.

- **Arsenomolybdate color reagent:**

  25.0g of ammonium molybdate was dissolved in 450ml of distilled water. 21ml of concentrated sulphuric acid was added slowly while mixing. To this, 3.0g disodium hydrogen arsenate (already dissolved in 25ml of water) was added. Mixed well, stored in amber coloured bottle at 37˚C for 48 hours for stabilization.

- **Lowry's reagent:**

  To 98.0ml of 4% sodium carbonate, 1ml of each 2% copper sulphate and 4% sodium –potassium tartarate were added and mixed.

- **Ninhydrine reagent:**

  Solution A: 1g of Ninhydrine powder in was added in 25ml of absolute alcohol.

  Solution B: 0.04g of stannous chloride was added in 25ml of citrate buffer of pH5.

  Both the solution A and B were mixed freshly.

- **Chromotropic acid reagent:**

  In 40ml of distilled water 0.448g of chromotropic salt was dissolved. Placed in ice bucket and to that 120ml of concentrated sulphuric acid and 60ml distilled water were added.

- **Copper reagent:**

  By mixing 1(M) acetic acid, 1(M) triethanolamine and 6.5% cupric nitrate in 1:9:10 ratios.

- **Ferric chloride reagent:**

  1.25g of ferric chloride was dissolved in 50ml orthophosphoric acid and stored in amber colored bottle. This serves as a stock solution. To prepare working standard 4ml of the stock solution was made up to 50ml with concentrated sulphuric acid (prepared freshly).

- **Ammonium molybdate reagent:**

  42% of ammonium molybdate solution in 1(N) sulphuric acid and 10% aqueous ascorbic acid solution were taken in the ratio of 6:1 in order to prepare this reagent (freshly prepared).

- **TBA-TCA-HCl Reagent:**

  0.37% Thiobarbituric acid, 15% Trichloroacetic acid and hydrochloric acid (0.25N) were mixed in 1:1:1 ratio to prepare this reagent.

- **5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent:**

  19.8mg of 5, 5'- dithiobis, 2-nitrobenzoic acid was dissolved in 100ml of 0.1% sodium nitrate solution to prepare this reagent.

- **ALT substrate:**

  0.532g alanine and 6.0mg α-ketoglutaric acid were added to 0.1ml of 1N NaOH prepared in 0.01 M phosphate buffer (pH 7.5). Final quantity was adjusted to 20ml with phosphate buffer (pH 7.5).

- **DNPH reagent:**

  10mg of dinitrophenyl hydrazine was added to 4.25ml conc. HCl and the final quantity was adjusted to 20ml with distilled water.

- **AST substrate:**

  0.35g aspartate and 6.0mg α-ketoglutaric acid were added to 0.1ml of 1N NaOH prepared in 0.01 M phosphate buffer (pH 7.0). Final quantity was adjusted to 20ml with 0.01M phosphate buffer (pH 7.0).

- **LDH buffered substrate:**

  125ml of glycine buffer (7.505g of glycine and 5.85g of sodium chloride were dissolved in about 900ml of distilled water and made up to 1 liter) and 75ml of 0.1N NaOH were mixed. To that 4.0g of lithium lactate was added, mixed and adjusted to pH 10.0.

- **NAD^+ solution:**

  10mg of NAD was dissolved in 2.14mg/dl (0.21M) NADH solution **(**0.71mg NADH was dissolved in 1.0ml of buffered substrate).

- **ALP substrate:**

  680mg of p-nitrophenyl phosphate was added to 8.0mg of $MgCl_2$ solution which was prepared by adding 30mg of $MgCl_2$ to 10ml glycine buffer.

- **Citrate buffer (pH4.9):**

  19.21g of citric acid, 180ml of 1N NaOH and 100ml of 0.1N HCl were mixed with 500ml of distilled water and final volume was adjusted to 1liter with distilled water after adjusting pH 4.9.

- **PNP working substrate solution:**

  0.4g of P-nitrophenyl phosphatase was dissolved in100ml of distilled water. Preparation of fresh working substrate: it was prepared by mixing equal parts of citrate buffer and PNP solution.

- **Dichromate acetic acid reagent:**

  5% potassium dichromate and glacial acetic acid were taken in 1: 3 ratios to prepare this reagent.

- **SOD substrate:**

  To 25ml of 0.2M potassium phosphate buffer(pH 7.0), 149mg of methionine, 4.93ml of nitroblue tetrazolium chloride (1mg/ml in 50mM potassium phosphate buffer, pH 7.0) and 0.63ml of riboflavin (1mg/ml in 0.05M potassium phosphate buffer, pH 7.0) were added and the volume was made up to 100ml with double distilled water.

- **HMG CoA reductase reagent mixture:**

  1ml water was mixed with 14ml phosphate buffer (0.1mol/l; pH 6.5), 0.4ml EDTA solution (0.1mol/l), 0.4ml cysteamine solution (0.1mol/l), 1.2ml 1% serum albumin and 1ml NADPH solution (5mmol/l)

3 Results:

The diabetes was monitored and confirmed by measuring the serum insulin and glycosylated haemoglobin concentrations. It is noted that due to induction of diabetes the concentration of insulin decreased significantly ($P<0.0001$) by 50% and 5 fold augmentation ($P<0.0001$) in glycosylated haemoglobin concentrations (Table 1). The supplementation of different fish oils viz. *Sardinella*, *Clarias* and *Rastrelliger* to the diabetic group of mice significantly ($F=145.85$ $P<0.0001$) elevated the concentration of serum insulin by 37-54% along with significant ($F=891.615$ $P<0.0001$) decrease in elevated level of glycosylated haemoglobin concentrations about by 45%.

3.1 Effect of supplementation of fish oil on concentration of biomolecules in non-diabetic and diabetic mice groups:

The changes in the concentration of biomolecules namely total carbohydrate, free sugar, total protein, free amino acid, triglyceride and free fatty acid in different tissues (serum, liver, kidney, heart, pancreas) of non-diabetic and diabetic mice groups due to supplementation with 10% fish oils (*Sardinella*, *Clarias*, *Rastrelliger*) for a period of one month are revealed in Tables 2-6.

Total carbohydrate: Supplementation of all three fish oils showed no significant changes in hepatic, cardiac and pancreatic concentration of total carbohydrate when compared to the control group of mice (NC group). However, about 25% increase in the concentration of total carbohydrate was noticed in serum ($F=2.542$ $P<0.05$) due to supplementation of fish oils (Table 3A). About 14% decrease ($P<0.0001$) in the concentration of carbohydrate in kidney tissue was noticed only

in SC and RC group of mice (Table 3A). Induction of diabetes by alloxan significantly (P<0.0001) enhanced the concentration of total carbohydrate in all liver, kidney, heart and pancreas tissues of mice by 45-80% (Tables 3B- 6B) along with 2.8 fold augmentation in serum total carbohydrate concentration (Table 2B). The supplementation of fish oil helped to markedly decrease the enhanced level of carbohydrate, approximately by 15-40% (P<0.0001) in the diabetic mice.

Free sugar: The supplementation of fish oils did not bring any significant alteration in the concentration of serum free sugar in non-diabetic mice, but significantly reduced (50%) the elevated level of serum free sugar in diabetic mice (Table 2). *Sardinella* oil supplementation brought down the level of free sugar, approximately by 15-20% in the liver, kidney, heart and pancreas tissue (P<0.05), whereas *Rastrelliger* oil brought down the concentration of free sugar about 20% in kidney tissue (P<0.05) of non-diabetic mice only (Table 2A). Besides 7.4-fold augmentation in the frees sugar level in serum (Table 2B), induction of diabetes also markedly elevated the concentration of free sugar, approximately by 60-100% in all other tissues like the liver, kidney, heart and pancreas as compared to NC group of mice (Tables 3B-6B). The dietary supplementation of *Sardinella* and *Rastrelliger* fish oils for 30 days to diabetic mice groups significantly (P<0.01-0.0001) brought down the elevated level of free sugar in all these tissues by 12-30%. However, the supplementation of *Clarias* oil brought down the free sugar level only in liver tissue of diabetic mice (Table 3B).

Total protein: Dietary supplementation of fish oils to the non-diabetic mice brought about 5-35% increase in the concentration of total protein (F=2.946-

4.884, $P<0.05$) in the liver and pancreas tissues only (Tables 3A, 6A). Induction of diabetes significantly ($P<0.0001$) brought down the level of total protein in all the tissues by 50-83% (Tables 2B-6B). The fish oil supplementation for the diabetic groups of mice resulted about 10% -2 fold elevation of reduced protein concentration ($P<0.0001$) in all the tissues (Tables 2B-6B) except in kidney tissue where supplementation of *Clarias* oil further brought down the level of protein.

Free amino acid: It is noted that the supplementation with fish oils significantly (F=10.454, $P<0.0001$) increased the free amino acid concentration in serum about by 50-60% (Table 2A), with about 30-40% decrease (F=5.810 $P<0.05$) in the heart (Table 5A) and with no significant changes in other tissues like liver, kidney and pancreas of non-diabetic mice. Induction of diabetes resulted in 70% - 7.6 fold augmentation ($P<0.0001$) of free amino acid concentration in all the tissues when compared to NC group of mice (Tables 2B - 6B). The augmented level of free amino acid in diabetic mice significantly lowered by 12-80% in all the tissues ($P <0.0001$) due to supplementation with fish oils for one month.

Triglyceride: The supplementation of fish oils to the non-diabetic groups of mice helped to decrease the concentration of triglycerides approximately by 25-30% (F=6.622, $P<0.0001$) only in liver (Table 3A). The concentration of triglycerides significantly ($P<0.001$-0.0001) augmented about 2 - 3 folds in all the tissues in alloxan induced diabetic mice (ND) when compared to control (NC) group of mice. The concentration of triglyceride significantly (F=3.27-36.42, $P<0.0001$) decreased by 10-65% in all the tissues upon supplementation of all the three fish

oils in the diabetic group of mice as compared to ND group of mice (Tables 2B-6B)

Free fatty acid: It is noted that the concentration of free fatty acid decreased significantly (P<0.0001) only in serum, liver and pancreas approximately by 10-50% of fish oils supplemented SC, CC and RC groups as compared to NC group of mice (Tables 2A, 3A, 6A). About 1.5 – 3 fold augmentation (P<0.0001) in the level of free fatty acid in all the tissues of diabetic mice (ND) was noticed as compared to NC group of mice (Tables 2B-6B). The fish oil supplementation for the diabetic groups of mice resulted 10-35% decrease (P<0.005-0.0001) in the elevated level of free fatty acids in all the tissues except in serum and heart tissues. Supplementation of *Clarias* oil in the diabetic mice did not show any significant change in the concentration of free fatty acids in serum and heart tissues (Tables 2B, 5B).

Table 1: Effect of dietary supplementation of fish oil on the concentration of serum insulin and glycosylated hemoglobin of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Parameters	NC	ND	SD	CD	RD	ANOVA
Insulin (ng/ml)	0.820 ±0.011	0.418 ±0.010	0.643 ±0.011	0.633 ±0.012	0.574 ±0.014	F=145.85 P<0.0001
Glycosylated hemoglobin (%)	4.28 ±0.34	21.1 ±0.52	11.6 ±0.54	12.01 ±0.53	11.7 ±0.29	F=891.65 P<0.0001

Table 2A: Effect of dietary supplementation of fish oil on the concentration of various biomolecules in serum of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	SC	CC	RC	ANOVA
Free sugar (mg/dl)	76.3 ± 11.5	84.3 ±13.1	97.3 ±6.7	91.5 ±2.7	F=0.919 NS
Total carbohydrate (mg/dl)	116.3 ± 4.5	149.2 ±13.2	145.03 ±11.6	146.03 ±6.59	F=2.542 *P*<0.05
Protein (g/dl)	2.94 ± 1.20	3.71 ±1.52	3.37 ±1.38	3.65 ±1.49	F=2.542 *P* <0.05
Free amino acid (mg/dl)	41.45 ± 5.11	62.9 ±4.22	65.7 ±5.28	74.8 ±2.24	F=10.454 *P* <0.0001
Triglyceride (mg/dl)	131.2 ±1.19	132 ±3.38	134.6 ±3.18	131.2 ±1.77	F=0.402 NS
Free fatty acid (g/dl)	4.61 ±0.17	3.80 ±0.26	4.17 ±0.24	3.87 ±0.12	F=3.172 *P* <0.05

Table 2B: Effect of dietary supplementation of fish oil on the concentration of various biomolecules in serum of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	ND	SD	CD	RD	ANOVA
Free sugar (mg/dl)	76.38 ± 11.5	566.1 ± 35.6	288.1 ± 15.4	298.2 ± 15.3	290 ± 8.5	F=78.05 *P* <0.0001
Total carbohydrate (mg/dl)	116.3 ± 4.5	336.9 ± 5.18	196.8 ± 5.02	244.2 ±10.5	228.7 ± 5.7	F=32.02 *P* <0.0001
Protein (g/dl)	2.94 ± 1.20	1.43 ± 0.58	2.38 ± 0.97	1.8 ± 0.73	2.24 ± 0.91	F=17.04 *P* <0.0001
Free amino acid (mg/dl)	41.45 ± 5.11	135.6 ± 3.13	64.95 ± 2.97	87.4 ± 3.55	64.5 ± 1.17	F=22.87 *P* <0.0001
Triglyceride (mg/ dl)	131.2 ± 1.19	398.2 ± 1.03	208.01 ± 1.51	227.7 ± 4.09	214 ± 2.93	F=24.98 *P* <0.0001
Free fatty acid (g/dl)	4.61 ± 0.17	8.64 ± 0.30	7.56 ± 0.34	7.72 ± 0.39	7.6 ± 0.23	F=25.85 *P* <0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 3A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in liver of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	SC	CC	RC	ANOVA
Free sugar	3.56 ±0.29	2.86 ±0.13	3.39 ±0.16	3.59 ±0.12	F=3.099 *P* <0.05
Total carbohydrate	3.74 ±0.19	3.14 ±0.26	3.68 ±0.14	3.20 ±0.22	F=2.220 NS
Protein	6.58 ±0.34	8.89 ±0.64	7.35 ±0.76	8.29 0.54	F=2.946 *P* <0.05
Free amino acid	0.151 ±0.010	0.119 ±0.010	0.147 ±0.013	0.125 ±0.09	F=2.043 NS
Triglycerides	2.97 ±0.22	2.05 ±0.11	2.24 ±0.16	2.20 ±0.11	F=6.622 *P* <0.001
Free fatty acid	6.75 ±0.31	5.50 ±0.108	5.13 ±0.22	5.16 ±0.20	F=11.104 *P* <0.0001

Table 3B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in liver of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	ND	SD	CD	RD	ANOVA
Free sugar	3.56 ± 0.29	7.05 ± 0.18	4.9 ± 0.19	5.88 ± 0.21	5.16 ± 0.20	F=33.9 *P* <0.0001
Total carbohydrate	3.74 ± 0.19	6.28 ± 0.15	4.89 ± 0.18	5.02 ± 0.27	4.90 ± 0.20	F=19.13 *P* <0.0001
Protein	6.58 ± 0.34	1.27 ± 0.101	2.20 ± 0.21	1.79 ± 0.35	2.18 ± 0.26	F=63.47 *P* <0.0001
Free amino acid	0.151 ± 0.010	1.30 ± 0.090	0.260 ± 0.011	0.447 ± 0.031	0.286 ± 0.011	F=116.27 *P* <0.00
Triglyceride	2.97 ± 0.22	8.77 ± 0.75	3.23 ± 0.48	3.97 ± 0.28	3.95 ± 0.16	F=29.97 *P* <0.0001
Free fatty acid	6.75 ± 0.31	10.79 ± 0.98	6.81 ± 0.16	7.38 ± 0.28	7.23 ± 0.17	F=11.9 *P* <0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 4A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in kidney of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	SC	CC	RC	ANOVA
Free sugar	3.18 ±0.108	2.49 ±0.106	2.97 ±0.22	2.64 ±0.13	F=4.343 *P* <0.05
Total carbohydrate	2.89 ±0.18	2.47 ±0.11	2.88 ±0.15	2.47 ±0.11	F=2.695 *P* <0.05
Protein	6.66 ±0.16	7.08 ±0.30	6.55 ±0.14	6.73 ±0.22	F=1.093 NS
Free amino acid	0.144 ±0.010	0.123 ±0.010	0.131 ±0.011	0.128 ±0.011	F=0.653 NS
Triglyceride	2.55 ±0.26	2.54 ±0.24	2.93 ±0.16	2.76 ±0.20	F=0.721 NS
Free fatty acid	5.16 ±0.27	4.50 ±0.39	4.70 ±0.53	4.66 ±0.17	F=0.582 NS

Table 4B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in kidney of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	ND	SD	CD	RD	ANOVA
Free sugar	3.18 ±0.108	5.36 ± 0.028	4.40 ± 0.02	5.09 ± 0.007	4.55 ± 0.0034	F=28.1 *P*<0.0001
Total carbohydrate	2.89 ±0.18	4.23 ± 0.25	3.17 ± 0.12	3.78 ± 0.12	3.59 ± 0.17	F=9.52 *P*<0.0001
Protein	6.66 ±0.16	2.01 ± 0.23	2.49 ± 0.13	1.67 ±0.11	2.21 ±0.23	F=138.3 *P*<0.0001
Free amino acid	0.144 ±0.010	0.484 ± 0.031	0.272 ± 0.025	0.265 ± 0.018	0.268 ± 0.012	F=33.7 *P*<0.0001
Triglyceride	2.55 ± 0.26	5.36 ± 0.85	3.73 ± 0.57	4.7 ± 0.72	4.18 ±0.25	F=3.27 *P*<0.0001
Free fatty acid	5.16 ± 0.27	8.2 ± 0.30	7.78 ± 0.73	6.77 ± 0.73	7.42 ± 0.33	F=9.411 *P*<0.001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 5A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in heart of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	SC	CC	RC	ANOVA
Free sugar	3.45 ± 0.17	2.70 ±0.22	3.32 ±0.102	3.28 ±0.13	F=4.016 *P*<0.05
Total carbohydrate	3.1 ± 0.11	2.74 ±0.19	3.10 ±0.11	2.96 ±0.14	F=1.260 NS
Protein	6.38 ± 0.27	7.27 ±0.50	6.17 ±0.81	6.63 ±0.40	F=0.795 NS
Free amino acid	0.145 ± 0.011	0.0895 ±0.011	0.0987 ±0.011	0.100 ±0.010	F=5.810 *P*<0.05
Triglyceride	3.74 ± 0.47	2.75 ±0.17	3.47 ±0.25	3.01 ±0.12	F=2.319 NS
Free fatty acid	3.60 ± 0.40	3.81 ±0.23	3.05 ±0.22	3.82 ±0.16	F=1.780 NS

Table 5B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in heart of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	ND	SD	CD	RD	ANOVA
Free sugar	3.45 ± 0.17	5.81 ± 0.22	4.86 ± 0.209	5.45 ± 0.13	5.07 ± 0.106	F=25.35 *P*<0.0001
Total carbohydrate	3.1 ± 0.11	5.19 ± 0.14	3.89 ± 0.14	4.12 ± 0.16	4.065 ± 0.19	F=22.01 *P*<0.0001
Protein	6.38 ± 0.27	1.10 ± 0.15	3.63 ± 0.14	1.67 ± 0.16	3.23 ± 0.12	F=140.5 *P*<0.0001
Free amino acid	0.145 ± 0.011	0.252 ± 0.017	0.174 ± 0.010	0.220 ± 0.014	0.218 ± 0.010	F=12.48 *P*<0.0001
Triglyceride	3.74 ± 0.47	7.02 ± 0.18	4.08 ± 0.87	5.36 ±0.37	4.88 ± 0.19	F=16.86 *P*<0.0001
Free fatty acid	3.60 ± 0.40	11.46 ± 1.7	7.58 ± 0.23	10.58 ± 0.23	8.09 ± 0.14	F=151.19 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 6A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in pancreas of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	SC	CC	RC	ANOVA
Free sugar	2.58 ± 0.11	2.09 ±0.13	2.35 ±0.12	2.28 ±0.12	F=2.739 *P*<0.05
Total carbohydrate	2.56 ± 0.16	2.27 ±0.12	2.47 ±0.11	2.35 ±0.10	F=1.011 NS
Protein	6.24 ± 0.13	7.15 ±0.24	6.60 ±0.12	7.09 ±0.24	F=4.884 *P*<0.01
Free amino acid	0.125 ± 0.010	0.124 ±0.0088	0.128 ±0.0080	0.123 ±0.0065	F=0.12 NS
Triglyceride	3.63 ± 0.28	2.79 ±0.49	3.97 ±0.38	3.64 ±0.19	F=1.970 NS
Free fatty acid	4.82 ± 0.34	3.24 ±0.13	4.05 ±0.12	3.67 ±0.34	F=6.559 *P*<0.05

Table 6B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg tissue) of various biomolecules in pancreas of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Biomolecules	NC	ND	SD	CD	RD	ANOVA
Free sugar	2.58 ± 0.11	4.17 ± 0.17	3.14 ± 0.13	3.75 ± 0.12	3.21 ± 0.16	F=18.54 *P*<0.0001
Total carbohydrate	2.56 ± 0.16	4.06 ± 0.16	3.04 ± 0.14	3.35 ± 0.11	3.07 ± 0.15	F=13.52 *P*<0.0001
Protein	6.24 ± 0.13	1.69 ± 0.12	2.68 ± 0.14	2.44 ± 0.107	2.50 ± 0.11	F=90.2 *P*<0.0001
Free amino acid	0.125 ± 0.010	0.403 ± 0.012	0.171 ±0.010	0.276 ± 0.011	0.205 ± 0.011	F=33.3 *P*<0.0001
Triglyceride	3.63 ± 0.28	9.00 ± 0.56	4.505 ± 0.33	5.26 ± 0.22	5.16 ± 0.14	F=36.42 *P*<0.0001
Free fatty acid	4.82 ± 0.34	6.28 ± 0.12	4.20 ± 0.15	4.44 ± 0.36	4.46 ± 0.14	F=11.164 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

3.2 Effect of supplementation of fish oil on the antioxidant and lipid peroxidation status in non-diabetic and diabetic mice groups:

Effect of supplementation of different fish oil on the concentration of antioxidant molecules like vitamin C, vitamin E and reduced glutathione, TBARS (lipid peroxidation product) along with the activities of antioxidant enzymes such as SOD and catalase and GGT (peroxidation enzyme) in different tissues (serum, liver, kidney, heart, pancreas) of non-diabetic mice and diabetic mice are revealed in Tables 7-10 and Figures 1-3 respectively.

Vitamin C: The supplementation of fish oil to the SC, CC and RC group of mice showed a 30-100% increase (P<0.05-0.001) in the concentration of vitamin C in all the tissues, except in the heart of the CC group of mice as compared to NC group of mice (Table 7A). The induction of diabetes resulted a significant (P<0.001-0.0001) decrease in concentration of vitamin C in all the tissues about by 55-65%. Supplementation of fish oils for the diabetic mice groups significantly (F=6.404-293.3 P<0.0001) increased the decreased level of vitamin C by 15-90% in all the tissues (Table 7B).

Vitamin E: The supplementation of *Sardinella* and *Rastrelliger* fish oil to the non-diabetic mice resulted about 14-30% increase (P<0.0001) in the concentration of vitamin E in serum, liver and kidney, whereas the *Clarias* oil supplementation showed significant (P<0.0001) increase in concentration of vitamin E in serum only (Table 8A). Induction of the diabetes led to decrease in the concentration of vitamin E significantly (P<0.0001) approximately by 20-45% in all the tissues. The *Sardinella* and *Rastrelliger* oil supplementation for the diabetic groups of mice led 10-55% elevations (P<0.0001) in the decreased level of vitamin E in all

the tissues. Whereas the *Clarias* oil supplementation brought a 40% increase (P<0.0001) in serum only (Table 8B).

Reduced glutathione: The concentration of reduced glutathione (Table 9A) significantly (F=9.861-33.340 P<0.0001) increased in all the tissue about by 30-70% upon supplementation of all three fish oils in non-diabetic groups of mice. The induction of diabetes in the ND group of mice markedly (P<0.0001) decreased the concentration of reduced glutathione level in all the tissues approximately by 40-60%. The supplementation of *Sardinella* and *Rastrelliger* fish oils in the diabetic group of mice significantly elevated the reduced glutathione concentration by 35-90% in all the tissues (F=11.42-43.91, P<0.0001). The elevation in the concentration of GSH in diabetic mice upon supplementation with *Clarias* fish oil is not significant (Table 9B).

Superoxide dismutase: The activity of the SOD is increased by 17-31% in serum, liver and kidney tissues (P<0.0001) upon supplementation of *Sardinella* and *Rastrelliger* fish oils to non-diabetic groups of mice (Figure 1A). Induction of diabetes significantly (P<0.0001) decreased the activity of enzyme by 30-45% in all the tissues. The supplementation of *Sardinella* and *Rastrelliger* fish oils to the diabetic mice (SD and RD group) significantly increased the activity of SOD by 15-40% in all the tissues (P<0.01-0.0001), whereas the *Clarias* fish oil supplementations (CD group of mice) increased the activity by 15-30% (P<0.01) in all the tissues except for serum (Figure 1B).

Catalase: The activity of the catalase increased significantly (F=456.1-5283 P<0.0001) in all the tissues by 40-70% (Figure 2A) upon supplementation of fish oils in non-diabetic mice. The alloxan induced diabetes resulted significant

(*P*<0.0001) decrease in enzyme activity by 5-30% in all the tissues. The supplementation of all the three fish oils for the diabetic groups of mice helped to elevate the decreased activity of catalase by 20-60% (F=197.787-1629.079 *P*<0.0001).

Thiobarbituric acid reactive substance: A significant 25-60% reduction (*P*<0.01-0.0001) in the concentration of TBARS in serum, liver and heart tissues was noticed upon dietary supplementation of *Sardinella* and *Rastrelliger* fish oils to non-diabetic mice, whereas *Clarias* oil supplementation brought down the TBARS concentration only in serum and liver tissues. The induction of diabetes significantly augmented the TBARS level in all the tissues approximately by 2 – 6 folds. Supplementation of fish oil significantly (F=15.77-40480 *P*<0.0001) brought down the elevated level of TBARS (Table 10B) in serum, liver, kidney and heart tissues by 10-70%.

Gamma glutamyl transpeptidase: The enzyme activity in all the tissues of *Sardinella* oil supplemented non-diabetic group of mice was decreased (*P*<0.0001) approximately by 15-30%, whereas 15-25% decreased (*P*<0.05-0.0001) activity was noticed with *Rastrelliger* oil supplementation in kidney, pancreas and heart and with *Clarias* oil supplementation in heart only (Figure 3A). The induction of diabetes significantly (*P*<0.05-0.0001) elevated the activity of GGT by 25-95% in all the tissues. The supplementation with respective fish oils to SD and RD group of mice significantly decreased the activity by 15-25% in all the tissues as compared with the ND (diabetic) group of mice. However, supplementation of *Clarias* oil in the diabetic mice reduced the activity by 5% (*P*<0.05) in liver and heart tissues only (Figure 3B).

Table 7A: Effect of dietary supplementation of fish oil on the concentration of vitamin C in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	SC	CC	RC	ANOVA
Serum (mg/dl)	144.7 ± 2.11	247.2 ±1.52	162.1 ±1.45	180.1 ±1.14	F=798.4 *P*<0.0001
Liver (mg/100mg)	1.80 ±0.25	3.56 ±0.40	2.52 ±0.29	2.82 ±0.41	F=4.365 *P*<0.05
Kidney (mg/100mg)	1.47 ±0.20	2.97 ±0.22	1.88 ±0.16	2.27 ±0.33	F=7.110 *P*<0.05
Heart (mg/100mg)	1.37 ± 0.14	1.96 ±0.14	1.57 ±0.11	2.16 ±0.075	F=8.365 *P*<0.001
Pancreas (mg/100mg)	1.30 ± 0.09	2.01 ±0.055	1.52 ±0.047	1.71 ±0.079	F=18.428 *P*<0.0001

Table 7B: Effect of dietary supplementation of fish oil on the concentration of vitamin C in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	ND	SD	CD	RD	ANOVA
Serum (mg/dl)	144.7 ± 2.11	66.2 ± 2	123.2 ±1.82	89.8 ± 1.19	92.3 ± 1.73	F=293.3 *P*<0.0001
Liver (mg/100mg)	1.80 ±0.25	0.708 ± 0.050	2.12 ± 0.28	1.48 ± 0.14	1.66 ± 0.12	F=7.46 *P*< 0.001
Kidney (mg/100mg)	1.47 ± 0.20	0.653 ± 0.075	1.402 ± 0.20	1.475 ± 0.072	1.35 ± 0.19	F=6.404 *P*<0.001
Heart (mg/100mg)	1.37 ± 0.14	0.625 ± 0.050	1.165 ± 0.18	1.163 ± 0.18	1.06 ± 0.15	F=7.526 *P*<0.0001
Pancreas (mg/100mg)	1.30 ± 0.09	0.541 ± 0.061	1.28 ±0.050	0.855 ± 0.067	1.18 ± 0.044	F=25.963 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 8A: Effect of dietary supplementation of fish oil on the concentration of vitamin E in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	SC	CC	RC	ANOVA
Serum (nmole/dl)	6300.3 ±12.2	7868 ±14.0	6756.1 ±13.5	7434 ±13.4	F=2751 *P*<0.0001
Liver (nmole/100mg tissue)	62.21 ±0.89	80.98 ±0.90	64.22 ±1.3	74.31 ±0.97	F=70.076 *P*<0.0001
Kidney (nmole/100mg tissue)	59.68 ±0.99	70.01 ±0.91	61.11 ±0.60	68.08 ±0.86	F=35.209 *P*<0.0001
Heart (nmole/100mg tissue)	55.74 ±1.5	61.2 ±0.77	59.5 ±2.1	61.1 ±3.2	F=1.430 NS
Pancreas (nmole/100mg tissue)	50.07 ±0.83	52.1 ±0.59	51.1 ±0.53	51.8 ±0.88	F=1.63 NS

Table 8B: Effect of dietary supplementation of fish oil on the concentration of vitamin E in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	ND	SD	CD	RD	ANOVA
Serum (nmole/dl)	6300.3 ±12.2	3330.3 ± 10.8	5217.8 ± 6.74	4713.5 ± 10.2	4776.5 ± 11.1	F=10602 *P*<0.0001
Liver (nmole/100mg tissue)	62.21 ±0.89	47.3 ± 1.18	56.7 ± 1.1	50.4 ± 1.02	52.4 ± 0.46	F=35.25 *P*< 0.0001
Kidney (nmole/100mg tissue)	59.68 ±0.99	44.46 ± 0.75	52.95 ± 0.72	49.79 ± 0.67	50.37 ± 0.64	F=64.324 *P*<0.0001
Heart (nmole/100mg tissue)	55.74 ± 1.5	42.3 ± 1.9	49.61 ± 2.6	47.88 ± 1.7	45.76 ± 2.2	F=9.323 *P*<0.001
Pancreas (nmole/100mg tissue)	50.07 ± 0.83	38.96 ± 0.88	47.21 ± 0.88	40.76 ± 0.88	44.93 ± 0.62	F=30.4 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 9A: Effect of dietary supplementation of fish oil on the concentration of reduced glutathione in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	SC	CC	RC	ANOVA
Serum (umole/dl)	2247.6 ± 80.4	3763.6 ±115	2913.1 ±139	2963.6 ±84.5	F=33.43 *P*<0.0001
Liver (umole/100mg tissue)	0.473 ±0.031	0.821 ±0.070	0.708 ±0.030	0.610 ±0.012	F=11.897 *P*<0.001
Kidney (umole/100mg tissue)	0.496 ±0.022	0.685 ±0.019	0.659 ±0.015	0.667 ±0.011	F=24.563 *P*<0.0001
Heart (umole/100mg tissue)	0.444 ± 0.012	0.772 ±0.046	0.737 ±0.027	0.753 ±0.025	F=26.283 *P*<0.0001
Pancreas (umole/100mg tissue)	0.330 ± 0.016	0.197 ±0.014	0.209 ±0.011	0.203 ±0.011	F=9.861 *P*<0.05

Table 9B: Effect of dietary supplementation of fish oil on the concentration of reduced glutathione in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	ND	SD	CD	RD	ANOVA
Serum (umole/dl)	2247.6 ± 80.4	1310.5 ± 112	1950.3 ± 50.6	1530 ± 74.0	1885.5 ± 77.6	F=11.42 *P*<0.0001
Liver (umole/100mg tissue)	0.473 ±0.031	0.217 ± 0.006	0.388 ± 0.022	0.250 ± 0.019	0.362 ± 0.013	F=26.51 *P*< 0.0001
Kidney (umole/100mg tissue)	0.496 ±0.022	0.207 ± 0.013	0.275 ± 0.032	0.299 ± 0.012	0.277 ± 0.031	F=17.4 *P*<0.0001
Heart (umole/100mg tissue)	0.444 ± 0.012	0.215 ± 0.011	0.250 ± 0.014	0.247 ± 0.013	0.296 ±0.019	F=43.91 *P*<0.0001
Pancreas (umole/100mg tissue)	0.330 ± 0.016	0.202 ± 0.009	0.430 ± 0.034	0.379 ± 0.019	0.321 ± 0.014	F=41.1 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 10A: Effect of dietary supplementation of fish oil on the concentration of TBARS in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	SC	CC	RC	ANOVA
Serum (umole/dl)	0.262 ± 0.0036	0.118 ±0.0027	0.107 ±0.0022	0.114 ±0.0016	F=2751.08 *P*<0.0001
Liver (umole/100mg)	0.268 ±0.018	0.192 ±0.016	0.206 ±0.011	0.204 ±0.016	F=4.400 *P*<0.01
Kidney (umole/100mg)	0.222 ±0.010	0.196 ±0.010	0.209 ±0.011	0.205 ±0.011	F=1.020 NS
Heart (umole/100mg)	0.247 ± 0.0075	0.174 ±0.016	0.215 ±0.014	0.181 ±0.015	F=5.763 *P*<0.01
Pancreas (umole/100mg)	0.330 ± 0.016	0.197 ±0.014	0.209 ±0.011	0.203 ±0.011	F=2.029 NS

Table 10B: Effect of dietary supplementation of fish oil on the concentration of TBARS in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Tissues	NC	ND	SD	CD	RD	ANOVA
Serum (umole/dl)	0.262 ± 0.0036	1.75 ±0.0052	0.505 ± 0.0018	0.584 ± 0.0055	0.558 ± 0.0026	F=40480 *P*<0.0001
Liver (umole/100mg)	0.268 ±0.018	0.581 ± 0.048	0.353 ± 0.012	0.516 ±0.0409	0.407 ± 0.020	F=15.77 *P*< 0.0001
Kidney (umole/100mg)	0.222 ±0.010	0.526 ± 0.026	0.362 ± 0.018	0.299 ± 0.019	0.327 ± 0.021	F=32.2 *P*<0.0001
Heart (umole/100mg)	0.247 ± 0.0075	0.581 ± 0.016	0.347 ± 0.014	0.221 ± 0.019	0.299 ± 0.015	F=92.02 *P*<0.0001
Pancreas (umole/100mg)	0.237 ± 0.012	0.450 ± 0.012	0.430 ± 0.034	0.450 ± 0.011	0.448 ±0.020	F=21.1 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

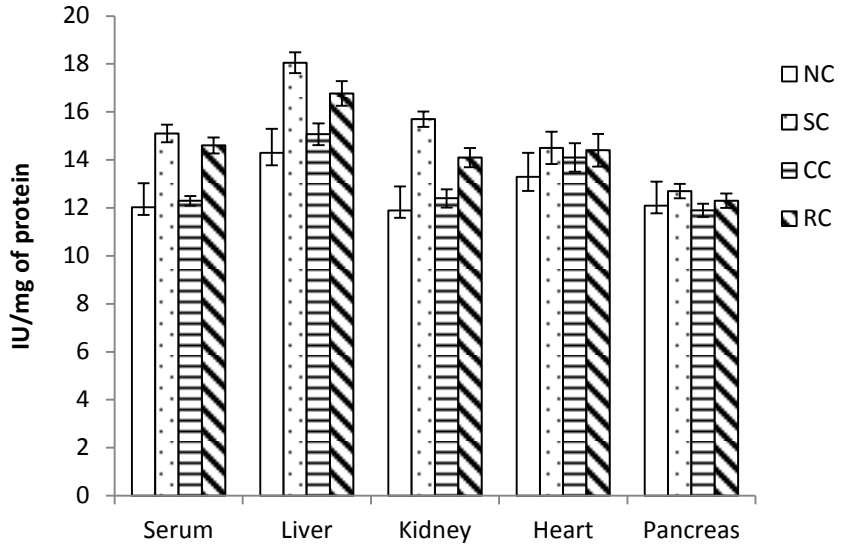


Figure 1A: **Changes in Superoxide dismutase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

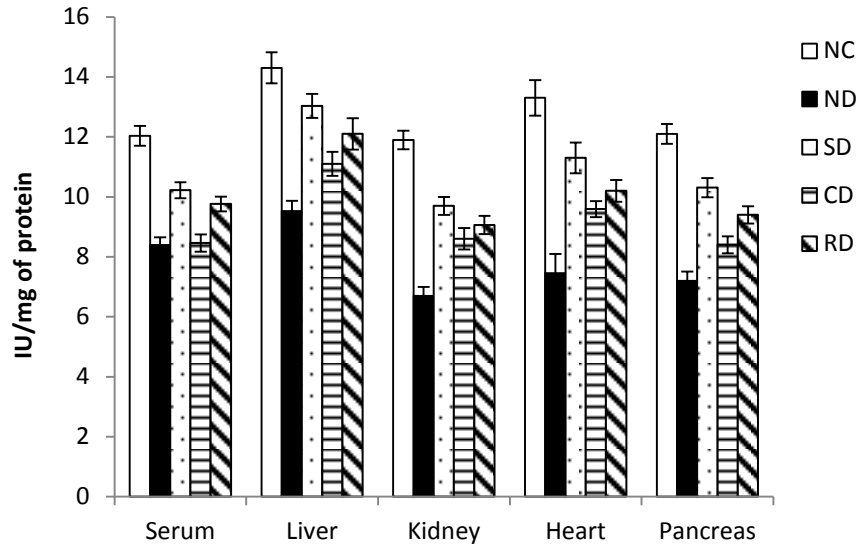


Figure 1B: **Changes in Superoxide dismutase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

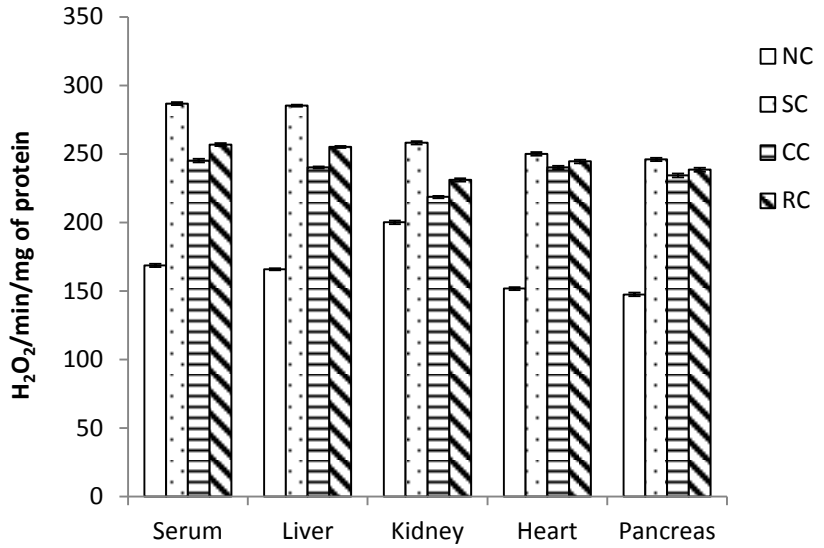


Figure 2A: **Changes in Catalase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

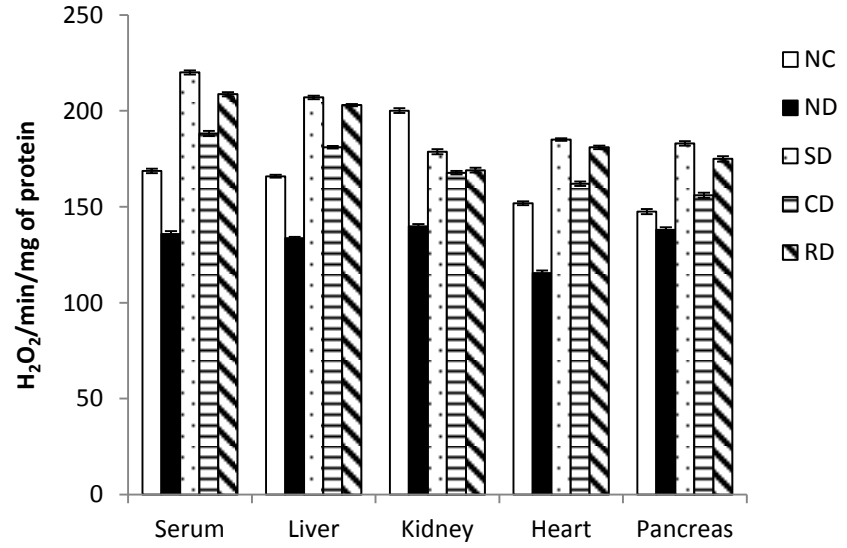


Figure 2B: **Changes in Catalase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

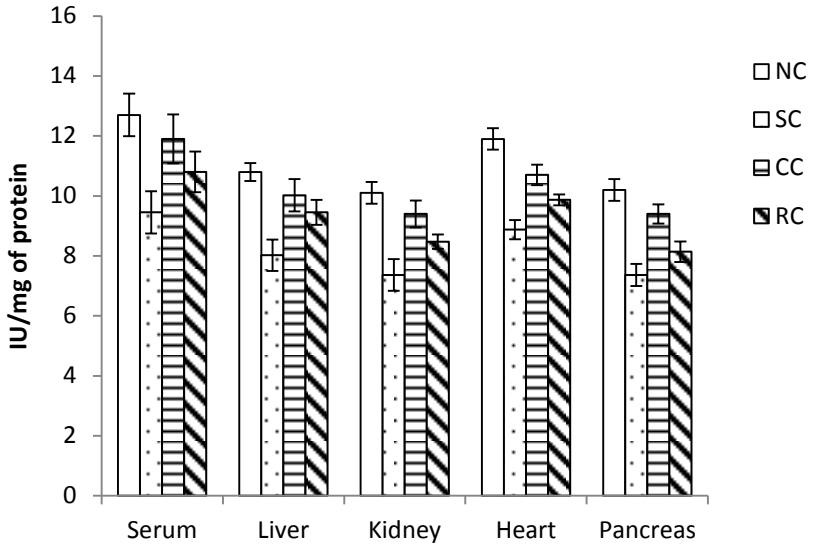


Figure 3A: **Changes in Gamma glutamyl transpepetidase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

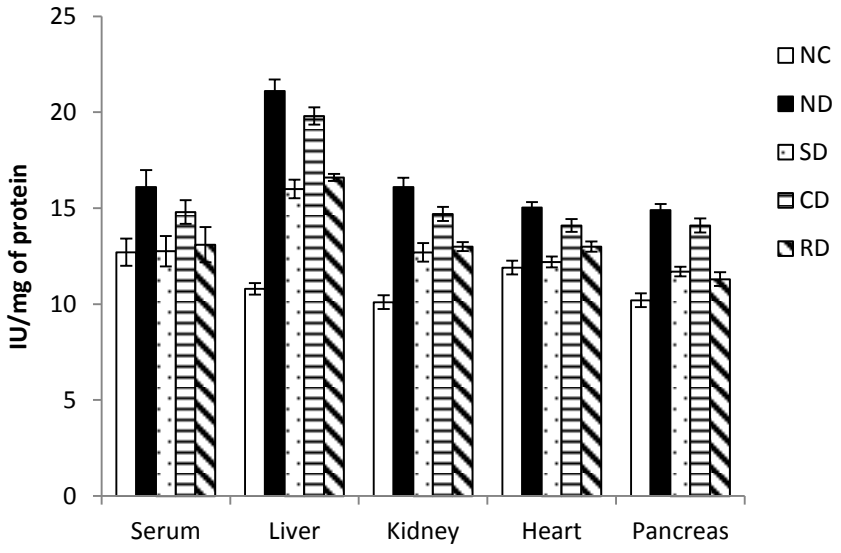


Figure 3B: **Changes in Gamma glutamyl transpepetidase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors**

3.3 Effect of supplementation of fish oil on activities of functional enzymes of non-diabetic and diabetic mice groups:

The changes in the activities of functional enzymes like ALT, AST, ALP, ACP and LDH in serum, liver, kidney, heart and pancreatic tissues of fish oil supplemented non-diabetic and diabetic groups were mentioned in Figures 4-8.

Alanine aminotransferase: A significant 10-35% reduction (P<0.01-0.0001) in the activity of ALT was noticed upon dietary supplementation of *Sardinella* oil in non-diabetic mice groups, whereas *Rastrelliger* oil supplementation brought down the activity (P<0.01) by 15-20% only in the liver and kidney. *Clarias* oil supplementation in non-diabetic mice showed no significant changes in the activity of this enzyme (Figure 4A). The induction of diabetes significantly (P<0.0001) increased the activity of enzyme by 75-100% in all the tissues. Upon supplementation of fish oil to the diabetic group of mice the activity was decreased significantly (P<0.001-0.0001) by 10-45% in all the tissues of except for heart tissue where supplementation of *Clarias* oil did not show any significant reductive effect (Figure 4B).

Aspartate aminotransferase: The activity of AST upon *Sardinella* fish oil supplementation in the non-diabetic group of mice decreased (P< 0.05-0.0001) approximately by 5-35% in all the tissues. However, supplementation of the other two fish oils helped to decrease (P< 0.01-0.0001) the enzyme activity by 5-30% in serum, heart and pancreas tissues only (Figure 5A). Alloxan induced diabetes resulted significant (P<0.0001) increase in activity of AST by 65-100% in all the tissues. The supplementation of fish oil for the diabetic groups, namely SD, CD

and RD groups of mice significantly (F=125.69-1716.5, *P*<0.0001) decreased the elevated activity of AST by 5-50% in all the tissues (Figure 5B).

Alkaline phosphatase: The supplementation of *Sardinella* oil to the non-diabetic SC group of mice decreased (*P*<0.05-0.0001) the enzyme activity approximately by 15-25% in all the tissues. It is can be also noted that in the *Rastrelliger* oil supplemented non-diabetic RC group of mice the activity was decreased by 15-20% in serum, liver and kidney only and *Clarias* oil supplementation to a CC group of mice reduced the activity of enzyme ALP about by 15% in kidney tissue only (Figure 6A). Up to about 2-fold augmentations (*P*<0.0001) in the activity of the enzyme was noticed upon induction of diabetes by alloxan (Figure 6B). Supplementation of fish oils for the diabetic groups of mice significantly (F=16.130-112.498 *P*<0.0001) brought down the augmented level of ALP by 15-35% in all the tissues.

Acid phosphatase: The *Sardinella* oil supplementation brought down (*P*<0.05-0.0001) the enzyme activity by 10-20% in all the tissues of non-diabetic group of mice, whereas about 10% decreased (*P*<0.01-0.0001) activity was noticed with *Rastrelliger* oil supplementation in kidney and heart tissues only. The *Clarias* oil supplementation showed 10-25% decrease in only serum, liver and heart tissue enzyme activity (Figure 7A). Induction of diabetes significantly increased the activity of ACP by 60-85% in all the tissues. The supplementation of *Sardinella* and *Rastrelliger* fish oils to diabetic group of mice significantly (*P*<0.001-0.0001) decreased the activity of enzyme about by 10-35% in all the tissues. The *Clarias* oil supplementation to the diabetic group of mice decreased (*P*<0.05-0.0001) the activity of enzyme by 5-10% in serum, kidney and heart tissues only (Figure 7B).

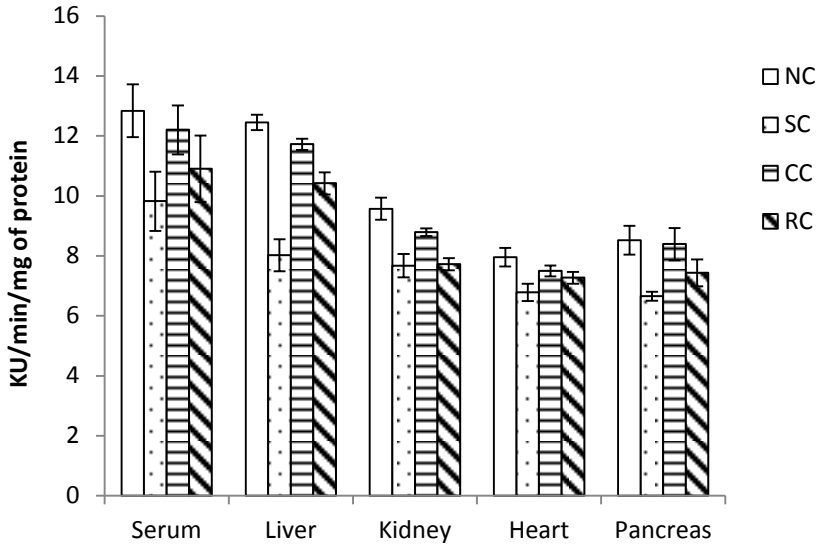


Figure 4A: **Changes in Alanine aminotransferase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

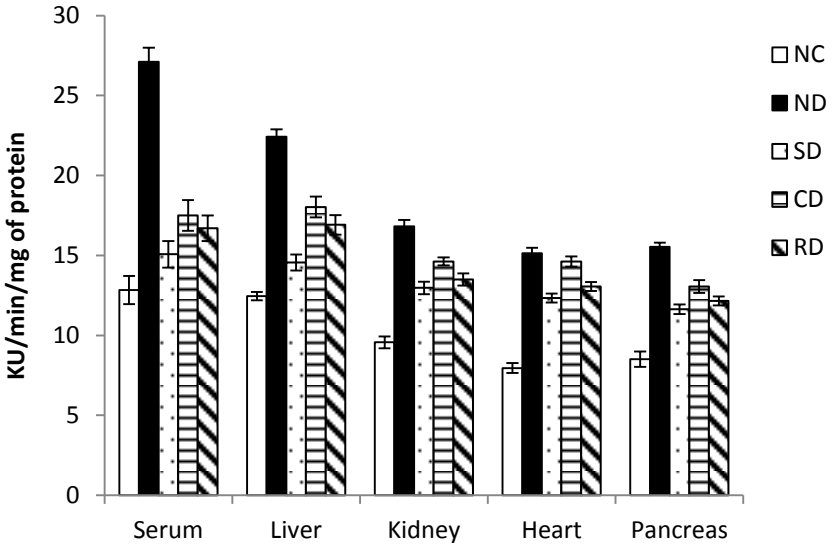


Figure 4B: **Changes in Alanine aminotransferase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard error**

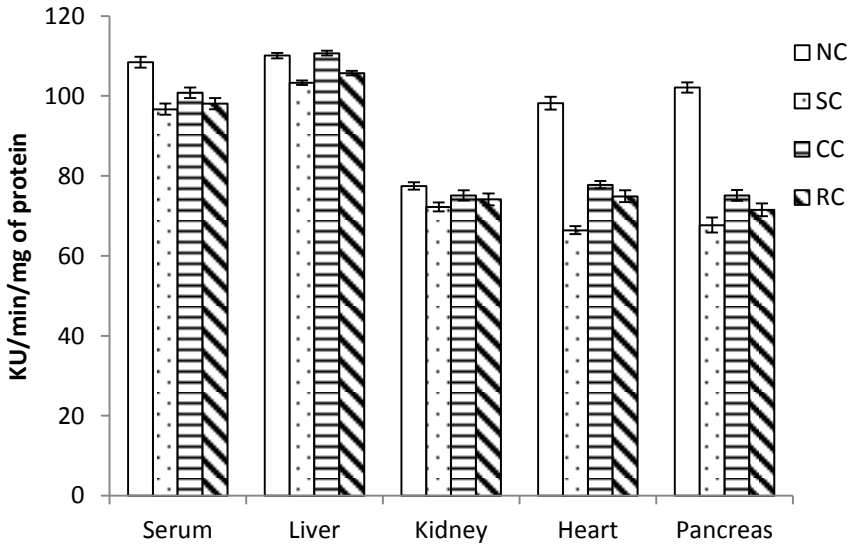


Figure 5A: **Changes in Aspartate aminotransferase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

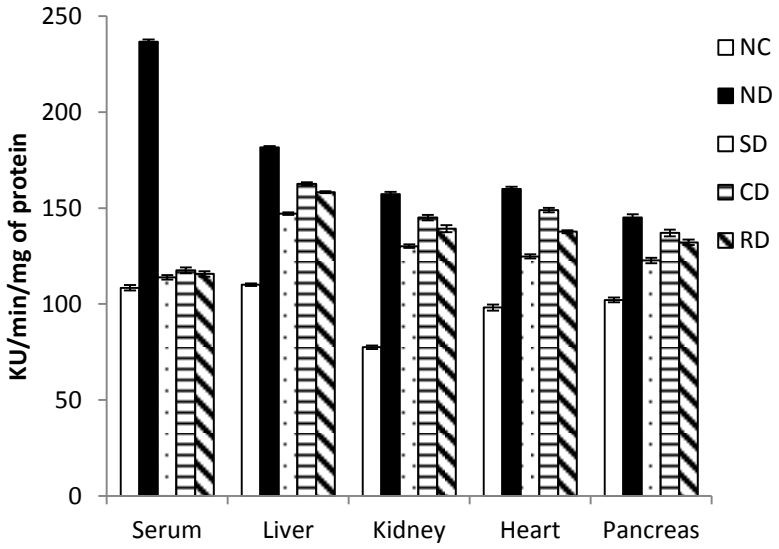


Figure 5B: **Changes in Aspartate aminotransferase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors**

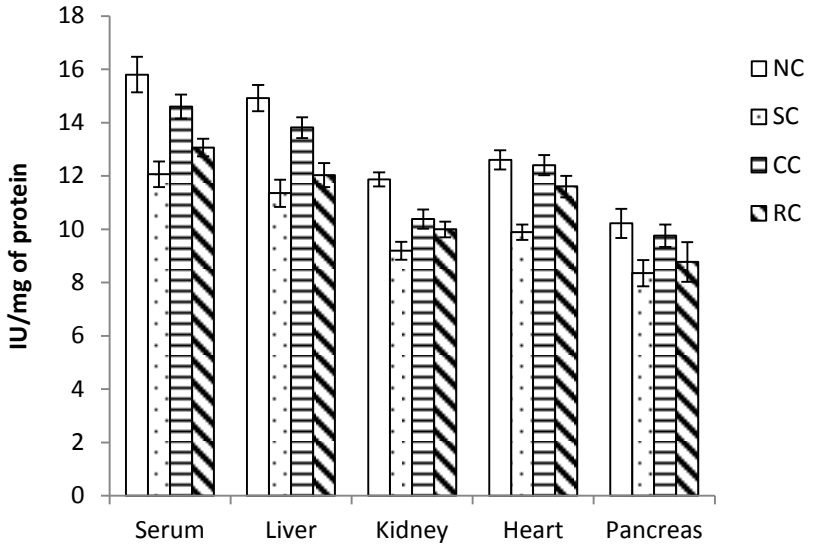


Figure 6A: **Changes in Alkaline phosphatase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

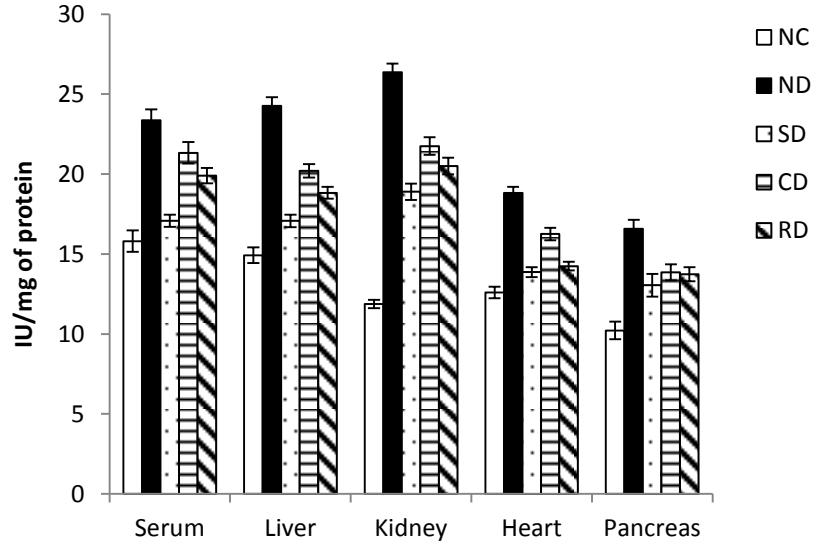


Figure 6B: **Changes in Alkaline phosphatase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

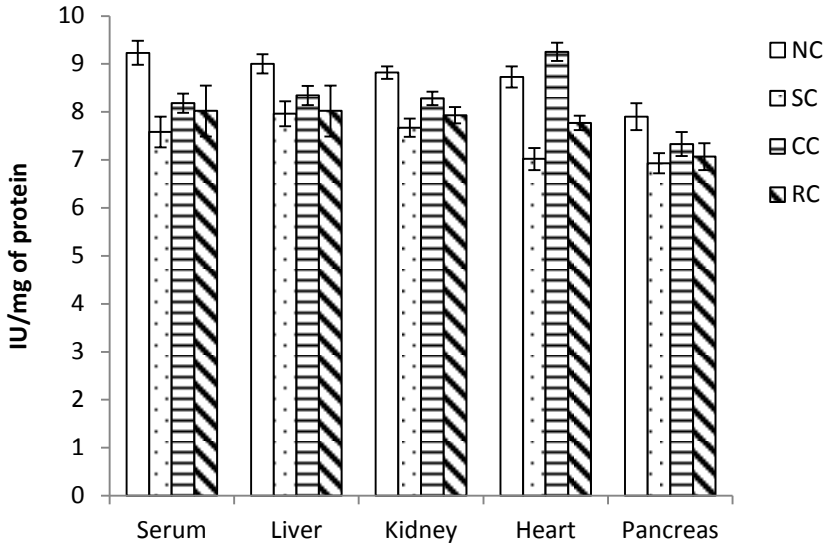


Figure 7A: **Changes in Acid phosphatase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

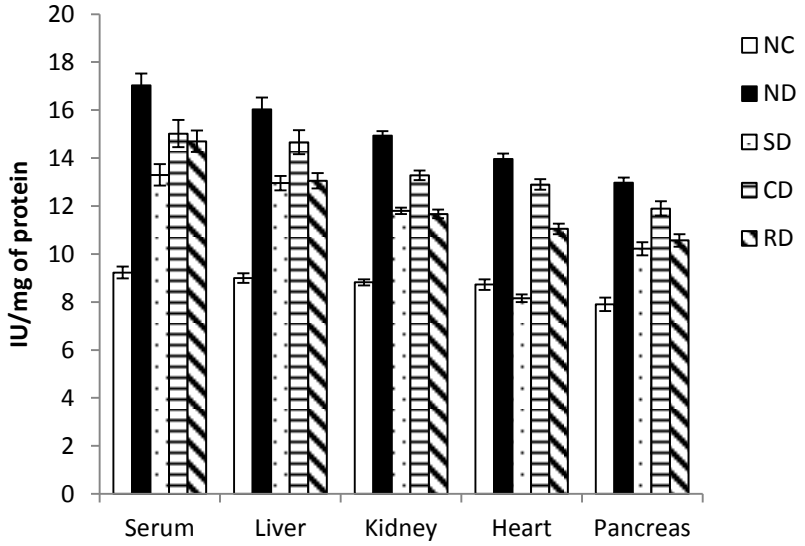


Figure 7B: **Changes in Acid phosphatase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.**

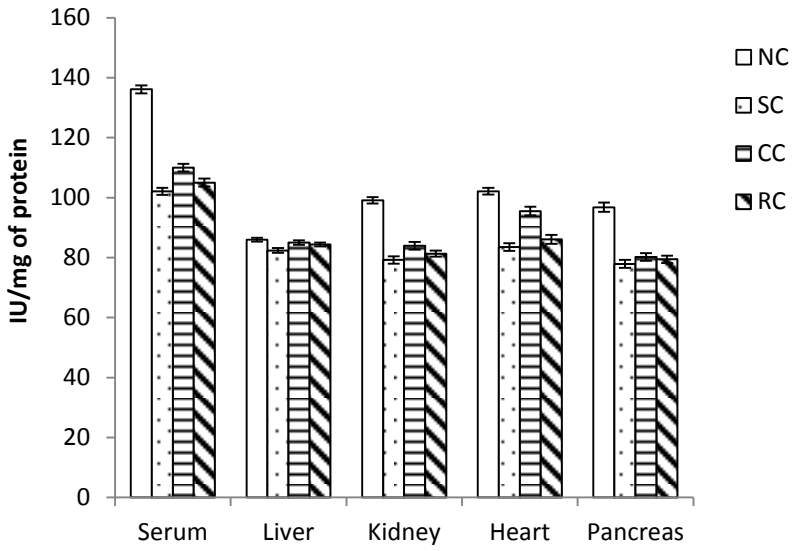


Figure 8A: Changes in Lactate dehydrogenase enzyme activity of various tissues in mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.

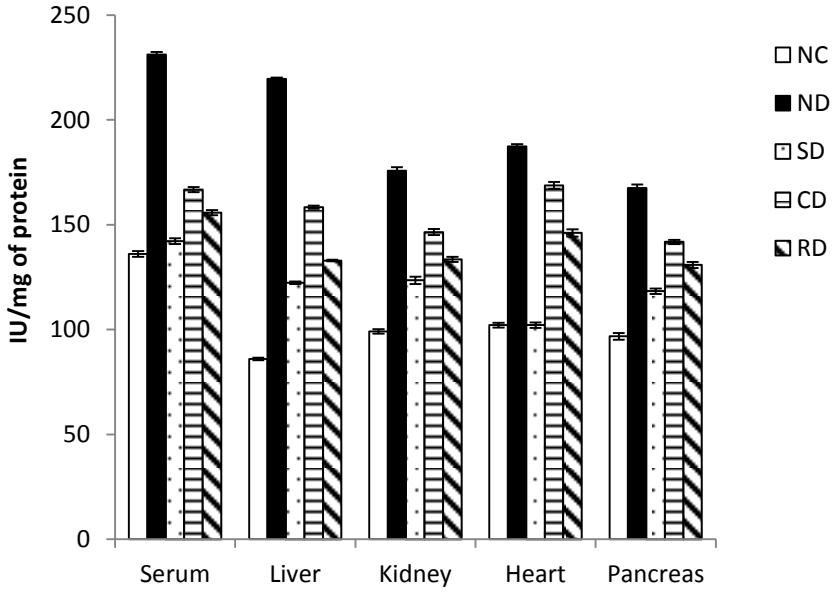


Figure 8B: Changes in Lactate dehydrogenase enzyme activity of various tissues in alloxan induced diabetic mice upon dietary supplementation of fish oils. Data represented as mean of six values and their standard errors.

Lactate dehydrogenase: The activity of LDH decreased significantly (F=41.759-142.986 $P<0.0001$) in fish oils supplemented non-diabetic SC, CC and RC group of mice about by 5-25% in all the tissues except for liver (Figure 8A). Up to 2.5 fold augmentation ($P<0.0001$) in the activity of enzyme in all the tissues was noticed due to induction of diabetes (Figure 8B). Supplementation of all three fish oils to the diabetic group of mice significantly (F=344.36-5742.56 $P<0.0001$) reduced the augmented activity of enzyme by 10-45% in all the tissues.

3.4 Effect of supplementation of fish oil on histological architecture of different tissues of diabetic mice group:

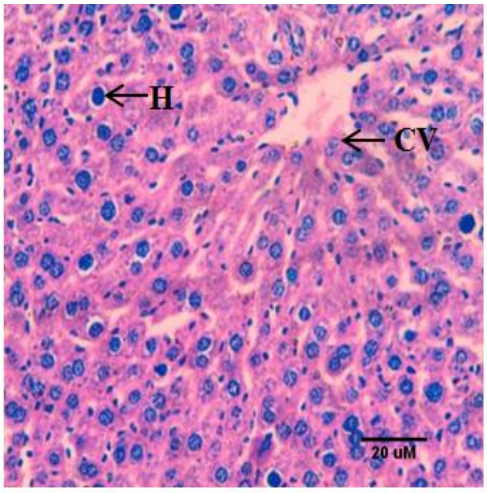
The changes in the histological architecture of liver, kidney, heart and pancreas tissues of fish oil supplemented diabetic groups were revealed in Plates 1-4.

Liver architecture: The control group of mice showed a normal liver architecture with sinusoidal cards of hepatocytes and in the centre of the lobule with central vein. The cells of the liver are arranged like the spokes of a wheel to form sheets (Plate 1A). Induction of diabetes completely distorted the liver structure due to inflammation in the hepatocytes resulted in congestion of the central vein and coagulative necrosis (Plate 1B). While the dietary supplementation of *Sardinella* and *Rastrelliger* oil helped to recover the normal arrangement of liver and aided to reduce the inflammation, there was no evidence of portal triditis, haemorrhage, necrosis or hepatocytolysis in this group (Plate 1C-1E). The supplementation of Clarias oil to the diabetic group of mice did not improve the liver histological structures significantly.

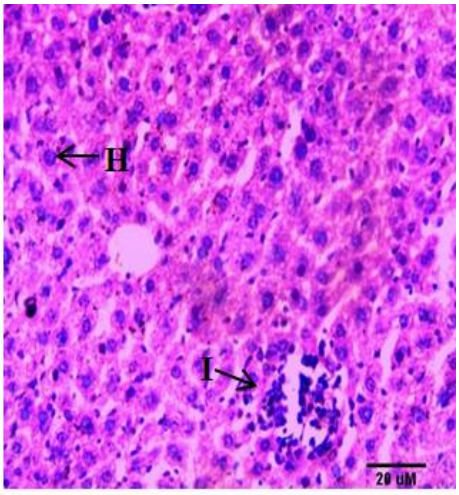
Kidney architecture: The histological architecture of the kidney in a control group of mice showed normal renal tubules and glomerulus (Plate 2A). Induction of diabetes by alloxan completely distorted the normal histological structure with enlargement of glomerular structure and a certain degree of inflammation (Plate 2B). Supplementation of all three fish oils helped to restore the normal structure of the kidney (2C-2E).

Heart architecture: The histological structures of heart of control mice exhibited the normal myocardium consisting of cardiomyocytes, cross-striated muscle cells, with one centrally placed nucleus and the cardiac muscle. Intercalated disc, which is specific junction between cardiac cells were observed (Plate 3A). The histological architecture of diabetic mice showed increased cytoplasmic vacuolization, myofibrillar loss and inflammation (Plate 3B). Supplementation of all the three fish oil to the diabetic mice helped heart to recover from these distortions. Only minor inflammation and degeneration were persisted (Plate 3C-3E).

Pancreas architecture: The normal histological structure of pancreas revealed with two intralobular ducts: smaller and larger, along with the Islets of Langerhans with lighter staining area. While the small intralobular ducts scattered throughout, larger intralobular ducts bounded by thick connective tissue (Plate 4A). Induction of diabetes in the ND group of mice (Plate 4B) resulted in complete deformation of the pancreas with the presence of the lymphocytic infiltrates in islets. Supplementation of fish oil to the SD, CD and RD (Plate 4C-4E) group of mice showed no much significant effect on restoring the normal pancreatic structure.



(A)



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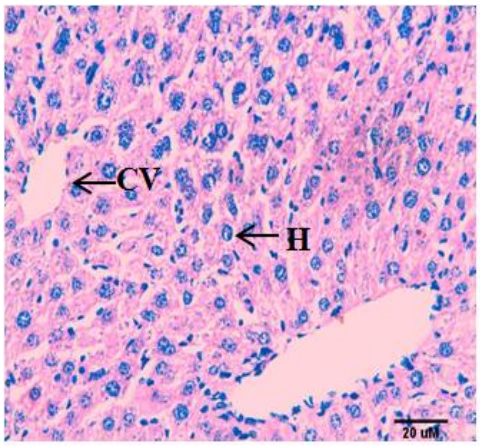

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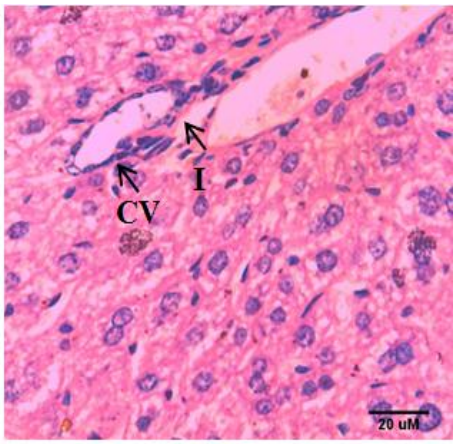

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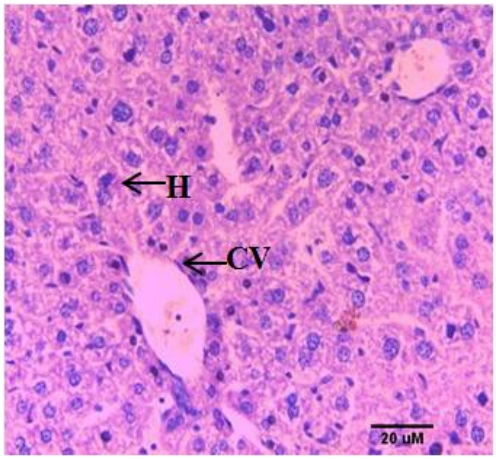


(E)

Plate 1: Histological changes in sample of liver tissues from (A) mice of control group showing normal liver architechure (B) mice of diabetic group showing deformed liver due to lipid accumulation (C) mice of SD group and (D) mice of CD (E) mice of RD group.

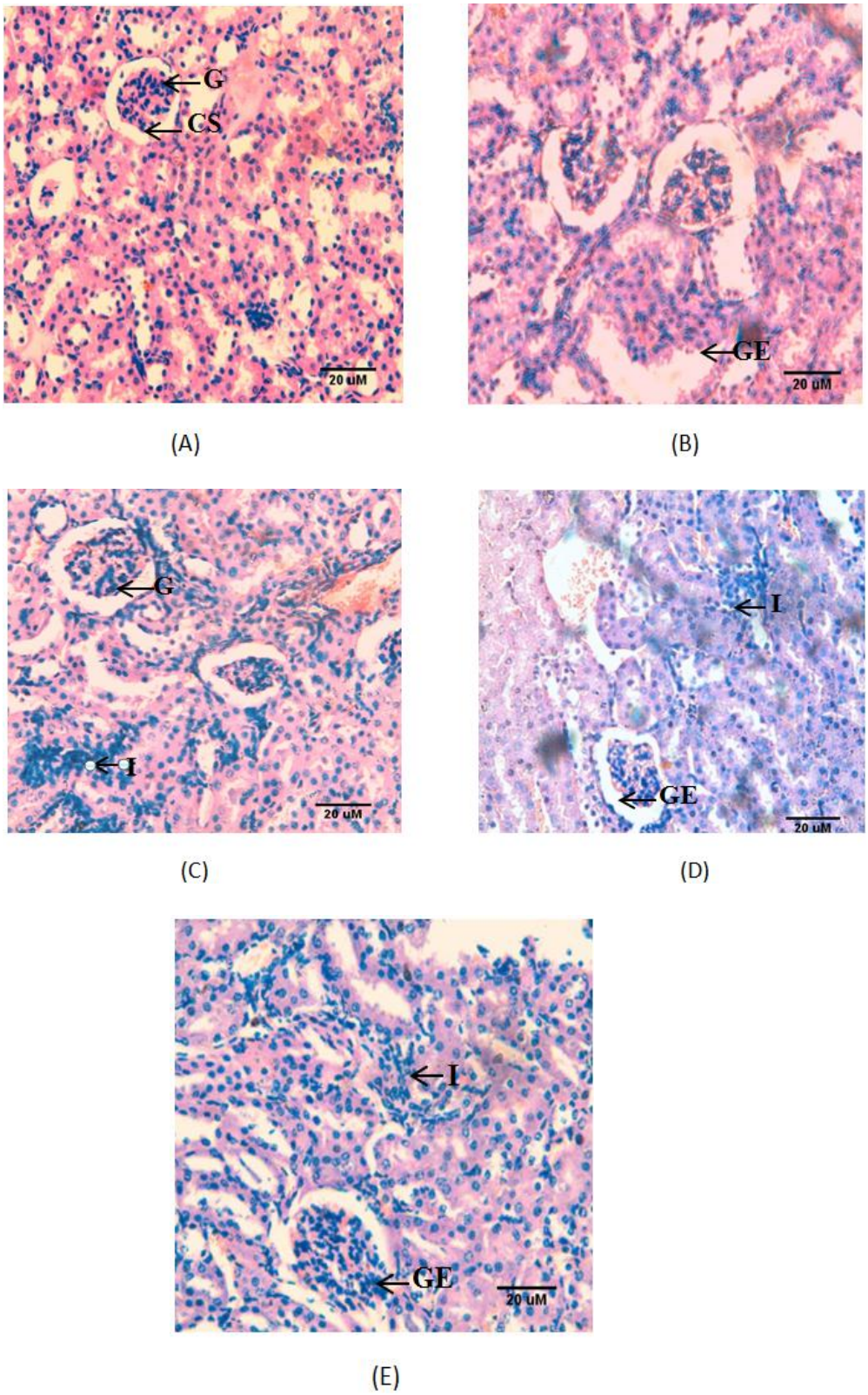


Plate 2: Histological changes in sample of kidney tissue from (A) control group mice showing normal renal corpuscle with glomeruli (B) diabetic mice showing glomeruli lesions (C) mice of SD group and (D) mice of CD (E) mice of RD group.

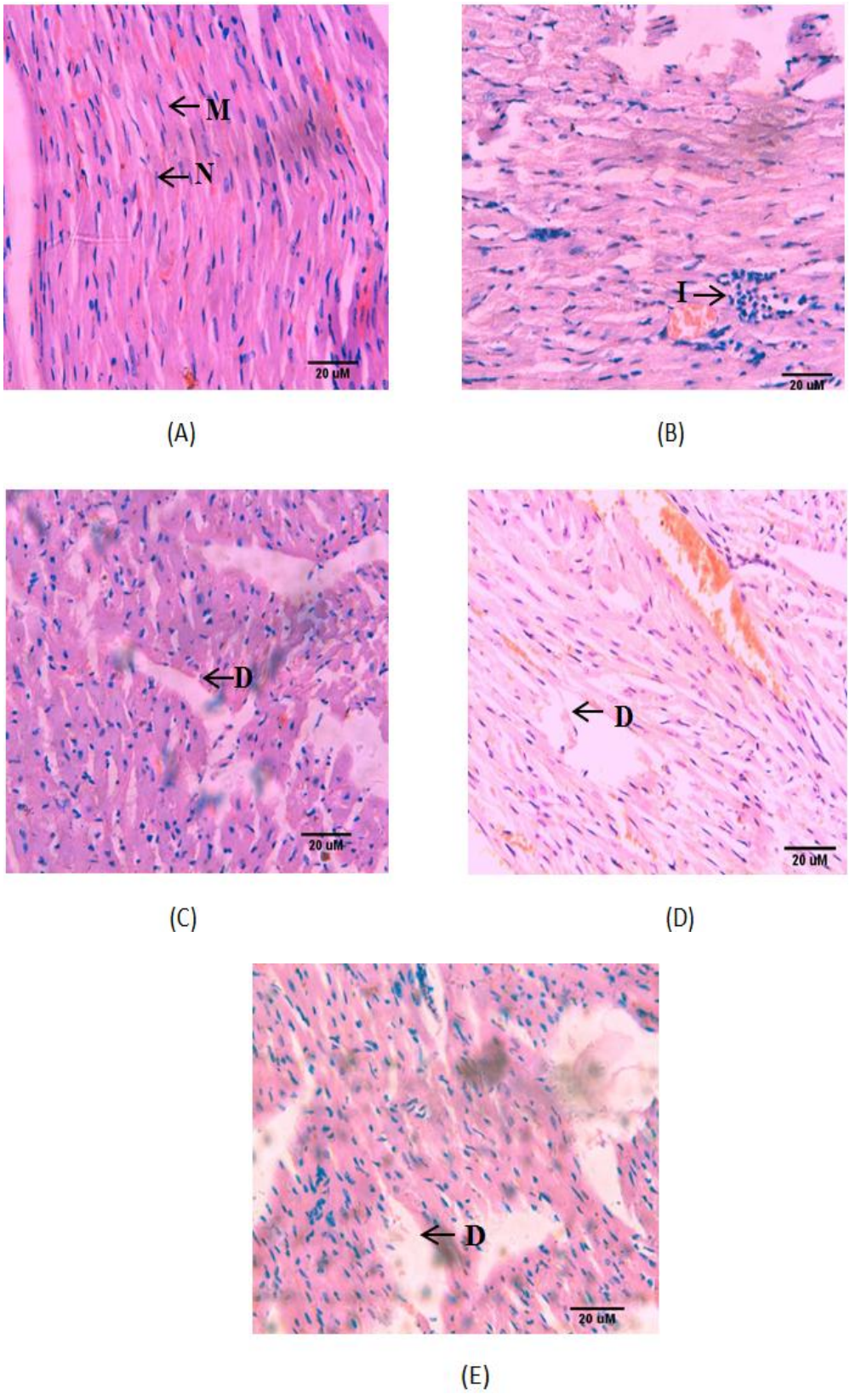


Plate 3: Histological changes in sample of heart tissue from (a) control mice showing linear arrangement of myofibrils (d) diabetic mice showing degenerated cardiomyocytes (c)mice of SD group and (d) mice of CD (e) mice of RD group

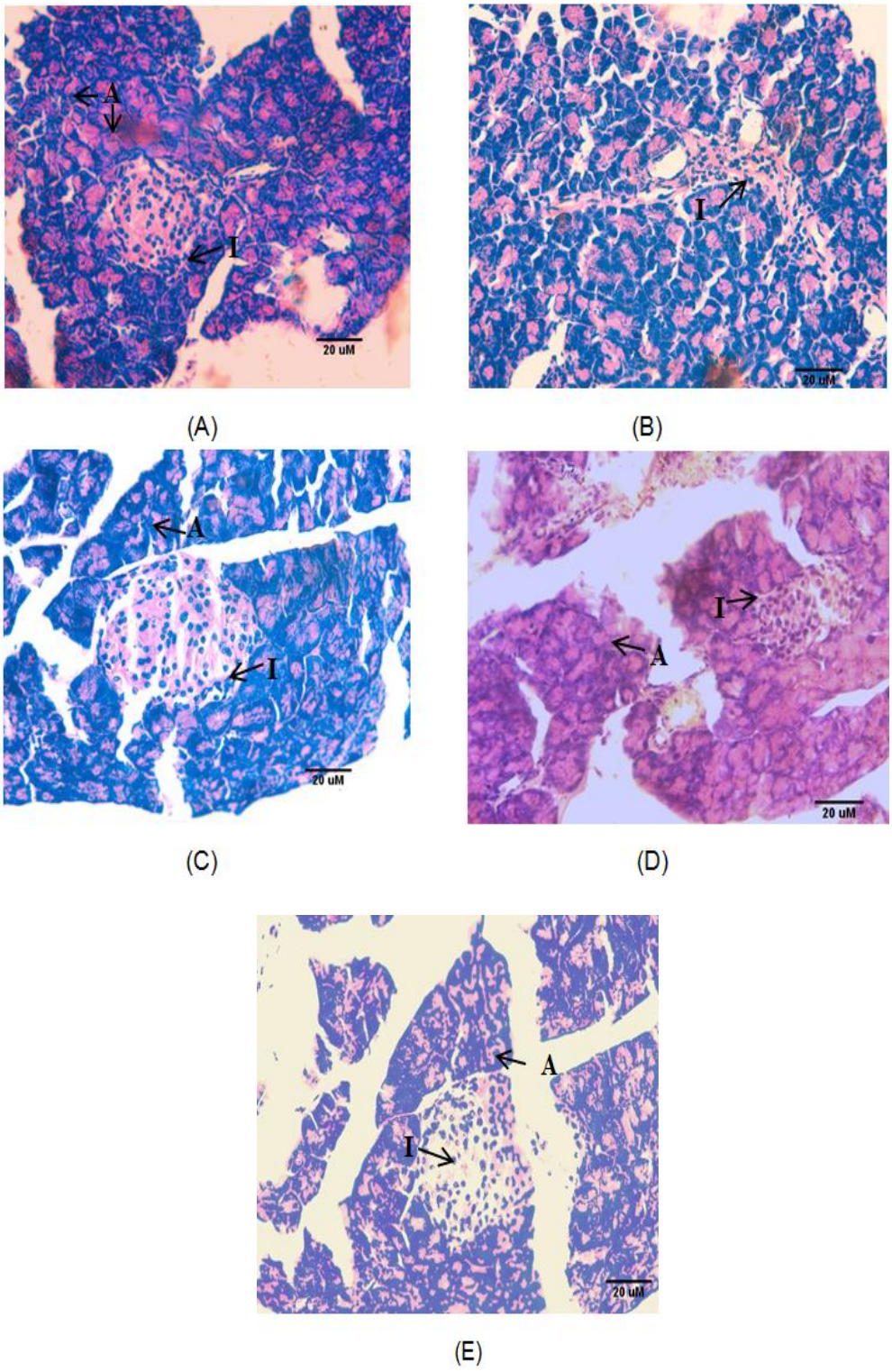


Plate 4: Histological changes in sample of pancreas tissues from (a) control mice showing normal acini and islet (b) diabetic mice showing infiltration (c) mice from SD group and (d) mice from RD (e) mice from CD group. I: islet, A: Acini

3.5 Effect of supplementation of fish oil on lipid profiles of non-diabetic and diabetic mice groups:

The changes in the concentration of serum cholesterol profile and tissue lipid profile of different tissues (serum, liver, kidney, heart, pancreas) of non-diabetic and diabetic mice groups, due to supplementation of 10% fish oils (*Sardinella, Clarias, Rastrelliger*) for a period of one month are revealed in Tables 11-15 and Figures 9-21.

3.5.1 Serum cholesterol profile:

The dietary supplementation of 10% fish oils for non-diabetic group of mice (Table 11A) for a period of 30 significantly ($P<0.001$) brought down the serum cholesterol levels by 30-45%, along with 15-60% augmentation in the concentration of HDL-cholesterol and 65-80% decrease in LDL cholesterol. However the VLDL cholesterol level remained unaltered. Induction of diabetes, enhanced ($P<0.001$) the serum cholesterol level by about 45% along with 2 fold augmentation in VLDL cholesterol level and 15% increase in LDL cholesterol level without any significant changes in HDL cholesterol. Supplementation of 10% fish oils (Table 11B) for a period of one month to diabetic group of mice significantly ($P<0.001$-0.0001) lowered the cholesterol by 55-60% which led to 45-80% decrease in LDL-cholesterol and VLDL-cholesterol. Supplementation of these oils also increased the HDL-cholesterol level by 43-64%.

Table 11A: Effect of dietary supplementation of fish oil on the concentration (mg/dl) of serum cholesterol profile of mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Parameters	NC	SC	CC	RC	ANOVA
Total Cholesterol	152.3 ±0.95	84.8 ±0.98	89.2 ±0.84	86.6 ±0.87	F=1281.79 *P*<0.0001
HDL-C	23.6 ±0.99	38.3 ±0.62	26.8 ±0.84	36.8 ±0.89	F=73.360 *P*<0.0001
VLDL-C	26.2 ±1.1	26.5 ±0.64	27.9 ±1.8	24.8 ±1.05	F=1.067 NS
LDL-C	102.2 ±1.8	20.2 ±1.2	34.2 ±1.5	25.1 ±1.05	F=690.299 *P*<0.0001
CH/HDL	6.45	2.21	3.32	2.36	

Table 11B: Effect of dietary supplementation of fish oil on the concentration (mg/dl) of serum cholesterol profile of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated

Parameters	NC	ND	SD	CD	RD	ANOVA
Total Cholesterol	152.3 ±0.95	219.1 ±0.88	99.7 ±0.90	101.9 ±0.98	105.05 ±0.78	F=3262.19 *P*<0.0001
HDL-C	23.6 ±0.99	22.6 ±0.54	37.3 ±0.62	24.3 ±0.54	32.5 ±0.63	F=188.859 *P*<0.0001
VLDL-C	26.2 ±1.1	79.6 ±1.3	41.5 ±1.2	45.5 ±1.6	42.7 ±1.7	F=188.86 *P*<0.0001
LDL-C	102.2 ±1.8	116.4 ±2.04	21.1 ±2.04	33.4 ±3.6	29.4 ±1.3	F=355.391 *P*<0.0001
CH/HDL	6.43	9.80	2.67	4.21	3.22	

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

3.5.2 Tissue lipid profile:

Glycerides: The changes in the concentration of total triglyceride on diabetic and fish oil supplemented control and diabetic mice have been mentioned in section 3.1. The relative concentration of glycerides estimated from the neutral lipid fractions has been mentioned below.

The supplementation of all three fish oils to the non-diabetic mice groups helped to decrease the relative concentration of triglycerides by 25-30% (F=6.622, P<0.0001) only in liver tissue. An increase of 15-50% in mono-glyceride and di-glyceride (P<0.05-0.0001 concentrations was noticed in all the tissues (Table 12A-15A). Due to induction of diabetes by alloxan the relative concentration of di-glyceride and triglyceride was increased by 30-100% (P<0.001-0.0001) along with 40-55% decrease (P<0.0001) in level of mono-glyceride in all the tissues. The relative content of di-glyceride and triglyceride significantly decreased about by 10-65% (F=3.27-36.42, P<0.0001) along with dramatic augmentation in level of mono-glycerides in all the tissues except for pancreas tissues of fish oil supplemented diabetic SD, CD and RD groups of mice (Table 12B-15B).

Free fatty acid: The changes in the concentration of total free fatty acid on diabetic and fish oil supplemented control and diabetic mice have been mentioned in section 3.1. The relative concentration of free fatty acid estimated from the neutral lipid fractions has been mentioned in Tables 12-15. It is noted that the relative concentration of free fatty acid decreased by 5-30% (F=5.81-35.05, P<0.0001) in the liver and pancreas in fish oils supplemented control groups (SC, CC and RC) of mice. Induction of diabetes significantly (P<0.01-0.0001) augmented the

concentration of free fatty acids in all the tissues approximately by 25-90% when compared to control group (NC) of mice. The fish oil supplementation to the diabetic SD, CD and RD groups of mice significantly decreased the elevated level of free fatty acids by 15-35% in all the tissues ($F=9.36\text{-}49.71$ $P<0.0001$).

Cholesterols: It is evident that the supplementation of *Sardinella* and *Rastrelliger* fish oils to the SC and RC groups of mice led to significant ($P<0.001\text{-}0.0001$) decrease in total cholesterol by 10-30% only in kidney, heart and pancreas tissues, however the supplementation of *Clarias* oil to the CC group of mice decreased the total cholesterol level approximately by 15% only in heart and pancreas tissues (Figure 9A). Induction of diabetes augmented the concentration of total cholesterol by 50% - 2.3 fold in all the tissues. Supplementation of fish oil for the diabetic mice groups decreased the total cholesterol concentration by 10-40% in all the tissues (Figure 9B). The changes in the relative concentration of cholesterol esters and free cholesterol estimated from neutral lipid fraction are represented in Tables 12-15.

The supplementation of *Sardinella* and *Rastrelliger* fish oils to the control group of mice resulted in 15-40% decrease in the relative concentration of cholesterol esters and free cholesterol in all the tissues, however *Clarias* fish oil supplementation decreased the concentration of cholesterol esters and free cholesterol by 5-30% ($P<0.01\text{-}0.0001$) only in liver, heart and pancreas (Table 12A-15A). Induction of diabetes significantly ($P<0.0001$) augmented the relative concentration of free cholesterol and cholesterol esters by 25-65% in all the tissues. Supplementation of fish oil for the diabetic mice groups significantly

(F=152.072-1164.519, *P*<0.0001) decreased the elevated level of free cholesterol and cholesterol esters about by 10-35% in all the tissues (Table 12B-15B).

Phospholipids: The concentration of total phospholipid in fish oil supplemented control SC, CC and RC group of mice increased significantly (F=30.164-125.04, *P*<0.0001) by 10-65% only in liver and heart tissues. In addition, Clarias oil supplementation significantly elevated the concentration of the total phospholipid by 5-15% in kidney and pancreas; Sardinella oil supplementation elevated the concentration by 20% (*P*<0.0001) in the pancreas (Figure 10A). Induction of diabetes resulted 45-55% reduction (*P*<0.000) in total phospholipid concentration in all the tissues. Supplementation of fish oils (Figure 10B) for the diabetic mice groups significantly increased the concentration phospholipid by 5-40% (*P*<0.001-0.0001) in all the tissues.

It is observed that the supplementation of fish oil to the control group of mice (SC, CC and RC) led to decrease the relative concentration of phophotidyl ethanolamine, sphigomylein by 5-10% (*P*<0.0001) and to increase (approximately 5% but highly significant) the relative concentration of phosphotidyl choline only in heart tissue. Besides, the supplementation of fish oil to the SC and RC group of mice decreased the concentration of phophotidyl ethanolamine and lysophospholipid by 5-10% (*P*<0.05-0.001) only in liver tissue (Figure 12A-15A). Induction of diabetes resulted in substantial changes in the relative concentration of phospholipid fractions with 5-45% increase (*P*<0.001-0.0001) in phophotidyl ethanolamine, sphigomylein and lysophospholipid and 10-40% decrease (*P*<0.05-0.0001) in phosphotidyl choline and phosphotidyl inositol+ phosphotidyl serine fraction when compared to control group of mice. The supplementation of fish

oils for the diabetic group of mice significantly increased the relative concentrations of phosphotidyl choline and phosphotidyl inositol fraction by 10-40% (P<0.05-0.0001) and decreased the concentration of phophotidyl ethanolamine, sphigomylein and lysophospholipid fractions by 10-45% (P<0.05-0.0001) in all the tissues except for liver (Figure 12B-15B).

Glycolipids: The supplementation of fish oil to the SC, CC and RC groups decreased significantly (F=7.046-19.011, P<0.001-0.0001) the concentration of total glycolipid by 5-15% in liver and heart tissues. It is also noted that supplementation of fish oil to the SC and RC group of mice decreased significantly the concentration of total glycolipid by 10-15% (F=3.488-13.132 P<0.05-0.0001) only in kidney and pancreas tissues (Figure 11A). Induction of diabetes resulted significant (P<0.0001) increase in the concentration of total glycolipid by 10-40% in all the tissues (Figure 11B). Supplementation of fish oil to the SD, CD and RD group of mice helped to significantly (F=11.414-60.836, P<0.0001) decrease the concentration of glycolipid by 5-15% in all the tissues (Figure 11B) except in heart.

Supplementation of fish oils to the control group of mice significantly (F=106.946-447.913, P<0.0001) increased the relative concentration of fraction I (not identified, Rf value 0.91) by 2 – 2.5 fold in all the tissues with 10-25% increase galactocerebroside only in kidney tissue (F=108.897, P<0.0001). It is also noted that there was significant (F=565.882-1244.937, P<0.0001) decrease in concentration of fraction II (not identified, Rf value 0.33) by 60-70% in all the tissue along with significant (F=13.105-72.287, P<0.0001) decrease in concentration of sphingolipid by 20-40% in the liver, kidney and heart tissues

(Figure 16A-19A). The change in total glycolipid concentration due to induction of diabetes was also reflected in the relative concentration of glycolipid fractions with 35% - 3.2 fold augmentation (P<0.0001) in fraction I and sphingolipid and 20-45% reduction (P<0.0001) in fraction II and galactocerebroside in all the tissues. The supplementation of fish oils to the diabetic (SD, CD and RD) group of mice resulted in the elevation of relative concentration of fraction I and galactocerebrosides fraction by 10% - 2.5 fold (P<0.001-0.0001) and 25-60% decrease (P<0.0001) in the relative concentration of fraction II and sphingolipid in all the tissues (Figure 16B-19B).

3.5.3 Effect of supplementation of fish oil on fatty acid profiles of tissues of diabetic mice groups:

15% - 2.5 fold augmentation in the level of myristic (14:0), palmitic (16:0), stearic acid (18:0), along with 5 -70% reduction in the level of palmetoleic (16:1), oleic acid (18:1), linoleic acid (18:2), gondoic acid (20:1), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5) and docosahexaenoic acid (22:6) was noticed in liver, kidney, heart and pancreas tissues of alloxan induced diabetic mice (Table 16A-D). Supplementation of fish oil to the SD, CD and RD diabetic groups of mice decreased the elevated level of myristic acid, palmitic acid, stearic acid by 10-55%, and also increased the level of oleic acid (18:1), linoleic acid (18:2), gondoic acid (20:1), dihomo-γ-linoleic acid (20:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5) and docosahexaenoic acid (22:6) concentration in all the tissues by 10%-3.7fold.

Table 12A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in liver of mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	SC	CC	RC	ANOVA
Cholesterol Esters	0.187 ±0.0013	0.151 ±0.0021	0.176 ±0.0031	0.155 ±0.0014	F=75.235 *P*<0.0001
Triglyceride	1.55 ±0.10	1.17 ±0.041	1.21 ±0.033	1.18 ±0.013	F=9.48 *P*<0.001
Free fatty acid	1.90 ±0.039	1.32 ±0.039	1.58 ±0.044	1.50 ±0.041	F=35.05 *P*<0.0001
Free cholesterol	0.155 ±0.0012	0.108 ±0.0022	0.128 ±0.0015	0.113 ±0.0017	F=148.493 *P*<0.0001
Monoglyceride	0.960 ±0.030	2.27 ±0.14	1.30 ±0.22	2.20 ±0.22	F=86.215 *P*<0.0001
Diglyceride	0.708 ±0.075	1.3 ±0.065	1.17 ±0.055	1.19 ±0.058	F=100.070 *P*<0.0001

Table 12B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in liver of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	ND	SD	CD	RD	ANOVA
Cholesterol Esters	0.187 ±0.0013	0.274 ±0.0021	0.196 ±0.0033	0.242 ±0.0025	0.205 ±0.0018	F=259.385 *P*<0.0001
Triglyceride	1.55 ±0.10	2.67 ±0.11	1.88 ±0.063	1.96 ±0.11	1.89 ±0.052	F=19.09 *P*<0.0001
Free fatty acid	1.90 ±0.039	3.67 ±0.17	2.21 ±0.063	2.61 ±0.092	2.24 ±0.044	F=49.71 *P*<0.0001
Free cholesterol	0.155 ±0.0012	0.230 ±0.0025	0.164 ±0.0024	0.180 ±0.0014	0.171 ±0.0021	F=251.010 *P*<0.0001
Monoglyceride	0.960 ±0.030	0.447 ±0.076	3.98 ±0.21	2.97 ±0.17	3.87 ±0.21	F=620.027 *P*<0.0001
Diglyceride	0.708 ±0.075	0.795 ±0.077	1.15 ±0.042	1.06 ±0.043	1.11 ±0.031	F=74.566 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 13A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in kidney of mice (*Mus musculus).)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	SC	CC	RC	ANOVA
Cholesterol Esters	0.162 ±0.0032	0.145 ±0.0037	0.167 ±0.0027	0.161 ±0.0025	F=8.884 *P*<0.001
Triglyceride	1.45 ±0.12	1.39 ±0.040	1.45 ±0.056	1.39 ±0.057	F=0.33 NS
Free fatty acid	1.52 ±0.036	1.42 ±0.026	1.50 ±0.032	1.49 ±0.026	F=1.87 NS
Free cholesterol	0.144 ±0.0027	0.118 ±0.0045	0.131 ±0.0029	0.124 ±0.0014	F=12.406 *P*<0.0001
Monoglyceride	0.905 ±0.016	2.04 ±0.023	1.18 ±0.022	2.07 ±0.028	F=664.096 *P*<0.0001
Diglyceride	0.676 ±0.022	1.02 ±0.024	1.08 ±0.032	1.11 ±0.024	F=59.733 *P*<0.0001

Table 13B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in kidney of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	ND	SD	CD	RD	ANOVA
Cholesterol Esters	0.162 ±0.0032	0.265 ±0.0020	0.171 ±0.0015	0.186 ±0.0015	0.172 ±0.0020	F=8.884 *P*<0.001
Triglyceride	1.45 ±0.12	2.52 ±0.11	1.92 ±0.088	2.01 ±0.039	1.78 ±0.10	F=15.17 *P*<0.0001
Free fatty acid	1.52 ±0.036	2.73 ±0.10	2.02 ±0.12	2.30 ±0.084	2.13 ±0.12	F=18.71 *P*<0.0001
Free cholesterol	0.144 ±0.0027	0.223 ±0.0021	0.152 ±0.0026	0.170 ±0.0027	0.163 ±0.0029	F=12.406 *P*<0.0001
Monoglyceride	0.905 ±0.016	0.420 ±0.023	3.33 ±0.026	2.41 ±0.031	3.25 ±0.032	F=664.09 *P*<0.0001
Diglyceride	0.676 ±0.022	0.734 ±0.030	0.969 ±0.023	0.985 ±0.032	0.978 ±0.034	F=59.733 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 14A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in heart of mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	SC	CC	RC	ANOVA
Cholesterol Esters	0.120 ±0.0018	0.0873 ±0.0012	0.104 ±0.0016	0.093 ±0.0018	F=81.033 *P*<0.0001
Triglyceride	1.32 ±0.042	1.28 ±0.011	1.31 ±0.056	1.29 ±0.031	F=0.31 NS
Free fatty acid	1.44 ±0.040	1.39 ±0.011	1.41 ±0.062	1.40 ±0.016	F=0.30 NS
Free cholesterol	0.126 ±0.0014	0.087 ±0.0018	0.096 ±0.0013	0.088 ±0.0015	F=132.03 *P*<0.0001
Monoglyceride	0.700 ±0.011	1.45 ±0.011	1.60 ±0.025	1.53 ±0.025	F=470.93 *P*<0.0001
Diglyceride	0.482 ±0.011	0.946 ±0.031	0.968 ±0.021	0.965 ±0.041	F=358.68 *P*<0.0001

Table 14B: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in heart of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	ND	SD	CD	RD	ANOVA
Cholesterol Esters	0.120 ±0.0018	0.197 ±0.0019	0.126 ±0.0010	0.148 ±0.0011	0.125 ±0.0011	F=81.033 *P*<0.0001
Triglyceride	1.32 ±0.042	1.94 ±0.031	1.47 ±0.042	1.52 ±0.032	1.51 ±0.041	F=36.69 *P*<0.0001
Free fatty acid	1.44 ±0.040	2.01 ±0.089	1.64 ±0.040	1.70 ±0.030	1.66 ±0.035	F=15.93 *P*<0.0001
Free cholesterol	0.126 ±0.0014	0.196 ±0.0015	0.125 ±0.0014	0.149 ±0.0012	0.134 ±0.0022	F=132.039 *P*<0.0001
Monoglyceride	0.700 ±0.011	0.390 ±0.013	2.79 ±0.023	2.28 ±0.022	2.54 ±0.024	F=470.932 *P*<0.0001
Diglyceride	0.482 ±0.011	0.560 ±0.022	0.759 ±0.037	0.800 ±0.033	0.789 ±0.025	F=358.680 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 15A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in pancreas of mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated.

Type of lipids	NC	SC	CC	RC	ANOVA
Cholesterol Esters	0.104 ±0.0012	0.062 ±0.0010	0.085 ±0.0013	0.073 ±0.0012	F=214.110 *P*<0.0001
Triglyceride	1.156 ±0.044	1.150 ±0.040	1.153 ±0.037	1.151 ±0.011	F=0.101 NS
Free fatty acid	1.32 ±0.025	1.20 ±0.018	1.203 ±0.023	1.201 ±0.031	F=5.81 *P*<0.01
Free cholesterol	0.107 ±0.0014	0.065 ±0.0013	0.075 ±0.0012	0.068 ±0.0011	F=226.504 *P*<0.0001
Monoglyceride	0.602 ±0.010	1.25 ±0.037	1.46 ±0.037	1.33 ±0.030	F=154.195 *P*<0.0001
Diglyceride	0.419 ±0.018	0.760 ±0.012	0.748 ±0.017	0.789 ±0.023	F=705.215 *P*<0.0001

Table 15A: Effect of dietary supplementation of fish oil on the concentration (mg/100mg) of neutral lipids in pancreas of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors were tabulated

Type of lipids	NC	ND	SD	CD	RD	ANOVA
Cholesterol Esters	0.104 ±0.0012	0.157 ±0.0018	0.107 ±0.0013	0.125 ±0.0011	0.108 ±0.0014	F=214.110 *P*<0.0001
Triglyceride	1.156 ±0.044	1.54 ±0.044	1.33 ±0.032	1.33 ±0.037	1.35 ±0.015	F=16.27 *P*<0.0001
Free fatty acid	1.32 ±0.025	1.66 ±0.063	1.42 ±0.034	1.47 ±0.031	1.45 ±0.036	F=9.36 *P* <0.0001
Free cholesterol	0.107 ±0.0014	0.173 ±0.0013	0.114 ±0.0015	0.124 ±0.0016	0.115 ±0.0014	F=226.504 *P*<0.0001
Monoglyceride	0.602 ±0.010	0.356 ±0.011	1.73 ±0.023	1.52 ±0.042	1.69 ±0.047	F=154.195 v<0.0001
Diglyceride	0.419 ±0.018	0.543 ±0.014	0.565 ±0.019	0.540 0.016	0.556 ±0.020	F=705.215 *P*<0.0001

NC-control, ND-diabetic, SC- *Sardinella* oil supplemented control, CC- *Clarias* oil supplemented control, RC- *Rastrelliger* oil supplemented control, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 16A: Effect of dietary supplementation of fish oil on the % concentration of fatty acid profile of liver of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Type of fatty acids	NC	ND	SD	RD	CD	ANOVA
C14:0	0.55±0.11	1.41±0.058	0.65±0.026	0.84±0.049	1.13±0.061	F=6.773 *P*<0.05
C16:0	21.10±0.29	39.31±0.91	29.21±0.55	30.28±0.76	29.9±1.6	F=36.7 *P*<0.0001
C16:1(cis)	1.06±0.23	2.01±0.20	3.98±0.24	4.17±0.27	3.63±0.15	F=43.4 *P*<0.0001
C16:1(trans)	3.6±0.29	4±0.57	3.11±0.10	3.47±0.30	3.98±0.29	NS
C18:0	8.63±0.34	11.81±0.64	10.74±0.48	10.91±1.2	11.56±0.16	F=3.17 *P*<0.05
C18:1(cis)	25.8±2.26	19.02±0.58	16.39±0.94	17.21±1.3	12.53±0.9	F=22.7 *P*<0.0001
C18:1(trans)	8.09+0.30	4.58±0.6	5.54±0.80	3.23±0.62	7.85±1.1	F=9.036 *P*<0.01
C18:2(n-6)	6.37±0.37	4.52±0.54	7.77±0.12	7.4±1.39	6.63±0.29	F=3.18 *P*<0.05
C20:1	0.93±0.031	0.46±0.12	0.63±0.034	0.82±0.19	0.85±0.053	NS
C20:3(n-6)	1.07±0.018	0.67±0.21	1.97±0.018	1.72±0.10	2.37±0.51	F=5.88 *P*<0.01
C20:4(n-6)	9.34±0.016	3.95±0.4	2.42±0.041	2.51±0.29	2.48±0.56	NS
C20:5(n-3)	4.61±0.055	2.39±0.061	9.05±0.30	9.20±0.67	8.98±0.48	F=23.4 *P*<0.0001
C22:5(n-3)	6.8±0.86	5.05±0.56	6.53±0.69	6.05±0.63	5.85±0.30	NS
C22:6(n-3)	1.31±0.33	0.81±0.058	2.01±0.104	2.04±0.44	2.25±0.41	F=9.05 *P*<0.01
Saturated	30.28	52.53	40.60	42.03	42.59	
Unsaturated	68.98	47.46	59.4	57.82	57.4	
n-3	17.45	9.81	17.59	17.29	17.08	
n-6	12.05	7.58	12.16	11.63	11.48	

NC-control, ND-diabetic, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 16B: Effect of dietary supplementation of fish oil on the % concentration of fatty acid profile of kidney of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Type of fatty acids	NC	ND	SD	RD	CD	ANOVA
C14:0	1.76±0.095	1.80±0.12	1.65±0.05	1.66±0.25	2.26±0.24	F=11.5 *P*<0.001
C16:0	24.1±0.70	30.54±0.86	26.8±0.25	27.47±0.48	28.47±0.36	F=27.7 *P*<0.0001
C16:1(cis)	0.58±0.012	2.03±0.11	2.24±0.16	3.87±0.26	2.56±0.26	F=76.2 *P*<0.0001
C16:1(trans)	5.7±0.22	5.56±0.31	4.27±0.20	3.17±0.73	5.69±0.34	F=7.5 *P*<0.01
C18:0	4.19±0.46	7.21±0.16	5.2±0.58	4.43±0.73	6.27±0.60	F=7.25 *P*<0.01
C18:1(cis)	32.10±0.58	26.16±0.40	28.92±2.9	29.7±1.01	26.21±0.35	F=6.7 *P*<0.01
C18:1(trans)	4.01±0.21	6.16±0.36	5.1±1.9	3.29±0.15	4.05±0.44	NS
C18:2(n-6)	7.02±0.41	6.40±0.32	6.81±0.43	8.2±0.32	8.95±0.73	F=4.8 *P*<0.05
C20:1	0.78±0.017	0.72±0.64	0.43±0.11	1.33±0.11	2.63±0.11	F=3.3 *P*<0.05
C20:3(n-6)	0.74±0.095	0.45±0.19	1.18±0.29	1.44±0.20	0.67±0.13	F=3.5 *P*<0.05
C20:4(n-6)	7.61±0.46	5.11±0.24	4.2±0.81	2.67±0.19	1.31±0.052	F=7.17 *P*<0.01
C20:5(n-3)	4.81±0.70	2.25±0.49	7.57±1.3	6.91±0.23	6.00±0.31	NS
C22:5(n-3)	4.03±0.28	3.16±0.30	2.79±0.12	3.01±0.92	2.91±0.71	NS
C22:6(n-3)	2.57±0.44	2.15±0.075	2.55±0.22	2.20±0.11	2.02±0.25	F=4.3 *P*<0.05
Saturated	30.05	41.55	33.65	33.56	37	
Unsaturated	69.95	58.15	66.06	65.79	63	
n-3	14.21	9.42	12.91	12.12	10.93	
n-6	12.57	10.12	12.19	12.31	10.93	

NC-control, ND-diabetic, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 16C: Effect of dietary supplementation of fish oil on the % concentration of fatty acid profile of heart of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Type of fatty acids	NC	ND	SD	RD	CD	ANOVA
C14:0	1.66±0.063	2.63±0.44	1.82±0.040	2.03±0.090	2.01±0.24	F=4.5 *P*<0.05
C16:0	24.48±0.42	31.46±0.84	20.14±0.48	22.43±0.53	25.07±0.59	F=24.6 *P*<0.001
C16:1(cis)	1.13±0.24	2.13±0.19	2.11±0.10	3.52±0.24	3.53±0.34	F=4.8 *P*<0.01
C16:1(trans)	5.86±0.34	5.28±0.49	6.34±0.16	6.03±0.48	6.96±0.64	F=40.3 *P*<0.0001
C18:0	8.05±0.76	12.85±1.14	15.1±0.10	12.84±0.65	12.88±0.33	F=3.0 *P*<0.05
C18:1(cis)	25.6±1.89	20.04±0.97	20.79±1.09	19.8±1.1	18.47±1.1	F=3.1 *P*<0.05
C18:1(trans)	5.33+0.45	4.42±0.23	4.22±0.46	4.01±0.51	2.61±0.41	F=9.5 *P*<0.01
C18:2(n-6)	9.73±0.28	6.58±0.30	7.79±0.17	7.33±0.43	7.24±1.0	F=5.1 *P*<0.01
C20:1	1.32±0.18	1.16±0.29	1.60±0.13	1.86±0.061	1.78±0.071	F=7.8 *P*<0.01
C20:3(n-6)	1.0±0.12	1.74±0.095	1.05±0.027	1.33±0.085	1.70±0.18	F=9.04 *P*<0.01
C20:4(n-6)	6.4±0.26	5.11±0.51	4.22±0.49	3.51±0.46	2.34±0.44	F=11.8 *P*<0.001
C20:5(n-3)	3.14±0.12	2.54±0.11	6.43±0.14	5.63±0.42	5.44±0.49	F=4.0 *P*<0.05
C22:5(n-3)	2.06±0.33	1.91±0.95	5.19±0.26	7.13±0.49	7.69±0.58	F=106 *P*<0.0001
C22:6(n-3)	4.24±0.30	1.36±0.047	3.20±0.096	2.15±0.075	1.38±0.21	F=244 *P*<0.0001
Saturated	34.19	46.94	37.06	37.3	39.96	
Unsaturated	65.81	52.27	62.4	62.3	59.14	
n-3	12.7	11.38	14.82	14.91	14.51	
n-6	13.87	7.86	13.06	12.17	11.28	

NC-control, ND-diabetic, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

Table 16D: Effect of dietary supplementation of fish oil on the % concentration of fatty acid profile of pancreas of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors were tabulated.

Type of fatty acids	NC	ND	SD	RD	CD	ANOVA
C14:0	1.78±0.098	3.07±0.36	2.29±0.52	2.33±0.29	2.01±0.24	F=3.06 *P*<0.05
C16:0	24.02±0.30	31.4±0.73	26.1±0.61	27.4±0.233	25.41±0.90	F=29.5 *P*<0.0001
C16:1(cis)	1.92±0.032	3.23±0.066	2.43±0.24	3.32±0.12	3.35±0.24	F=4.4 *P*<0.05
C16:1(trans)	5.89±0.16	5.62±0.27	6.53±0.23	6.66±0.18	6.93±0.29	F=16.2 *P*<0.0001
C18:0	12.05±0.92	14.1±1.55	11.7±0.78	12.1±0.40	12.3±0.25	NS
C18:1(cis)	24.1±0.54	20.3±0.36	23.4±0.51	22.06±0.078	23.6±0.50	F=13.19 *P*<0.001
C18:1(trans)	6.2±0.54	5.42±0.49	4.28±0.13	4.59±0.19	3.41±0.088	F=13.7 *P*<0.0001
C18:2(n-6)	10.9±0.41	6.92±0.64	8.01±0.20	8.66±0.51	7.37±1.13	F=6.8 *P*<0.01
C20:1	1.05±0.016	0.773±0.075	1.09±0.048	0.926±0.074	0.783±0.01	F=54.2 *P*<0.0001
C20:3(n-6)	1.05±0.027	0.51±0.040	0.556±0.03	0.453±0.033	0.557±0.04	F=36.8 *P*<0.0001
C20:4(n-6)	4.47±0.32	3.77±0.33	1.88±0.33	1.51±0.25	1.64±0.072	F=5.3 *P*<0.01
C20:5(n-3)	2.08±0.11	1.18±0.085	6.76±0.19	6.96±0.46	6.78±0.14	F=18.9 *P*<0.0001
C22:5(n-3)	1.72±0.064	1.18±0.086	1.56±0.048	1.46±0.24	1.5±0.15	F=149 *P*<0.0001
C22:6(n-3)	3.12±0.060	1.40±0.041	2.51±0.35	2.19±0.10	2.45±0.12	F=17.5 *P*<0.0001
Saturated	37.85	48.57	40.09	41.83	39.72	
Unsaturated	62.85	51.43	59.91	58.17	60.28	
n-3	9.31	11.38	14.82	14.91	14.51	
n-6	14.03	10.86	13.06	12.17	11.28	

NC-control, ND-diabetic, SD- *Sardinella* oil supplemented diabetic, CD- *Clarias* oil supplemented diabetic, RD- *Rastrelliger* oil supplemented diabetic

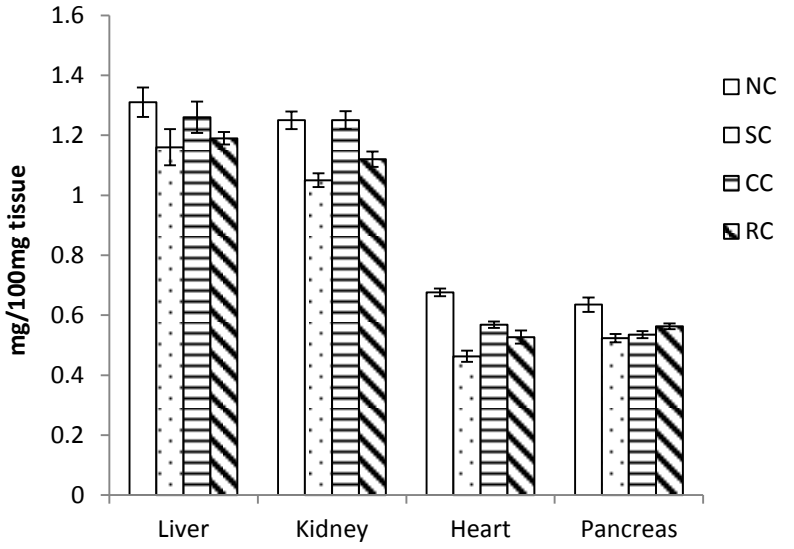


Figure 9A: Effect of dietary supplementation of fish oil on the concentration of total cholesterol in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

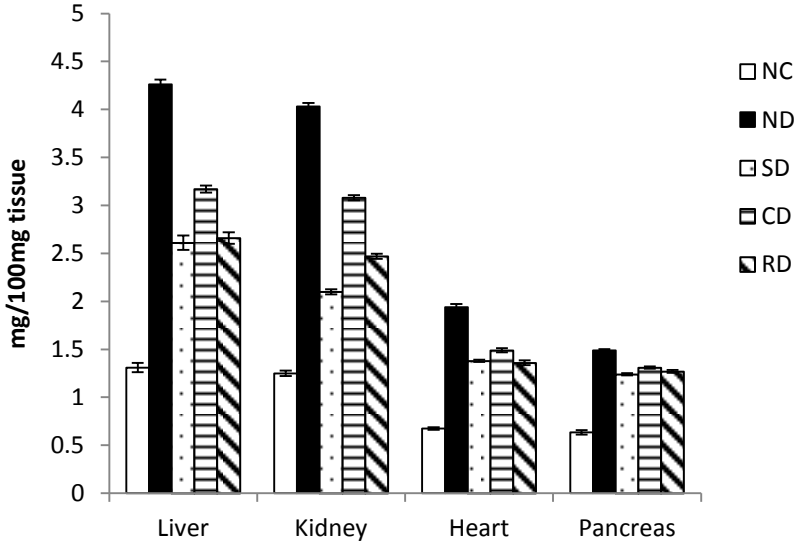


Figure 9B: Effect of dietary supplementation of fish oil on the concentration of total cholesterol in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

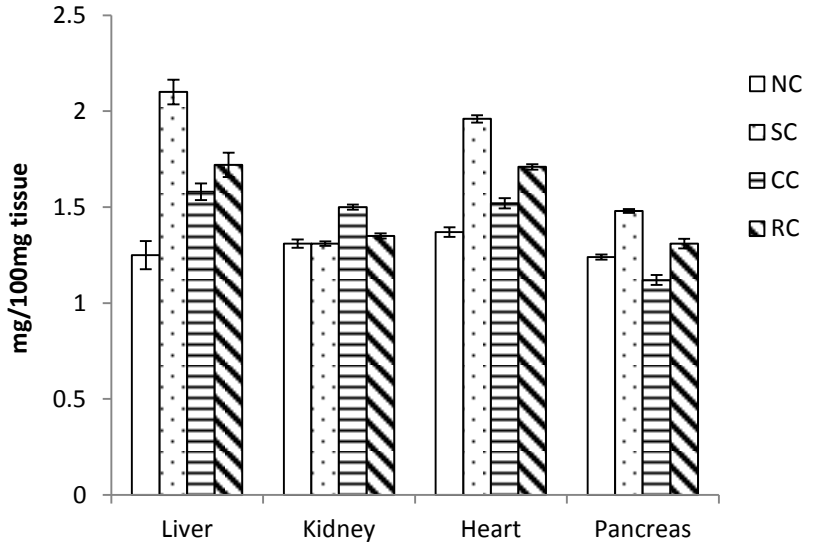


Figure 10A: Effect of dietary supplementation of fish oil on the concentration of total phospholipid in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

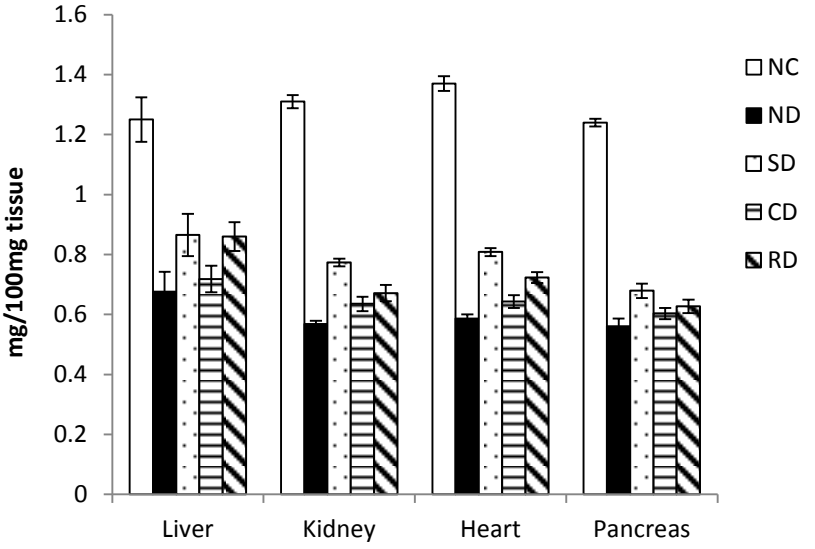


Figure 10B: Effect of dietary supplementation of fish oil on the concentration of total phospholipid in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

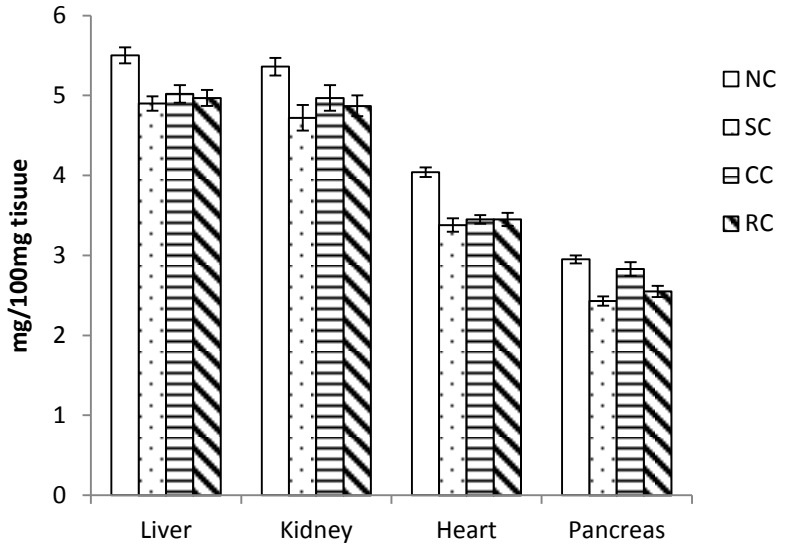


Figure 11A: Effect of dietary supplementation of fish oil on the concentration of total glycolipid in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

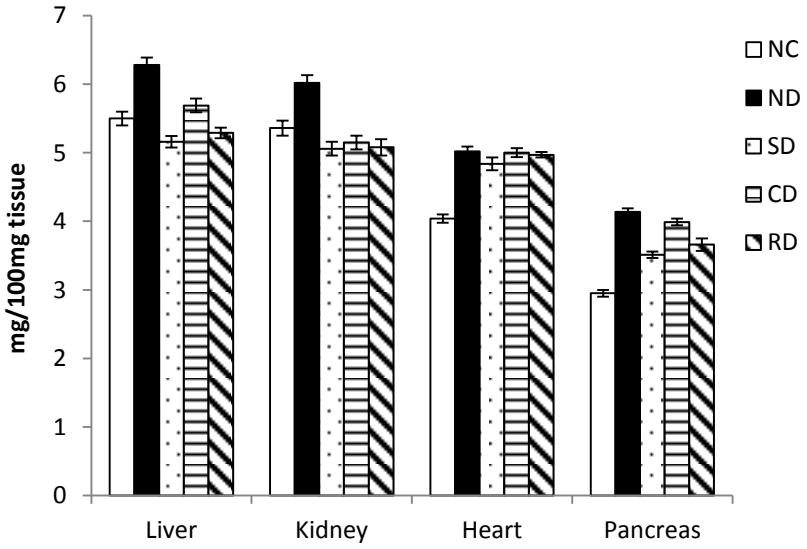


Figure 11B: Effect of dietary supplementation of fish oil on the concentration of total glycolipid in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

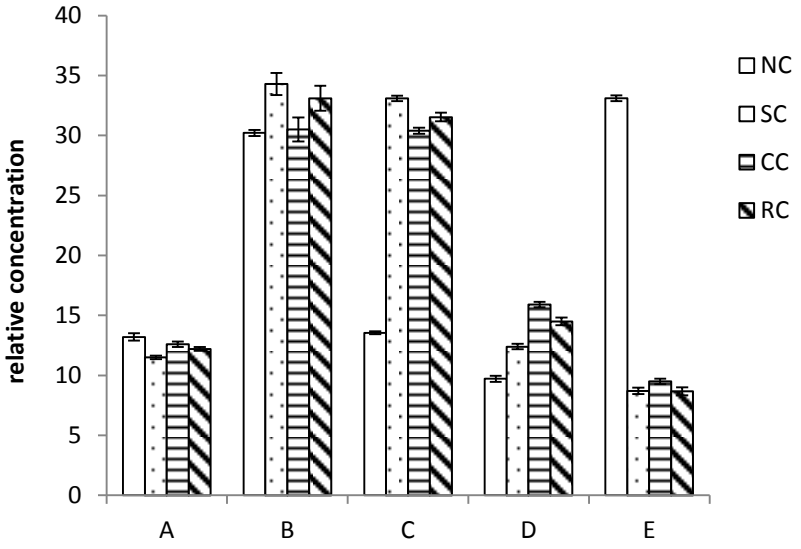


Figure 12A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in liver of mice (*Mus musculus*).Mean values of six individual mice and their standard errors.

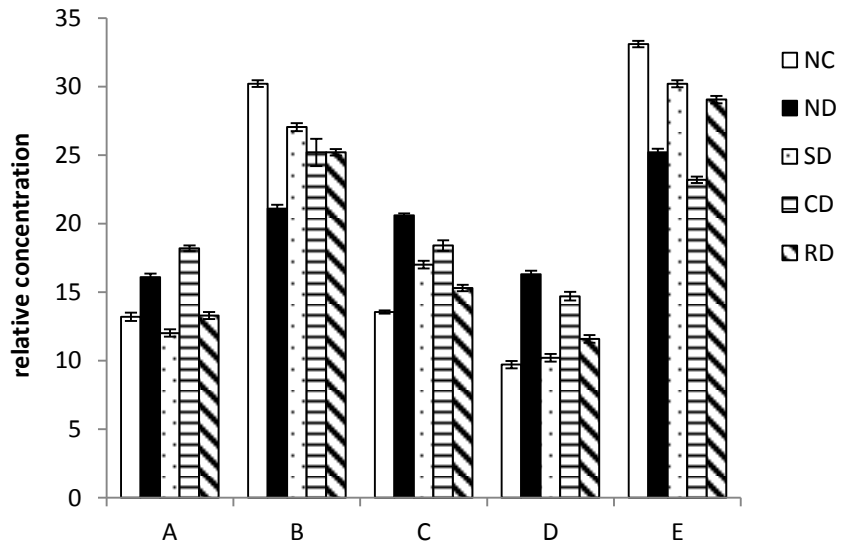


Figure 12B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in liver of alloxan induced diabetic mice (*Mus musculus)*. Mean values of six individual mice and their standard errors.

A: Phosphotidyl ethanolamine, B: Phosphotidyl choline, C: Sphingomyelin, D: Lysophospholipid, E: Phosphotidyl inisitol+Phosphotidyl serine.

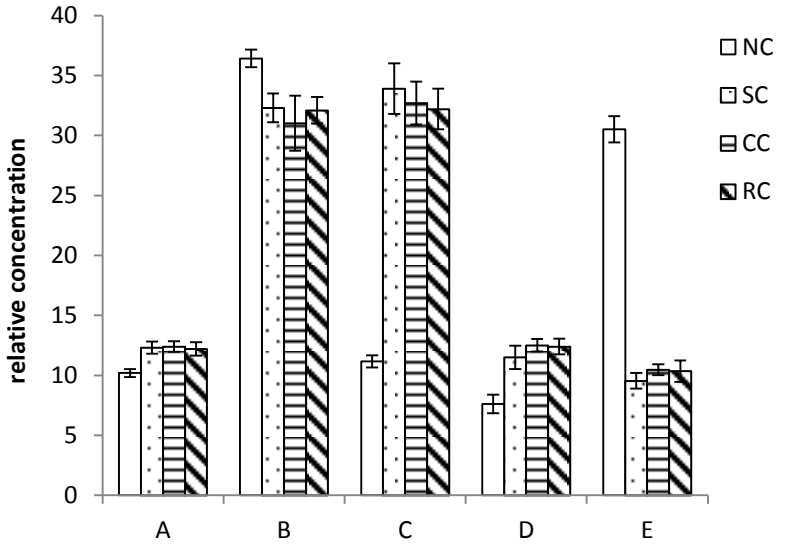


Figure 13A: Effect of dietary supplementation of fish oil on relative concentration of (%) various phospholipid fractions in kidney of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

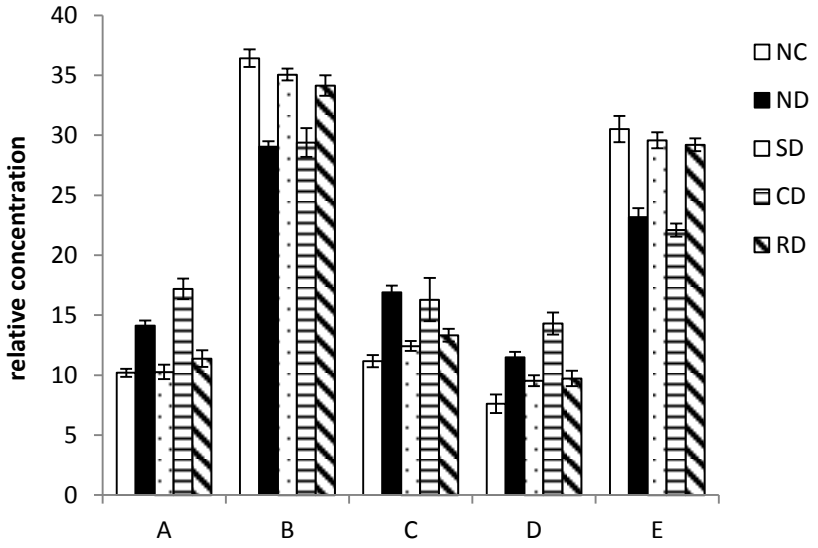


Figure 13B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in kidney of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Phosphotidyl ethanolamine, B: Phosphotidyl choline, C: Sphingomyelin, D: Lysophospholipid, E: Phosphotidyl inisitol+Phosphotidyl serine.

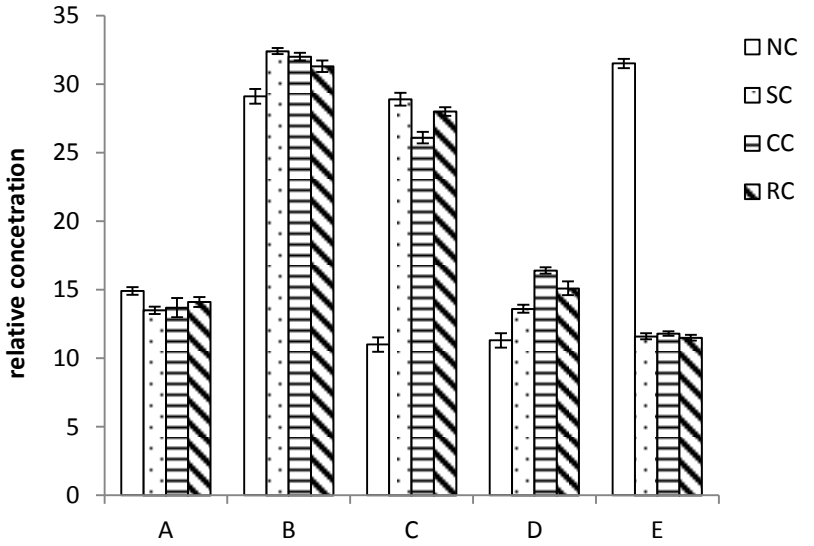


Figure 14A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in heart of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

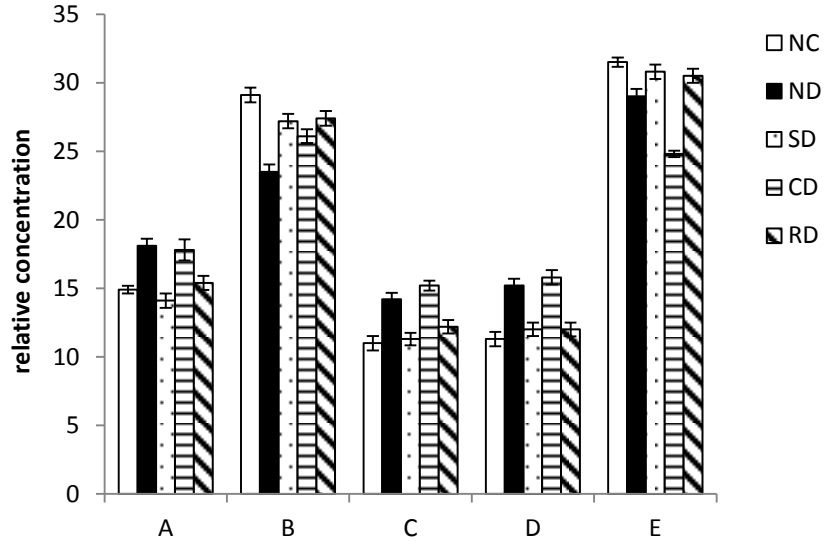


Figure 14B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in heart of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Phosphotidyl ethanolamine, B: Phosphotidyl choline, C: Sphingomyelin, D: Lysophospholipid, E: Phosphotidyl inisitol+Phosphotidyl serine.

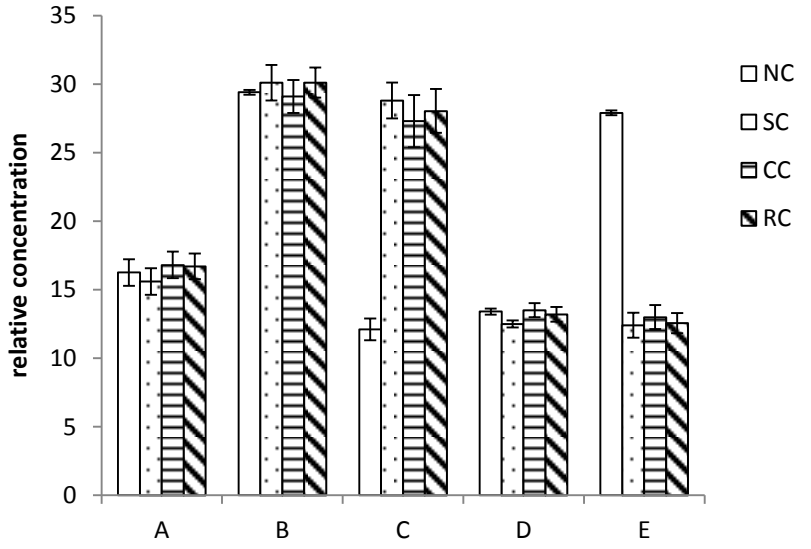


Figure 15A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in pancreas of mice (*Mus musculus*). Mean values of six individual mice and their standard errors)

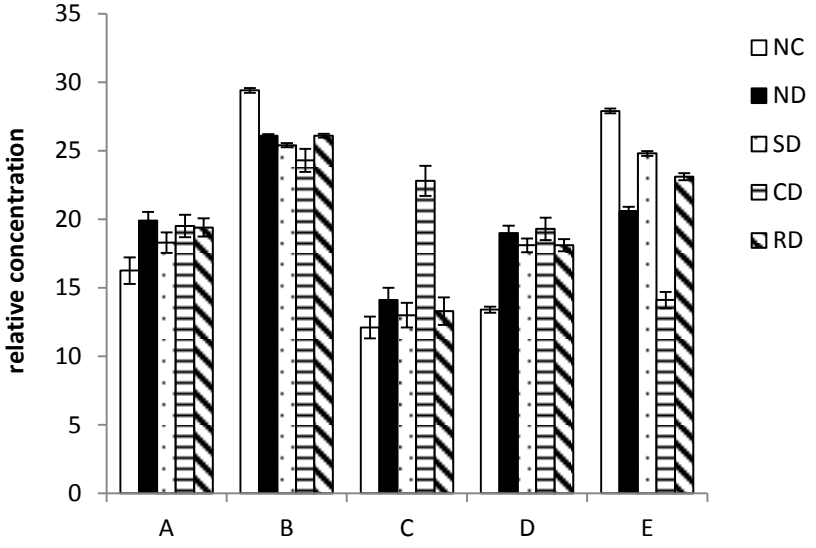


Figure 15B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various phospholipid fractions in pancreas of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Phosphotidyl ethanolamine, B: Phosphotidyl choline, C: Sphingomyelin, D: Lysophospholipid, E: Phosphotidyl inisitol+Phosphotidyl serine.

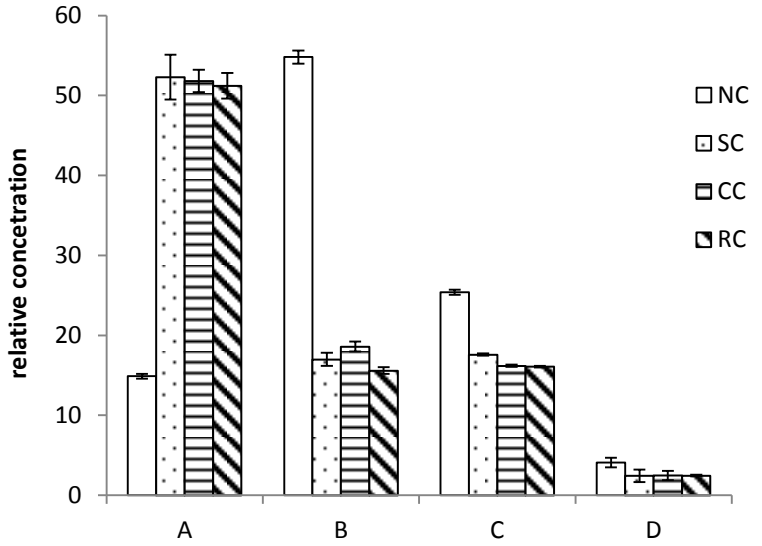


Figure 16A: Effect of dietary supplementation of fish oil on relative concentration of (%) various glycolipid fractions in liver of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

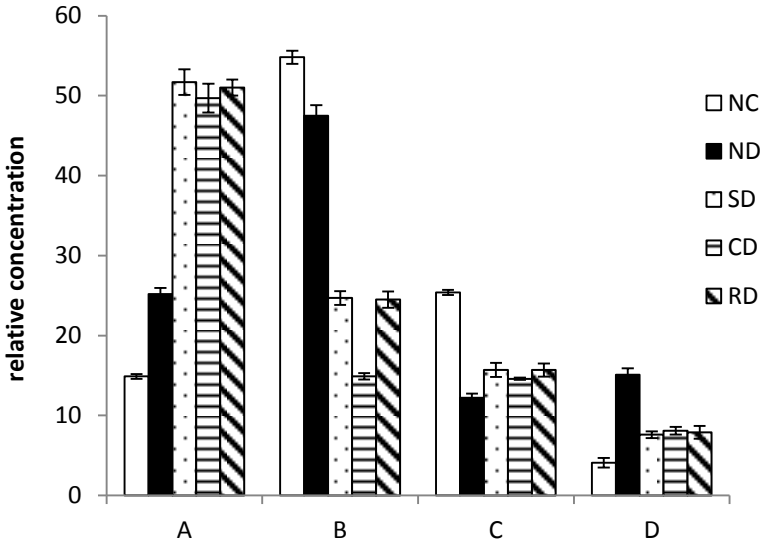


Figure 16B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in liver of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Unknown 1 (RI=0.9), B: Unknown 2 (RI=0.32), C: Galactocerebrocide, D: Sphingolipid.

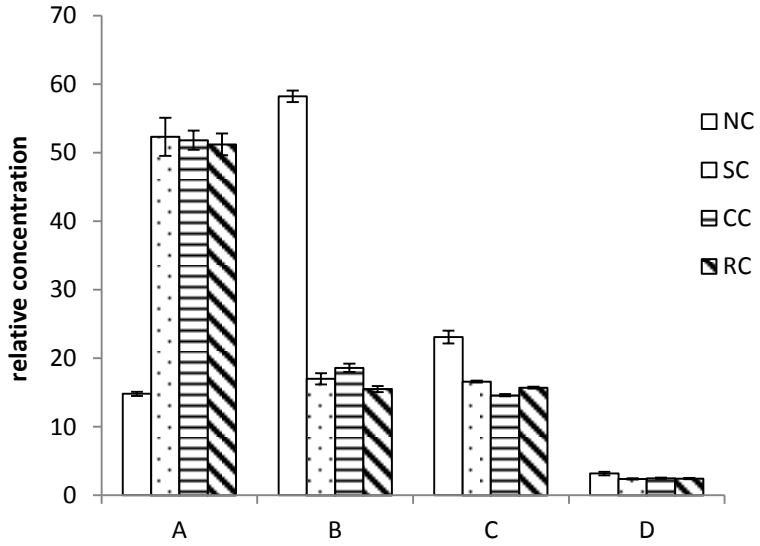


Figure 17A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in kidney of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

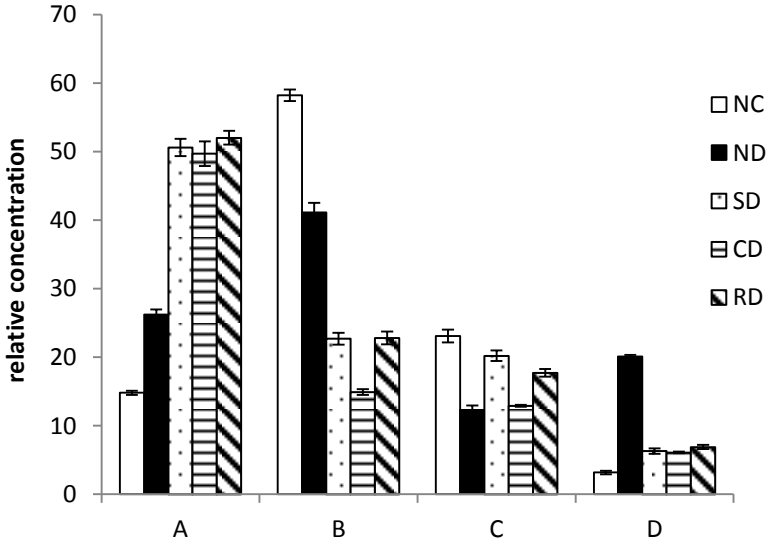


Figure 17B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in kidney of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Unknown 1 (RI=0.9), B: Unknown 2 (RI=0.32), C: Galactocerebrocide, D: Sphingolipid.

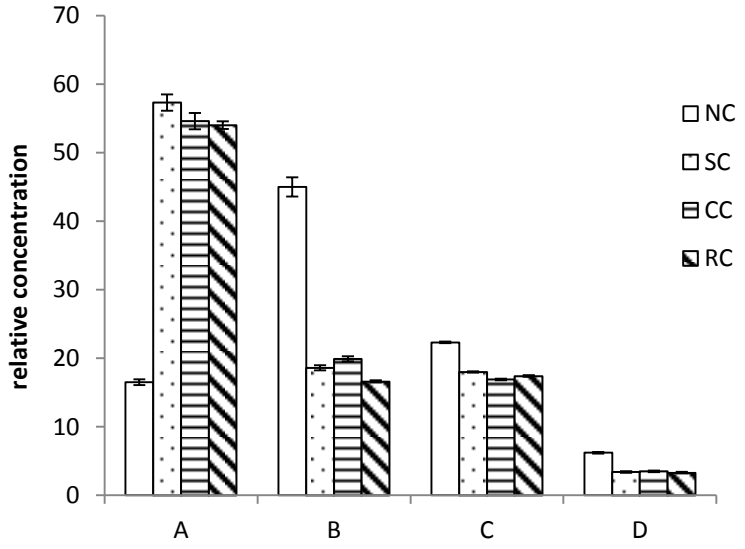


Figure 18A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in heart of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

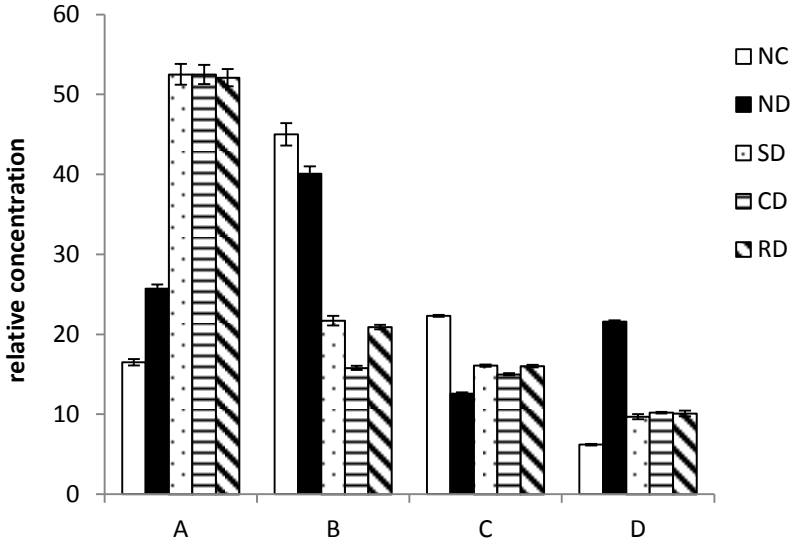


Figure 18B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in heart of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Unknown 1 (RI=0.9), B: Unknown 2 (RI=0.32), C: Galactocerebrocide, D: Sphingolipid.

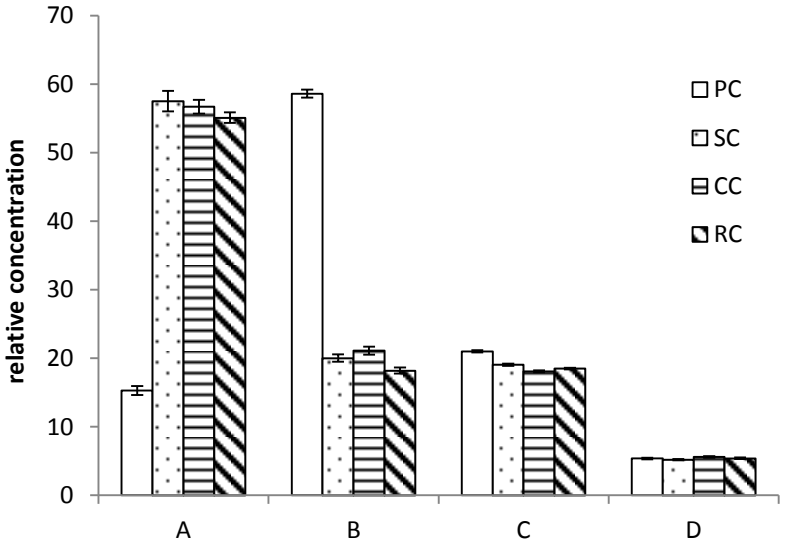


Figure 19A: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in pancreas of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

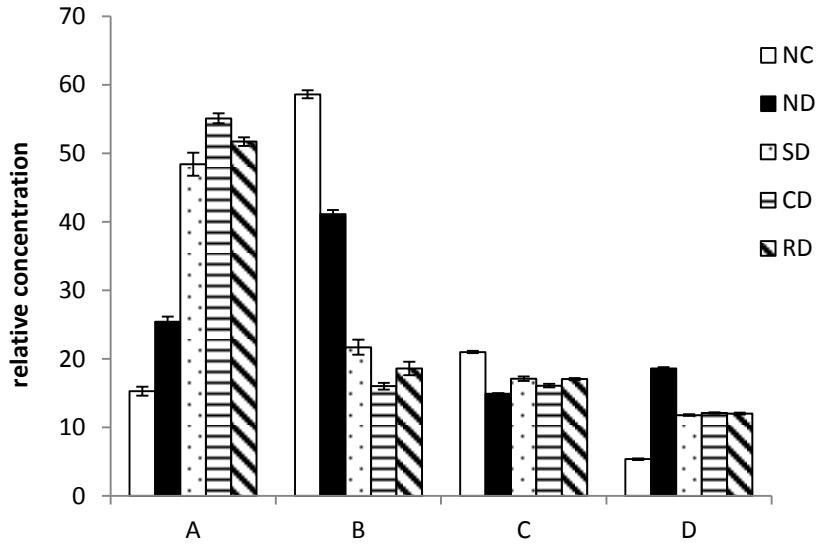


Figure 19B: Effect of dietary supplementation of fish oil on the relative concentration of (%) various glycolipid fractions in pancreas of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

A: Unknown 1 (RI=0.9), B: Unknown 2 (RI=0.32), C: Galactocerebrocide, D: Sphingolipid.

3.5.4 Effect of supplementation of fish oil on membrane fluidity of diabetic mice groups:

DPH anisotropy vale indirectly provides the information about the fluidity of the cell membrane. The hindrance of the rotation of the DPH probe in the membrane lipid bilayer will enhance the anisotropy value. Therefore the anisotropy value is inversely related to the membrane fluidity (Roy *et al*., 1999). Increase in anisotropy reflects more highly constrained motion and lowered fluidity while a decrease in anisotropy reflects less highly constrained motion and increased fluidity. DPH-Anisotropy values for liver, kidney, heart and pancreas tissues of diabetic mice groups are presented in Figure 20A-D.

Induction of diabetes significantly ($P<0.0001$) elevated the anisotropy by 40% - 1.7 fold, which indicates that the fluidity of the membrane is significantly decreased in diabetic mice tissues when compared to control group of mice. The *Sardinella* and *Rastrelliger* fish oil supplementation in diabetic mice groups decreased ($P<0.0001$) the anisotropy by 10-35% in all the tissues, whereas the *Clarias* oil supplementation decreased ($P<0.0001$) the anisotropy approximately by 10-20% in the liver, heart and pancreas only, which signifies that fish oil supplementation helps to regain the fluidity of membrane in diabetic mice groups.

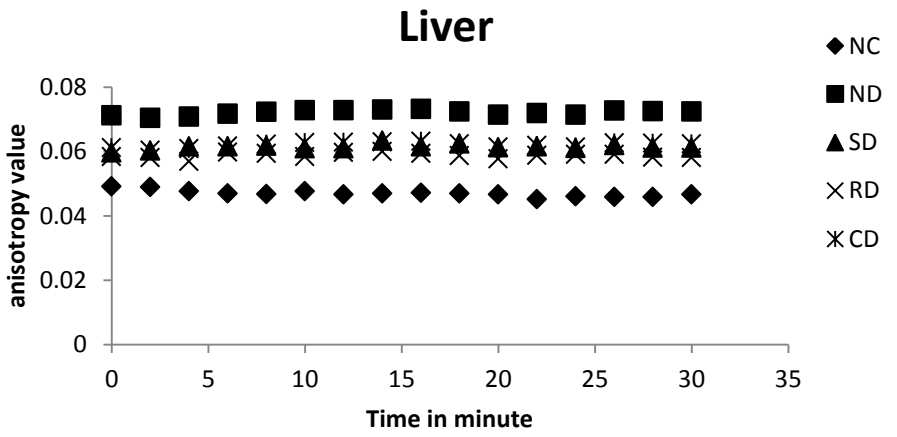


Figure 20A: Effect of supplementation of fish oil on tissue membrane fluidity of liver in alloxan induced diabetic mice (*Mus musculus*)

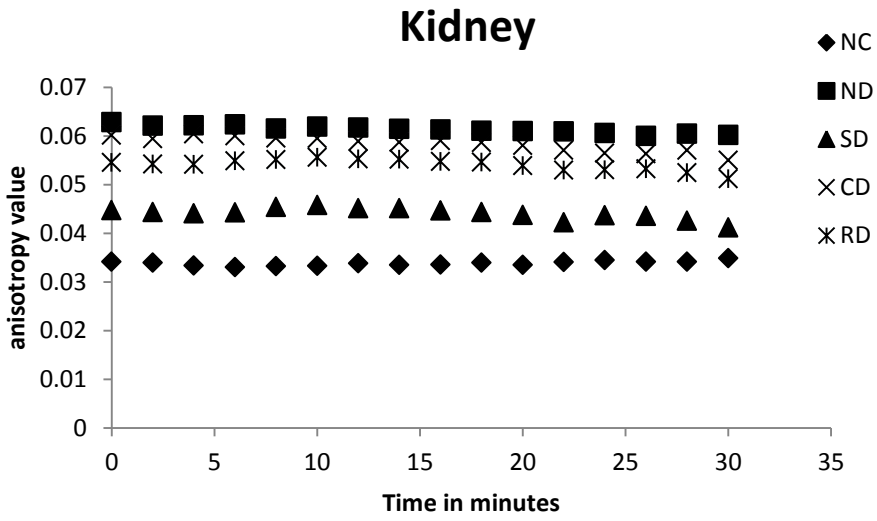


Figure 20B: Effect of supplementation of fish oil on tissue membrane fluidity of kidney in alloxan induced diabetic mice (*Mus musculus*)

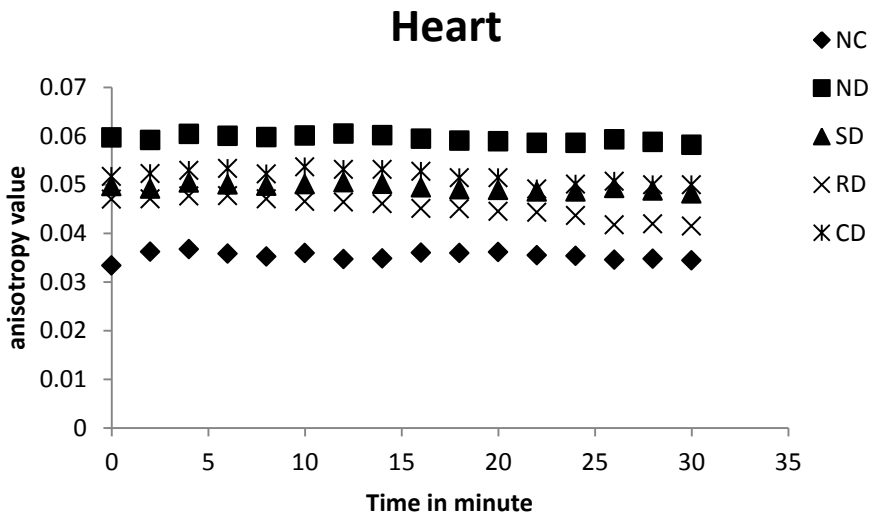


Figure 20C: Effect of supplementation of fish oil on tissue membrane fluidity of heart in alloxan induced diabetic mice (*Mus musculus*)

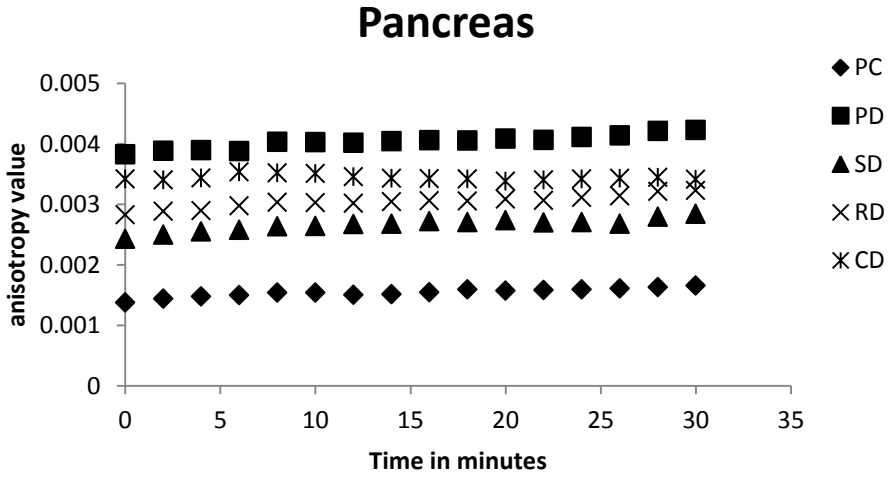


Figure 20D: Effect of supplementation of fish oil on tissue membrane fluidity of pancreas in alloxan induced diabetic mice (*Mus musculus*)

3.5.5 Effect of supplementation of fish oil on the activity of enzymes (lipid metabolism) of control and diabetic mice groups:

HMG-CoA synthase: HMG-CoA synthase enzyme is an intermediate in cholesterol synthesis and ketogenesis. It catalyses the condensation of 3 acetyl CoA to form HMG-CoA.

The *Sardinella* oil supplementation to the non-diabetic group of mice decreased the activity of enzyme about by 10-20% in all the tissues (P<0.01-0.0001), whereas the *Rastrelliger* and *Clarias* oil supplementation decreased the activity of enzyme by 5-15% in serum, liver and heart only (Figure 21A). Induction of diabetes significantly (P<0.0001) augmented the enzyme activity by 25-55% in all the tissues. The supplementation *Sardinella* oil to the diabetic group of mice reduced (P<0.0001) the activity of enzyme by 15-25% in all the tissues. The augmented activity of enzyme decreased by 10-25% in all the tissues (P<0.0001) except for pancreas in *Rastrelliger* and *Clarias* oil supplemented diabetic group of mice (Figure 21B).

HMG-CoA reductase: HMG-CoA reductase is a rate-controlling NADH and NADPH dependent enzyme involved in cholesterol biosynthesis. It reduces HMG-CoA to mevalonic acid.

Supplementation of fish oils to the non-diabetic mice group significantly (F=135.984-555.707, P<0.0001) decreased the activity of the enzyme by 10-35% in all the tissues (Figure 22A). Induction of diabetes increased the activity of the enzyme significantly (P<0.0001) by 40-80%. Supplementation of fish oils to the diabetic group of mice significantly (F=458.674-2280.415, P<0.0001) decreased the elevated activity of an enzyme (Figure 22B) by 5-35% in all the tissues.

Glucose-6-phosphate dehydrogenase: Biosynthesis of any lipid molecule needs the reducing power of NADP not the reducing power of NAD. Glucose-6-phosphate dehydrogenase enzyme through phosphogluconate pathway it supplies NADPH to the cells.

Supplementation of all the three fish oil to the non-diabetic group of mice increased the activity of enzyme approximately (P<0.001) by 15-45% in serum, kidney and pancreas only (Figure 23A). In addition, supplementation of *Sardinella* oil enhanced the activity in heart tissue by 10% (P<0.01). Induction of diabetes significantly (P<0.0001) decreased the enzyme activity by 20-60% in all the tissues. The supplementation *Sardinella* fish oil to the diabetic group of mice significantly (P<0.0001) augmented the enzyme activity by 25-90% in serum, heart, kidney and pancreas tissues. However, the *Rastrelliger* oil supplementation showed 20-90% increase (P<0.001- 0.0001) in the activity of enzyme in all the tissues except for the liver. In addition, 10-30% increased (P<0.001- 0.0001) activity was noticed in serum, heart, kidney tissues upon supplementation of *Clarias* oil in the diabetic group of mice (Figure 23B).

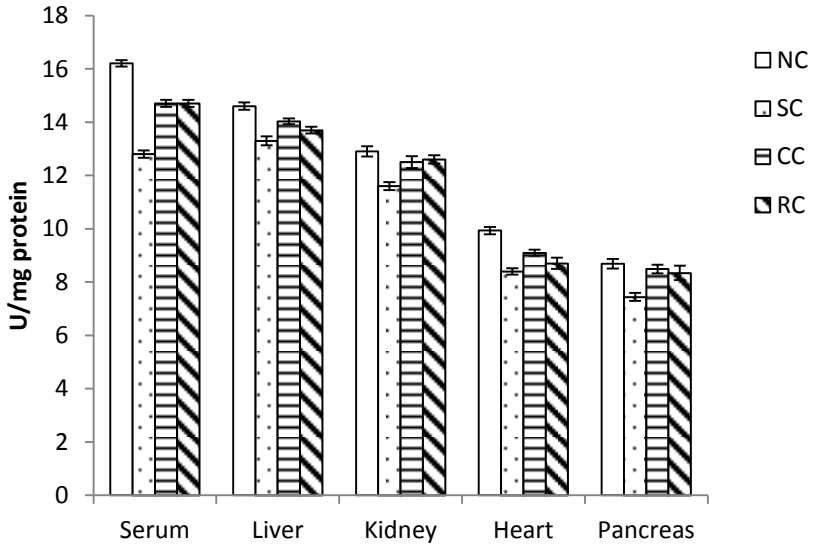


Figure 21A: Effect of dietary supplementation of fish oil on the concentration of enzyme HMG Co A synthase in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

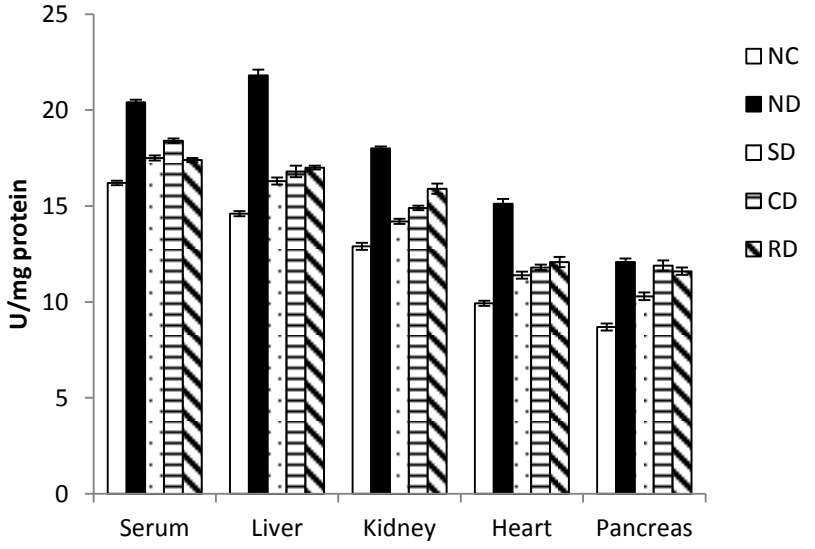


Figure 21B: Effect of dietary supplementation of fish oil on the concentration of enzyme HMG Co A synthase in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

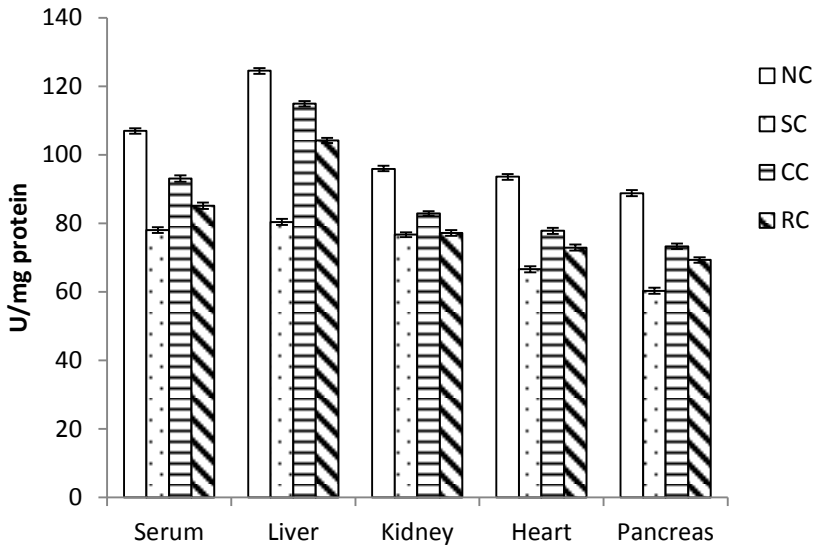


Figure 22A: Effect of dietary supplementation of fish oil on the concentration of enzyme HMG Co A reductase in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

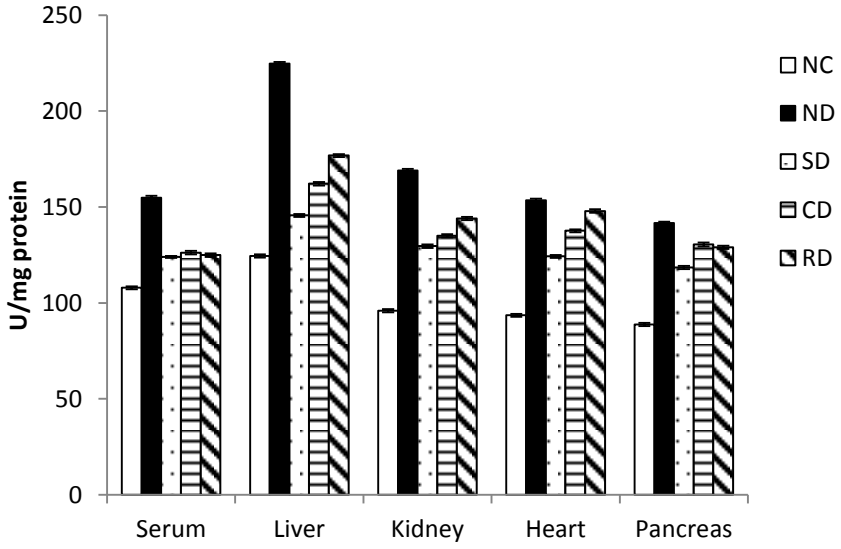


Figure 22B: Effect of dietary supplementation of fish oil on the concentration of enzyme HMG Co A reductase in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

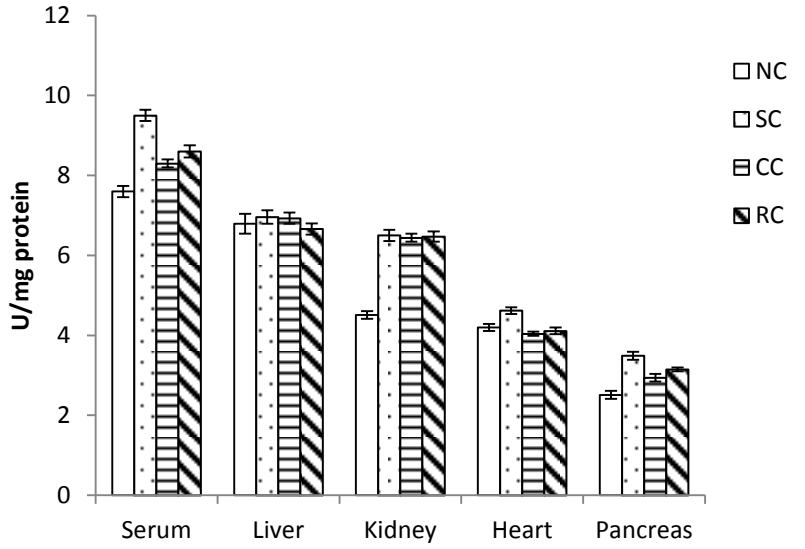


Figure 23A: Effect of dietary supplementation of fish oil on the concentration of enzyme G6PD in various tissues of mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

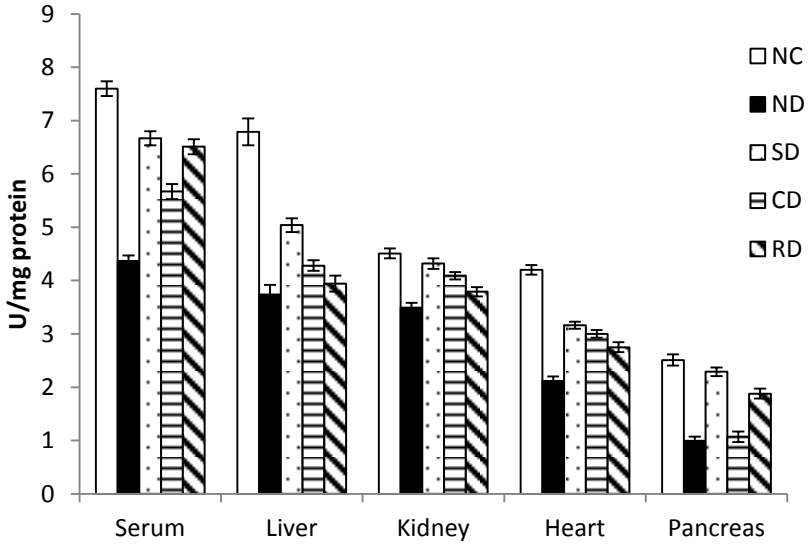


Figure 23B: Effect of dietary supplementation of fish oil on the concentration of enzyme G6PD in various tissues of alloxan induced diabetic mice (*Mus musculus*). Mean values of six individual mice and their standard errors.

3.6 Effect of supplementation of fish oil on some cytokine and regulatory gene expressions of diabetic mice:

The relative quantity of gene expression was quantified by comparing the amount of gene of interest band with a reference band of β-actin (housekeeping gene). The quantification is done with the help of BioRad Image lab software.

Cytokine expression:

TNFα which is a cell signalling protein and IL1α which is a protein of the interleukin family are responsible for production of inflammation. It is noted that induction of diabetes significantly (P<0.0001) increased the gene expression of TNFα and IL1α by 30-80% in the liver, kidney and heart tissues when compared to control. Supplementation of fish oils to the SD, CD and RD group of mice significantly (F=807.66-9920.14, P<0.0001) decreased the TNFα and IL1α expression by 30-60% in the above mentioned tissues (Plate 5, Figure 24A-B).

Regulatory genes expression:

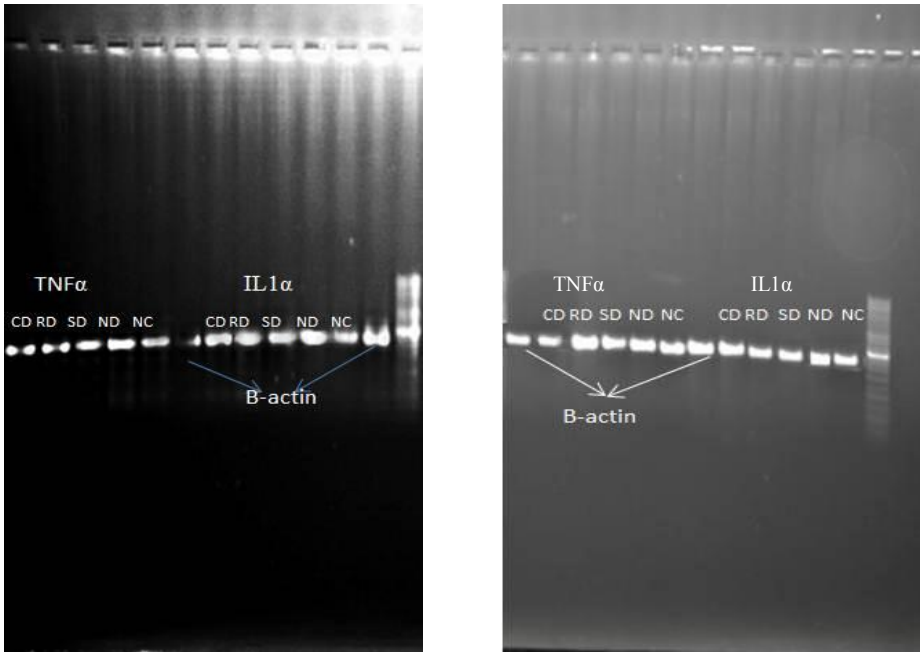
GLUTs (GLUT2 and GLUT4) are the membrane proteins which facilitate the transport of glucose over a membrane liver to blood. Induction of diabetes in the ND group of mice significantly (P<0.001) decreased the expression of GLUT2 (Plate 6a) and GLUT4 (Plate 6b) by 25-35% in liver tissues when compared to control group of mice (NC). Supplementation of fish oil to the SD, CD and RD group of mice significantly increased (P<0.0001) the expression of GLUT2 and GLUT4 by 5-10% in liver tissue (Table 17A)

Cyclooxygenase (COX1 and COX2) is the key enzymes required for conversion of 20 carbon chain fatty acid to form prostanoids including

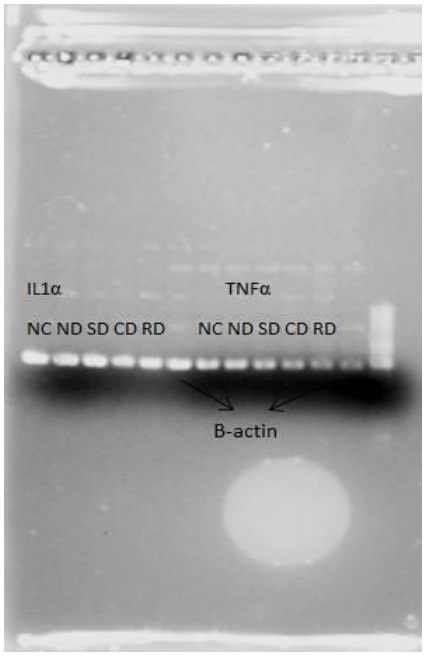
thromboxane and prostaglandins. Induction of diabetes in the ND group of mice elevated (P<0.001) the expression of COX2 nearly by 70% (Plate 6c), with no notable changes in expression of COX1 (plate 6d) in the liver. Supplementation of fish oil to the SD, CD and RD group of mice significantly decreased the expression COX by 10-35% (P<0.01-0.0001) in liver tissue (Table 17A).

Sterol regulatory element binding protein 1 is involved in the enhancement of transcription of genes encoding enzymes of cholesterol and fatty acid biosynthesis and uptake. Induction of diabetes in the ND group of mice significantly (P<0.001) increased the expression of SREBP1 (Plate 7c) by 70% in liver tissue when compared to control group of mice. Supplementation of fish oil to the SD, CD and RD group of mice significantly decreased (P<0.0001) the expression of SREBP1 nearly by 25-30% in liver tissue (Table 17A).

The insulin (Ins1 and Ins2) gene provides instruction for the production of pancreatic hormone insulin, which is necessary to maintain blood glucose level. The protein encoded by the glucagon (GCG) gene is cleaved into four different peptides, one of which produces pancreatic hormone glucagon that counteracts the glucose lowering action of insulin. Due to induction of diabetes in the ND group of mice the Ins1 (Plate 6a) and Ins2 (Plate 6b) gene expression decreased significantly (P<0.0001) by 56-60% and along with a 55% increase in expression of the glucagon gene (Plate 6e) in the pancreas. The supplementation of all the three fish oils for the diabetic groups of mice resulted in 55-100% significant (F=629-4833 P<0.0001) increase in both Ins1 and Ins2 expression along with 10-15% decrease (F=466.2 P<0.0001) in glucagon expression (Table 17B).



(a) (b)



(c)

Plate 5: Agarose gel electrophoresis showing the amplification of TNF α and IL1α cytokines in **(A)** liver (**B)** Kidney and **(C)** Heart. NC: Control mice, ND: Diabetic mice, SD: Diabetic mice supplemented with *Sardinella* fish oil, CD: Diabetic mice supplemented with *Clarias* fish oil, RD: Diabetic mice supplemented with *Rastrelliger* fish oil.

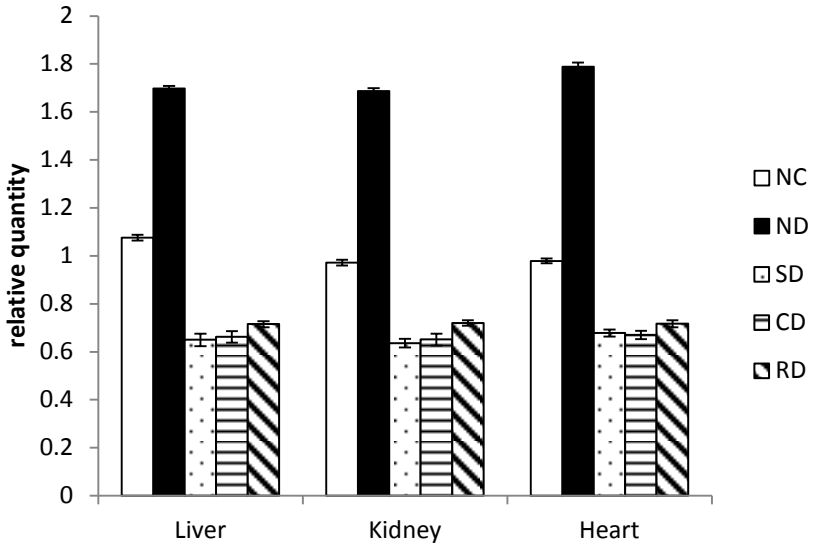


Figure 24A: Effect of fish oil treatment on the (relative quantity) gene expression of tissue TNFα upon induction of diabetes. Mean values of 3 individual mice and their ±SE)

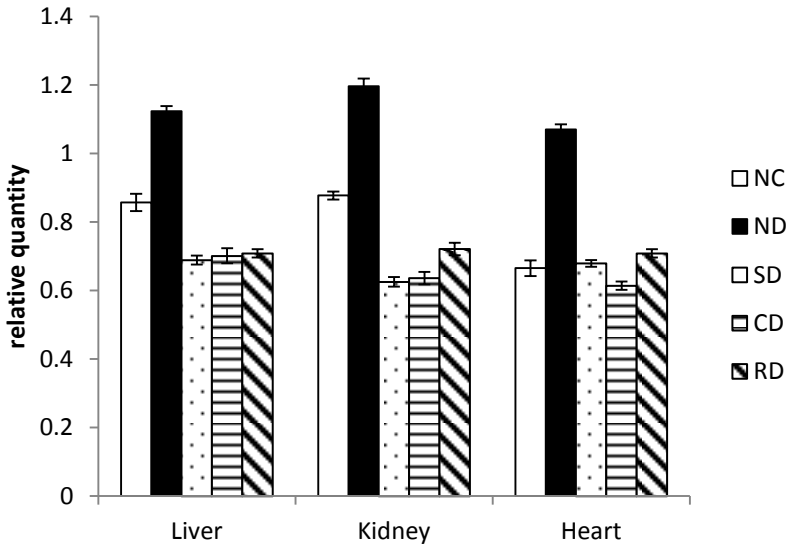
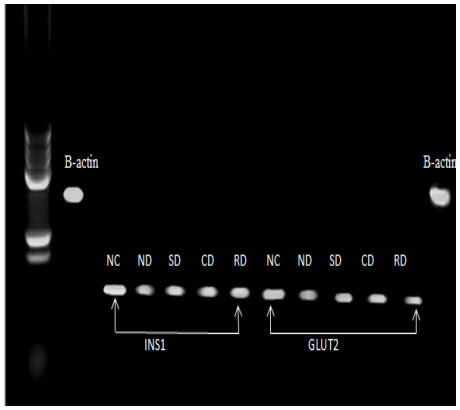
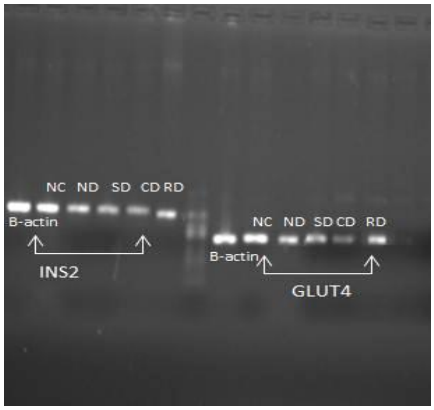


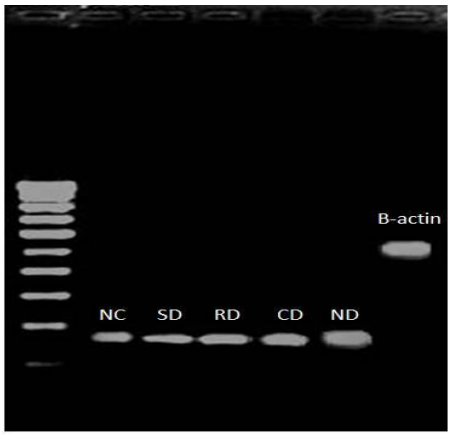
Figure 24B: Effect of fish oil treatment on the (relative quantity) gene expression of tissue IL1α upon induction of diabetes. Mean values of 3 individual mice and their ±SE.



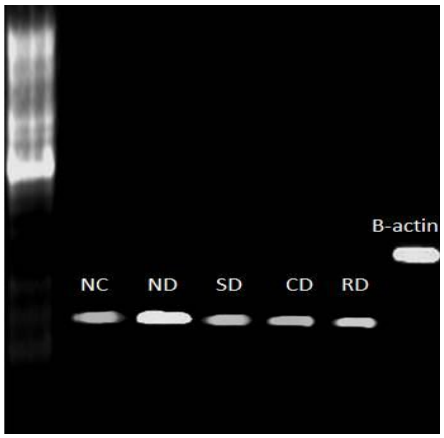
(a)



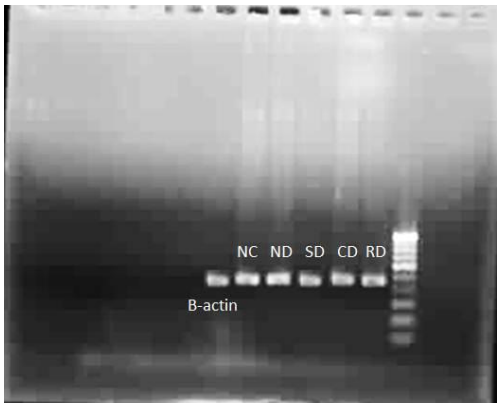
(b)



(c)



(d)

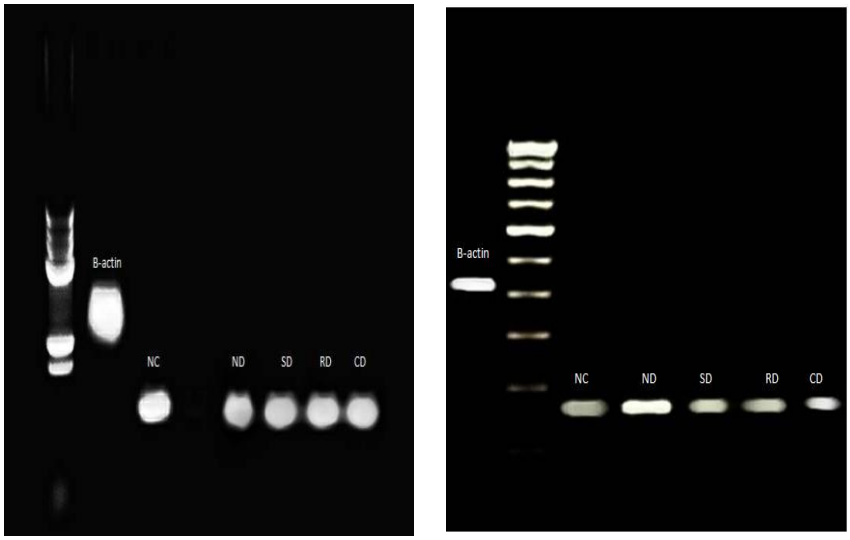


(e)

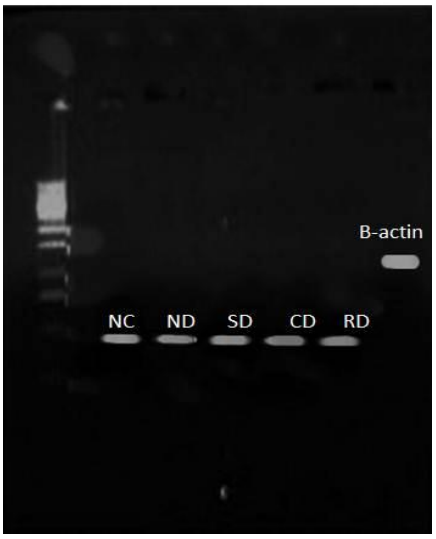
Plate 6: Agarose gel electrophoresis showing the amplification of **(A)**: Ins1 and GLUT2 **(B)** Ins2 and GLUT4 **(C)** COX1 **(D)** COX2 and **(E)** Glucagon. NC: Control mice, ND: Diabetic mice, SD: Diabetic mice supplemented with *Sardinella* fish oil, CD: Diabetic mice supplemented with *Clarias* fish oil, RD: Diabetic mice supplemented with *Rastrelliger* fish oil.

PPARα a member of the ligand-activated nuclear receptor superfamily have a significant role in the metabolism of lipid and glucose homeostasis which is an important regulator of cardiac metabolism. Due to induction of diabetes the PPARα (Plate 7a) expression in heart significantly (P<0.0001) decreased by 25%. The supplementation of fish oil to the SD, CD and RD group of mice significantly (57.35 P<0.0001) increased the PPARα expression in heart by 10-15% (Table 17C).

TGFβ is a multifunctional cytokine which acts as a key mediator of glomerular and tubulointerstitial pathobiology in chronic kidney diseases. Induction of diabetes resulted in nearly 95% increase (P<0.0001) in expression of TGFβ (Plate 7c) in kidney tissue. The supplementation of fish oils for the diabetic groups of mice significantly (F=5560.91 P<0.0001) brought down the elevated level by 45% (Table 17C).



(a) (b)



(c)

Plate 7: Agarose gel electrophoresis showing the amplification of **(A)**: PPARα heart **(B)** TGFβ in kidney **(C)** SREBP1c in liver. NC: Control mice, ND: Diabetic mice, SD: Diabetic mice supplemented with *Sardinella* fish oil, CD: Diabetic mice supplemented with *Clarias* fish oil, RD: Diabetic mice supplemented with *Rastrelliger* fish oil.

Table 17A: Effect of fish oil treatment on the (relative quantity) of COX1, COX2, GLUT1, GLU2 and SREBP1 gene expression in liver upon induction of diabetes. Mean values of six individual mice and their standard errors were tabulated

Genes	**NC**	**ND**	**SD**	**CD**	**RD**	**ANOVA**
GLUT2	0.806 ±0.037	0.576 ±0.024	0.626 ±0.023	0.599 ±0.011	0.619 ±0.026	F=12.49 *P*<0.001
GLUT4	0.821 ±0.024	0.536 ±0.011	0.604 ±0.0091	0.597 ±0.010	0.601 ±0.0063	F=61.04 *P*<0.0001
COX1	1.023 ±0.035	1.131 ±0.043	0.970 ±0.010	0.978 ±0.0076	1.002 ±0.011	F=6.045 *P*<0.01
COX2	0.673 ±0.0089	1.176 ±0.039	0.770 ±0.017	0.775 ±0.014	0.810 ±0.011	F=83.218 *P*<0.0001
SREBP1	0.667 ±0.010	1.151 ±0.046	0.808 ±0.017	0.828 ±0.015	0.855 ±0.014	F=52.620 *P*<0.0001

Table1 17B: Effect of fish oil treatment on the (relative quantity) gene expression of Ins1, Ins2 (insulin) and Gcg (Glucagon) in pancreas upon induction of diabetes. Mean values of six individual mice and their standard errors were tabulated.

Gene	**NC**	**ND**	**SD**	**CD**	**RD**	**ANOVA**
Ins 1	1.031 ±0.0055	0.419 ±0.0059	0.870 ±0.0040	0.719 ±0.0026	0.860 ±0.0035	F=4833.56 *P*<0.0001
Ins 2	1.027 ±0.0034	0.446 ±0.0069	0.753 ±0.0048	0.696 ±0.0094	0.738 ±0.0020	F=629 *P*<0.0001
Gcg	0.419 ±0.0059	0.667 ±0.010	0.569 ±0.0039	0.586 ±0.0035	0.560 ±0.0074	F=466.26 *P*<0.0001

Table1 17C: Effect of fish oil treatment on the (relative quantity) gene expression of TGFβ and PPARα in kidney and heart respectively upon induction of diabetes. Mean values of six individual mice and their standard errors were tabulated

Gene	**NC**	**ND**	**SD**	**CD**	**RD**	**ANOVA**
TGFβ (Kidney)	0.763 ±0.0052	1.573 ±0.0062	0.825 ±0.0029	0.853 ±0.0029	0.877 ±0.0043	F=5560.94 *P*<0.0001
PPARα (Heart)	1.082 ±0.023	0.774 ±0.010	0.903 ±0.014	0.854 ±0.010	0.868 ±0.022	F=57.35 *P*<0.0001

4. Discussion:

Lipids are healthy nutritional constituents with the possible influence on human health, reducing risk of diseases and improving quality of life. The fats and lipids are common components of food. They perform essential roles like energy production and storage, act as chemical messengers. Beyond these basic nutritional functions, they have physiological benefits and/or reduce the risk of chronic disease (Alabdulkarim *et al*., 2012). The important components of lipids are fatty acids, which are one of the main macronutrient serving both structural and metabolic functions. Among fatty acids the long chain omega-3 and omega-6 PUFAs has effect on various physiological processes (Ander *et al*., 2003). The effect of omega-3 and omega-6 fatty acids varies according to the type of target cells and organs involved, as well as their respective amounts in the diet. These two are essential and have different, i.e. antagonistic effects on inflammation and their effects can vary according. The balance of omega-3 and omega-6 EFAs is important for good health and normal development. The omega-6 PUFAs are cosmopolitan in nature makes it dominant in our diet making less consumption of omega-3 PUFAs (Surliker and Roy, 2010). Marine fishes are the most effective means of omega-3 PUFA supplementation. The omega-3 fatty acids are vital in human nourishment is in no doubt and are undoubtedly essential throughout life (Simopoulos, 2002c). The another essential role of omega-3 PUFA is prevention and modulation of certain diseases like coronary heart disease, essential fatty acid deficiency in infancy, autoimmune disorders, Crohn disease, mild hypertension and rheumatoid arthritis which are common in Western civilization (Connor, 2000).

A large number of studies related with consumption of omega-3 PUFAs have shown positive health benefits (Riediger *et al*., 2009). On the other hand, a few studies have also suggested that higher dietary fat was significantly associated with some chronic inflammatory diseases (Patterson *et al*., 2012) and metabolic syndrome (Narasimha *et al*., 2016). However, as we mentioned earlier (section 1.7) there is a conflict and lack of clear information regarding the effects of natural diets containing different types of fatty acids. The three fish oil source of diets used in present study mainly differed in their saturated and unsaturated fatty acid composition and also in their omega-3 and omega-6 PUFAs content. *Sardinella* fish oil contained 54% of unsaturated fatty acid in which 40% are omega-3 PUFA when compared to other two fish oils i.e. from *Rastrelliger* and *Clarias*. However, *Clarias* oil contained better omega-6 PUFA composition (i.e. 15.3%) when compared to *Rastrelliger* and *Sardinella* fish oil (Table B).

4.1. Effect of dietary supplementation of fish oil on mice:

We observed the beneficial effects of dietary supplementation of lipids in the form of fish oils on the saline treated control mice groups (SC, CC and RC). The concentration of total carbohydrate, free amino acid and triglyceride was not much affected by supplementation of fish oils. However, there was 15-20% decrease in free sugar in all the tissues with 10-50% decrease in free fatty acid concentration and 5-35% increase in protein concentrations in serum, liver and pancreas tissues (Table 2A-5A) of 10% fish oil supplemented control mice groups. All these changes indicated the shift in the energy yielding process as the diet is enriched with PUFA, which influence utilization of fat for the purpose of energy (Mieczkowska *et al.,* 2001).

The vitamin C which acts as an antioxidant and controls the lipid peroxidation (Bendich *et al*., 1986; Barrita and Sánchez, 2013), vitamin E a fat soluble antioxidant stops the production of ROS produced due to oxidation of fat (Herrera and Barbas, 2001; Packer *et al*., 2001) and reduced glutathione, which is mostly identified for minimizing the lipid peroxidation and such targets which are produced due to oxidative stress (Kerksick and Willoughby, 2005). The concentration of vitamin C and GSH augmented by 30-100% in all the tissues of fish oil supplemented groups, whereas vitamin E level is increased by 15-30% in serum, liver and kidney tissues only (Table 7A-9A). The increased (30-70%) activity of SOD and catalase (Figure 1A-2A) indicates the elevation of antioxidant status in all the tissues due to the dietary supplementation with fish oils. These antioxidants are involved in the protection against peroxides, free radicals and other toxic constituents. There was 15-60% reduction in concentration of TBARS (Table 10A) along with the activity of GGT (Figure 3A) due to dietary fish oil supplementation which indicates the protection of tissues by reducing ROS level. This is supported by a previous study based on olive oil enriched diets which have been reported to reduce in vitro generation of ROS in phagocytic cells and prevent the damage by ROS and lower the production of proteolytic enzymes (Puerta, 2004). Moreover, there were 5-35% decreases were observed in the activity of enzymes like AST, ALT, ALP and LDH in all the tissues, whereas activity of the ACP was decreased by 10-25% in serum, kidney and heart tissues only (Figure 4A-6A) which indicates fish oil supplements have a positive effect on metabolism (Tirosh, 2015). Dietary supplementation of fish oil results in accumulation of n-3 PUFA in various tissues enriched antioxidant status might protect the tissue from lipid peroxidation (Wohler *et al*., 2003).

It is reported that n-3 PUFA rich diet leads to lower hepatic fat content (Chen *et al*., 1992). In this study the dietary supplementation of fish oil decreased the content of TG by 25-30% along with 10-50% decrease in the relative concentration of FFA, TC (including CE and free cholesterol), with 15-50% increase in MG and DG concentration in all the tissue (Table 12A-15A). It also altered the serum LDL and VLDL concentrations which were markedly decreased by 60-80% in 43-64% elevation in HDL-C level, which might be due to the inhibition of triacylglycerol and VLDL synthesis (Harris *et al*., 1990). Earlier it has been reported that the large doses of fish oil helps to reduce the LDL synthesis and plasma LDL concentrations (Illingworth *et al*., 1984). The clearance of chylomicron triglycerides is accelerated by n-3 PUFA in humans, which efficiently reduces triglyceride levels in serum (Park and Harris, 2003). Neschen *et al*. (2002) reported that in rat liver n-3 PUFA can inhibit the *de novo* lipogenesis which results in reduction of the activity of acetyl Co A carboxylase. Reduction in the level of total cholesterol in the fish oil supplemented group of mice is due to a noteworthy 10-35% decrease in activity of HMG CoA reductase and HMG CoA synthase along with 15-45% increase in G6PD activity in all the tissues. It has been reported that PUFA supplementation leads to a reduction in the HMG-Co A which is a rate limiting step in cholesterol biosynthesis (Roy *et al*., 2008), supported by other studies which shows that omega-3 PUFAs have a positive impact on cholesterol synthesis (Choi *et al*., 1989; Soria *et al*., 2002). Sekiya *et al*. (2003) reported that hepatic lipogenesis can be regulated by dietary n-3 and n-6 PUFA by decreasing sterol regulatory element binding protein-1 in the liver which was also supported by our findings. It is also observed that the supplementation of fish oil lowered the concentration of glycolipid by 5-15%

(Figure 11A) and elevated the phospholipid level by 10-65% in all the tissues (Figure 10A) which also effected the relative concentration of glycolipid and phospholipid fractions. A study conducted to check the effect of EPA on phospholipid synthesis had reported that triglycerol synthesis is inhibited by EPA, which also stimulates the phospholipid synthesis and utilizes glycolipid as a contributor for energy yielding process, which improves cellular signalling and messaging (Benner *et al*., 1990; Pujari and Roy, 2010).

4.2. Ameliorative effect of supplementation of fish oil on diabetic mice:

Prolong Alloxan administration to the mice architecturally distorted liver, kidney, heart and pancreas. Diabetic mice showed liver with the accumulation of lipid in hepatocytes, kidney with deformation, glomerular expansion, heart with degeneration, inflammation and deformation of the pancreas with the presence of the lymphocytic infiltrates in islets. This could be ascribed to impaired metabolism of fatty acids resulting in increased quantities of fat within liver cells (Oršolić *et al*., 2012) and successive effects of hyperglycemia, which persuades degenerative changes in tissues may be due to the augmented ROS generation (Livshits and Pflueger, 2012) and diminished defense of antioxidant system (Cai *et al*., 2002).

It is also noted that induction of diabetes in the control diet fed ND groups lead to destruction of insulin producing beta cell of pancreatic islet, with 60-100% increase in level of free sugar, which is might be due to low insulin (Sharma and Garg, 2009) and increased concentration of glycosylated hemoglobin by 5 fold, which might be due to deprived regulation of glucose metabolism (Koenig *et al*.,

1976) or which might be due to gluconeogenesis and glycogenolysis (Petersen *et al*., 2004). The diabetes also resulted in a significant decrease in protein concentration by 50-80% along with an increase in free amino acid, triglyceride and free fatty acid levels by 30%-2.3 fold in all the tissues (Table 2B-6B). The decrease in protein level with increase in amino acid concentration in diabetes is may be due to increase in catabolism of muscle protein i.e. increase in net breakdown of protein rather than decline in protein synthesis due to profound effect of insulin deficiency (Charltron and Nair, 1998; Møller and Nair, 2008).

Diabetes significantly increased the activities of AST, ALT, ALP, ACP and LDH by 45%-1.5fold in all the tissues when compared to non-diabetic control (Figure 4B-8B) which directly related to changes in metabolism where in these enzymes are involved (Udaykumar *et al*., 2009). The ALP which is a membrane bound enzyme and ALT, AST which are mitochondrial and cytosolic enzymes are known to be the pointer of liver impairment. In the present study the elevation in the activities of ALT, AST in diabetic mouse tissues indicated the tissue impairment and mitochondrial membrane dysfunction and elevation in ALP activity might be due to the disturbance in the transport of metabolites (Navarro *et al*., 1993; El-Demerdash *et al*., 2005). In the diabetic mice the increased LDH, ACP activity might be due to necrosis of liver (Soltan, 2012).

The diabetes also results in alterations in the lipid peroxidation and antioxidant status. SOD and catalase, which are free radical scavenging enzymes (Figure1B-2B) and GSH (Table 9B) which counterbalance free radical facilitated damage and acts as an endogenous antioxidant (Bauche *et al*., 1994) along with

vitamin E, vitamin C, antioxidants (Table 7B-8B) are decreased by 5-65%, with 10%-5.6 fold rise in TBARS (Table 10B) and GGT (Figure 3B) concentration in all the tissues of diabetic mice. The reason for this change is might be excessive lipid peroxidation and the generation of free radicals. Oxidative stress triggered by extreme production of superoxide and an inequity in antioxidant enzymes or increased consumption might be the reason for a reduction in the level of biomolecules and enzymatic antioxidant status in tissues of diabetic mice (Qujeq and Rezvani, 2007).

Supplementation with PUFAs rich fish oils to alloxan induced diabetic mice helped to recover the architectural tissue damage, which was also reflected in biochemical composition and enzyme activity of these tissues. Fish oil supplementation in diabetic mice ensued significant reduction of glycosylated hemoglobin and free sugar by 4-7fold along with elevation in the level of insulin by 50% (Table 1) as shown by our results indicates the role of fish oil in increasing insulin action and in regulating glucose metabolism (Gordon, 1965; Lam *et al*., 2003). Which is might be due to mechanism based on substituting of fuel with increased glucose utilization and reduced fatty acid accessibility and enhancing the effect of insulin, the cycle involved with glucose–fatty acid could also be the reason (Aguilera *et al*., 2004). The 10-65% decrease in concentration of biomolecules like triglyceride and free fatty acid with 10-80% decrease in free amino acid level, resulting 10% -2 fold increases in concentration of protein was observed in all the tissue of fish oils supplemented diabetic groups (Table 2B-6B). This may be due to the fact that as the diets were enriched with PUFA proteins were saved from energy yielding processes. The PUFA composition of a diet

influences the utilization of fat for energy yielding processes (Mieczkowska *et al.*, 2001), might be also due to the EPA and DHA present in fish oil are found to stimulate the synthesis of protein and limit the protein breakdown, they also have triglyceride lowering effects (Shacky, 2006; Kamolrat and Gray, 2013)

The significant decrease by 5-95% of functional enzyme activity (ALT, AST, ALP, ACP and LDH) in all the tissues was provoked by supplementation of fish oils, which is held by previous findings, which proves that in experimental animals oral feeding of oils rich in ω-3 EPA and DHA helps to prohibit the development of induced diabetes mellitus (Ikemoto *et al*., 1996) which also postulates the effect of fish oil on activity, mainly regarding to gluconeogenesis and ketogenesis (Soltan, 2012).

In the present study, the antioxidant status of fish oil supplemented diabetic mice also improved. The concentration of antioxidants like GSH, Vitamin E and Vitamin C (Table 7B-9B) and activity of antioxidant enzymes SOD, catalase (Figure 1B-2B) are elevated by 15%-1.9fold and TBARS (Table 10B) and GGT (Figure 3B) lipid peroxidation variables decreased significantly by 10-70% with the supplementation of fish oils in all the tissues. This indicates that omega-3 PUFAs rich fish oils are having a useful effect on attenuation of oxidative stress and antioxidant potential which is in support with previous results, which had authenticated that omega3 PUFAs present in edible oils shows anti-inflammatory effect signifying their use in the treatment of diabetes or hyperglycemia (Medeiros *et al*., 2005).

4.3. Dietary supplementation of fish oil on lipid metabolism in diabetic mice:

In the diabetes type1 the reckonable lipid deformities observed is mostly due to insulin deficiency. The vital role in the regulation of lipid metabolism is played by insulin (Vergès, 2001). In adipose tissue, insulin inhibits the hormone-sensitive lipase, thus shows an anti-lipolytic action, decreasing the discharge of free fatty acids from adipose tissue in the circulation and stimulating storage of triglycerides in the adipocytes. Defect in insulin action and hyperglycemia, or additional utilization of fat from the adipose tissue because of under consumption of glucose might be the cause of lipid anomalies observed in diabetes (Ginsberg, 1996; Goldberg, 2001).

It is noted that due to induction of diabetes, serum TG, TC, VLDL-C and LDL-C levels were increased by 15%-2 fold (P<0.001) and with a decrease in HDL level (Table 11B). Diabetes also led 10%-2.3 fold elevation in tissue total cholesterol and total glycolipids concentration (Figure 9B and 11B) with 45-55% decrease in total phospholipid concentration in all the tissues (Figure 10B). The concentration of neutral lipid fractions tissues in the alloxan induced diabetic were also varied which was in agreement with previous reports regarding alteration in these parameters in diabetic condition (Sharma and Garg, 2009; Qi *et al*., 2008). Induction of diabetes in the mice group fed with control diet led to a significant elevation in the relative concentration of diglycerides, triglycerides by 30-100%, with 40-45% decrease in monoglycerides in all the tissues (Table 12B-15B). One of the most important organs for the metabolism of lipids and lipoproteins is liver (Luo *et al*., 2010). Diabetes damages liver hepatocyte structures as supported by histological study, which alters lipid metabolism in liver. Reduction in

concentration of total phospholipid by 45-55% (Figure 10B) and increase in concentration of phospholipid fractions like phosphatidyl ethanolamine, lysophospholipid inositol along with decrease in phosphatidyl choline and phosphatidyl inositol+ serine in all the tissues (Figure 12B-15B) is related to positive correlation between decrease in insulin and phospholipid fractions and clearly stipulates that the membrane has lost its functional capability which may arise from alteration in the ingestion of precursors, modification in cellular metabolism related with the biosynthesis and catabolism of the phospholipids (Choy *et al*., 1997; Zeghari *et al*., 2000).

HMG-CoA reductase, which is the rate-controlling enzyme of the mevalonate metabolic pathway that yields cholesterol and other isoprenoids and the HMG-CoA synthase, which catalyses reaction to produce HMG-CoA, which is intermediate of cholesterol synthesis, are increased by 25-80% (Figure 21B-22B) in the diabetic mouse tissues in the present study. The over activation is might be due to prolonged insulin deficiency and exhaustion of substrate for gluconeogenesis and the TCA cycle (Young *et al*., 1982; Wang *et al*., 2005). Glucose-6-phosphate-dehydrogenase acts as catalyst for the production of 6-phosphogluconate from glucose-6-phosphate with a simultaneous oxidation of $NADP^+$ to NADPH which maintains sufficient levels of glutathione in it reduced form and helps to overcome oxidative stress (Oni *et al*., 2005). We have noticed the decline in activity of G6PD by 20-60% in all the tissues (Figure 23B) of the diabetic group of mice, which is in accordance with previous studies which shown that the decreased activity of G6PD results in oxidative impairment, cellular

dysfunction and eventually organ damage (Zhang *et al*, 2000; Xu *et al*, 2005; Diaz-Flores *et al*., 2006).

The changes in lipid profile were also reflected in membrane fluidity which is decreased in diabetic mice tissues when compared with the control mice group (Figure 20A-20D) which can be ascribed to a sophisticated ordering of the hydrocarbon region phospholipid molecules in the membranes, i.e. to lower membrane lipid packing. Hyperlipidemia is reported as one of the factor contributory for increased lipid peroxidation (Kesavulu *et al*., 2002), the free radicals generated in this process attack membrane PUFA result of which lipid peroxides are produced leads to decreased membrane permeability (Saravanan *et al*., 2006). Also lower PUFA content in cell membranes leads to variations of the phospholipids, fatty acid arrangement and physicochemical properties of plasma membranes and reducing their fluidity (Novgorodtseva *et al*, 2011). Our observations are in agreement with previous studies by Bryszewska et al. (1986), Watala et al. (1987), Candiloros et al. (1995) and Waczulíková et al. (2000), who observed decreased fluidity in erythrocytes of diabetic patients, along with a noteworthy change in the arrangement of phospholipid molecules in the membrane lipid bilayer.

The fish oils rich with omega-3 PUFA also helped to decrease significantly the level of serum TG, TC, VLDL-C and LDL-C and tissue TG, TC along with the increase in level of serum HDL-C. It also elevated in all the tissue phospholipid concentration by 5-40% (P<0.001-0.0001) concentration along with 5-35% decrease (P<0.0001) in total cholesterol, total glycolipid (Figure 9B-11B)

concentration as shown by our results in agreement with previous reports indicates that oil rich with omega-3 PUFA might play an important role in the triglyceride-lowering effect that has been mostly attributed to reduced hepatic synthesis of VLDL (Mozaffarian and Wu, 2011; Soltan, 2012). Omega-3 fatty acid subdues hepatic lipogenesis and decreases circulating TG level (Storlien *et a*l., 2007). The fish oil rich with omega-3 reduces synthesis of cholesterol and may decrease cholesterol absorption (Nestel, 1990 and 2000) by LDL removal and particularly fish oils down-regulates the LDL receptor in hepatic cells, but the mechanisms by which the HMG-CoA reductase and synthase are regulated by fatty acids are unclear (El-Sohemy and Archer, 1999). The supplementation with fish oils also helped to improve the concentration of G6PD in different tissues.

The supplementation with fish oils also aided to recover the changed neutral lipid, glycolipid and phospholipid profiles of all the tissues. Dietary supplementation of fish oil significantly reduced in all the tissue cholesterol (both free and esterified cholesterol) along with triglyceride concentration (Table12B-15B). The decrease in the cholesterol level with fish oil supplementation might be due to reduced activity of HMG CoA reductase and HMG CoA synthase, which is also observed in our study (Roy *et al.*, 2008). It is supported by the substantial evidence that increased intake of omega-3 PUFAs especially EPA and DHA has an affirmative impact on the metabolism of fat (Soria *et al*., 2002). Earlier it has been reported that supplementation of dietary lipid in the form of fish oil alters the lipoprotein metabolism and reduces triglyceride levels (Harris *et al*., 1993). The cell signalling properties of these fatty acids with alteration of proteins involved in gene expression of in the fatty acid oxidation and triglyceride secretion can be

ascribed to PUFA supplementation (Friedberg *et al*., 1998). It is known that insulin and cytokines regulate lipids and lipoprotein metabolism and also decreases lipolysis *in vivo* could also be the reason (Cheung *et al*., 1990). Our data on a study of cytokine expression also support this result.

The membrane fluidity index depends on two main factors: the level, the arrangement and the proportion of PUFA in the membrane and level of membrane cholesterol. Increase in PUFA results in fluidizing the membrane while a rise in cholesterol toughens the membrane by reducing fluidity index (Simopoulos and Cleland, 2003). The increase in membrane fluidity in fish oils treated diabetic mice (Figure 20A-20D) is may be due to PUFAs present in fish oil gets assimilated into cellular membranes, where they can mediate their action and increase the fluidity of the membrane and modify the subcellular location of proteins (Matesanz *et al*., 2010). Supplementation of fish oils with higher EPA and DHA content to the diabetic mice group might help to restore the assembling of the phospholipid molecules in the membrane lipid bilayer and the glycolipid arrangement of the extracellular face of the cell membrane (Fox *et al*., 2006). The omega-3 PUFA might be responsible for proteolytic effect and regulation of lipid metabolism by regulating key genes SREBP1 and PPARα (Abbott *et al*., 2012), however, the exact mechanism of regulation of the membrane phospholipid need to be explored.

4.4. Dietary supplementation of fish oils on gene expression in diabetic mice:

Traditionally, diabetes was not believed to be a disease related to the immune system, however, there is increasing evidence supporting a role for

inflammation in diabetes. In the pathogenesis of diabetes through increased inflammation and fibrosis affecting vascular system the inflammatory cells, cytokines, and profibrotic growth factors, including TGF-*β*, TNF-*α*, connective tissue growth factor (CTGF), monocyte chemo attractant protein-1 (MCP-1), interleukins-(IL-1, IL-6, IL-18) and cell adhesion molecules (CAMs) have been involved (Elmarakby and Sullivan 2012; Francés *et al*., 2013). TNF-α and IL-1 are beneficial when produced appropriate quantities, but the overproduction of these may result in inflammation (Simopoulos, 2002). In the present study about 30-60% increase in inflammatory cytokines TNFα and IL1α expression was observed in liver, kidney and heart tissues (Figure24A-24B) along with a 95 % increase in TGFβ expression in kidney (Table 17C) of alloxan induced diabetic mice group. TGFβ is a multifunctional cytokine which acts as a key mediator of glomerular and tubulointerstitial pathobiology in chronic kidney diseases. In response to inflammation and infection, one of the main cytokines released in the inflammatory processes is TNF-*α*, produced primarily macrophages and lymphocyte immune system cells, which can trigger signalling pathways related to cell survival, apoptosis, inflammation and cell differentiation (Littlejohn *et al*., 2003; McFarlane *et al*., 2002). The activation of NFκB or the initiation of caspases activation is promoted by the binding of TNF-*α* to TNF-R1 which has a major role in the implementation of programmed cell death or apoptosis (Srinivasan and Ramarao 2007). NFκB stimulates the expression of genes encoding cytokines like TNF-*α*, Inteleukins, INF-γ, CM-CSF and CAMs, chemokine receptors and inducible enzymes (e.g., COX- 2, iNOS). The early event which contributes to the disease process in the liver during inflammation is an increase in TNF- α in type 1 diabetes (Francés *et al*., 2013).

Animal and human studies have shown that production of cytokines can be reduced by n-3 fatty acids (Calder, 2003). In our study the expression of IL1α, TNFα and TGFβ in the dietary fish oil supplemented groups lowered by 35-60% (P<0.001) in spite of alloxan induced diabetes. It is specified by the studies that omega-3 PUFA and their explicit lipid mediators can diminish the process of activation of inflammation and also supplementation of fish oil reduces the production of TNF-α and IL-1 in mononuclear cells (Weylandt and Kang, 2005; Schmocker *et al*., 2007, Pujari and Roy, 2012). It has been reported that high concentrations of n-3 PUFA leads to a decreased production of TNF-α by reducing the activation of nuclear factor kappa B (Babcock *et al*., 2002). Our data on a study of cytokine expression suggests that reduced action of cytokines results in improved lipid metabolism and insulin action.

The expression of Ins1 and Ins2 decreased by 55-60% in the pancreas tissue (Table 17B) of diabetes induced mice, which might be due to inflammation of the pancreatic islet, resulting in the preferential destruction of insulin producing β-cells to varying degrees by the rigorous action of auto reactive T-cells and monocytic cells (Mathis *et al*., 2001). Diabetes also resulted in 60% elevation in glucagon expression (Table 17B). The reduced insulin secretion in diabetes is may be due to an inflammatory response termed "insulitis," the incursion of the islets by mononuclear cells results in loss of most β-cells after prolonged periods of diabetes type 1 (Klöppel *et al*., 1985). The functional impairment evolves to β-cell death after prolonged exposure to IL-1β + Interferon-γ and/or tumor necrosis factor (TNF)-α, but not to either cytokine alone (Eizirik and Mandrup-Poulsen, 2001). This also results in failure to suppress glucagon secretion and intra-islet

paracrine mechanisms results in the hyper secretion of glucagon (Hussein et al., 2000). The mechanism underlying the hyperglucagonemia in diabetes suggests that glucose regulates the glucagon secretion. Secondarily prolonged defect in insulin secretion, defects in the endocrine system and α-cell abnormality might be the reason for the increased glucagon secretion (Luyckx, 1975, Flachs *et al*., 2014). Studies suggest that contradictory glucagon hyper secretion in hyperglycemic condition may be dependent on the dose-responding U-shaped glucose regulation and secretion (Salehi *et al*., 2006).

The supplementation of 10% fish oils to the SD, CD and RD groups elevated the decreased expression of Ins1 and Ins2 by 55-100% in the pancreas, which was also reflected in serum insulin concentration, with nearly 10-15% decrease in expression of glucagon. Substantial evidence shows that the dietary fat subtypes play major role in insulin action. Saturated fatty acid intake is strongly linked to the development of obesity and insulin resistance due to poor oxidization and mobilization by lipolytic stimuli, which in return impairs membrane function by increasing the gene expression associated with adipocyte proliferation, while PUFAs shows contrasting action (Storlien *et al*., 2000). There is strong evidence related to animal models that PUFA consumption is associated with enhanced insulin action more directly and decreased adiposity (Storlien *et al*., 1996). The action of omega-3 PUFA is based on metabolizing of incretin glucagon-like peptide-1 which increases endogenous insulin secretion (Iwase *et al*., 2015). The in vitro experiments offered evidence that changes in the fatty acid composition of membrane phospholipids influences the action of insulin, changing both insulin binding and action. In overall, if saturated fatty acids (SFA) in membrane

phospholipid increases, it results in the most deleterious effect (Grunfeld *et al*. 1981; Field *et al*., 1988) further, some studies proposed unsaturated omega-3 fatty acids in higher concentration, perhaps mainly beneficial (Sohal *et al*., 1992; Clandinin *et al*., 1993).

The expression of the GLUT2 minimally decreased by 28% along with a 34 % decrease in GLUT4 expression in the liver of alloxan induced diabetic mice, which show the existence of a β cell-specific control of expression, an observation consistent with previous reports. The β-cell glucose unresponsiveness and insulin inadequacy is associated with loss of GLUT2 and GLUT4 expression (Thorens *et al*., 1992; Okuno *et al*., 1995). The supplementation of 10% fish oil helped to slightly increase the expression of glucose transporters in the liver. This observation suggests that a diet rich with PUFA induces changes in lipid composition of the membrane (Table 12B-19B) and enhances the membrane fluidity (Figure 20A-20D) which might increase the glucose transporter inherent activity (Storlien *et al*., 1987; Richter *et al*., 1998).

The increase in expression of COX-2 and not much changes in expression of COX-1 gene was observed in the liver of diabetic mice. COX-1, sub serve housekeeping functions, expressed constitutively in most cells, is the main source of prostanoids (Dubois *et al*., 1998). The up regulation of COX-2 in diabetes, is may be due to inflammatory stimuli, hormones and growth factors (Ricciotti and FitzGerald, 2012). The supplement of fish oils contains EPA, which acts as the natural COX inhibitor, inhibits both COX-1 and COX-2 activity. EPA inhibits AA metabolism and acts as alternate substrate for COX. The omega 6 prostaglandin

PGH_2 which is produced by conversion of AA by COX is replaced by EPA, which gets converted to n-3 homologue PGH_3 (James *et al*., 2000; Cleland *et al*., 2006). The changes in the level of various prostaglandins of 2 and 3 series in the diabetic mice need to be confirmed.

The SREBP1 which enhance transcription of genes encoding enzymes of fatty acid and cholesterol bioproduction and uptake is well known **(**Proctor *et al*., 2006). Induction of diabetes also increased the expression of SREBP-1 which is may be due to regulation of expression of main mediator (IRS-1 and IRS-2) in insulin signalling pathways (Shimomura *et al*., 1999; Kohjima *et al*., 2008). In addition to this the depletion in the hepatic omega-3 long chain PUFA may favour the proteolytic release of membrane bound SREBP-1c and its nuclear abundance by altering membrane lipid composition (Lombardo and Chicco, 2006; Pettinelli *et al*., 2009). The diet rich with the saturated and monounsaturated fatty acids has a petite influence as LXR antagonists, while omega-6 PUFAs are more persuasive LXR antagonists than are omega-3 PUFAs, PUFAs down regulate hepatic lipogenic gene expression by serving as antagonists for LXR (Schultz *et al*., 2000; Yoshikawa *et al*., 2002; Stulnig *et al*., 2002; Seo *et al*., 2004). The omega-3 PUFA regulates key gene SREBP1, which might be responsible for proteolytic effect and regulation of lipid metabolism (Abbott *et al*., 2012) which is being reflected in the tissue level decrease in TG, FFA, TC (Figure 9B, Table 2B-6B).

PPARα a member of the ligand-activated nuclear receptor superfamily have a significant role in the metabolism of lipid and glucose homeostasis (Park *et al*., 2006). A noticeable decrease in the expression of PPAR-α which mediates

fatty acid oxidation was observed in heart, which is in agreement with previous reports (Proctor *et al*, 2006; Hu *et al*., 2013) The studies have shown that the down regulation of PPAR-α is may be due to the high glucose concentration or regulation of expression by reactive oxygen species (Dewald *et al*., 2005). Supplementation of fish oil to the SD, CD and RD group of mice significantly increased the expression of PPAR-α in heart. The dietary supplementation of PUFA is known to stimulate B-oxidation and reduce plasma triacylglycerol by inducing the expression of PPAR-α target gene (Takeuchi *et al*., 2001). PUFA up regulates the hepatic fatty acid oxidation and energy expenditure along with a decrease in apolipoprotein expression, release of VLDL particles and by shifting the energy yielding process though the very long chain unsaturated fatty acids, such as DHA. DHA also acts as a good activator of PPAR-α, which regulates lipid metabolism (Madsen *et al*., 2005).

In conclusion, the supplementation of fish oil rich with omega-3 PUFA alleviates hyperglycemia through improvement of insulin action by regulating glucose metabolism and glucose uptake. Besides, the present study firmly and significantly throws light on the valuable contribution of supplementation with fish oils rich with PUFA on antioxidant property, beneficial effect on attenuation of oxidative stress. The long term supplementation of fish oils enriched with omega-3 PUFAs is having the anti-inflammatory potential to arrest cellular damage and may be useful in the management of oxidative stress induced tissue damage caused by prolonged diabetes. Increased intake of omega-3 PUFAs especially EPA and DHA has an affirmative impact on the metabolism of fat in diabetic mice, also helps to improve the membrane fluidity which is dependent on

level, composition and the percentage of PUFA and level of membrane cholesterol. Long chain omega 3 PUFA present in the fish oil and their mediators can also diminish the process of activation of inflammation by reducing the expression of these cytokines. The dietary supplementation of fish oil rich with omega-3 PUFA would be effective in prevention of diabetes and diabetes related complications.

The fish oil supplementation elevates the insulin secretion and decreases glucagon production. Despite advances in our understanding, further experiments are needed to understand the action of fish oil on β-cell and α-cell receptor of the pancreas and regulate the secretion of these hormones. It is clear from the study that fish oil has effect on the insulin hormone secretion, but further studies are essential to understand the effect of fish oil on insulin resistance in type2 diabetes. The specific effects of eicosapentanoic acid versus docosahexanoic acid on diabetes and the relative merits of oily fish rich with omega-3 PUFA also require further investigation. In order to produce an authentic formulation of fish oil, one has to identify the factors that mediate β-cell preservation and provide hope for future treatment options for diabetes.

5. Summary:

The fats are one of the important dietary nutrients affecting health and growth. They are mainly used for energy, act as signalling molecules and also as vital constituents of cellular membranes. The fatty acid is the major component of fats. The polyunsaturated fatty acids of the omega-3 and omega-6 groups are labelled as essential and play an important role in the biological systems for maintaining integrity of cell membrane, growth and development. These long chain omega-3 and omega-6 PUFAs produce a distinct type of metabolites, each of which has very different effects in the body and act in an antagonistic manner. The healthy diet should comprise the balanced ratio of omega-3/omega-6. Marine omega-3 PUFAs, have been found to have beneficial effects against several diseases, including cardiovascular disease, mental illness, obesity, inflammatory diseases and cancer. However, the effects are unclear in diabetes and there is no clear conclusion about the net benefits of administering omega-3 PUFA in diabetic patients. Hence we tested the hypothesis that, the dietary fish oil rich in EPA and DHA might also help in reversing the metabolic changes and prevent the tissue damages due to prolonged diabetes. We selected three sources of dietary fats namely laboratory extracted fish oils, from *Sardinella longiceps*, *Clarias batrachus* and *Rastrelliger kanagurta*, rich with omega-3 PUFAs but to a variable degree. The main objectives of the research work was to check the effect of fish oil on biochemical composition, antioxidant profile, enzyme activity along with the evaluation of lipid metabolism and the study of expression of some cytokines and regulatory genes in the diabetic induced mice.

Diabetes was induced in the Swiss albino mice, *Mus muscululs* by intrapetrioneal injection of Alloxan (100 mg / kg body weight in 20 µl saline) and the diabetic conditions was maintained by repetitive injection of alloxan after every 5^{th} day. Both the diabetic and control group of mice (injected with 20 µl saline injection) was fed *ad lib*. with commercial pellet feed blended with 10% of fish oil for a period of 30 days. The concentration of various biomolecules like free sugar, total carbohydrate, total protein, free amino acid, triglycerides and free fatty acids, antioxidant and lipid peroxidation molecules (vitamin C, vitamin E, GSH, TBARS) along with the activity of functional enzymes like AST, ALT, ACP, ALP and LDH were monitored in different tissues like the serum, liver, kidney, heart and pancreas. The serum lipid profile and lipid and fatty acid profile in the tissues like liver, kidney, heart and pancreas were also evaluated. The expression of cytokines like TNFα a and IL1α was evaluated in the liver, kidney and heart tissues, along with some tissue specific regulatory genes (TGFβ, PPARα, SREBP1, GLUT2, GLUT4, Ins1, Ins2, Gcg, COX1 and CoX2) expression was also evaluated.

The main objective of our study included finding the physiological response to alloxan induced diabetes in mice and the ameliorative effect of supplementation of various fish oils rich with omega-3 PUFA on the diabetic mice. Upon supplementation of fish oils to the diabetic mice groups showed improved concentration of insulin, with decrease in concentration of glycosylated haemoglobin and free sugar. There was also increase in concentration of protein, along with decrease in level of total carbohydrate, free amino acid, triglyceride and free fatty acid. Augmentation in level of TBARS and in activity of gamma

glutammyl transpeptidase upon induction of diabetes was much lower in fish oil supplemented groups. The supplementation of fish oil also increased the concentration of vitamin C, vitamin E, reduced glutathione along with the activity of superoxide dismutase and catalase in diabetic mice. In spite of induction of diabetes the supplementation of fish oil showed conserved architecture of liver, kidney, heart and pancreas with reduced inflammation, minimal degeneration, which was also reflected in activity of functional enzymes.

It is well known that the profiles of lipids are greatly prejudiced by the *de novo* synthesis and the dietary intake of lipids of specific type and composition. To recognize the precise lipid molecule involved in pathogenesis of any disease, a study of analysis of tissues lipid composition provides better vision. There is also growing evidence which suggests that disturbance in the lipid metabolism is also frequently found in the severer forms of the diabetes. Hence in this study we attempted to analyse the alteration in lipid composition due to induction of diabetes and also the effect of dietary supplementation of fish oil. Dietary consumption of lipid in the form of fish oil, helped to greatly improve the lipid profile. Alleviation in the concentration of DHA and EPA long chain fatty acid concentration was also found in diabetic mice supplemented with fish oils.

Inflammation also plays an important role in pathogenesis of diabetes. Prostaglandins (PG) produced by PUFA plays a key role in the generation of the inflammatory response. About 33-47% decrease in the expression of TNF-α, IL1-α and TGF-β was observed in diabetic mice supplemented with fish oils. PUFA present in fish oil may diminish inflammation by regulating expression of

inflammatory cytokines. There was also significant increase in expression of glucose transporter, Ins1 and gene involved in fatty acid oxidation (PPAR-α) along with decrease in expression of COX-1 and COX-2 which are main source of prostanoids and gene encoding enzymes of cholesterol and fatty acid biosynthesis (SREBP1c).

Finally it can be concluded that 10% supplementation of fish oil with long chain fatty acids helps to maintain health of *Mus musculus* in improved way. Our study showed that dietary supplementation of fish oils rich with long chain polyunsaturated fatty acids significantly alleviates the alloxan induced diabetes which was reflected in the composition of biochemical molecules, activity of enzymes, histological study of tissues along with the lipid profiles and expression of cytokines, glucose transporter, insulin, genes involved in lipid metabolism and prostaglandin synthesis. The *Sardinella* oil supplementation showed better protective effect when compared to *Clarias* and *Rasttrelliger* oil supplementation which might be due to the higher concentration of omega-3 fatty acids especially EPA and DHA in *Sardinella* oil. Overall results supports the concept that dietary fish oils rich in omega-3 PUFA may be of therapeutic benefit in patients with diabetes.

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The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Free sugar:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PD vs SD	< 0.0001	<0.0001	0.003	0.007	0.001
PD vs RD	< 0.0001	<0.0001	0.014	0.049	0.001
PD vs CD	< 0.0001	0.007	0.777	0.609	0.020
CD vs PC	< 0.0001	<0.0001	<0.0001	<0.0001	0.008
CD vs SD	0.996	0.034	0.042	0.161	0.595
CD vs RD	0.998	0.184	0.167	0.565	0.682
RD vs PC	< 0.0001	0.000	<0.0001	<0.0001	0.141
RD vs SD	1.000	0.918	0.959	0.914	1.000
SD vs PC	< 0.0001	0.002	0.000	<0.0001	0.184
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Total carbohydrate:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	0.001	0.002	< 0.0001	0.001
PD vs RD	< 0.0001	0.001	0.094	0.000	0.001
PD vs CD	< 0.0001	0.002	0.373	0.001	0.020
CD vs PC	< 0.0001	0.002	0.008	0.001	0.008
CD vs SD	0.000	0.993	0.111	0.837	0.595
CD vs RD	0.470	0.995	0.930	0.999	0.682
RD vs PC	< 0.0001	0.004	0.048	0.002	0.141
RD vs SD	0.016	1.000	0.419	0.941	1.000
SD vs PC	< 0.0001	0.004	0.754	0.013	0.184
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Triglyceride:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	0.007	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	0.035	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	0.114	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	0.897	0.006	< 0.0001
CD vs PC	< 0.0001	0.496	0.053	0.008	0.018
CD vs SD	< 0.0001	0.753	0.211	0.047	0.520
CD vs RD	0.004	1.000	0.484	0.440	1.000
RD vs PC	< 0.0001	0.513	0.716	0.280	0.029
RD vs SD	0.436	0.768	0.980	0.723	0.647
SD vs PC	< 0.0001	0.993	0.954	0.933	0.390
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Free fatty acid:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs SD	0.115	< 0.0001	0.003	< 0.0001	0.000
PD vs RD	0.137	0.000	0.031	< 0.0001	0.000
PD vs CD	0.220	0.000	0.069	0.128	0.003
CD vs PC	< 0.0001	0.891	0.030	< 0.0001	0.429
CD vs SD	0.996	0.925	0.688	< 0.0001	0.825
CD vs RD	0.999	0.999	0.996	< 0.0001	0.848
RD vs PC	< 0.0001	0.956	0.066	< 0.0001	0.948
RD vs SD	1.000	0.975	0.877	0.619	1.000
SD vs PC	< 0.0001	1.000	0.363	< 0.0001	0.959
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Protein:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PC vs CD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PC vs RD	0.012	< 0.0001	<0.0001	0.012	< 0.0001
PC vs SD	0.062	< 0.0001	<0.0001	0.062	< 0.0001
SD vs PD	0.001	0.144	0.034	0.001	< 0.0001
SD vs CD	0.048	0.828	0.389	0.048	0.654
SD vs RD	0.951	1.000	0.819	0.951	0.856
RD vs PD	0.003	0.152	0.277	0.003	0.001
RD vs CD	0.199	0.842	0.945	0.199	0.996
CD vs PD	0.363	0.655	0.693	0.363	0.003
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Free amino acid:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	0.002	< 0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001	0.012	< 0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001	0.441	< 0.0001
CD vs PC	< 0.0001	0.001	<0.0001	0.004	< 0.0001
CD vs SD	0.001	0.042	0.993	0.120	< 0.0001
CD vs RD	0.515	0.102	0.999	0.371	0.001
RD vs PC	< 0.0001	0.214	<0.0001	0.214	0.000
RD vs SD	0.052	0.993	1.000	0.962	0.245
SD vs PC	0.001	0.410	<0.0001	0.553	0.047
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Vitamin C:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	0.004	0.003	0.000	< 0.0001
PC vs CD	< 0.0001	0.768	0.998	0.603	0.000
PC vs RD	< 0.0001	0.986	0.981	0.233	0.663
PC vs SD	< 0.0001	0.773	1.000	0.610	0.999
SD vs PD	< 0.0001	0.000	0.003	0.007	< 0.0001
SD vs CD	< 0.0001	0.169	0.998	1.000	0.001
SD vs RD	< 0.0001	0.474	0.983	0.953	0.788
RD vs PD	< 0.0001	0.014	0.010	0.036	< 0.0001
RD vs CD	0.860	0.962	0.999	0.955	0.012
CD vs PD	< 0.0001	0.062	0.006	.007	0.016

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Reduced glutathione:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	< 0.0001	<0.0001	< 0.0001
PC vs CD	0.000	< 0.0001	< 0.0001	<0.0001	< 0.0001
PC vs RD	0.094	0.005	< 0.0001	<0.0001	< 0.0001
PC vs SD	0.227	0.046	< 0.0001	<0.0001	< 0.0001
SD vs PD	0.001	< 0.0001	0.002	0.008	< 0.0001
SD vs CD	0.039	0.001	0.041	0.200	0.000
SD vs RD	0.989	0.888	0.917	1.000	0.304
RD vs PD	0.003	0.000	0.019	0.009	0.004
RD vs CD	0.104	0.006	0.213	0.209	0.033
CD vs PD	0.514	0.770	0.765	0.579	0.899

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for TBARS:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	<0.0001	<0.0001
PD vs SD	< 0.0001	0.000	< 0.0001	<0.0001	0.964
PD vs RD	< 0.0001	0.005	< 0.0001	<0.0001	0.999
PD vs CD	< 0.0001	0.594	< 0.0001	<0.0001	1.000
CD vs PC	< 0.0001	< 0.0001	0.001	0.001	0.989
CD vs SD	< 0.0001	0.010	0.761	<0.0001	<0.0001
CD vs RD	0.001	0.135	0.583	0.119	<0.0001
RD vs PC	< 0.0001	0.034	< 0.0001	0.131	<0.0001
RD vs SD	< 0.0001	0.752	0.089	0.009	0.884
SD vs PC	< 0.0001	0.339	0.021	0.735	<0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Vitamin E:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	< 0.0001	<0.0001	< 0.0001
PC vs CD	< 0.0001	< 0.0001	< 0.0001	0.006	< 0.0001
PC vs RD	< 0.0001	< 0.0001	< 0.0001	0.153	0.002
PC vs SD	< 0.0001	0.005	< 0.0001	0.434	0.136
SD vs PD	< 0.0001	< 0.0001	< 0.0001	0.005	< 0.0001
SD vs CD	< 0.0001	0.001	< 0.0001	0.235	< 0.0001
SD vs RD	< 0.0001	0.034	0.136	0.965	0.318
RD vs PD	< 0.0001	0.008	< 0.0001	0.023	0.000
RD vs CD	0.002	0.591	0.008	0.579	0.012
CD vs PD	< 0.0001	0.199	0.349	0.402	0.548

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Alanine amino transferase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	0.002	< 0.0001	0.000
CD vs PC	0.016	0.016	<0.0001	0.016	< 0.0001
CD vs SD	0.396	0.396	0.030	0.396	0.064
CD vs RD	0.975	0.975	0.230	0.975	0.405
RD vs PC	0.060	0.060	<0.0001	0.060	< 0.0001
RD vs SD	0.746	0.746	0.846	0.746	0.834
SD vs PC	0.486	0.486	<0.0001	0.486	< 0.0001
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Aspartate amino transferase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	0.001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	0.766	< 0.0001	0.003
CD vs PC	0.000	< 0.0001	<0.0001	0.000	< 0.0001
CD vs SD	0.312	< 0.0001	0.000	0.312	< 0.0001
CD vs RD	0.851	0.005	0.012	0.851	0.172
RD vs PC	0.006	< 0.0001	<0.0001	0.006	< 0.0001
RD vs SD	0.871	0.060	0.500	0.871	0.001
SD vs PC	0.052	< 0.0001	<0.0001	0.052	< 0.0001
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Superoxide dismutase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PC vs CD	< 0.0001	0.000	<0.0001	0.000	< 0.0001
PC vs RD	< 0.0001	0.016	<0.0001	0.001	< 0.0001
PC vs SD	0.001	0.282	0.000	0.062	0.002
SD vs PD	0.001	< 0.0001	<0.0001	<0.0001	< 0.0001
SD vs CD	0.002	0.043	0.170	0.122	0.003
SD vs RD	0.787	0.633	0.611	0.506	0.364
RD vs PD	0.018	0.003	0.000	0.005	0.000
RD vs CD	0.027	0.507	0.897	0.896	0.200
CD vs PD	1.000	0.116	0.003	0.039	0.057

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Alkaline phosphatase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	<0.0001	0.001
PD vs RD	< 0.0001	< 0.0001	<0.0001	<0.0001	0.013
PD vs CD	< 0.0001	< 0.0001	<0.0001	0.000	0.017
CD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	0.001
CD vs SD	0.000	0.000	0.004	0.000	0.851
CD vs RD	0.451	0.223	0.407	0.003	1.000
RD vs PC	0.000	< 0.0001	<0.0001	0.018	0.001
RD vs SD	0.019	0.071	0.184	0.935	0.905
SD vs PC	0.580	0.019	<0.0001	0.096	0.013
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Gamma glutamyl transpeptidase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	3.403	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs SD	3.338	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs RD	3.003	< 0.0001	0.000	0.001	< 0.0001
PD vs CD	1.238	0.204	0.191	0.261	0.401
CD vs PC	2.165	< 0.0001	<0.0001	0.000	< 0.0001
CD vs SD	2.100	< 0.0001	0.012	0.003	< 0.0001
CD vs RD	1.765	0.000	0.047	0.116	0.000
RD vs PC	0.400	< 0.0001	0.000	0.150	0.028
RD vs SD	0.335	0.802	0.975	0.496	0.918
SD vs PC	0.065	< 0.0001	0.001	0.938	0.157
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Acid phosphatase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs SD	< 0.0001	0.001	0.0001	<0.0001	< 0.0001
PD vs RD	0.011	0.001	<0.0001	<0.0001	< 0.0001
PD vs CD	0.036	0.244	<0.0001	0.009	0.259
CD vs PC	< 0.0001	< 0.0001	<0.0001	0.0001	< 0.0001
CD vs SD	0.091	0.100	<0.0001	<0.0001	0.001
CD vs RD	0.986	0.135	<0.0001	<0.0001	0.005
RD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
RD vs SD	0.232	1.000	0.988	0.000	0.900
SD vs PC	< 0.0001	< 0.0001	<0.0001	0.866	< 0.0001
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Lactate dehydrogenase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
CD vs SD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
CD vs RD	< 0.0001	< 0.0001	<0.0001	<0.0001	0.000
RD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
RD vs SD	< 0.0001	< 0.0001	0.000	0.392	< 0.0001
SD vs PC	0.022	< 0.0001	<0.0001	<0.0001	< 0.0001
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Catalase:

Contrast	Difference	Liver	Kidney	Heart	Significant
SD vs PD	74.125	< 0.0001	<0.0001	<0.0001	Yes
SD vs PC	41.817	< 0.0001	<0.0001	<0.0001	Yes
SD vs CD	26.150	< 0.0001	<0.0001	<0.0001	Yes
SD vs RD	4.667	< 0.0001	<0.0001	< 0.0001	Yes
RD vs PD	69.458	0.001	<0.0001	<0.0001	Yes
RD vs PC	37.150	< 0.0001	<0.0001	<0.0001	Yes
RD vs CD	21.483	< 0.0001	0.918	0.918	Yes
CD vs PD	47.975	< 0.0001	<0.0001	< 0.0001	Yes
CD vs PC	15.667	< 0.0001	<0.0001	<0.0001	Yes
PC vs PD	32.308	< 0.0001	<0.0001	<0.0001	Yes
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for HMG-CoA reductase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001	0.000	< 0.0001
CD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
CD vs SD	0.318	< 0.0001	<0.0001	<0.0001	< 0.0001
CD vs RD	0.730	< 0.0001	<0.0001	<0.0001	0.754
RD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
RD vs SD	0.951	< 0.0001	0.002	<0.0001	< 0.0001
SD vs PC	< 0.0001	< 0.0001	<0.0001	<0.0001	< 0.0001
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for HMG-CoA synthase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001	< 0.0001	0.589
PD vs SD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001	< 0.0001	0.977
CD vs PC	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
CD vs RD	< 0.0001	0.984	<0.0001	0.581	0.897
CD vs SD	< 0.0001	0.255	0.004	< 0.0001	0.000
SD vs PC	< 0.0001	0.000	<0.0001	< 0.0001	0.000
SD vs RD	0.581	0.531	0.086	0.125	0.002
RD vs PC	< 0.0001	< 0.0001	0.000	< 0.0001	< 0.0001
LSD-value:			0.375		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Glucose-6-phosphate dehydrogenase:

Contrast	Serum	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PC vs CD	< 0.0001	< 0.0001	<0.0001	< 0.0001	< 0.0001
PC vs RD	< 0.0001	< 0.0001	0.025	< 0.0001	0.001
PC vs SD	0.000	< 0.0001	0.571	< 0.0001	0.463
SD vs PD	< 0.0001	0.000	<0.0001	< 0.0001	< 0.0001
SD vs CD	0.000	0.001	0.004	0.012	< 0.0001
SD vs RD	0.915	0.039	0.426	0.643	0.035
RD vs PD	< 0.0001	0.206	0.001	< 0.0001	< 0.0001
RD vs CD	0.001	0.636	0.175	0.226	< 0.0001
CD vs PD	< 0.0001	0.921	0.179	0.000	0.978
Tukey's d critical value:			4.153		

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for total cholesterol:

Contrast	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs RD	< 0.0001	< 0.0001	0.002	0.444
CD vs SD	< 0.0001	< 0.0001	0.015	0.093
SD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs RD	1.000	< 0.0001	0.924	0.883
RD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for cholesterol esters:

Contrast	liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs SD	< 0.0001	0.000	< 0.0001	< 0.0001
CD vs RD	< 0.0001	0.001	< 0.0001	< 0.0001
RD vs PC	< 0.0001	0.016	0.210	0.434
RD vs SD	0.040	0.995	0.994	0.997
SD vs PC	0.064	0.038	0.098	0.643

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for free cholesterol:

Contrast	Liver	Kidney	Heart	pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs SD	< 0.0001	0.000	< 0.0001	< 0.0001
CD vs RD	0.012	0.291	< 0.0001	0.000
RD vs PC	< 0.0001	0.000	0.006	0.001
RD vs SD	0.059	0.036	0.005	0.995
SD vs PC	0.026	0.167	1.000	0.003

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for total glycolipid:

Contrast	Liver	Kidney	Heart	Pancreas
PD vs SD	< 0.0001	< 0.0001	0.338	< 0.0001
PD vs RD	< 0.0001	< 0.0001	0.983	< 0.0001
PD vs PC	< 0.0001	0.005	< 0.0001	< 0.0001
PD vs CD	0.002	0.000	1.000	0.373
CD vs SD	0.006	0.978	0.449	< 0.0001
CD vs RD	0.048	0.993	0.997	0.006
CD vs PC	0.618	0.749	< 0.0001	< 0.0001
PC vs SD	0.136	0.978	< 0.0001	< 0.0001
PC vs RD	0.551	0.494	< 0.0001	< 0.0001
RD vs SD	0.889	1.000	0.647	0.363

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for monoglyceride:

Contrast	Liver	Kidney	Heart	Pancreas
SD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs CD	< 0.0001	< 0.0001	< 0.0001	0.000
SD vs RD	0.726	0.241	< 0.0001	0.716
RD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs CD	< 0.0001	< 0.0001	< 0.0001	0.003
CD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for diglyceride:

Contrast	Liver	Kidney	Heart	Pancreas
SD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs PD	< 0.0001	< 0.0001	< 0.0001	0.183
SD vs CD	0.071	0.995	0.129	0.112
SD vs RD	0.656	0.999	0.400	0.894
RD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs PD	< 0.0001	< 0.0001	< 0.0001	0.642
RD vs CD	0.626	1.000	0.959	0.484
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PD	< 0.0001	< 0.0001	< 0.0001	0.999
PD vs PC	0.092	0.625	0.001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Unknown 1 (glycolipid):

Contrast	Liver	Kidney	Heart	Pancreas
SD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs CD	0.676	0.676	0.792	0.009
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs RD	0.001	0.001	< 0.0001	< 0.0001
RD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs PC	0.013	0.013	0.002	0.001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for Unknown 2 (glycolipid):

Contrast	Liver	Kidney	Heart	Pancreas
PC vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs RD	0.000	0.000	0.000	< 0.0001
SD vs CD	0.000	0.000	0.000	< 0.0001
CD vs RD	1.000	1.000	1.000	0.975

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for galactocerebroside (glycolipid):

Contrast	Liver	Kidney	Heart	Pancreas
RD vs PD	< 0.0001	< 0.0001	< 0.0001	0.001
RD vs CD	< 0.0001	0.057	< 0.0001	1.000
RD vs PC	0.473	0.187	< 0.0001	< 0.0001
RD vs SD	1.000	0.003	0.974	0.001
SD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SD vs CD	< 0.0001	< 0.0001	< 0.0001	0.002
SD vs PC	0.591	< 0.0001	< 0.0001	< 0.0001
PC vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs CD	0.000	0.974	< 0.0001	< 0.0001
CD vs PD	< 0.0001	< 0.0001	< 0.0001	0.001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for sphingolipid (glycolipid):

Contrast	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs RD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs CD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs SD	0.001	0.004	0.004	0.494
CD vs RD	0.603	0.002	0.784	0.949
RD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs SD	0.040	< 0.0001	0.133	0.892
SD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for phosphotidyl ethanolamine (phospholipid):

Contrast	Liver	Kidney	Heart	Pancreas
CD vs SD	0.835	0.014	< 0.0001	< 0.0001
CD vs PC	< 0.0001	0.009	< 0.0001	< 0.0001
CD vs RD	0.733	0.157	< 0.0001	< 0.0001
CD vs PD	0.987	0.794	0.000	< 0.0001
PD vs SD	0.933	0.001	< 0.0001	< 0.0001
PD vs PC	< 0.0001	0.001	< 0.0001	< 0.0001
PD vs RD	0.824	0.014	< 0.0001	< 0.0001
RD vs SD	0.274	0.799	< 0.0001	< 0.0001
RD vs PC	< 0.0001	0.680	< 0.0001	< 0.0001
PC vs SD	< 0.0001	1.000	< 0.0001	0.996

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for sphingomyelin (phospholipid):

Contrast	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	< 0.0001	< 0.0001	0.000
PD vs RD	< 0.0001	0.001	< 0.0001	0.000
PD vs CD	< 0.0001	0.000	< 0.0001	< 0.0001
CD vs PC	< 0.0001	0.303	< 0.0001	< 0.0001
CD vs SD	< 0.0001	0.997	< 0.0001	< 0.0001
CD vs RD	0.011	0.926	0.942	< 0.0001
RD vs PC	< 0.0001	0.069	< 0.0001	0.006
RD vs SD	0.289	0.775	< 0.0001	1.000
SD vs PC	< 0.0001	0.492	0.034	0.005

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for lysophospholipid (phospholipid):

Contrast	Liver	Kidney	Heart	Pancreas
PD vs PC	< 0.0001	0.001	< 0.0001	< 0.0001
PD vs SD	< 0.0001	0.167	< 0.0001	< 0.0001
PD vs RD	< 0.0001	0.245	< 0.0001	< 0.0001
PD vs CD	< 0.0001	0.485	0.000	< 0.0001
CD vs PC	< 0.0001	0.044	< 0.0001	< 0.0001
CD vs SD	< 0.0001	0.956	< 0.0001	< 0.0001
CD vs RD	< 0.0001	0.989	< 0.0001	< 0.0001
RD vs PC	< 0.0001	0.118	< 0.0001	< 0.0001
RD vs SD	< 0.0001	0.999	0.998	0.008
SD vs PC	0.000	0.179	< 0.0001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for phosphotidylcholine (phospholipid):

Contrast	Liver	Kidney	Heart	Pancreas
PC vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PC vs SD	< 0.0001	0.563	< 0.0001	< 0.0001
PC vs CD	< 0.0001	0.186	< 0.0001	< 0.0001
PC vs RD	< 0.0001	0.115	< 0.0001	< 0.0001
RD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RD vs SD	< 0.0001	0.845	0.008	0.000
RD vs CD	0.961	0.999	0.176	0.438
CD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CD vs SD	< 0.0001	0.939	< 0.0001	0.000
SD vs PD	< 0.0001	< 0.0001	< 0.0001	< 0.0001

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for phosphotidylinisitol (phospholipid):

Contrast	Liver	Kidney	Heart	Pancreas
SD vs PD	< 0.0001	0.004	< 0.0001	0.013
SD vs CD	< 0.0001	0.064	< 0.0001	0.282
SD vs PC	< 0.0001	0.867	< 0.0001	0.970
SD vs RD	0.432	0.997	0.038	0.985
RD vs PD	< 0.0001	0.009	< 0.0001	0.013
RD vs CD	< 0.0001	0.009	< 0.0001	0.004
RD vs PC	< 0.0001	0.967	< 0.0001	0.785
PC vs PD	< 0.0001	0.041	< 0.0001	0.055
PC vs CD	< 0.0001	0.368	< 0.0001	0.631
CD vs PD	0.006	0.763	0.567	0.580

The significant difference between diabetic groups based on Tukey's HSD test for insulin and glucagon gene expression in pancreas:

Contrast	Insulin1
PC vs PD	< 0.0001
PC vs CD	< 0.0001
PC vs RD	< 0.0001
PC vs SD	< 0.0001
SD vs PD	< 0.0001
SD vs CD	< 0.0001
SD vs RD	0.429
RD vs PD	< 0.0001
RD vs CD	< 0.0001
CD vs PD	< 0.0001
4.654	

The significant difference between diabetic groups based on Tukey's HSD test for TGFβ (kidney) and PPARα (Heart) gene expression:

Contrast	TGFβ	PPARα
PD vs PC	< 0.0001	< 0.0001
PD vs SD	< 0.0001	0.001
PD vs CD	< 0.0001	0.028
PD vs RD	< 0.0001	0.021
RD vs PC	< 0.0001	< 0.0001
RD vs SD	< 0.0001	0.064
RD vs CD	0.026	0.892
CD vs PC	< 0.0001	< 0.0001
CD vs SD	0.009	0.241
SD vs PC	< 0.0001	< 0.0001

The significant difference between diabetic groups based on Tukey's HSD test for SREBP1 gene expression in liver:

Contrast	SREBP1	Glut2	Cox1	Cox2
PD vs PC	0.484	0.000	0.091	<0.0001
PD vs SD	0.343	0.021	0.011	<0.0001
PD vs CD	0.323	0.003	0.014	<0.0001
PD vs RD	0.296	0.660	0.039	<0.0001
RD vs PC	0.187	< 0.0001	0.979	0.007
RD vs SD	0.047	0.003	0.910	0.682
RD vs CD	0.026	0.001	0.965	0.761
CD vs PC	0.161	0.175	0.758	0.043
CD vs SD	0.020	0.705	1.000	1.000
SD vs PC	0.141	0.025	0.644	0.054
Tukey's d critical value:				

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for TNFα gene expression:

Contrast	Liver	Kidney	Heart
PD vs SD	< 0.0001	< 0.0001	<0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001
PD vs PC	< 0.0001	< 0.0001	<0.0001
PC vs SD	< 0.0001	< 0.0001	<0.0001
PC vs CD	< 0.0001	< 0.0001	<0.0001
PC vs RD	< 0.0001	< 0.0001	<0.0001
RD vs SD	0.004	0.000	0.001
RD vs CD	0.017	0.001	0.000
CD vs SD	0.882	0.714	0.749
Tukey's d critical value:			4.654

The significant difference between diabetic groups for each tissue based on Tukey's HSD test for IL1α gene expression:

Contrast	Liver	Kidney	Heart
PD vs SD	< 0.0001	< 0.0001	<0.0001
PD vs CD	< 0.0001	< 0.0001	<0.0001
PD vs RD	< 0.0001	< 0.0001	<0.0001
PD vs PC	< 0.0001	< 0.0001	<0.0001
PC vs SD	< 0.0001	< 0.0001	0.470
PC vs CD	< 0.0001	< 0.0001	0.001
PC vs RD	< 0.0001	< 0.0001	0.002
RD vs SD	0.087	< 0.0001	0.026
RD vs CD	0.848	0.000	<0.0001
CD vs SD	0.364	0.901	<0.0001
Tukey's d critical value:			4.654