ROLE OF THERMALLY OXIDIZED EDIBLE OILS ON THE DEVELOPMENT OF HIGH FRUCTOSE INDUCED INSULIN RESISTANCE AND ASSOCIATED EVENTS THAT LINK TO HEPATOSTEATOSIS AND COLON CARCINOGENESIS

Thesis submitted to

UNIVERSITY OF CALICUT

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DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

(Faculty of Science)

By

ARUNAKSHARAN N., M.Sc., B.Ed., CSIR-JRF (NET)

under the guidance of

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AMALA CANCER RESEARCH CENTRE,
THRISSUR, KERALA, INDIA

August 2018

DECLARATION

I hereby declare that the thesis entitled **"ROLE OF THERMALLY OXIDIZED EDIBLE OILS ON THE DEVELOPMENT OF HIGH FRUCTOSE INDUCED INSULIN RESISTANCE AND ASSOCIATED EVENTS THAT LINK TO HEPATOSTEATOSIS AND COLON CARCINOGENESIS"** is based on the original work carried out by me at Department of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala, under the guidance of Dr. C.R. Achuthan, Assoc. Professor, Dept. of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala. The thesis has been subjected to plagiarism check and no part thereof has been presented for the award of any other degree, diploma or other similar titles.

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ARUNAKSHARAN N.

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ABBREVIATIONS

Akt	- Protein kinase B
ALP	- Alkaline phosphatase
ALT	- Alanine transaminase
ANOVA	- Analysis of Variance
AR	- Aldose reductase
AST	- Aspartate transaminase
ATR	- Attenuated total reflection
AUC	- Area under the curve
CD	- Conjugated diene
CDD	- Chronic degenerative diseases
CFAM	- Cyclic fatty acid monomer
CO	- Coconut oil
CRC	- Colorectal cancer
CT	- Conjugated triene
CVD	- Cardiovascular disease
FTIR	- Fourier-transform infrared spectroscopy
GCMS	- Gas chromatography–mass spectrometry
GGT	- Gamma glutamyl transferase
GPx	- Glutathione peroxidase
GR	- Glutathione reductase
GSH	- Reduced glutathione
HDL	- High Density lipoprotein

HFr	- High fructose diet
HHE	- 4- Hydroxy hexenal
HOMA	- Homeostatic model assessment
IL-6	- Interleukin-6
IR	- Insulin resistance
LC-MS	- Liquid chromatography- mass spectroscopy
LD	- Lard
LDH	- Lactate dehydrogenase
LDL	- Low density lipoprotein
MCFA	- Medium chain saturated fatty acid
MCT	- Medium chain triglycerides
MDA	- Malondialdehyde
MO	- Mustard oil
MUFA	- Monounsaturated fatty acid
NADPH	- Nicotinamide adenine dinucleotide phosphate
NAFLD	- Nonalcoholic fatty liver disease
NBT	- Nitro blue tetrazolium
NRF-2	- Nuclear factor erythroid 2–related factor 2
ODC	- Ornithine decarboxylase
OGT	- Oral glucose tolerance
PCR	- Polymerase chain reaction
PPAR-Ɣ	- Peroxisome proliferator activated receptor gamma
PUFA	- Polyunsaturated fatty acid

Q-TOF	- Quadrupole time of flight
ROS/RNS	- Reactive oxygen/nitrogen species
SDH	- Sorbitol dehydrogenase
SFA	- Saturated fatty acid
SO	- Sunflower oil
SOD	- Superoxide dismutase
TBA	- Thiobarbituric acid
TBARS	- Thiobarbituric acid reactive substances
TC	- Total cholesterol
TCO	- Thermally oxidized coconut oil
TG	- Triacylglycerol
TLC	- Thin layer chromatography
TLD	- Thermally oxidized lard
TMO	- Thermally oxidized mustard oil
TNF-α	- Tumor necrosis factor-α
TSO	- Thermally oxidized sunflower oil
VLDL	- Very low density lipoprotein

"Let food be thy medicine and medicine be thy food"

There has been a large shift from the infectious diseases to non-communicable or degenerative diseases in the present century. Often these disorders are closely associated with daily lifestyle of people and hence it is popular as Lifestyle diseases or lifestyle associated diseases. An array of chronic degenerative diseases exists, which include cardiovascular diseases and stroke, obesity and type-2 diabetes, various forms of cancers, neurodegenerative diseases as well as diseases associated with drug abuse. The predominant risk factors that lead to the development of degenerative diseases include unhealthy food habits, lack of physical activity, disturbed biological clock due to stress and substance abuse.

Among the risk factors mentioned, unhealthy dietary habit seems to be universally applicable and hence the most important. Healthy diet is often regarded as the medicine, and hence the quality and composition of the diet determines its biological effects. Several diets, especially Mediterranean diet, that contain natural antioxidants from sea weeds and high content of omega-3 fatty acids, are well-known for healthy lifestyle. On the contrary, dietary practices such as western diet, which is rich in sugar and fried fats, or high calorie junk food, are unhealthy.

The main constituents of Western diet or junk foods are fats and sugars, especially fried foods and sugar sweetened beverages. Mainly animal fat especially of chicken, beef or pork are the content in these food items, which are rich sources of long chain saturated fatty acids. Increased intake of dietary fats, especially red meat risks dyslipidemia and associated disorders in body. Current trends show an increased use of repeatedly heating or reusing of edible oils and fat in the fast food industry. Thermal oxidation of oil/fat induces various chemical and physical changes in them and makes them unhealthy for dietary purpose. However, due to lack of knowledge and negligence, industries reuse these oils several times and are being consumed by people regularly.

In addition to these, high content of fructose present in sugar sweetened beverages (6-7% of the drink) further raise the health risk of the dietary regimen. Fructose or other sugars induce insulin resistance in the body. Since, insulin resistance is crucial in the development of type-2 diabetes, obesity and metabolic syndrome such as fatty liver

and also various forms of cancers, the high fat/ high sugar diet possibly raise the risk factor.

In Indian scenario, consumption rate of sugar sweetened beverages and fried foods are being considerably increased. In addition, epidemiological studies indicated an increasing trend for the incidences of nonalcoholic fatty liver disease and colorectal cancer. Hence, it is necessary to evaluate the effect of common edible oils used for frying purposes with respect to general health. Thus, the present study has chosen coconut oil, mustard oil, sunflower oil, and lard (pork fat) as well as their thermally oxidized products for evaluation. The study aims to evaluate the effect of above mentioned dietary oils/fats on high fructose diet induced insulin resistance and subsequent changes that correlated with atherogenic dyslipidemia, hepatosteatosis, and changes in colorectal epithelia.

Abstract

Over the recent decades, there has been a considerable increase in the incidence of various chronic degenerative diseases such as cancers, cardiovascular diseases, fatty liver, and neurological disorders. Obesity and associated insulin resistance are reported to be the driving forces behind each of these disorders. Further, lack of physical activity and substance abuse aggravates these ailments. It is generally considered that the dietary pattern, especially the intake of fat and sugars, have close association with obesity and insulin resistance and epidemiological evidence have indicated that dietary regimen which containing sugar sweetened beverages and fried fats can induce insulin resistance and metabolic syndrome. In Indian scenario, several different edible oils are used for cooking purposes, where deep frying is a common event. The commonly used edible oils in Indian populations are medium chain saturated fatty acid rich coconut oil (CO), monounsaturated fat rich mustard oil (MO), polyunsaturated fatty acid containing sunflower oil (SO) and animal long chain saturated/ polyunsaturated fat containing lard (LD). Thus, the present study evaluated the possible health hazard of these edible oils or their thermally oxidized forms together with a high fructose diet (which is the predominantly used sugar in beverages) upon consumption. The experimental diet consisted of fructose (60%), fat source (10%), protein (20%) and various other micronutrients. Among these, in each experimental animal group, the fatty acid source was supplemented with either of these aforementioned edible oils or their respective thermally oxidized forms (TCO, TMO, TSO, and TLD).

The physico-chemical analysis of the edible oils observed significant changes during thermal oxidation. The unsaturated fatty acid rich edible oils were more prone to oxidative modification and generated various lipid peroxidation products such as conjugated diene (CD), thiobarbituric acid reactive substances (TBARS), and aldehydes. On contrary, MCFA rich CO was more resistant to these oxidative modifications; however, spectroscopic studies indicated the possible formation of triglyceride polymerization products during thermal oxidation in TCO. Supporting our results, previous studies had reported the formation of the cyclic fatty acid monomers (CFAM) during the thermal oxidation of edible oils including SO (Romero *et al.*, 2006). These molecules generated by thermal oxidation and polymerization were shown to interfere with various metabolic processes such as enzyme activities and lipid metabolism in the body (Lamboni *et al.*, 1998; Martin *et al.*, 1997). Thus our study went on to analyze the physiological effects of these edible oils on high fructose induced metabolic

dysregulation in animals that may have a possible link to hepatosteatosis and colon carcinogenesis.

High fructose diet has been used as a model for metabolic syndrome in animals, especially Wistar rats. Fructose intake impairs glucose metabolism and induces hepatic lipogenesis, subsequently leading to the development of hepatosteatosis (the beginning stage of Nonalcoholic fatty liver disease (NAFLD)). In our study, dietary supplementation with TCO had no significant effect on high fructose induced insulin resistance and glucose intolerance (as depicted by HOMA indices); whereas, dietary TSO and TLD intake significantly ($p<0.01$) exacerbated in these animals. The hyperglycemia associated with insulin resistance had been reported to trigger the polyol pathway enzymes. In accordance with these, TSO, TLD and TMO containing diet fed animals had a significant increase in the activities of aldose reductase and sorbitol dehydrogenase, however the activation was comparatively lower in TCO containing diet fed animals. Insulin resistance is often associated with hepatic and peripheral dyslipidemia; likewise, the TLD containing diet fed animals had significantly elevated serum and hepatic triglycerides as well as reduced HDLc levels. Though there was the development of dyslipidemia in TCO, TMO and TSO fed groups, the intensity was comparatively lower. It is expected that the increased dyslipidemic status in TLD may be due to the cholesterol content present in the lard; whereas being vegetable oils, SO, MO and CO lack raw cholesterol. The hepatic redox balance, especially reduced glutathione level (GSH) as well as activities of superoxide dismutase (SOD) and catalase, was also diminished in HFr diet fed animals. Supplementation of thermally oxidized edible oils exacerbated these changes to a more severe extent, with the order TSO>TLD>TCO>TMO. In contrast with this, grading of NAFLD indicated a higher incidence of microvesicles and lipogranuloma in TCO containing diet fed rats followed by TSO, TLD and TMO.

A similar redox imbalance was also induced by HFr diet in the colon epithelial tissues of rats; where thermally oxidized edible oils significantly augmented the effects. However, in contrast with the pattern observed in the hepatic tissue, the toxic insults were more profound in the TLD and TSO containing diet fed animals, which was followed by TMO; TCO containing diet fed animals. These animals had the least effect on their colon epithelial tissues. This oxidative imbalance was well corroborated with the elevated LDH and GGT activities in colon tissues of these animals ($p<0.01$). Apart from the oxidative stress, chronic inflammatory condition as indicated by the overexpression of IL-6 and TNF-α, was observed in the colon epithelial tissues of TLD, TSO and TMO containing diet fed animals, whereas

CO, SO and TCO had very little effects. Together with this, diminished expression of PPARγ and increased Wnt-1 and Akt expression ($p<0.05$) also indicated a possible cell proliferative/ survival potential. Reports have indicated that thermal oxidation of linoleic acid, the predominant fatty acid in SO and LD, generates pro-inflammatory molecules such as 9- and 13-hydroxyoctadecadienoic acids (9-HODE and 13-HODE) (Mabalirajan *et al.*, 2013; Patwardhan *et al.*, 2010); which are present in high levels in the neoplastic colorectal tissues (Silverman *et al.*, 1996). Apart from the linoleic acid oxidation products, the cholesterol present in Lard is also susceptible to oxidative modifications, yielding various oxysterols (Orczewska-Dudek *et al.*, 2012); these molecules have been found to promotes the carcinogenic process in colorectal tissues (Rossin *et al.*, 2017). Thus, the study concludes that together with the redox imbalance and chronic inflammation, altered expression of cell proliferation/ survival genes in the colon epithelial tissues of animals fed with TLD and TSO diet possibly indicates a pro-carcinogenic risk over long run.

The study thus observed significant oxidative damages in the edible oils during thermal treatment which are directly proportional to their unsaturation. Further, consumption of these thermally oxidized oils, especially those with higher level of unsaturation TSO&TLD) have been shown to exacerbate the HFr induced insulin resistance and glucose intolerance. Together with this, increased dyslipidemia and oxidative imbalance were also evident in the hepatic and colon epithelial tissues of experimental animals. TCO exacerbated the HFr induced hepatosteatosis; on the other hand TSO, TLD and TMO were more damaging to colorectal tissues. The study thus proposes a close association of thermally oxidized oils and high sugar diet with the hepatosteatosis and initiation events of colon carcinogenesis; in addition, it provides a possible mechanistic basis for the epidemiological correlation between the western diet and the chronic degenerative diseases. In view of these, high dietary fried foods and sugar sweetened beverages by pre-diabetic/ diabetic people are cautioned.

Chapter 1

Review of Literature

1.1 INTRODUCTION

Non-communicable diseases (NCDs) are becoming a concern for public health as well as socio-economic sectors all over the world, especially in developing countries like India. Chronic degenerative diseases or lifestyle associated diseases contribute to a major portion of NCDs. Cardiovascular diseases, diabetes mellitus and cancers account for about 59% of the annual mortality and approximately 45.9% of global disease morbidity. They share common risk factors such as obesity, hyperglycemia, insulin resistance and dyslipidemia. In addition, each of these diseases acts as an independent risk factor for the development of other NCDs. Drug abuse; alcoholism and smocking are also known risk factors for NCDs.

Obesity and insulin resistance are the two complementary and predominant risk factors which drive these NCDs. Under obese conditions, increased oxidative damages to cellular macromolecules occur, which in turn contribute to the dysregulation of various metabolic pathways in the body. Further, chronic inflammatory conditions prevailing in the obesity and insulin resistance drive the changes associated with cell cycle regulation and proliferation. These changes lead to the development of maladaptations in various tissues of the body, thereby triggering the onset and progression of diseases including fatty liver, hypertension and cancers. Since the dietary changes are important determinants of obesity and insulin resistance, the changes in dietary habits may have a predominant role in these diseases.

Changes associated with modern lifestyle are increasing in the developing countries (Naicker *et al.*, 2015). Among these, food habits such as westernized diet and lack of physical activity are the major concerns. Mediterranean diets which are rich sources of antioxidants and polyunsaturated fats reduce the risk of developing lifestyle associated diseases (Caretto and Lagattolla, 2015). On the contrary, a plethora of scientific evidence from epidemiological, ecological and interventional studies have reported that western diets, rich in saturated fats and sugars, increases the NCD risk in populations (Ezzati and Riboli 2013; Hariharan *et al.*, 2015; Manzel *et al.*, 2014). Experimental evidence has indicated that a high fat- high sugar diet accelerated cancer progression (Healy *et al.*, 2016; Kimura and Sumiyoshi, 2007), cardiovascular (DiNicolantonio *et al.*, 2016) and neurodegenerative diseases (Freeman *et al.*, 2014) in animal models. Changes associated with the culinary system are important factors

that control the biological effects of food. There is could be due to a shift from the earlier culinary processes such as boiling and baking to fast food cultures of deep frying in the recent years. Studies have come up with the observations that deep fried foods especially thermally oxidized edible oils increase the risk for various metabolic and lifestyle associated diseases (Gadiraju *et al.*, 2015; Narayanankutty *et al.*, 2016a).

1.2 OBESITY, INSULIN RESISTANCE AND TYPE-2 DIABETES

As indicated earlier, the incidence of various degenerative disorders are increasing every year, and each of them acting as an independent risk factor for the remaining. In these, the obesity is the primary and pace-setting condition for the development of subsequent disorders. The prevalence of obesity has gained much attention since its widespread occurrence, irrespective of age, sex or races. About two third of the world population suffers the risk of obesity. The obesity is closely related to the development of type-2 diabetes (T2D). Globally, around 171 million peoples are diabetic at present, and it is expected to be 366 million by the next 10 years.

Insulin resistance is the common factor that is linking obesity and Type 2 diabetes (T2D). The majority of all diabetes, i.e. about 85-95% is T2D. The prevalence of diabetes for all age-groups worldwide is estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al.*, 2004). Prevalence of T2D is higher in underdeveloped countries and lower in developed countries. India has the most diabetic patients, with a current figure of 50.8 million, followed by China with 43.2 million.

Usually, under physiological condition, the pancreatic beta cells produce insulin which helps in the uptake of glucose by cells and later proceeding to the metabolic conversion products. Even though there is a mild insulin resistance, the beta cells increase insulin secretion to overcome the hyperglycemia induced by lack of insulin sensitivity. However, in individuals with obesity, increased levels of non-esterified fatty acids (NEFA) exist in the serum, which in turn induces beta cell dysfunction and thereby increasing the susceptibility for type 2 diabetes (Mas *et al.*, 2010; Mihalik *et al.*, 2010). The elevated NEFA level during obesity causes a competitive inhibition of glucose metabolism by the enzymes pyruvate dehydrogenase, hexokinase and phosphofructokinase. Further, the increased level of lipolytic byproducts including

DAG and acetyl CoA inhibits Phosphoinositide 3-kinase (PI3K) activation, mediated by the phosphorylation of Insulin receptor substrate 1 and 2 (IRS-1, 2). This results in the reduced insulin receptor signaling and thereby inducing insulin insensitivity. Studies have also reported that treatment with Acipimox, an agent blocking lipolysis, has been considerably increased glucose uptake and metabolism (Fulcher *et al.*, 1993; Saloranta *et al.*, 1993).

Changes in the adipocyte microenvironment are another important factor that promotes the obesity-induced type-2 diabetes. The secretions from adipocytes include NEFA, glycerols derivatives, leptin and adiponectin as well as various pro-inflammatory immunocytokines. Retinol-binding protein 4 (RBP4) is one of the common and most important adipocytokine, which is known to induce insulin resistance by increasing tumour necrosis factor alpha (TNF-α) and leptin expression. Further, it also regulates PI3K signalling and phosphoenolpyruvate carboxykinase enzyme activity and thereby promoting insulin insensitivity. Adiponectin is another factor, however, which has a protective role in insulin resistance. It enhances 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptors (PPARs) dependent fatty acid oxidation and thereby increasing insulin sensitivity.

Tumour necrosis factor alpha (TNF-α) is another molecule which acts dependent on Retinol-binding protein-4 (RBP4). TNF-α together with pro-inflammatory cytokines IL-6 and monocyte chemoattractant protein-1 (MCP-1) induce peripheral and hepatic insulin resistance. These molecules induce chronic inflammation by enhancing the expression of JNK/IKK/NF-kB pathways, which in turn reduce insulin sensitivity and beta cell functioning. Inducible nitric oxide synthase (iNOS) also acts as an independent promoting factor for insulin resistance under obese conditions.

1.2.1 Type-2 Diabetes contribute to oxidative stress and inflammation

Type-2 diabetes conditions are a suitable platform for the development of oxidative radicals and thereby the development of inflammation. Hyperlipidemia and hypertriglyceridemia also can cause chronic inflammatory conditions (Xu *et al.*, 2003). It is evident that the level of Lipoprotein lipase (LPL) also associated with hypertriglyceridemia, hypercholesterolemia and cancer (Mutoh *et al.*, 2009; Mutoh *et al.*, 2006). The role of fatty aldehyde dehydrogenase (FALDH) in the regulation of

oxidative stress is also evident (Demozay *et al.*, 2004), FALDH is an enzyme that acts on the fatty aldehydes and converts them to less toxic carboxylic acids. Under several human cancers and T2D, aldehyde dehydrogenases are underexpressed (Traverso *et al.*, 2002).

1.2.2 Fat and insulin resistance

Body fat deposition and distribution determine the insulin sensitivity, for instance, lean individuals have higher insulin sensitivity than obese individuals. In lean individuals, the body fats are distributed peripherally; whereas in obese individuals fat distribution is largely abdominal or central (Carey *et al.*, 1996; Cnop *et al.*, 2002).

The roles of dietary fat in the development of insulin resistance and associated complications have also been studied for a long time. A high-fat diet has shown to enhance the expression of PI3K/AKT (Protein kinase B), which eventually leads to the development of insulin resistance (Yang *et al.*, 2014a). The study also revealed that the reversal of genes associated with glycolysis and gluconeogenesis by the up-regulation of Nuclear factor (erythroid-derived 2)-like 2 (NRF2)/ antioxidant response element (ARE) expression can help to ameliorate the high fat-induced insulin resistance. Further, consumption of high-fat diet throughout pregnancy period has shown to reduce the insulin receptor tyrosine kinase activity, leading to the decreased insulin sensitivity in the adult offspring (Elton *et al.*, 2002).

The saturated, monounsaturated, and omega 6 fats have shown to develop insulin resistance in rats; whereas a diet rich in omega 3 fats could effectively improve the insulin resistance (Albert *et al.*, 2014; Storlien *et al.*, 1991; VanWinden *et al.*, 2017). Among these, saturated fats are shown to reduce insulin action; in comparison, monounsaturated fats, as well as ω-6 PUFA, have a lesser influence on insulin resistance development (Koska *et al.*, 2016; Riccardi *et al.*, 2004). Recent studies have also indicated that not all saturated fatty acid impair insulin resistance, among that medium chain saturated fats are shown to have insulin-sensitizing effects (Silvia *et al.*, 2009).

1.2.3 Fats and chronic inflammation

Inflammation is part of the normal host response to infection and injury. However, excessive or inappropriate inflammation contributes to a range of acute and chronic

human diseases including diabetes, cancer, Alzheimer's, and atherosclerosis. Inflammation is characterized by the production of inflammatory cytokines, eicosanoids (prostaglandins, thromboxanes, leukotrienes, and other oxidized derivatives), other inflammatory agents (e.g., reactive oxygen species), and adhesion molecules.

Evidence from a number of studies has reported that SFA stimulates an inflammatory response by a pathway involving Toll-like receptors (TLR). TLR have significant roles in the development of inflammation and insulin resistance. Studies had shown that the TLR deficient mice were failed to develop the insulin resistance modulated by the pro-inflammatory pathways (Saberi *et al.*, 2009). Further, SFA also acts through in TLR independent manner by the production of reactive oxygen species (ROS). ROS are responsible to activate the nucleotide-binding domain, leucine-rich repeat containing family, pyrin domain-containing 3 (NLRP3) inflammasome (Dostert *et al.*, 2008). NALP3 forms a complex with apoptotic speck protein (ASC), called NALP3-ASC inflammasome complex, which controls the cleavage of IL-1β from pro-IL-1β (Franchi *et al.*, 2009). As the larger amounts of IL-1β is secreted, which leads to the decreased insulin sensitivity in the tissues (Nov *et al.*, 2010).

In contrast to the saturated fat, Omega 3 fatty acids are acting both directly and indirectly on the Lipoxidase mediated inflammatory mechanism. The n-3 fatty acid replaces arachidonic acid (an eicosanoid substrate) and thereby inhibits the arachidonic acid catabolism. They act indirectly through modulation of the major inflammatory genes and thus reducing the production of inflammatory molecules in the body. Resolvins are a family of anti-inflammatory molecules generated from the omega 3 fatty acids, which can also effectively inhibit the pro-inflammatory activity in the body (Hong and Lu, 2013).

1.3 FRUCTOSE: ROLE IN OBESITY AND INSULIN RESISTANCE

Fructose is a common natural hexose sugar found mostly in fruits. Though it is less consumed in Indian traditional scenario, fructose has been a predominant sugar in Western diet (Lambertz *et al.*, 2017). Since fructose is sweeter compared to equivalent amounts of table sugar and glucose, the use of fructose is promoted as a bulk sweetener (Ramirez, 1996). High fructose corn syrup is the major source of fructose for artificial sweetening purpose, and the use of which has been considerably

increased over the last three decades. Dietary fructose has been associated with various types of health issues and the majority of fructose intake was in the form of sugar-sweetened beverages or soft drinks containing high fructose corn syrup (HFCS) (Rippe and Angelopoulos, 2013). Compared to other sweeteners such as glucose/ sucrose, the metabolism of fructose is highly varied and unchecked, making it a more aggressive and health damaging sugar (**Figure 1.1**).

A volume of information is available on the role of high fructose intake and the pathophysiology of various metabolic syndromes, primarily NAFLD (Bantle, 2009). Compared to other organs, the liver is primarily affected by the consumption of fructose and the difference is due to the presence of fructose specific transported protein GLUT 5. Tissues like adipocytes, kidney and intestine also possess a low level of GLUT 5. Intestinal GLUT5 helps in the trans-luminal transport of fructose to the portal vein and thereby contributing to the hepatic accumulation of fructose.

1.3.1 Hepatic metabolism of fructose

Compared to glucose, fructose metabolism has been entirely controlled in the hepatic tissue by fructokinase (Km: 0.5 mM), however, the former can be metabolized by glucokinase uniformly in almost all tissues (Km: 10mM) (Rizkalla, 2010). Further, the hepatic metabolism of fructose and other hexose sugars are highly varied. Primarily, the fructose metabolism is independent of insulin levels and also it can bypass the glycolytic pathway (Basciano *et al.*, 2005).

Hepatic metabolism of glucose begins with the conversion to glucose-6-phosphate by glucokinase, followed by subsequent conversions into fructose -6-phosphate. The key regulatory step in glycolysis is as the level of Phosphofructokinase, an enzyme that controls the conversion of fructose -6-phosphate to fructose 1,6-bisphosphate, is inhibited by ATP as well as citrate. Later, fructose 1,6-bisphosphate is converted to pyruvate by series of enzymatic reactions prior to the entry of TCA cycle.

On the contrary to glucose, fructose is rapidly converted to triose-Phosphates by an insulin-independent pathway. Since fructokinase bypass the regulatory step in glycolysis controlled by phosphofructokinase, feedback inhibition by ATP or citrate is negotiated in fructose metabolism and thereby enabling continuously entering the glycolysis (Sun and Empie, 2012). The lower Km value of fructokinase for fructose

also aids this rapid conversion. Majority of triose phosphates produced from fructose is converted to glucose and later to glycogen via gluconeogenesis pathway. A portion continues the classical glycolytic pathway and still another portion is converted to lactic acid and enters circulation (Jang *et al.*, 2018). In addition to these, a fraction is used for the synthesis of free fatty acids which in turn increases the re-esterification of fatty acids and VLDL (Schwarz *et al.*, 2015). In conclusion, rapid and uncontrolled metabolism of fructose produces glucose, lactic acid and pyruvate, which provides large quantities of glycerol and acyl portions for hepatic synthesis of triglyceride which is incorporated to VLDL particles.

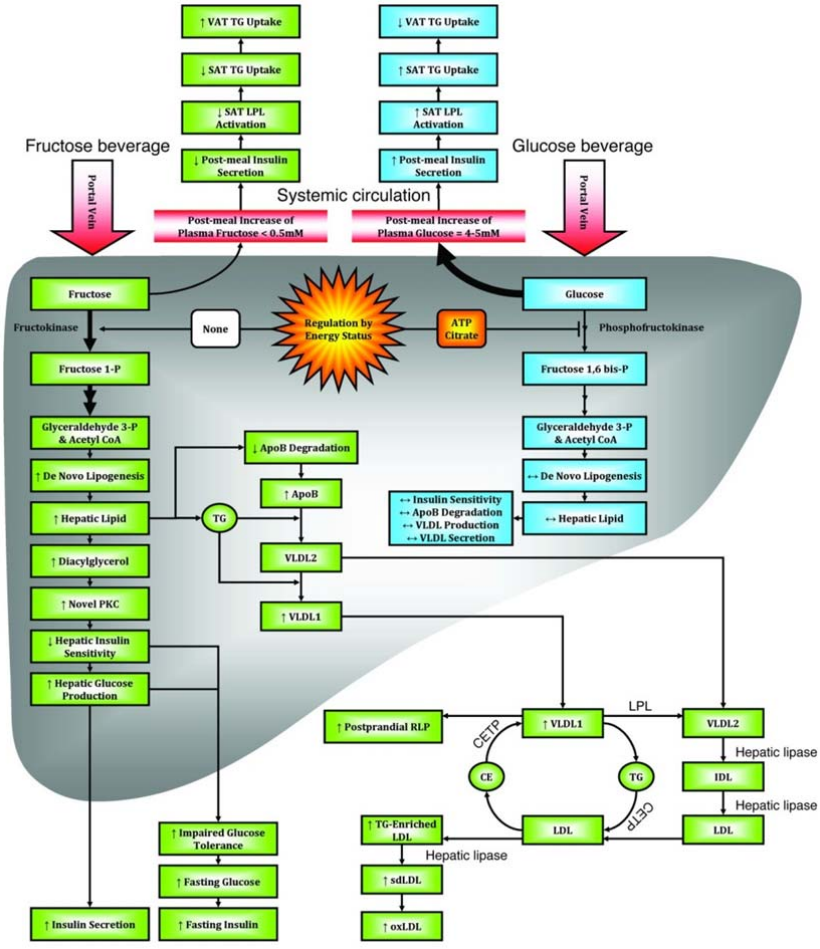


Figure 1.1 Hepatic metabolism of fructose and glucose (Stanhope *et al.*, 2009)

1.3.2 Fructose consumption and insulin resistance

Insulin resistance is a condition in which the cells lack the sensitivity even though insulin is present in sufficient quantities. It is a common risk factor for several

degenerative diseases (Roberts *et al.*, 2013). Several preclinical and epidemiological studies have indicated the close association of fructose intake with the development of hepatic and muscle insulin resistance (Stanhope, 2016). A significant decrease in glucose uptake and insulin sensitivity is observed in animals fed with a diet containing 40- 60% fructose (Baena *et al.*, 2016; Wong *et al.*, 2016). Chronic intake of fructose diet has also been shown to reduce the β-cell mass by increasing apoptotic cells (Zhang *et al.*, 2017). Moreover, it has been reported that in the hepatic tissue of fructose-fed rats insulin receptor phosphorylation has been found reduced (Bezerra *et al.*, 2000). Further, decreased phosphorylation of tyrosine residues in the insulin receptors and IRS-1 has also observed in the neural tissues of fructose-fed hamsters.

The initial metabolite of fructose, the fructose-1-phosphate is shown to activate mitogen-activated protein kinase (MAPK8) (Liu *et al.*, 2000). Further, the diacylglycerol another product of fructose metabolism activates the hepatic protein kinase C (Geraldes and King, 2010). Both these enzymes together inactivate IRS-1 via phosphorylation of its serine residues, which subsequently results in the development of insulin resistance (Aguirre *et al.*, 2002).

1.3.3 Fructose consumption and obesity

Increased fructose intake either as raw fructose or in the form of HFCS has been reported to be promoting adiposity and obesity (Lakhan and Kirchgessner, 2013). Laboratory studies in animal models have shown that high fructose intake increases body weight compared to the equal amount of sucrose feeding. Moreover, prolonged fructose intake of fructose results in an increased abdominal adiposity and increased triglycerides in the blood. It has been reported that fructose also contributes to increased food consumption by modulating central nervous system. Fructose stimulates AMPK activity by reducing malonyl CoA levels in the hypothalamus, which in turn drives food intake (Cha *et al.*, 2008). Further, free fatty acids produced as a result of fructose metabolism are incorporated to VLDL, which causes dyslipidemia and subsequently increase the risk of obesity (Crescenzo *et al.*, 2014).

1.3.4 Fructose and fatty liver

Lack of inhibition on fructose metabolism results in constitutive activation of glycolysis and which in turn leads to a substrate overload in the form of acetyl CoA in

liver (Softic *et al.*, 2016). The citrate formed from the acetyl CoA forms the substrate for de novo lipid biosynthesis. Further, malonyl CoA formed by the dimerization of acetyl CoA inhibits mitochondrial β-oxidation of fats (Foster, 2012). This overwhelmed production of lipids and diminished lipid clearance results in intrahepatic accumulation of lipids, resulting in lipotoxicity and hepatosteatosis.

1.4 NON ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Nonalcoholic fatty liver disease or generally known as the fatty liver is the most a frequent form of the chronic hepatic disorder, which is characterized by the accumulation of triacylglycerol primarily in the liver (Pagano *et al.*, 2002). It is also described as the hepatic manifestation of metabolic syndrome, estimating for about 24-42% of the general population globally and 9-32% in India (Fung *et al.*, 2015; Kalra *et al.*, 2013) (**Figure 1.2)**.

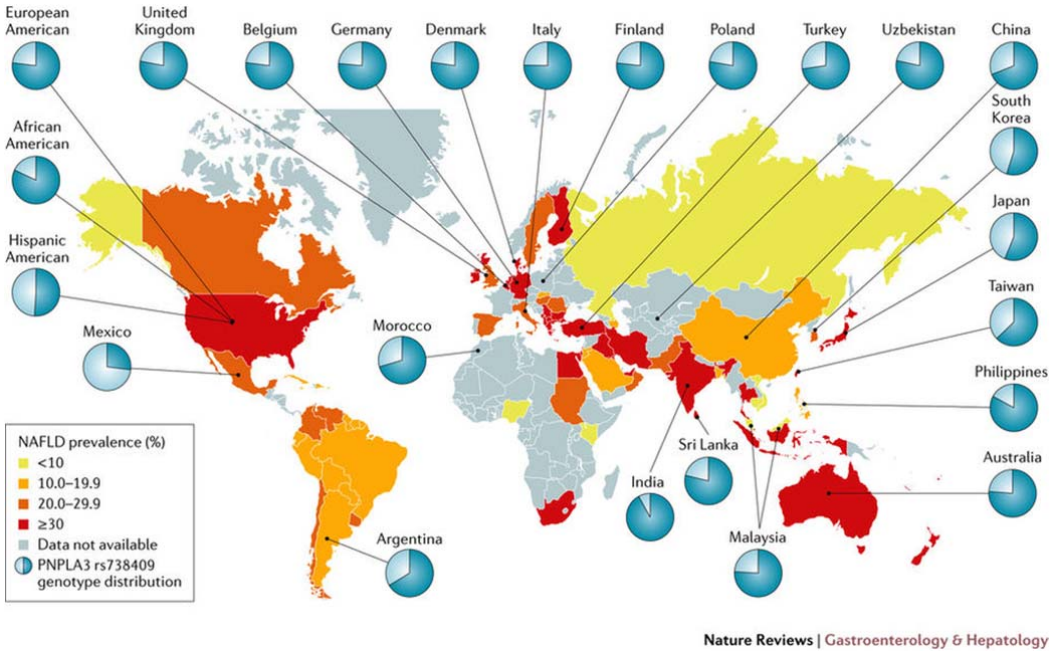


Figure 1.2 Prevalence of nonalcoholic fatty liver disease (NAFLD) (Copyright Order No. 4319171150086) (Younossi *et al.*, 2018)

NAFLD is a broad terms that comprises of simple accumulation of lipids, known as steatosis or hepatosteatosis and nonalcoholic steatohepatitis (NASH) (advanced steatosis with inflammatory activation). These conditions often progress to cirrhosis and ultimately up to hepatocellular carcinoma. Insulin resistance, impaired

carbohydrate and lipid metabolism, obesity and chronic inflammation are the hallmark of hepatosteatosis (Huang, 2009). In the past few decades, the severity of NAFLD has paralleled to that of type-2 diabetes and insulin resistance associated with obesity.

1.4.1 The 'Two-Hit' and "Multiple-Hit" theories of NAFLD

The two hit theory explains the onset and progression of NAFLD, where the initial or first hit involves the excessive lipid influx in the form of increased de novo lipogenesis, hepatic lipid accumulation, and reduced lipid clearance due to the inhibition of β-oxidation of fatty acids in the mitochondria. These processes are aided by insulin resistance and hyperglycemia, especially in fructose consumption (Dowman *et al.*, 2010). At this stage, the steatosis is reversible by co-ordinated treatments and physical exercise.

The second hit, which is less prevalent and involves a further progression of hepatosteatosis in nonalcoholic steatohepatitis. Micronutrient deficiencies, associated redox imbalance and chronic oxidative stress accelerate the second hit in patients. In this condition, increased cytokine activation from hepatic stellate cells leads to lobular inflammation, hepatocellular ballooning and peri-sinusoidal fibrosis (Huang, 2009). This condition usually progresses to cirrhosis within a few years, if untreated.

The two-hit theory of NAFLD was formed from a limited and narrow aspect view as well as it failed to represent the complexity of NAFLD. Compared to the cell culture/ animal models, human NAFLD is more complex with the synergistic or additive interaction of multiple parallel factors (Paschos and Paletas, 2009). Thus, a multiple-hit hypothesis has been proposed which incorporates the genetic predispositions for NAFLD together with the exogenous factors promoting the disease.

The "Multiple-hit" hypothesis provides an overall view of the various risk factors (hits) that regulate the onset as well as the progression of NAFLD. In addition to the lipid accumulation in the hepatocytes (first hit) and subsequent redox imbalance as well as a chronic inflammatory cascade (second hit) that drives the NAFLD, the role of dietary habits, genetic predisposition and environmental factors have been considered in the multiple-hit hypothesis. As described in the previous sections, insulin resistance and high sugar consumption increase the risk for NAFLD. Insulin

resistance increases the de novo lipogenesis and adipocyte lipolysis mediated fatty acid influx to the liver. Further, intake of a high-fat diet also induces hepatic lipotoxicity by increasing hepatic triglyceride accumulation (Described in detail in the coming section). In addition to these dietary factors and gut-microbiota also influence the fatty liver incidence by regulating fatty acid absorption in the intestine (Ma *et al.*, 2017). The genetic predisposition or epigenetic modifications influence the NAFLD by regulating the lipotoxicity (apoptotic) pathways and hepatic stellate cell activation (Buzzetti *et al.*, 2016).

1.4.2 Metabolic and dietary risk factors

Other than genetic predispositions, several exogenous factors influence the development of hepatosteatosis. Among these, dietary habits, metabolic factors and sedentary lifestyle have prime importance. Increased adiposity, obesity and insulin resistance are the basic risk factors involved in the onset of NAFLD. Changes in the hepatic inflammatory cytokine/ adipokine interplay are also a necessary factor in NAFLD (Streba *et al.*, 2015).

Diet rich in fats and sugar as in western countries are known to promote the lifestyle disorders including the development of hepatosteatosis (Mirmiran *et al.*, 2017). It has been also verified that reduced intake of dietary fats and lowered intestinal absorption of cholesterol have brought down the frequency of hepatosteatosis (Ushio *et al.*, 2013) indicating the role of dietary fats in the development of the disease. High-fat diet feeding is known to induce changes in lipid metabolism thereby leading to hepatosteatosis (Meli *et al.*, 2013; VanSaun *et al.*, 2009; Yang *et al.*, 2014b). Fructose, which is known to up-regulate the hepatic lipogenesis also becomes an independent risk factor for hepatosteatosis. High fructose diet is widely being used as an experimental dietary model to induce insulin resistance (Prakash *et al.*, 2011; Prakash *et al.*, 2014; Singh *et al.*, 2015b). As reports indicate, consumption of fructose enhances hepatic lipid biosynthesis and thereby leading to hyperlipidemic conditions (Moore *et al.*, 2014; Samuel, 2011). Epidemiological studies have also reported higher rates of mortality associated metabolic disorders and fructose consumption (Ruff, 2015; Singh *et al.*, 2015a).

1.4.3 Dietary fat and NAFLD

The liver is the predominant site of lipid metabolism, which makes a close association of dietary lipids with hepatic disorders including NAFLD. Numerous studies have identified the beneficial and adverse effects of dietary fats on the initiation and progression of NAFLD. Mainly long chain saturated fatty acids have been described as the deleterious forms compared to unsaturated. In vitro studies have identified the role of palmitic acid in inducing mitochondrial dysfunction and subsequent development of lipotoxicity and NAFLD like symptoms (García-Ruiz *et al.*, 2015; Yao *et al.*, 2011). A study by Zhang *et al.* (2012) has shown that compared to linoleic acid (PUFA), palmitic acid induces endoplasmic reticulum (ER) stress and leading to the development of lipotoxicity in hepatoma cells. In animal models, high fat diet has been shown to mimic a condition of human over-nutrition and lack of physical activity (Kakimoto and Kowaltowski, 2016).

Animal model studies have also confirmed the development of hepatosteatosis under high-fat diet feeding (Kakimoto and Kowaltowski, 2016). Increased plasma homocysteine levels have been observed under animal models of hepatosteatosis, which is partly contributed by the reduced homocysteine breakdown enzyme activities under high-fat feeding (Bravo *et al.*, 2011). Further, increased production of hepatic triglycerides and very low-density lipoprotein (VLDL) mediated by the 5-hydroxytryptamine-2 receptor (5-HT 2) is also noted under high-fat feeding (Li *et al.*, 2018). Compared to these studies, where a high cholesterol/ fat was used, intake of polyunsaturated (Borengasser *et al.*, 2012) and monounsaturated fatty acids (Hanke *et al.*, 2013) have shown to ameliorate the hepatosteatosis in animals. On contrary, saturated fats have shown to increase the endoplasmic reticulum stress and thereby exacerbating diet-induced hepatosteatosis (Wang *et al.*, 2006). However, later studies have indicated a relation of the chain length of saturated fatty acids with NAFLD, where medium chain saturated fatty acids (MCFA) has been shown to offer a mitigating effect (Ronis *et al.*, 2013; Wang *et al.*, 2017). Further, there observed a correlation between western diet and NAFLD incidence (Roberts *et al.*, 2015), where the high fat and fried foods have been described as the culprits (Mollard *et al.*, 2014).

1.4.4 Insulin resistance and fatty liver

Under the diabetic condition, an increased incidence of NAFLD has been reported and the possible involvement of insulin resistance is suggested. Insulin has significant

involvement in the regulation of hepatic lipogenesis process by regulating the activation of transcription factors such as sterol regulatory element-binding protein (SREBP-1). SREBP-1 is a transcriptional factor which activates the de novo lipogenesis in the liver, which is regulated by an insulin receptor- insulin receptor substrate-2 (Schreuder *et al.*, 2008). During insulin resistance, the IRS-2 is down-regulated, subsequently resulting in the overexpression of SREBP-1 and thereby increased de novo lipogenesis. Together with this, reduced beta-oxidation of fatty acids under insulin resistance is also contributing towards the hepatic lipid accumulation (Postic and Girard, 2008a; Postic and Girard, 2008b).

1.4.5 Association of NAFLD with colorectal carcinogenesis

There are very limited reports on the association of non-alcoholic fatty liver disease and risk of colorectal cancer. Reports have indicated the spectrum of NAFLD that ranges from simple steatosis to steatohepatitis, cirrhosis and even to the development of hepatocellular carcinoma. However, evidence has indicated that the burden of NAFLD is also broad to extra-hepatic diseases, including colon tumours (Adams *et al.*, 2017; Kim *et al.*, 2018). According to the study by Kim *et al.* (2018), a 15 fold increase in hepatocellular carcinoma has been observed in patients with NAFLD; further, there is a 2 fold increase for colorectal cancers in men and 1.9 fold increase for breast cancer in women.

Both NAFLD and colorectal cancers share common metabolic risk factors such as insulin resistance, obesity and associated inflammation (**Figure 1.3**) (Sanna *et al.*, 2016; Tilg and Moschen, 2014). Previously, studies conducted by Shen *et al.* (2014a) and Ding *et al.* (2015) has also reported a close association of NAFLD with that of colorectal cancers. However, in these studies NAFLD was diagnosed based on the liver function marker enzymes, thus the studies were not conclusive. Recent studies by Pan *et al.* (2017) and Mantovani *et al.* (2018) further confirmed the association of NAFLD and CRC; where the diagnosis of NAFLD was carried out through ultrasonography, liver biopsy as well as by biochemical liver function marker assays, and therefore found more reliable. Though clinical and epidemiological evidence suggests an association between NAFLD and colon cancer, there is no clear information on the mechanism is available yet.

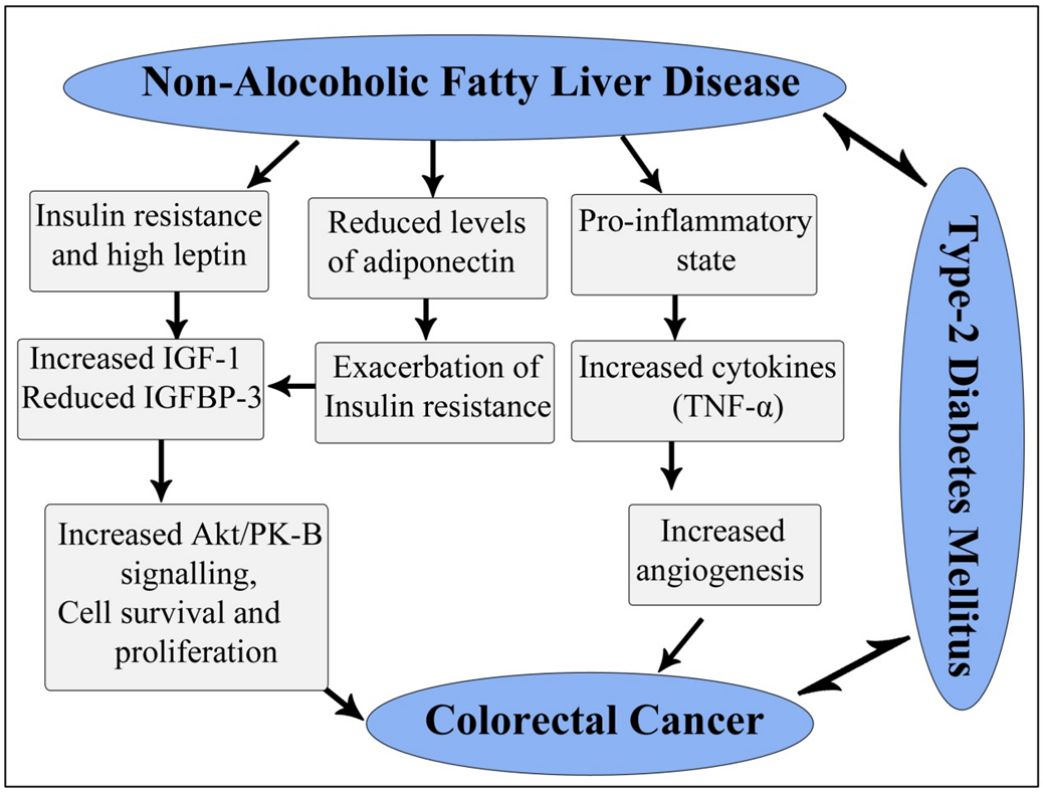


Figure 1.3 Association of type-2 diabetes, non-alcoholic fatty liver and colorectal cancers (Adapted from http://dx.doi.org/10.1155/2012/637538)

1.5 COLORECTAL CANCERS

Genetic analysis shows that essentially colon and rectal tumours are genetically same cancer and hence known as colon cancer or colorectal cancers. CRC accounts for over 9% of all cancer incidences (**Figure 1.4**). It is the third most common cancer worldwide and the fourth most common cause of death. In the Indian scenario, CRC is the fourth common cancer type. Overall, the lifetime risk of developing colorectal cancer is about 1 in 20 (5%). This risk is slightly lower in women than in men. CRC is highly prevalent in the countries of Australia, New Zealand and countries of Western Europe and lower in Africa. In 2008, 1.23 million new cases of colorectal cancer were clinically diagnosed, and that it killed 608,000 people globally (Ferlay *et al.*, 2010). Global colon cancer status in men and women are shown in **Figure 1.5 & 1.6**.

Estimated Deaths

Males				Females		
Lung & bronchus	84,590	27%		Lung & bronchus	71,280	25%
Colon & rectum	27,150	9%		Breast	40,610	14%
Prostate	26,730	8%		Colon & rectum	23,110	8%
Pancreas	22,300	7%		Pancreas	20,790	7%
Liver & intrahepatic bile duct	19,610	6%		Ovary	14,080	5%
Leukemia	14,300	4%		Uterine corpus	10,920	4%
Esophagus	12,720	4%		Leukemia	10,200	4%
Urinary bladder	12,240	4%		Liver & intrahepatic bile duct	9,310	3%
Non-Hodgkin lymphoma	11,450	4%		Non-Hodgkin lymphoma	8,690	3%
Brain & other nervous system	9,620	3%		Brain & other nervous system	7,080	3%
All Sites	**318,420**	**100%**		**All Sites**	**282,500**	**100%**

Figure 1.4 Global death rates associated with various cancers (WHO, 2015 data)

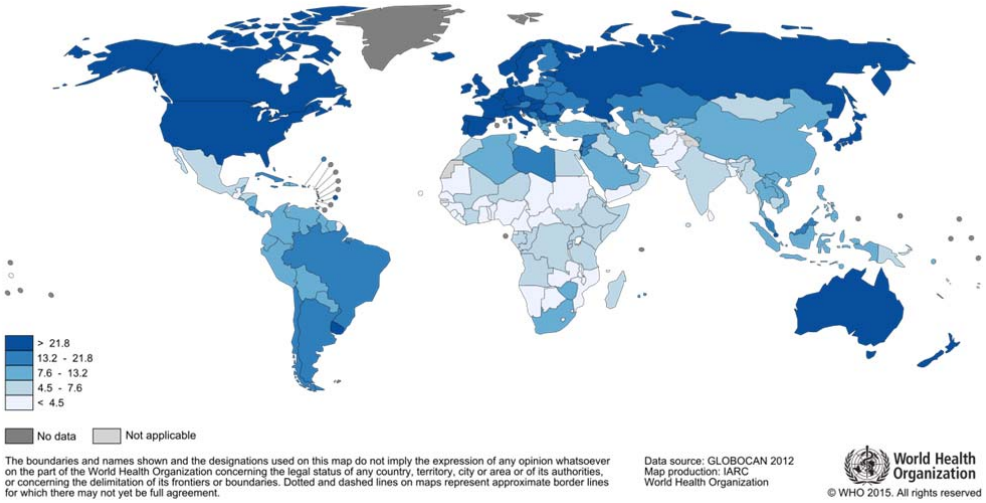


Figure 1.5 Worldwide colorectal cancer incidences in men (WHO, 2015 data)

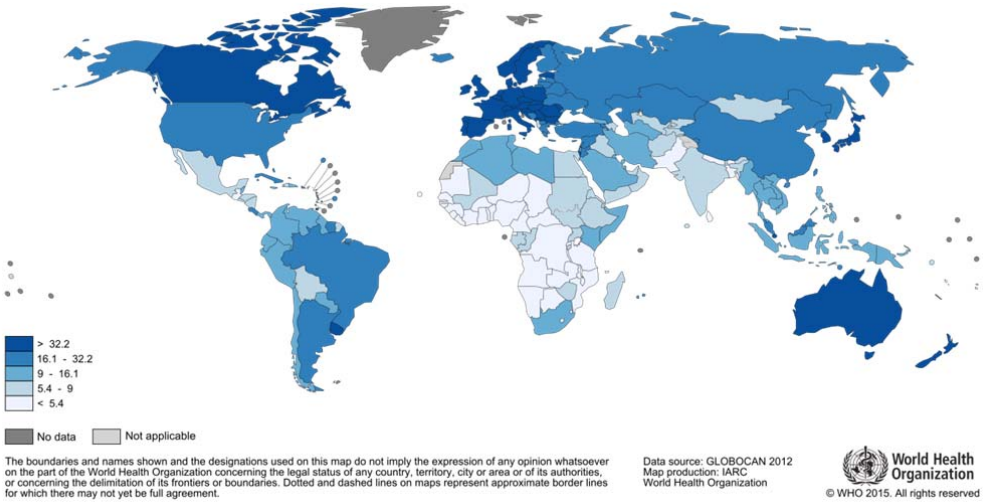


Figure 1.6 Worldwide colorectal cancer incidences in women (WHO, 2015 data)

1.5.1 Dietary fat in Colon cancer

Several studies have shown that the dietary fat has a direct role in the development and progression of colon cancers. The saturated fat has shown to sustain the induction of Benzopyrene biotransformation enzymes, leading to the generation and accumulation of B(a)P metabolites in the liver and colon rather than the unsaturated fats (Diggs *et al.*, 2013). This, in turn, leads to DNA and protein adducts formation in the colon which eventually increases the risk of colon cancer development. The animal model studies conducted by Reddy et al (Reddy, 2002) explains the possible role of dietary fat composition on the tumorigenesis and progression. The study reveals that saturated and omega 6 fats rich diet may have a role in the progression of colon cancers in rats, whereas the DHA and EPA composed diet shown to reduce the risk. The study was supported by the finding that elevated ras-p21 expression and increased colon cancer incidences in animals fed a diet rich in omega 6 fats, on the counterpart the omega 3 fats rich diet showed to increase the membrane localization of RAS (a small GTPase)- p21 (cyclin-dependent kinase inhibitor) and decreased expression of COX-2 leading to decreased tumor occurrence (Singh *et al.*, 1997a). The composition of the fat is also an important factor, which promotes the CRC. The level of heme in the fat is shown to be in direct correlation with colon cancer risks (Sesink *et al.*, 2000).

Bile acids appear to be of particular importance in colon cancer. The bile acid deoxycholic acid (DOC) is increased in the colonic contents of humans in response to a high-fat diet (Reddy *et al.*, 1980). Studies show that exposure of colon cells to high-level concentrations of DOC increases the formation of reactive oxygen species, causing oxidative stress and also that exposure of cells to bile acids increases DNA damage (Payne *et al.*, 2009). DOC can increase DNA damage in colonic cells and also reported to elevate the levels of NF-kB and AP-1. It is also reported that COX-2 signalling is activated by DOC and a high-fat diet, which have a significant role in the proliferation and invasiveness of colon cancer cells (Zhu *et al.*, 2012). Studies conducted by Reddy et al also confirms the role of fat in the activation of deoxycholic acid and lithocholic acid (Reddy, 1981; Reddy *et al.*, 1977). Amount and type of dietary fats have a central role in the etiology of colorectal cancer under Azoxymethane induced animal models (Reddy and Maeura, 1984).

Role of fat in cancer metastasis is not studied in detail. But available studies suggest a possible relation of dietary fat and composition with cancer invasiveness. Breast cancer metastasis is shown to be increased with a high-fat diet through cyclooxygenase mediated activity (Rose and Connolly, 1997). In contrast with the omega 6 fatty acids, Eicosapentaenoic acid, an omega 3 PUFA are shown to inhibit the human breast cancer invasions (Senzaki *et al.*, 1998). However, in the colorectal cancers, there was an increase in the metastatic rate and fat consumption. In contrast with the breast cancer metastasis, consumption of fat have irrespective of its composition found to increase the metastasis to liver, in the animal models(Griffini *et al.*, 1998; Klieveri *et al.*, 2000). High fat feeding was also shown to elevate the levels of growth factors, transcription factors and the expression of genes involved in the inflammation, angiogenesis, and cellular proliferation in the CT26 colon cancer cells (Park *et al.*, 2012). Even though more references are not available, by considering the possible roles of fats in inflammatory and oxidative stress signalling, it can be said that high-fat consumption can lead to increased rate of colorectal cancer metastasis, especially under the favourable conditions prevailing under diabetes.

1.5.2 Association of T2D and colorectal cancers

Increased cancer incidences are reported in diabetic people. The obesity and associated chronic inflammation result in the induction of insulin resistance, thereby leading to T2D (Usui and Tobe, 2011). People with T2D have also been shown to be at a greater risk for developing colon cancer (Djiogue *et al.*, 2013; Yang *et al.*, 2005), even though the reason is not fully understood. A recent study says that diabetic patients are at a 38% higher risk of developing colon cancer than those do not have diabetes (Yuhara *et al.*, 2011).

Diabetes and colorectal cancers have several risk factors in common, even though the exact pathophysiology remains unknown (Giovannucci *et al.*, 2010). It is suggested that the factors that may link T2D with colorectal cancer may be hyperinsulinemia, hyperglycemia or higher levels of molecules like IGF-1, NF-kB etc. One of the possible mechanisms is the elevated levels of insulin in Type 2 diabetes, affecting cells and promoting the growth of tumours (Giovannucci, 1995). A direct role for insulin to induce cancer cell proliferation and metastasis is also been suggested (Zhang *et al.*, 2010). If this holds true, diabetic patients receiving insulin will have a

higher propensity for cancer development. To date, no such information is available (Tseng, 2012). Another study explains that the level of IGF-1 and overexpression of its receptor IGF-1R promotes colorectal cancer (Shiratsuchi *et al.*, 2011). It is proposed that an elevated level of blood glucose is another factor that may induce colon cancer (Michaud *et al.*, 2005). All these factors enhance colon cancers, still, the actual signalling molecule that may drive these changes to colon carcinogenesis is still not clear.

One of the possible mechanisms is the elevated levels of insulin in diabetes, which affects cell division and apoptosis, leading to tumour promotion (Giovannucci, 1995). A direct role for insulin to induce cancer cell proliferation and metastasis is also been identified (Wang *et al.*, 2013). However, under such a condition, those diabetic patients who are undergoing insulin treatment will have higher chances for colon cancer development, yet no such information is available (Tseng, 2012). Elevated levels of IGF-1 and overexpression of its receptor IGF-1R under diabetes can another factor that promotes colorectal cancer incidences (Shiratsuchi *et al.*, 2011). In addition, western diets which are high in insulinotropic dairy have contributed to lifestyle diseases through activation of IGF-1 and insulin signalling (Melnik *et al.*, 2011). Although all these factors promote colonic neoplasias, it remains still unclear that which of the molecules initiate the changes that lead to colon carcinogenesis.

Supporting these evidence, fatty aldehyde dehydrogenases which are involved in the detoxification of fatty aldehydes are found to be reduced under diabetes, insulin resistance and cancers (Demozay *et al.*, 2004; Traverso *et al.*, 2002). In chronic diabetes, hyperplasia and hypertrophy in the colorectal epithelial tissues have also been observed (Zoubi *et al.*, 1995). In addition, enhanced uptake of sugars and proteins (Fedorak, 1990) and increased synthesis of cholesterol and triacylglycerol (Feingold *et al.*, 1990) in the colon was also observed. These changes in colonic epithelium may be attributed to the oxidative damages occurred in the tissue.

1.5.3 Roles of fats in linking diabetes and colon cancer

As we know, diabetes itself provides an inflammatory condition, in addition to the above when the fatty acids of saturated and n- 6 class is ingested, it will make the situation more complicated. The possible role of insulin resistance, hyperglycemia,

hypertriglyceridemia, and in addition to these the levels of fatty acids and their derivatives can contribute to increased mutation rates.

The dietary fatty acids can affect the bile acid production, which may also alter the inflammatory and stress condition prevailing in diabetes. The polyunsaturated and monounsaturated fat has shown to increase the expression of cholesterol 7 alpha-hydroxylase (CYP7alpha1) and thereby increasing the secretion of bile acids, whereas saturated fat-rich diet reduced the bile acid pool by decreasing the same (Li *et al.*, 2005). The secondary bile acids which are a known to induce cellular damages and may also produce genotoxicity and can increase the mitochondrial reactive oxygen species, and also known to promote colorectal cancers (Nagengast *et al.*, 1995). Studies have reported that the levels of secondary bile acids and 12 alpha-hydroxylase (CYP8B1) was increased in patients with type two diabetes mellitus (Haeusler *et al.*, 2013).

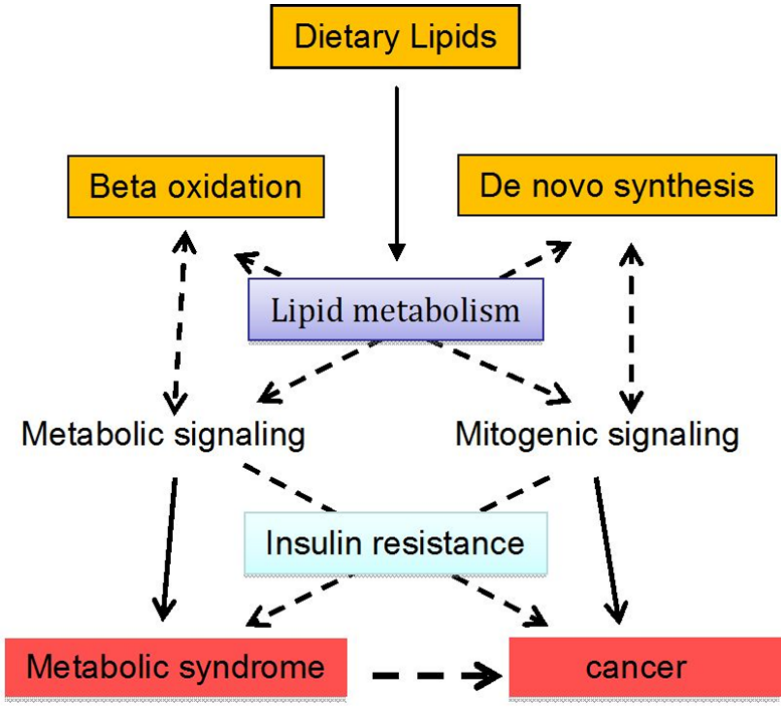


Figure 1.7 Lipid Metabolism, Metabolic Syndrome, and Cancer (Hu *et al.*, 2013)DOI: 10.5772/51821

Under the diabetic condition, consumption of higher n- 6 fat may lead to the increased secretion of insulin leading to impaired insulin metabolism and finally to insulin resistance (Lardinois *et al.*, 1987; Storlien *et al.*, 1986). The increased insulin resistance may further activate downstream molecules such as Phospholipase A2, leading to the release of the membrane-bound fatty acids. The free fatty acids especially the n- 6 fats, acts as the substrates for inflammatory signal amplification mediated by lipoxygenase and cyclooxygenase (**Figure 1.7**). In addition to the above, this may result in the enhanced production of reactive oxygen and other free radicals (Berry, 2001). The dietary modification induced changes along with the genetic predisposition for cancer may enhance the risk of developing and promoting colon cancers.

1.6 COOKING OILS AND THERMAL OXIDATION

1.6.1 Cooking oils

Edible oils are the important source of essential and non- essential fatty acids to the body. Different types of edible oils are being used all over the world, which differ in their fatty acid composition, micronutrient status, phenolic content as well as several other physicochemical and health properties. Based on the nature of fatty acids present, the edible oils are divided into saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Among the saturated fat-rich oils, there is medium chain saturated fat (MCSFA) containing oil (coconut oil) and long-chain saturated fat containing oil (palm oil). Similarly, based on the nature of fatty acids present, PUFA are again divided into omega 3 fat (fish and shark liver oil) and omega 6 fats (sunflower oil).

1.6.1.1 Coconut oil

Coconut oil (CO) is the generally consumed form of edible oil in south India. It is rich in medium chain saturated fat reaching up to 90% of the total, with lauric acid as the predominant fatty acid (50%). Coconut oil possesses several health benefits, it includes neuroprotection (Nafar and Mearow, 2014) and improvement of lipid metabolism in pre-menopausal women (Feranil *et al.*, 2011). At the same time, reports indicate an increased incidence of cardiovascular diseases when saturated fat intake is high indicating its possible health hazard (Sakata and Shimokawa, 2013; Siri-Tarino

et al., 2010; Wallstrom *et al.*, 2012). Also, coconut oil as a fatty acid source in high fructose diet induces hyperglycemia and oxidative stress in murine (Shawky *et al.*, 2014) and lead to hepatosteatosis like symptoms (Narayanankutty *et al.*, 2016b).

1.6.1.2 Mustard oil

Mustard oil is among the most common edible oil in India, which is rich in monounsaturated fats especially, erucic acid. Biological effects of dietary mustard oil have been identified. In acute myocardial infarction patients, mustard oil has shown to induce placebo effect and effectively modulates the lipid metabolism and reducing oxidative stress (Singh *et al.*, 1997b). It has been also shown that mustard oil possesses anti-mutagenic effect as indicated by a reduction in chromosomal breaks (Choudhury *et al.*, 1997) and chemopreventive effects on colon tumours possibly due to its polyunsaturated fat content (Dwivedi *et al.*, 2003). Though the beneficial effects have been reported, because of the higher monounsaturated fatty acid content, these fats raise some health concerns also. It has been documented that dietary mustard oil can induce hyperalgesia (Jiang and Gebhart, 1998) and neurologic inflammation (Banvolgyi *et al.*, 2004). In addition, the MO has shown to enhance hepatic pre-neoplastic foci development in rats (Shukla and Arora, 2003). Consumption of mustard oil increase the serum triglycerides with a reduction in mitochondrial cardiolipin content (Sen and Gupta, 1980). Due to high erucic acid contents, it is also known to induce cardiac fibrosis and vacuolation (Gopalan *et al.*, 1974).

1.6.1.3 Sunflower oil

Sunflower oil (SO) is one among the commonly consumed edible oil in various parts of India. They are had gained much popularity due to their high polyunsaturated fatty acid content. About 63% of the total fatty acid content is formed by linoleic acid. Sunflower oil is shown to reduce hyperlipidemia in rats with fatty liver by improving hepatic lipid metabolism, in comparison with palm oil (Go *et al.*, 2015). Similar observations also made by Trautwein *et al.* (1999) and Trautwein *et al.* (1997). However, recent studies have indicated that deep frying of the SO increases its peroxide contents as well as the free fatty acid and polymerized triglycerides (Carbonera *et al.*, 2014). Further, several animal model studies have also indicated that consumption of thermally oxidized sunflower oil (TSO) over a short period of

time, increases the oxidative damage in the body (Olivero David *et al.*, 2010; Wang *et al.*, 2016).

1.6.1.4 Lard (Pork fat)

Lard is an animal fat which is rich in monounsaturated fatty acids, often used in cooking as well as baking purposes. In addition to these triglycerides, it contains considerable amounts of cholesterol also as it is of animal origin, which makes its biological effects different from other plant-derived PUFAs. Lard has shown to accentuate the obesity associate changes including insulin resistance and accumulation of visceral as well as subcutaneous fat mass, compared to the hydrogenated vegetable oils (Kubant *et al.*, 2015). The oil is often used as a component in high-fat-diet-induced hepatosteatosis by inducing mitochondrial dysfunction (Apolzan and Harris, 2012; Lionetti *et al.*, 2014; Pranprawit *et al.*, 2013; Vendel Nielsen *et al.*, 2013). In addition to its effects on hepatic tissue, high-fat diets that contain lard as the primary source of fat leads to thyroid dysfunction, which is not reversible even after replacement from the modified diet for 6 weeks (Shao *et al.*, 2014). Similarly, neurological problems including impairment of hippocampal-dependent place recognition memory are noted in the short-term feeding of lard and sucrose (Beilharz *et al.*, 2014).

Biological functions of these oils are varied, which includes physiological, nutritional and pharmacological functions. Based on the chemical nature of the fatty acids, they differ in their biological activities also. Among these fatty acids, PUFA and MUFA are well described for its health promoting effects such as hypolipidemic, hypoglycemic, anticancer and lipid trafficking abilities. On the other hand, SFA is often described as unhealthy especially relating to cardiovascular health. However, recent studies indicated that compared to long chain saturated fatty acids (LCSFA), MCSFA is being emerged as a nutraceutical.

Though these edible oils are healthy in their fresh form, thermal oxidation or deep frying of these fats are known to generate noxious products that cause deleterious changes in them. This makes the edible oils not suitable for edible purposes and makes health problems. There are several physical and chemical changes involved during the process of deep frying. Under these frying conditions, the temperature,

atmospheric oxygen as well as the moisture content of oil makes up a unique reaction system, where the triglycerides and free fatty acids become the reactants.

1.6.2 Chemistry of thermal oxidation

During the process of frying, the edible oils are exposed to a high temperature usually above 160^{o}C. Previously, Choe and Min (2007) and Warner (1999) have well described the chemistry of edible oils during thermal oxidation. The oxidation of lipids during the process of frying is a complex process that is aided mainly by heat and also by free radicals. As shown in figure 1, the common chemical changes that are taking place during the thermal oxidation process include hydrolysis, oxidation and polymerization reactions (**Figure 1.8**) (Warner, 1999). The primary lipid peroxidation products formed during the process of deep frying include peroxides, conjugated diene structures. Due to their reduced stability and high reactivity, they undergo auto-decomposition yielding highly toxic secondary oxidation products such as aldehydes and ketone bodies. Ammouche *et al.* (2002) have reported that the thermal oxidation of the sunflower oil increases its peroxide value and free fatty acids content. Studies have also observed elevated levels of triacylglycerol polymers and dimers in these oils (Bastida and Sánchez-Muniz, 2001). Recently, Romero *et al.* (2006) have reported the presence of high levels of cyclic fatty acid monomers (CFAM) in thermally oxidized edibles oil, especially that contain unsaturation.

1.6.2.1 Oxidation

Oxidation reactions are aided by the atmospheric oxygen, which reacts under high temperature with the unsaturation of oils. It is a process that is similar to auto-oxidation, however resulting in a rapid increase in the levels of epoxides and peroxides of saturated or monounsaturated nature. It results in the increase in peroxide values, colour viscosity and polymers of triglycerides. Other than temperature, the presence of metals, reactive radicals and light also increase the oxidative modifications in oils. Compared to the saturated fatty acid rich oils, unsaturated fatty acid containing edible oils are more prone to oxidative modifications (**Figure 1.9**).

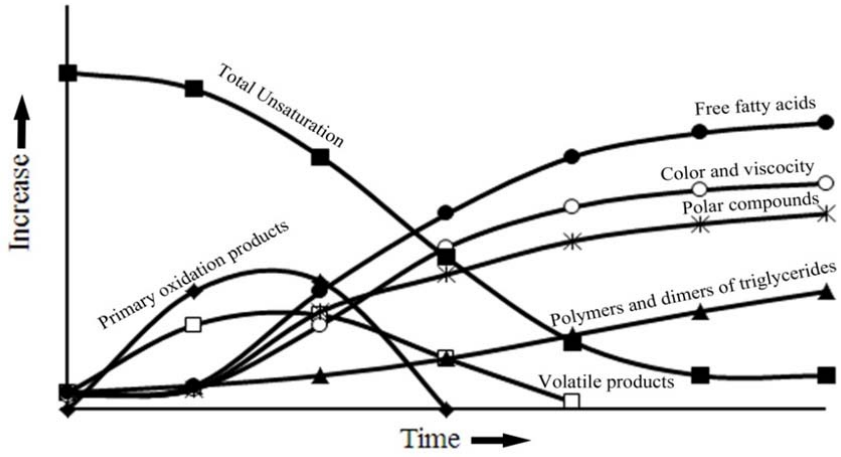


Figure 1.8 Physicochemical changes in the edible oils with respect to time of thermal oxidation (Choe and Min, 2007)

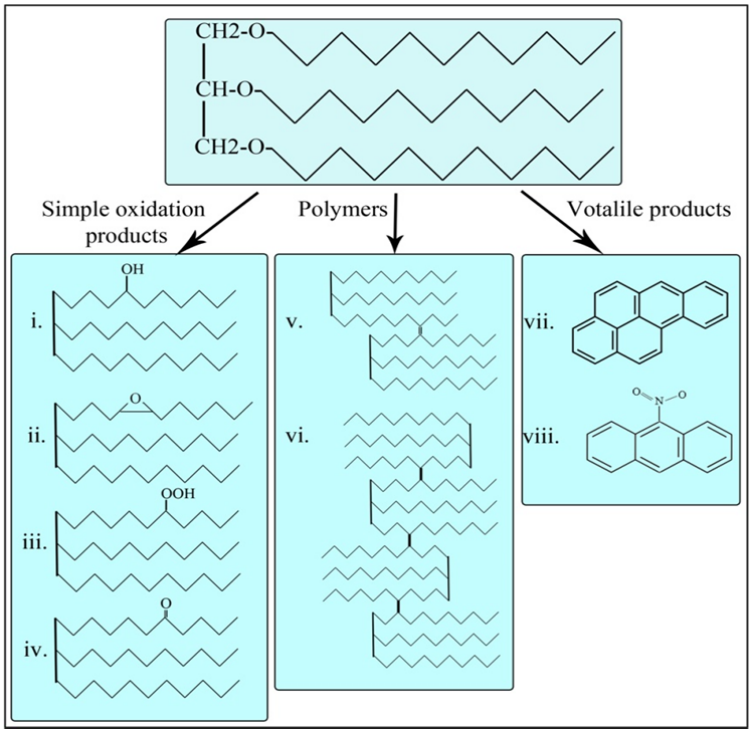


Figure 1.9 Common products formed during the thermal oxidation of oils (Choe and Min, 2007)

1.6.2.2 Hydrolysis

Other than the atmospheric oxygen, moisture content present in the oil as well as atmosphere interacts with the fatty acids. This results in a series of complex chemical

reactions resulting in the formation of diglycerides and free fatty acids, this results in the increase in acid value and polar contents (**Figure 1.9**).

1.6.2.3 Polymerization and isomerization

Polymerization reactions are the results of crosslinking of two or more triglycerides in thermally oxidized oils. In addition, thermal oxidation results in the formation of high molecular weight compounds that are formed by cyclization (formation of cyclic structures such as CFAMs) or dimerization (formation of intra-strand crosslinking between two different fatty acids). Usually saturated fatty acid rich oils undergo polymerization reactions during the high-temperature treatments (**Figure 1.9**).

1.6.3 Fried oils and health problems

As discussed above, the usual physicochemical nature of edible oils and fats drastically changes during the process of deep frying. Though consumption of the oils seems to be beneficial in various aspects of health, the deep fried oils are reported to be unhealthy. This is due to the newly formed reactive as well as toxic molecules and their metabolism in the body, which is being discussed in the coming sessions.

1.6.3.1 Fried oils and its association with hypertension and cardiovascular diseases

Cardiovascular health is often associated with the type and nature of the edible oil consumed. Several edible oils are found to be protective in cardiovascular diseases. In Mediterranean diets a reduced cardiovascular disease risk is observed, where olive and sunflower oil are the predominant forms (Guallar-Castillon *et al.*, 2012); whereas an independent study conducted by Djoussé *et al.* (2015) indicated that, people following a western dietary style are at high risk for developing coronary heart disease especially heart failure. The use of fried oils and fats in western diets, as well as the lack of dietary antioxidants as in the Mediterranean diet, could be the possible explanation for this observation. A volume of studies has identified a positive association between fried oil intake and hypertension (Kamisah *et al.*, 2016; Kamisah *et al.*, 2015; Leong *et al.*, 2010). In a cross-sectional anthropometric study, there observed an increased association between hypertension and intake of thermally oxidized sunflower oil, especially that is rich in polar compounds (Soriguer *et al.*, 2003). Similar results are also shown by an independent study carried out by Kang and Kim (2016). Reduction in the vasorelaxation ability of thoracic aorta occurs

during prolonged consumption of fried oils, which could be another factor that leads to hypertensive disorders (Leong *et al.*, 2009). Vascular inflammatory changes are often associated with reduced vasorelaxation ability, which is partially mediated by endothelial VCAM-1 (vascular cell adhesion molecule-1) and ICAM (intercellular cell adhesion molecule). Corroborating with these, there are reports that consumption of repeatedly heated soybean and palm oil increases the VCAM-1 and ICAM levels in rats (Ng *et al.*, 2012a; Ng *et al.*, 2012b). Hence, it can be ascertained that the alterations in the vascular thickening and vascular inflammation leads to hypertensive disorders during thermally oxidized edible oil feeding.

Results from the clinical or cohort studies also indicated an association of various cardiovascular disease risk factors to the thermally oxidized oil consumption. The positive association between a western style diet and acute myocardial infarction is also noticed in INTERHEART study (Iqbal *et al.*, 2008). However, a separate study by Sayon-Orea *et al.* (2014) observed increased adiposity as well as hypertension during thermally oxidized oil intake, whereas no positive association with metabolic syndrome. Further, the risk for developing the adiposity/obesity is directly related to the frequency of intake of fried food in a Mediterranean diet (Sayon-Orea *et al.*, 2013). Supporting the above, results from a Spanish cohort study showed an increased risk for general and central obesity in thermally oxidized sunflower oil intake (Guallar-Castillon *et al.*, 2007) however, it has no association with coronary heart diseases associated mortality (Guallar-Castillon *et al.*, 2012).

1.6.3.2 Fried oils and its association with obesity, insulin resistance and dyslipidemia

Role of thermally oxidized edible oils has been studied in the development of obesity and insulin resistance (**Table 1.1**). A study conducted by Guallar-Castillón *et al.* (2007) have observed a positive correlation between thermally oxidized oil intake and the development of obesity in Spanish populations. Obesity is often associated with the reduced glucose tolerance and insulin resistance. Liao *et al.* (2008) indicated that consumption of 20% frying oil induces hyperinsulinemia and insulin resistance in rats. On the contrary, observations by Chao *et al.* (2007) indicate that rats fed with fried oil (20%) have reduced insulin secretion thereby resulting in glucose intolerance, which is mediated through PPAR alpha signalling. Later studies revealed that

consumption of fried oil induces oxidative damages to the islets of the pancreas, thereby reducing the insulin secretion, which might be responsible for the observed glucose intolerance developed in the animals (Chiang *et al.*, 2011). With regards to the adiposity, controversial results exist. Bautista *et al.* (2014) observed an increase in abdominal adiposity in animals fed with a fried canola oil diet over a period of 10 weeks. On the contrary, studies conducted by Chiang *et al.* (2011) and Chao *et al.* (2007) shown that the fried oils are less adipogenic compared to the fresh oils.

Table 1.1 Effect of different thermally oxidized edible oils consumption on the lipid profile in animal model experiments.

Edible oil	Dose and Duration	Changes in lipid profile	Reference
Palm oil	10% of diet for 30 days	Increase in TC and LDL, reduction in TG and HDL	Falade *et al.* (2015)
Palm oil	15% oil and 2% cholesterol for 4 months	Increase in TC, TG and LDL, reduced HDL	Adam *et al.* (2008)
Sesame, Groundnut and Coconut oil	20% oil for 2 months	Increased TC and LDL, reduced HDL, TG and VLDL	Srinivasan and Pugalendi (2000)
Peanut, Sesame and Coconut oil	20% oil for 20 weeks	Increased TC, reduced TG and HDL	Narasimhamurthy and Raina (1999)
Sunflower oil	5% oil for 90 days	Increase in TC & reduced TG	Ammouche *et al.* (2002)
Palm oil	15% oil for 20 weeks	Reduced TC and an increase in TG levels	Kamsiah *et al.* (2001)

Obesity and insulin resistance are often linked with serum and hepatic dyslipidemia. Similarly, a number of studies have identified the dyslipidemic properties of fried oils, which is given in Table 1. General tendency observed in these studies include the hike in serum total cholesterol and LDL levels, with a subsequent reduction in the HDLc and triglycerides level. However, some studies also indicated the higher level of triglycerides in the fried oil fed animals (Kode *et al.*, 2005), which may be attributed to the increased free fatty acid levels in these oils as well as the lipase inhibitory

activities of CFAMs derived from the oxidized edible oils. Together with the presence of CFAM, the duration of feeding fried oils also depends on these trends, where long-term feeding increase the TG levels in the experimental animals (Adam *et al.*, 2008; Kamsiah *et al.*, 2001).

1.6.3.3 Fried oils and its association with genotoxicity, mutagenesis and cancer

Fried oils generate a volume of toxic substances which are both volatile and non-volatile in nature. Mainly these compounds come under the classes of polycyclic aromatic hydrocarbons (PAHs), nitro polycyclic aromatic hydrocarbons and aromatic amines (**Table 1.2**).

Table 1.1 Mutagenic or carcinogenic oxidation products formed during thermal oxidation of various edible oils

Edible Oils	**Mutagen/ carcinogen**	**Reference**
Safflower, olive, coconut, mustard, vegetable and corn oils	benzo[a]pyrene, dibenz[a,h]anthracene, benzo[b]fluoranthene, benzo[a]anthracene	Chiang *et al.* (1999b)
Sunflower, sesame, palm oil, soya, canola, mustard and peanut oils	Benzo[a]pyrene, Benz[a]anthracene and chrysene	Alomirah *et al.* (2010)
Soybean and Peanut oil	Benz[a,h]anthracene and benz[a]anthracene	Chiang *et al.* (1997)
Lard, sunflower and vegetable oils	2-naphthylamine and 4-aminobiphenyl	Chiang *et al.* (1999a)
Lard, soybean and peanut oils	1-nitropyrene and 1,3-dinitropyrene	Wu *et al.* (1998)
Peanut oil	trans-trans-2,4-decadienal	Wu and Yen (2004b)
Peanut oil	trans-trans-2,4-decadienal, trans-trans-2, 4-nonadienal, trans-2-decenal and trans-2-undecenal	Wu *et al.* (2001)
Lard, Soybean and sunflower oil	trans-trans-2,4-decadienal, trans-trans-2,4-nonadienal, trans-2-decenal and trans-2-undecenal	Dung *et al.* (2006)

Preliminary studies on Salmonella SV50 strains indicated the genotoxic effects of soybean and rapeseed oil fumes, however, the addition of BHC reduced these genotoxic effects (Chen *et al.*, 1992; Qu *et al.*, 1992). Later, cell culture-based studies also shown the DNA damaging and apoptotic effect of fried oil fumes (Che *et al.*, 2014; Lin *et al.*, 2002). Further studies conducted by Chiang *et al.* (1999b) indicated the presence of various toxic and carcinogenic compounds such as benzo[a]pyrene and benzo[a]anthracene in safflower, olive, coconut, mustard, and corn oils. Trans-trans-2,4-decadienal, a derivative during frying of peanut oil, is shown to induce genotoxicity mediated by the formation of reactive oxygen species and reduction of cellular glutathione content (Wu and Yen, 2004a). 1-nitropyrene and 1,3-dinitropyrene are the derivatives of lard, soybean and peanut oils (Wu *et al.*, 1998). DNA damage induced by these compounds is well evident from the increased formation of 8-hydroxy-2'-deoxyguanosine and subsequent expression of Human 8-oxoguanine DNA glycosylase-1 in cells (Cherng *et al.*, 2002). Pro-inflammatory and proliferative ability of these compounds are also reported (Chang *et al.*, 2005). Further, it has been shown that these fumes improve cell viability of lung cancer cells by increasing the anti-apoptotic gene c-IAP2 and downregulating p21 and caspase 3 (Hung *et al.*, 2005; Hung *et al.*, 2007).

Animal model studies have also supported these observations, where the consumption of repeatedly heated coconut oil induce hepatic foci and pre-neoplastic lesions in rats treated with diethylnitrosamine (Srivastava *et al.*, 2010a). Similarly to these, boiled sunflower and mustard oil are shown to have genotoxic and carcinogenic effects in murine models (Shukla and Arora, 2003; Srivastava *et al.*, 2010b). Compared to these studies where the fried oils are administered with a pro-carcinogen, our recent study has shown that a diet containing high fructose diet with fried coconut and mustard oils induce lipotoxicity and fatty liver incidence in male Wistar rats (Narayanankutty *et al.*, 2016a). The toxic effects are also mediated by the upregulation of polyol pathway activation as well as by altering redox balance.

Clinical evidence for the association of fried oil intake and cancer risk are also available. Cooking oil fumes are reported to have a higher correlation with lung cancer incidence (Lee and Gany, 2013; Metayer *et al.*, 2002; Xue *et al.*, 2016). A possible explanation for this is come up with the observations that fried oil fumes

inactivated DNA strand break repair systems such as ataxia-telangiectasia mutated (ATM) gene (Shen *et al.*, 2014b). Further, fried oil intake showed a positive correlation with cancers of the larynx (Bosetti *et al.*, 2002), oropharynx and oesophagus (Galeone *et al.*, 2005). Prostate cancer risk is also associated with dietary practices (Lippi and Mattiuzzi, 2015), where a high intake of white fish especially those following the methods of frying, broiling and grilling heightened the risk, whereas no risk is observed while using low temperatures (Joshi *et al.*, 2012); studies by Stott-Miller *et al.* (2013) also supported this hypothesis.

It is thus concluded that even though cooking and frying are usual processes involved in the preparation of food items, it has been associated with increased damages to oils and fats. Repeated heating of these edible oils, especially of unsaturated nature, are known to generate a wide array of compounds which are genotoxic, mutagenic and carcinogenic in nature. Saturated fatty acids upon thermal oxidation yields polymerized triglycerides and cross-linked compounds, which induce toxic insults to the body. Thus, consumption of high amounts of fried oil containing products as part of a changing lifestyle such as fast foods and junk foods may increase the risk of metabolic disorders including diabetes, fatty liver and various forms of cancers.

Chapter 2
Materials and methods

2.1 MATERIALS

2.1.1 Chemicals

Name of Chemical	Manufacturer
Acetic acid	Merck (New Jersey, United States)
Acetone	-do-
Agarose	Sisco Research Laboratories (India)
Ascorbic acid	Merck (New Jersey, United States)
Bovine Serum Albumin (BSA)	Sisco Research Laboratories (India)
Chloroform	Merck (New Jersey, United States)
Copper sulfate penta hydrate	-do-
Diethyl pyro carbonate (DEPC)	-do-
Dithiobis (2-nitro benzoic acid) (DTNB)	Sisco Research Laboratories (India)
Dithiothreitol (DTT)	-do-
Ethidium bromide	-do-
Ethylene diamine tetra acetic acid	-do-
Ferrous ammonium sulfate	-do-
Folin's Ciocalteau reagent	Sisco Research Laboratories (India)
Formaldehyde	-do-
Gel loading dye	Promega (Wisconsin, United States)
Glutathione oxidized (GSSG)	Sisco Research Laboratories (India)
Glutathione reduced (GSH)	-do-
Hydrochloric acid	Merck (New Jersey, United States)
Hydrogen peroxide	-do-
Isoamyl alcohol	-do-
Isopropanol	-do-
Methanol	Spectrochem (Mumbai, India)
Nicotinamide adenine dinucleotide Phosphate oxidized (NADP)	Sisco Research Laboratories (India)
Nicotinamide adenine dinucleotide Phosphate reduced (NADPH)	-do-
Nitrobluetetrazolium (NBT)	-do-

PCR Master mix	Promega (Wisconsin, United States)
Potassium chloride (KCl)	Merck (New Jersey, United States)
Potassium dihydrogen phosphate	-do-
Silica gel 60 F254 TLC plate	-do-
Sodium acetate	-do-
Sodium Azide	-do-
Sodium bicarbonate	-do-
Sodium dihydrogen phosphate dehydrate	-do-
Sodium dodecyl sulfate	-do-
Sodium hydroxide	-do-
Sodium potassium tartrate	-do-
Sulfuric acid	-do-
Thiobarbituric acid (TBA)	Hi-Media (Mumbai, India)
Tris Buffer	Merck (New Jersey, United States)
Tris-HCl	-do-
Trizol reagent	Invitrogen (California, United States)
β-mercaptoethanol	Merck (New Jersey, United States)

2.1.2 Diagnostic kits and reagents

Name of the Diagnostic Kit	**Manufacturer**
Alanine transaminase kit	Span Diagnostics, Surat, India
Alkaline phosphatase kit	Span Diagnostics, Surat, India
Aspartate transaminase kit	Span diagnostics, Surat, India
Hemoglobin kit	Agappe Diagnostics Ltd
Gamma glutamyl transferase kit	Agappe Diagnostics Ltd
Glucose kit	Agappe Diagnostics Ltd
High-density lipoprotein kit	Euro Diagnostic Systems, India
Rat Interleukin- 6 (IL-6)	PeproTech, Germany
Rat Insulin	Mercodia, Uppsala, Sweden
Total bilirubin kit	Span Diagnostics, Surat, India
Total cholesterol	Span Diagnostics, Surat, India
Total protein	Span Diagnostics, India

Triglycerides kit	Euro Diagnostic Systems, India

2.1.3 Instruments

Name of the Instrument	Make
Deep freezer (-70& -20ºC)	Remi Laboratory Instruments, India
Double distillation Unit (Quartz)	Borosil, India
Electronic balance	Schimadzu, Genzo Shimadzu
Electrophoresis unit	Genei, Bangalore, India
ELISA Reader	ThermoScientific, Waltham, USA
FTIR spectrophotometer	PerkinElmer, Massachusetts, USA
Gel documentation system	Remi Laboratory Instruments, India
High-speed cooling centrifuge	Remi Laboratory Instruments, India
Horizontal Laminar flow hood	CleanAir, Chennai, India
Hot air oven	Rotex Instruments Pvt Ltd, India
Incubator	Beston Instruments, India
Inverted microscope	Magnus INVI, Bangalore , India
LC-MS/MS	Agilent biotech, California, USA
Microcentrifuge	Tarsons Products Private Limited
Multi dispenser	Eppendorf, Hamburg, Germany
PCR (Thermal cycler)	Eppendorf, Hamburg, Germany
pH meter	Eutech, Waltham, USA
Phase contrast microscope	Magnus INVI, Bangalore, India
Quantitative real-time PCR	Applied Biosystems, Waltham, USA
Tissue homogenizer	Yorco Scientific, Chennai, India
Ultra low Deep freezer (-80ºC)	New Brunswick, Eppendorf, Germany
UV/Visible Spectrophotometer	PG Instruments Ltd; Systronics India
Vacuum concentrator	Eppendorf, Hamburg, Germany

2.1.4 Software

Name of the Software	Purpose
Adobe Photoshop CS 5.0,	Image processing
Endnote X5	Reference Manager

Graphpad prim 7.0	Statistics & Graph preparation
Graphpad Instat 3.0	Statistical analysis
IS Capture 3.6.6	Microscope image capture
Applied Biosystems 7300 software	Real-time PCR analysis
MS Word 2010	Text preparation
MS Excel 2010	Calculation
MS Powerpoint 2010	Presentation and artworks
Adobe acrobat DC Pro	Preparation and edit PDF files
Image J 1.48	Biological image processing
UV Win spectrophotometer software	Spectroscopic measurement

2.1.5 Edible oils and thermal oxidation

Coconut oil (KLF), mustard oil (RK enterprise), sunflower oil (Gold winner), and lard was purchased from the local market and kept under refrigerated conditions in order to avoid auto-oxidation. A portion of these oils was used for thermal oxidation according to the methods described by González-Muñoz *et al.* (2003). Briefly, 100 mL each of the edible oils was heated in a conical flask for one hour (with intermittent shaking) and the process was repeated for 6 hours. The thermally oxidized oils were kept at $^{-}20\ ^{o}C$ to avoid further oxidative changes.

2.1.6 Animals

Male Wistar rats weighing 140-160g were purchased from the Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur. They were housed in the animal house facility of Amala Cancer Research Centre, Thrissur in polypropylene cages with proper bedding. They were fed on a non-purified rat chow from Sai Durga Feeds (Bangalore, India) and filtered water for two weeks to get acclimatized. All the experimental procedures had prior permission from the institutional animal ethical committee, Amala Cancer Research Centre [Approval No. ACRC/IAEC/15/06-(2)] and all procedures were humanly adhering to the strict regulations of Committee for the Purpose of Control and Supervision of Experiment on Animals, Ministry of Environment, Forest, and Climate change, Govt. of India.

2.2 METHODOLOGY

2.2.1 EXPERIMENTAL DESIGN AND MODIFIED DIETS

Experimental animals were divided into different groups, each containing eight rats; the grouping and respective diet composition is as shown in Table 2.1. Animals were maintained in their respective diets for a period of 30 weeks. All animals had free access to their respective food and water throughout the experimental period.

Table 2.1 Composition of the experimental diets and reference diet (normal).

Components (g/100 g)	**Experimental diet**			
	Reference diet	**HFr**	**Fresh oil (CO/MO/SO/LD)**	**Thermally oxidized oil (TCO/ TMO/ TSO/ TLD)**
Protein	20.0	20.0	20.0	20.0
Corn starch	60.0	0.0	0.0	0.0
Fructose	0.0	60.0	60.0	60.0
Ground nut oil	10.0	10.0	–	–
Fresh unheated oil	–	–	10.0	–
Thermally oxidized oil	–	–	–	10.0
Vitamin	1.5	1.5	1.5	1.5
Mineral	3.5	3.5	3.5	3.5
Fiber	5.0	5.0	5.0	5.0
Total (g)	**100**	**100**	**100**	**100**

At the end of 30 weeks, animals fasted overnight and oral glucose tolerance was measured as per standard protocols as described previously (Narayanankutty *et al.*, 2016). Next day, animals were euthanized under CO_2 anesthesia, blood was collected in EDTA free tubes and serum was separated by centrifugation (7500g, 20 min). The liver and colon tissues were excised, washed in ice-cold saline, a portion was cut

immediately and preserved in buffered formalin (8%) for histological analysis and the remaining tissues were frozen under -80 ^{o}C until use.

2.3. PHYSICOCHEMICAL PARAMETERS

2.3.1 Thin later chromatography (TLC)

Initially, TLC plates (10x10 cm) were cleaned by a pre-run using the solvent system and dried in the oven. The edible oils (10 µL) were diluted using acetone to 1.0 mL and 10 µL from this were spotted on the TLC plate approximately 1 cm above the bottom surface. The solvent system used was hexane: ethyl acetate: formic acid (85:15:1 v/v/v). The plates were then visualized under UV light and later developed under iodine exposure for 10 minutes.

2.3.2 UPLC-Q-TOF-MS analysis

Ultra high pressure liquid chromatographic (UPLC) analysis was carried out using Acquity UPLC H class (Waters) system with a diode-array detector (DAD). For the analysis, a C18 column with dimensions 50 mm × 2.1 mm × 1.7 µm (Waters, USA) was used. Gradient elution was conducted using the mobile phase acetonitrile (A) and methanol (B) (flow rate 0.5 mL/ min) as follows;

0-1 min	-	0 % A
1-3 min	-	15 % A
3-5 min	-	30 % A
5-7 min	-	50 % A
7-9 min	-	80% A
9-10 min	-	100 % A

Detection was achieved at a wavelength of 210 - 360 nm.

The mass spectral data was obtained using Xevo G2 (Waters, USA) Quadruple – Time-of-Flight (Q-TOF) system. MS specifications were; capillary voltage of 2.5 kV (-ve and +ve ionization mode) and cone voltage of 30 V. The source and desolvation temperatures were 135^{o}C and 35^{o}C and the respective gas flows were 50- 900 Ltr/ h.

2.3.3 GC-MS analysis

GC-MS analysis was carried out using a GC model-7890A (Agilent Technologies, USA). The column used for the study was DB-5 with dimensions 30 m x 0.25 mm x 0.25 µm. The MS detector used was Agilent, 5975C Inert XL MSD. The injection volume was 1.0 µL and the injection temperature was 250^{o}C. The Column Oven initial Temperature was 60^{o} C with an increment at a rate of 5^{o}C/ min. The detector was set at a temperature of 250^{o}C.

2.3.4 Thiobarbituric acid reactive substances (TBARS) (Pegg, 2004)

Principle: Lipid carbonyls formed during thermal oxidation of edible oils react with thiobarbituric acid under acidic conditions to form pink colored complex, which is estimated as the absorbance at 532 nm.

Procedure: Approximately 100 µL of edible oil was mixed with 200 µL each of butanol and sodium dodecyl sulfate (8%), 1.25 mL of thiobarbituric acid (0.8% in butanol) and 1.25 mL of 20% acetic acid (pH 3.5). The mixture was incubated in a boiling water bath for 1 hour and cooled. The absorbance was measured at 532 nm against a reagent blank. A standard was prepared similarly using malondialdehyde (MDA) and plotted on graph, which was used for the calculation of slope.

2.3.5 Conjugated diene (CD) and conjugated triene (CT) (Pegg, 2004)

Principle: CD and CT are the initial lipid peroxidation products formed, that have characteristic UV absorbance maxima at 234 and 269 nm.

Procedure: The oils samples (30 µL) were diluted to 3.0 mL using isooctane and the absorbance of each sample was measured at 234 and 269 nm. Sample with absorbance above 0.800 was appropriately diluted and the dilution factor was applied while calculation. The level of CD and CT are calculated using the molar extinction coefficient of linoleic acid hydroperoxide (2.525×10^4 M^{-1} cm^{-1}) and expressed as nmoles of linoleic acid hydroperoxide equivalents/ Kg of oil.

2.3.6 p- Anisidine value (Tompkins and Perkins, 1999)

Principle: The levels of non-volatile aldehydes and ketones were detected by the condensation reaction of p-Anisidine to form Schiff bases. The products formed have a characteristic absorption at 350 nm.

Procedure: About 500 mg of the fresh and thermally oxidized edible oils were mixed with 24.5 mL of isooctane in a 25-mL Erlenmeyer flask. The initial absorbance (Ab) was measured against isooctane blank at 350 nm. Then, 1 mL of p-Anisidine (0.25% in glacial acetic acid) was mixed with 5 mL of the initial oil-isooctane mixture and incubated for 10 minutes. The absorbance (As) of the sample was again measured against the reagent blank. The p-Anisidine value is calculated using the formula;

$$\text{p-Anisidine value} = 25 \times \frac{\text{As-Ab}}{\text{mass of sample}}$$

2.4 DETERMINATION OF INSULIN RESISTANCE

2.4.1 Fasting blood glucose level

Principle: Glucose oxidase initially oxidize glucose to gluconic acid and H_2O_2, and the peroxide then reacted with phenol and aminoantipyrene (AP) to form red colored quinonimine dye.

$$Glucose + O_2 \rightarrow Gluconic\ acid + H_2O_2$$

$$2\ H_2O_2 + \text{Phenol} + 4\text{AP} \rightarrow \text{Red Quinonimine} + 4H_2O$$

Procedure: Approximately 10 µL of lysis free serum was mixed with 1 mL of reagent and incubated for 5 minutes. The color developed was measured using a spectrophotometer set at 505 nm. The concentration of blood glucose was estimated using the formula;

$$\text{Fasting blood glucose}\left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\text{OD of Sample}}{\text{OD of Standard}} \times \text{Conc. of Standard}$$

2.4.2 Fasting plasma insulin level

Principle: The reaction depends on the selective biding of insulin to its antibody immobilized on the ELISA plate and subsequent determination of the bound insulin using ABTS coloring kit.

Procedure: Pre-coated ELISA plate was procured from Mercodia (Uppsala, Sweden). The hemolysis free plasma samples 100 µL were added to each well and incubated for 10 minutes. The wells were washed and secondary antibody was added (100 µL) and

further incubated for 30 minutes. After incubation, the unbound antibodies were washed off and ABTS coloring reagent was added. After 1 hour of incubation, the color formed was read at 450 nm using a ELISA reader (Thermoscientific, USA). The concentration of insulin was calculated as;

$$\text{Conc. of insulin } \left(\frac{\text{pmols}}{\text{L}}\right) = \frac{\text{OD of sample}}{\text{Slope of the standard curve}}$$

2.4.3 Oral glucose tolerance

Procedure: The animals were fasted overnight and initial fasting glucose was measured using commercially available strips (One Touch select, India). The animals were administered 2 g/Kg glucose orally and blood glucose was measured at 30, 60, 90, and 120 minute intervals. The graph was plotted using blood glucose level against the time and the area under the curve was calculated using MS office excel 2010.

2.4.4 Calculation of HOMA indices (Levy *et al.*, 1998)

The Homeostasis Model Assessment (HOMA) estimates steady state beta cell function (%B), insulin resistance (IR) and insulin sensitivity (%S), as percentages of a normal reference population. The three indices were calculated as follows;

$$\text{HOMA \%B} = \frac{20\times \text{Fasting plasma insulin (pM)}}{\text{Fasting plasma glucose (mM)- 3.5}}$$

$$\text{HOMA IR} = \frac{\text{Fasting plasma insulin (pM)}\times \text{Fasting plasma glucose (mM)}}{22.5}$$

$$\text{HOMA \%S} = \frac{1}{\text{HOMA IR}} \times 100$$

2.5 DETERMINATION OF LIPID PROFILE

2.5.1 Serum total cholesterol

Principle: Cholesterol esters in serum are hydrolysed by cholesterol esterase (CHE).The free cholesterol produced is oxidized by cholesterol oxidase to form Cholest 4en-3-one and hydrogen peroxide (H_2O_2). The hydrogen peroxide oxidatively couples with 4- aminoantipyrine (4-AP) and phenol in the presence of peroxidase (POD) to yield a red chromophore.

$$\text{Cholsterol ester} + H_2O \rightarrow \text{Free cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \rightarrow \text{Cholesterol-3-one} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + 4\text{AP} \rightarrow \text{Quinonimine} + 4H_2O$$

Procedure: From unhemolysed serum sample, 10 µL was added to 1 mL of reagent containing Phenol (26 mmol/L), Cholesterol esterase (1000 U/L), Cholesterol oxidase (300 U/L), Peroxidase (650 U/L) and 4-Aminophenazone (0.4 mmol/L) in 90 mmol/L of PIPES buffer (pH 6.9). This was mixed well and incubated at 37 °C for 5 min and read against reagent blank at 505 nm.

$$\text{Cholesterol } \left(\frac{\text{mg}}{\text{dL}}\right) = \left(\frac{\text{Ab of sample}}{\text{Ab of standard}}\right) \times \text{Conc. of Standard}$$

2.5.2 Serum Triglycerides

Principle: Triglycerides in sample when incubated with lipoprotein lipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol - 3- phosphate (G3P) is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2), which in turn reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red coloured dye:

$$\text{Triglycerides} + H_2O \rightarrow \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol 3 phosphate} + \text{ADP}$$

$$\text{G3P} + O_2 \rightarrow \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{p-Chlorophenol} + 4\text{AP} \rightarrow \text{Quinone} + H_2O$$

Procedure: From unhemolysed serum sample, 10 µL was added to 1 mL of reagent containing p- chlorophenol (2 mmol/L), lipoprotein lipase (150000 U/L), glycerol kinase (500 U/L), glycerol 3-oxidase (3500 U/L), ATP (0.1 mmol/L) and 4-Aminophenazone (0.1 mmol/L). This was mixed well and incubated at 37°C for 5 min and read against reagent blank at 505 nm.

$$\text{Triglycerides } \left(\frac{\text{mg}}{\text{dL}}\right) = \left(\frac{\text{Ab of sample}}{\text{Ab of standard}}\right) \times \text{Conc. of Standard}$$

2.5.3 Serum High Density Lipoprotein

Principle: HDL-c is released using a detergent that solubilizes only HDL. HDL-c interacts with cholesterol esterase, cholesterol oxidase and chromogens to give colour. Non HDL lipoproteins like LDL, VLDL and chylomicrons are inhibited from reacting due to absorption of detergents on their surface.

Procedure: From unhemolysed serum sample, 10 µL was added to 450 µL of reagent 1 containing DSBmT (1mM) and Cholesterol oxidase (1000 U/L) in GOOD buffer (pH 7). This was incubated for 5 minutes at 37°C after which 150 µL of reagent 2 containing Cholesterol esterase (1500 U/L), Detergent (2%), Ascorbic oxidase (3000 U/L), Peroxidase (1300 U/L) and 4-Aminoantipyrine (1mM) in GOOD buffer (pH 7). This was mixed well and incubated at 37°C for 5 min and read against reagent blank at 650 nm.

$$\text{HDL cholesterol } \left(\frac{\text{mg}}{\text{dL}}\right) = \left(\frac{\text{Ab of sample}}{\text{Ab of standard}}\right) \times \text{ Conc. of Standard}$$

2.5.4 Low density lipoprotein and atherogenic index

Since all the values obtained are expressed in mg/dL, the LDL value was calculated from these results by the formula by Friedewald *et al.* (1972):

$$\text{LDL cholesterol=Total cholesterol-HDLc-} \left(\frac{\text{Triglycerides}}{5}\right)$$

2.5.5 Extraction of liver lipids

Approximately,1 gm of liver tissue was homogenized with 20 mL of chloroform and methanol (2:1) (239). The homogenate was mixed well, kept at room temperature for 20 minutes. It was centrifuged for 15 minutes at 1500 rpm. The precipitate was discarded and the supernatant was then washed with 4 mL of 0.9 % sodium chloride. The reaction mixture was vortexed and again centrifuged for 15 minutes. From the two layers formed, the upper layer was discarded, the lower layer separated and evaporated to dryness. To this, 2 mL of alcohol: acetone (1:1) mixture was added.

After thorough mixing, it was centrifuged at 1800 rpm for 10 minutes, transferred into clean tubes and stored below -20ºC. This was used for estimation of cholesterol and phospholipid content of liver tissue.

2.5.6 Total liver cholesterol

Lipid extract (100 µL) from tissue homogenate was taken and evaporated to dryness. To this 50 µL of alcohol: acetone (1:1) and 4.950 µL of 0.05% ferric chloride-acetic acid reagent and 3 mL sulphuric acid were added (Manalil *et al.*, 2015). The reaction mixture was kept for 20-30 minutes at room temperature and absorbance was read at 570 nm. Value was obtained from standard graph of cholesterol.

2.6 ESTIMATION OF LIVER FUNCTION MARKERS

2.6.1 Aspartate transaminase (AST) activity (Reitman and Frankel, 1957)

Principle: Aspartate aminotransferase (AST), also known as Glutamate Oxaloacetate Transaminase (GOT) catalyzes the transamination between L-aspartate and α-ketoglutarate resulting in the formation of oxaloacetate and L-glutamate. In the assay system, the unstable oxaloacetate will get coupled to 2,4-Dinitrophenylhydrazine (2, 4-DNPH) color reagent forming an equivalent hydrazone. The absorbance of the resultant brown colored complex can be determined at 505 nm under alkaline conditions.

Procedure: Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. To each test tube, 250 µL of buffered aspartate - α- ketoglutarate substrate (pH 7.4) was added. 50 µL of serum and 50 µL of working pyruvate standard (8 mM) were added to the test and standard respectively and mixed well. All the tubes were incubated at 37ºC for 60 min, followed by the addition of 250 µL of 2,4-DNPH color reagent. Then, 50 µL distilled water and 50 µL of each serum sample was added to the blank and the serum control, respectively and incubated at room temperature for 20 min. Then, 2.5 mL of NaOH solution (0.4 N) was added to all tubes, mixed thoroughly and the absorbance was spectrophotometrically determined against distilled water as blank at 505 nm within 15 min. The enzyme activity was calculated using the formula:

$$\text{AST activity } \left(\frac{\text{IU}}{\text{L}}\right) = \frac{\text{Ab Test-Ab Control}}{\text{Ab Standard-Ab Blank}} \times \text{Conc. of Standard}$$

2.6.2 Alanine transaminase (ALT) activity (Reitman and Frankel, 1957)

Principle: Alanine aminotransferase/glutamate pyruvate transaminase (GPT) catalyzes the formation of pyruvate and L-glutamate by the transamination between L- alanine, and α-ketoglutarate. In the assay system, the unstable pyruvate formed will get coupled with 2,4 – Dinitrophenylhydrazine (2, 4-DNPH) color reagent to form a corresponding hydrazone, The absorbance of the resulting brown colored complex can be spectrophotometrically determined at 505 nm under alkaline conditions.

Procedure: Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. To each test tube, 250 µL of buffered alanine - α- ketoglutarate substrate (pH 7.4) was added. 50 µL of serum and 50 µL of working pyruvate standard (8 mM) were dispensed into the test and standard respectively, and thoroughly mixed. All the tubes were incubated at 37ºC for 30 min, followed by the addition of 250 µL of 2,4-DNPH color reagent. Then, 50 µL of distilled water and 50 µL of each serum sample were added to the blank and the serum control, respectively. The mixture was incubated at room temperature for 20 min. Then, 2.5 mL of NaOH solution (0.4 N) was added to all test tubes, mixed properly and the absorbance was spectrophotometrically determined against distilled water in a spectrophotometer at 505 nm within 15 min. The enzyme activity was calculated using the formula:-

$$\text{ALT activity } \left(\frac{\text{IU}}{\text{L}}\right) = \frac{\text{Ab Test-Ab Control}}{\text{Ab Standard-Ab Blank}} \times \text{Conc. of Standard}$$

2.6.3 Alkaline phosphatase (ALP) activity (Kind and King, 1954)

Principle: Alkaline phosphatase in serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. In alkaline medium, the phenol thus formed reacts with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide. The absorbance of the resulting orange-red colored complex can be measured at 510 nm. The color intensity is proportional to the enzyme activity.

Procedure: The working solution for the substrate was prepared by reconstituting buffered substrate provided in the kit using 2.2 mL of water. Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. 0.5 mL of working buffered substrate and 1.5 mL of distilled water was dispensed to all the test tubes, mixed well and incubated at 37ºC for 3 min. 50 µL of serum into and 50 µL of phenol standard were dispensed into the test and standard respectively, mixed well and incubated for 15 min at 37ºC. Thereafter, 1 mL of chromogen reagent was added to all tubes. Serum (0.05 mL) was added to the corresponding control tubes and mixed well. The absorbance was read against distilled water in a spectrophotometer at 510 nm within 15 min. The enzyme activity was calculated using the formula:

$$\text{ALP activity } \left(\frac{\text{IU}}{\text{L}}\right) = \frac{\text{Ab Test-Ab Control}}{\text{Ab standard-Ab Blank}} \times 10 \times 7.1$$

2.7 MARKERS OF PROLIFERATION AND TOXICITY

2.7.1 Gamma glutamyl transferase (GGT) activity

Principle: Gamma glutamyl-3- carboxy p-nitroanilide and glycylglycine combine to form 5-amino-2-nitrobenzoic acid, which has a characteristic absorption at 405 nm.

GLUPA-C+Glycylglycine →L-γ glutamylglycine+5 amino2-nitrobenzoic acid

Procedure: About 25 µL of sample was mixed with 500 µL of reagent 1 in the kit and immediately read the change in absorbance at 405 nm for 175 seconds. Change in absorbance per minute (ΔA/min) was calculated and the activity is estimated as;

$$\text{GGT activity } \left(\frac{\text{IU}}{\text{L}}\right) = \Delta\text{A per minute} \times 2211$$

2.7.2 Lactate dehydrogenase (LDH) activity (McQueen, 1975)

Principle: Lactate dehydrogenase catalyzes the reduction of pyruvate with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance, which is proportional to the LDH activity in serum.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-Lactate} + \text{NAD}^+$$

Procedure: Working reagent was prepared by mixing reagent 1 [Tris buffer (pH 7.4, 80 mM), pyruvate (1.6 mM) and sodium chloride (200 mM)] with reagent 2 [NADH (240 µM)] in 4:1 ratio. 10 µL of serum was added to 1 mL of the working reagent, mixed well and incubated for 1 min at 37^0C. The change in absorbance was measured per minute for 3 min at 340 nm. The enzyme activity was calculated using the formula;

$$\text{LDH activity} = \left(\frac{\Delta \text{OD}}{\text{min}}\right) \times 16030$$

2.7.3 Ornithine decarboxylase (ODC) activity (Ngo *et al.*, 1987)

Principle: The reaction is based on the principle that the product of enzymatic activity of ODC, putrescine reacts with 2, 4, 6 trinitrobenzene sulfonic acid (TNBS) to form a colored product, which has an absorbance at 420 nm.

Procedure:

Putrescine standards were prepared in 0.1 M phosphate buffer (pH 7.0). One mL of 4 N NaOH was added and mixed vigorously to 0.5-mL aliquots of standards. Two milliliters of were mixed vigorously for 20 s with a Vortex mixer. After the emulsions were centrifuged for 5 min at 2000 rpm, 1 mL of the upper (organic) phase was transferred to test tubes 8.0) and were mixed briefly. One milliliter of 8.0) and were mixed briefly. One milliliter of 10 mM TNBS dissolved in 1-pentanol was added to each tube, and the samples were mixed for 20 s with a Vortex mixer; 2 mL of DMSO were then added to each tube and the samples were mixed for an additional 20 s. The phases were separated by centrifuging he sample for 5 min at 2000 rpm, and the absorbance of the organic phase of each sample was measured against a reagent blank at 420 nm.

2.8 ESTIMATION OF POLYOL PATHWAY ENZYMES (Jang *et al.*, 2010)

2.8.1 Aldose reductase (AR) activity

Principle: The reaction is based on the oxidation of the DL-glutaraldehyde by AR enzyme using NADPH. The extinction coefficient of NADPH is used for the calculation.

Procedure: AR activity was assayed in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.4 mM of ammonium sulfate (0.4 mM), EDTA (0. mM), DL-glutaraldehyde (20 µM), and NADPH (25 µM). The reaction was initiated by the addition of 100 µ*L* tissue sample, and the rate was measured as the change in absorbance at 340 nm for 2 minutes.

2.8.2 Sorbitol dehydrogenase (SDH) activity

Principle: The reaction is based on the oxidation of the substrate by SDH enzyme using NADH. The extinction coefficient of NADH is used for the calculation.

Procedure: SDH activity was measured in100mM triethanolamine buffer (pH 7.4). The reaction was based on the oxidation of NADH (12mM) by SDH using fructose (1.1M) as a substrate in the tissue sample at 340 nm for 2 minutes.

2.9 ANALYSIS OF TISSUE REDOX PARAMETERS

2.9.1 Preparation of tissue homogenate

The tissues were collected and washed thoroughly in ice-cold saline, followed by heparinized PBS to remove complete traces of blood. A 25% tissue homogenate was prepared in 0.1M Tris buffer (pH 7.4) using an electrical Polytron homogenizer. The homogenate was centrifuged at 10,000g for 30 minutes at 4^{o}C to yield clarified supernatant, which was used for the subsequent biochemical assays in tissues, including lipid peroxidation (Ohkawa *et al.*, 1979) and protein carbonyls (Levine *et al.*, 1990). The enzymatic and non-enzymatic antioxidants such as GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), catalase (Beers and Sizer, 1952) and GR (Carlberg and Mannervik, 1975) were also assayed.

2.9.2 Reduced glutathione (GSH) content (Moron *et al.*, 1979)

Principle: GSH is measured by its reaction with DTNB, which yields a yellow colored complex with maximum absorption at 412 nm.

Procedure: About 200 µL of clarified tissue homogenate was mixed with 50 µL of 25% TCA (final concentration of 5% or less) and kept at 25^{o}C for 5 minutes. Further, the mixture was centrifuged at 3000 g for 10 min and from the supernatant, 100 µL was mixed with 900 µL of 0.2 M sodium phosphate buffer (pH 8.0) and 2.0

mL DTNB (0.6 mM). The mixture was then incubated at room temperature for 20 minutes and absorbance was measured at 412 nm. A standard curve for reduced glutathione was plotted and using the slope of the curve, concentration of GSH was calculated and expressed as nmol/mg protein.

2.9.3 Catalase activity (Beers and Sizer, 1952).

Principle: The catalase activity was assayed by measuring the decomposition of H2O2 which has an absorption maximum at 240 nm and this absorption decreases with the decomposition of H2O2. The difference in extinction per unit time is used to measure the catalase activity.

Procedure: Clarified tissue homogenate (100 µL) was mixed with 2.8 mL of 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by adding 100 µL of 0.3% H_2O_2 solution. The activity was recorded as the decrease in absorbance at 240 nm 30 seconds interval for 3 min. The catalase activity was calculated using the molar extinction coefficient of hydrogen peroxide (43.6 $mM^{-1}cm^{-1}$) and the specific activity at 25 ºC was expressed as mmoles of H_2O_2 consumed/min/mg of protein.

$$\text{Catalase activity } \left(\frac{\text{U}}{\text{mg protein}}\right) = \frac{\text{Ab at}\frac{240}{\text{min}} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}$$

2.9.4 Superoxide dismutase (SOD) activity (McCord and Fridovich, 1969)

Principle: The assay principle lies in the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radical, generated during the reaction of photo reduced riboflavin with oxygen.

Procedure: Tissue supernatant (100 µL) was mixed with 2550 µL of 67 mM phosphate buffer (pH 7.8). Then 100 µL NBT (0.15mM) and 200 µL KCN (0.0015%) was added. To that 50 µL riboflavin (0.12 mM) was added and initial OD was measured at 560 nm. Then the test tubes were uniformly illuminated for 15 minutes under an incandescent lamp and again the OD was measured at 560 nm.

Percentage inhibition was calculated as the percentage change of absorbance of the test with that of control. The concentration of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and

was expressed as U/mg protein.

$$Y=\frac{100\times50\times\text{mg protein}}{\%\text{inhibition}}\text{ x } 1/100$$

The results are expressed as SOD (U/mg protein) = 1/ Y

2.9.5 Glutathione peroxidase (GPx) activity (Hafeman *et al.*, 1974)

Principle: Glutathione peroxidase depletes H_2O_2 in presence of glutathione (GSH). The remaining GSH is measured using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), which gives a colored complex.

$$H_2O_2 + 2\,GSH \rightarrow GSSG + 2\,H_2O$$

Procedure: Reaction mixture contained 100 µL (5 mM) GSH, 100 µL of 0.1 M phosphate buffer (pH 7.0), 100µL sodium azide (25mM), 100 µL tissue extract and 100 µL of H_2O_2 (1.2 mM) in a total volume of 2.5mL.The reaction mixture was incubated at 37^0C for 6 minute. Reaction was stopped by addition of 2.0 mL of 1.67% meta- phosphoric acid and centrifuged for 15 minutes at 2500 rpm. To about 2mL of supernatant, added 2mL of 0.1 M phosphate buffer containing DTNB (1mM). Incubation was carried out at 37ºC for 10 minutes and OD was measured at 412 nm. A sample without the tissue was processed in the same way and was kept as the blank. The activity is expressed as U/mg protein.

$$\text{GPx activity } \left(\frac{\text{U}}{\text{mg protein}}\right)=\frac{\text{OD of Blank-OD of Sample}\times1000}{\text{mg protein}\times10}$$

One unit of enzyme activity was defined as a decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

2.9.6 Glutathione reductase (GR) activity (Racker, 1955)

Principle: The activity of GR was estimated by the amount of NADPH consumed in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The reaction catalyzed by GR follows:

$$GSSG+NADPH+H^{+} \rightarrow 2GSH+NADP^{+}$$

Procedure: The reaction mixture contains 100 µL EDTA (10 mM), 100 µL GSSG (10 mM), 50 µL of serum and sodium phosphate buffer (1 M, pH 7.0) in a total volume of 900 µL. It was then incubated for 5 min at 37 °C, followed by the addition of 100 µL NADPH (2 mM) and decrease in optical density was measured at 340 nm for 5 min with an interval of one minute. The activity of GR was calculated using the molar extinction coefficient of 6.22 mm-1cm-1 and expressed as nanomoles of NADPH consumed /min/mg protein.

2.9.7 Thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.*, 1979)

Principle: Lipid peroxidation products, especially malondialdehyde (MDA), produced during peroxidation of polyunsaturated fatty acids (PUFAs) can react with thiobarbituric acid (TBA) reagent under acidic conditions to form a pink colored complex, which has an absorption maximum at 532 nm.

Procedure: About 200 µL of tissue homogenate was mixed with 600 µL of distilled water, 200 µL sodium lauryl sulfate (8%), 1.5 mL each of 20% acetic acid (pH 3.5) and thiobarbituric acid (0.8%). The mixture was further incubated at 100°C for 1hr in a boiling water bath. The samples were cooled and mixed with 4 mL of pyridine: butanol (15:1) and centrifuged at 3000 g for 10 min. The clarified supernatant was collected and read at 532 nm against a reagent blank and the concentration of lipid peroxidation products were calculated from the standard curve prepared using MDA and expressed as nmol of MDA/mg protein.

2.9.8 Estimation of total protein (Kingsley, 1939)

Principle: In alkaline solution, proteins form a blue-violet colored complex together with copper ions. The intensity of the color formed is directly proportional to the quantity of total proteins present in the sample.

Procedure: To 1 mL of Biuret reagent, 10 µL of sample (serum or tissue sample) and 10 µL of protein standard (6 g/dL) were added into the test and standard reaction systems respectively, properly mixed and incubated at 37^{0}C for 5 min. The absorbance was measured at 555 nm within 60 min. The serum total protein was

calculated using the formula:

$$\text{Total protein } \left(\frac{\text{g}}{\text{dL}}\right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard}$$

2.10 HISTOPATHOLOGICAL ANALYSIS

The tissues (liver and colon) were washed in PBS and then fixed in 8% buffered formalin. The fixed tissue was dehydrated in a series of alcohol with ascending strength (10-100%). Further, clearing was done in xylene and impregnated with molten wax. Blocks were prepared and serial sections were taken using an automated microtome at the thickness of 5µM. The serial sections were spread on fresh glass slides and rehydrated using decreasing grades on alcohol. Histological staining was carried out using hematoxylin and eosin in Coplin Jar. The slides were again dehydrated and permanent slides were prepared using DPX mountant. The slides were dried and visualized under Magnus INVI microscope (200X magnification) and photographed using IS Capture version 3.6.6.

Scoring of hepatosteatosis was carried out according to the standard method (Brunt *et al.*, 1999). The grade values 1, 2 and 3 represented low, moderate and severe hepatic damages respectively.

2.11 GENE AND PROTEIN EXPRESSION ANALYSIS

2.11.1 RNA isolation from tissue

Diethyl pyro carbonate (DEPC) treated glass and plastic wares were used for RNA isolation and cDNA synthesis. Approximately, 50 mg of the colon tissue from euthanized animals were collected and washed with PBS. About 1.0 mL of TRIzol® reagent (Thermo Fisher, USA) was added and homogenized. The homogenate was incubated at room temperature for 5 minutes in order to separate nucleo-protein complexes. To this mixture, about 200 µL of chloroform was added and mixed well. The samples were further incubated for 3 minutes and then centrifuged at 12,000 × g at 4°C for 15 minutes. The upper aqueous phase containing the RNA was removed to a new tube and 500 µL of chilled isopropanol was added and allowed to precipitate at -20^{o}C. The precipitated RNA was collected by centrifuging at 12,000 × g for 10 minutes (4°C). The supernatant was discarded and RNA pellet was resuspended in in

1 mL of 75% ethanol. The resuspended RNA was again centrifuged for 5 minutes at 7500 × g at 4°C and dissolved in 20 µL of RNase free water and stored at -20° C. The integrity of RNA was analyzed by agarose gel electrophoresis and quantified by measuring the absorbance ratios of 260/280.

2.11.2 cDNA synthesis from RNA

Accurately a 4 µg of RNA sample was used for the synthesis of cDNA using Verso First strand cDNA synthesis kit (Thermo Fisher Scientific, United States). The reaction mixture was as follows;

Component	**Quantity (µL)**
5X cDNA synthesis buffer	4.0
dNTP Mix	2.0
Anchored oligo dT	1.0
RT Enhancer	1.0
Verso Enzyme Mix	1.0
Template (RNA)	5.0
Water, nuclease-free	6.0
Total	20.0

The cDNA synthesis was carried out using a thermal cycler set at 42°C for 45 minutes. Finally inactivation of enzyme was done by a 65°C cycling for 1 minute.

2.11.3 Real time quantitative PCR

Selective amplification of the target genes was done in an Applied Biosystems 7300 qPCR system. The composition of PCR mix was as follows (20 µL);

Components	**Quantity (µL)**
PowerUp™ SYBR™ Green Master Mix (2X)	10
Forward and reverse primers	2
DNA template	4
Nuclease-Free Water	4
Total	20

The master mix was then subjected to amplification in the thermal cycler with the following temperature settings.

Steps	Temperature	Time
Step 1	95°C	2.0 min
Step 2	95°C	30 sec
Step 3	60°C	45 sec
Go to step 2 and repeat 39 cycles		
Step 5	60°C	1.0 min

The sequences of the primers used were as follows.

Gene	Forward primer	Reverse primer
Akt	TCACCTCTGAGACCGACACC	ACTGGCTGAGTAGGAGAACTGG
IL-6	CCACTGCCTTCCCTACTTCA	TGGTCCTTAGCCACTCCTTC
P53	GACTTCTTGTAGATGGCCATGG	ATGGAGGAGTCACAGTCGGATA
PPAR gamma	GCGGAGATCTCCAGTGATATC	TCAGCGACTGGGACTTTTCT
Wnt	ATAGCCTCCTCCACGAACCT	GGAATTGCCACTTGCACTCT
Beta catenin	GCAATCAGGAAAGCAAGCTC	TCAGCACTCTGCTTGTGGTC
Beta actin	AGATTACTGCCCTGGCTCCT	ACATCTGCTGGAAGGTGGAC

Applied Biosystems real time PCR software package was used to calculate the Ct values and ΔΔCt values calculated to determine the fold change in expression (Livak and Schmittgen, 2001). A positive ΔΔCT value indicates down-regulation and a negative ΔΔCT value indicates upregulation (Yuan *et al.*, 2008).

$$\Delta\Delta C_T = \left(C_{T\,(Target)} - C_{T\,(Actin)}\right)^{Normal} - \left(C_{T\,(Target)} - C_{T\,(Actin)}\right)^{Treated}$$

2.11.4 Detection of PCR products (Data Not included in result)

The PCR product (10 µL) was resolved in 1.2 % agarose gel by electrophoresis.

Reagents: A 10X TAE buffer was prepared by mixing 48.4 g of Tris base, 11.42 mL of glacial acetic acid and 20 mL of 0.5 M EDTA and making up to 1 L using nuclease free double distilled water.

Procedure: Electrophoresis apparatus was cleaned and set up without leakage an appropriate gel comb was placed. A 1.2% agarose gel was prepared by mixing 0.9 g agarose in 60 mL 1X TAE buffer and 0.5µg/mL ethidium bromide (1 µL) was added. The gel was dissolved by gentle heating and then carefully poured into the gel tray without trapping air bubbles and allowed to solidify. The comb was carefully removed without disturbing the solid gel and tank was filled with 1X TAE buffer. The samples were loaded (10 µL) with the gel loading dye (2 µL) in individual wells and electrophoresis was performed at 60V. The gel was taken out, blotted and observed under UV/ Vis gel documentation system.

2.11.5 Serum IL-6 level

Principle: The reaction depends on the selective biding of IL-6 to its antibody immobilized on the ELISA plate and subsequent determination of the bound IL-6 using ABTS kit.

Procedure: ELISA plates were coated with IL-6 by incubating a mixture of 10 µL of primary antibody and 200 µL of coating buffer in each well of a 96-well plate (PeproTech, Hamburg, Germany). The hemolysis free plasma samples 100 µL were added to each well and incubated for 10 minutes and then the wells were washed with washing buffer. Further, the secondary antibody was added (100 µL) and incubated for another 30 minutes. After incubation, the unbound antibodies were washed off and ABTS coloring reagent was added. After 1 hour of incubation, the color formed was read at 450 nm using ELISA reader (Thermoscientific, USA).

2.12 STATISTICAL ANALYSIS

The results of the animal study were represented as Mean ± standard deviation of six animals per group. All in vitro studies were carried out in three independent experiments, each in duplicate and the data were represented as Mean ± standard deviation. Statistical analysis of the results was done by One-way ANOVA followed by Dunnett's post hoc test was performed using performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California USA).

Chapter 3

Physico-chemical changes in edible oil during thermal oxidation

3.1 Introduction

Edible oils are the important dietary components and a predominant source of different fatty acids. Chemically, oils are triacylglycerol, where one glycerol moiety is bound to three fatty acid molecules, and upon enzymatic action they release free fatty acids. Composition of each edible oil is different based on the nature of fatty acids attached to the glycerol, they may be either saturated, monounsaturated or polyunsaturated and having a length of short, medium or long. Depending on these fatty acid variations in the triacylglycerol composition, the biological effects of each of the edible oil vary. In addition, edible oils also contain several other bioactive components such as polyphenols and sterols, which are strong antioxidant compounds.

Edible oils are mainly used for cooking purposes, which involves rapid and repeated heating at higher temperatures. During the process of frying, the edible oils are exposed to high temperature usually above 160^{o}C. Previously, Choe and Min (2007) and Warner (1999) have well described the chemistry of edible oils during thermal treatments, these include hydrolysis, oxidation and polymerization reactions. Changes associated with thermal treatment also determine the biological effects of these edible oils after consumption.

Thermal oxidation, which is aided by the atmospheric oxygen, is a process that is similar to auto-oxidation, however resulting in a rapid increase in the levels of epoxides and peroxides of saturated or monounsaturated nature. It results in the increase in peroxide values, color, viscosity and polymers of triglycerides. Other than temperature, presence of metals, reactive radicals and light also increases the oxidative modification in oils. Compared to the saturated fatty acid rich oils, unsaturated fatty acid containing oils are more prone to oxidative modifications.

In addition moisture content present in the oil as well as atmosphere can interact with the triglycerides resulting in the hydrolysis. This results in a series of complex chemical reactions resulting in the formation of diglycerides and free fatty acids, thereby increasing the acid value and polar content of the oil.

Polymerization reactions are crosslinking of two or more triglycerides, which often takes place during thermal oxidation of saturated fatty acid rich oils. This may results

in formation of high molecular weight compounds. Further, cyclization (formation of cyclic structures such as CFAMs) and dimerization (formation of intra strand crosslinking between two different fatty acids) reactions are also taking place during the deep frying process of oils. Bastida and Sánchez-Muniz (2001) have observed elevated levels of triacylglycerol polymers and dimers in sunflower oil. Recently, Romero *et al.* (2006) have reported the presence of high levels of cyclic fatty acid monomers (CFAM) in thermally oxidized edibles oil, especially that contain unsaturation.

The primary lipid peroxidation products formed during the process of deep frying of poly unsaturated fatty acids include peroxides, conjugated diene structures. Due to their reduced stability and high reactivity, they undergo auto-decomposition yielding highly toxic secondary oxidation products such as aldehydes and ketone bodies. Ammouche *et al.* (2002) have reported that thermal oxidation of the sunflower oil increases the peroxide value and free fatty acids content.

Many of the edible oils are reported to contain polyphenols of varying structure and composition (Konsoula and Liakopoulou-Kyriakides, 2010). Among the various phenolic contents, lignans are the most resistant to thermal oxidation, whereas simple phenolic acids and complex phenolics undergo oxidative changes gradually (Carrasco-Pancorbo *et al.*, 2007). However, during thermal oxidation, there occurs a considerable reduction in their phenolic content (Brenes *et al.*, 2002; Gómez-Alonso *et al.*, 2003). The polyphenolic loss is also dependent on the fatty acid composition of the oils, where an oil rich in PUFA shows higher loss in phenolics during thermal oxidation (Brenes *et al.*, 2002). A portion of the phenolic compounds also undergo oxidation resulting in the formation of oxidized phenolics (Armaforte *et al.*, 2007).

Oxidative modifications of other bioactive components such as beta carotene also occur during deep frying. Zeb and Murkovic (2013) have indicated that during thermal oxidation of corn oil, β-carotene oxidation occurs and generates 8′-apo-β-carotenal, 5,6-epoxy-8′-apo-β-carotenal, β-carotene-2,2′-dione and all-E-5,6-epoxy-β-carotene. These oxidation products further activates the oxidation of triglycerides to form epidioxy bis-hydroperoxides and hydroxy bis-hydroperoxides.

India is a country with diverse food types depending on the regional specificity, likewise, the edible oils and fats chosen for the culinary purposes also varies. Coconut

oil, which is a medium chain saturated fatty acid rich edible oil, is the common ingredient for cooking purpose in Southern parts of India like Kerala, Tamil Nadu and Karnataka. Mustard oil, which is monounsaturated fatty acid rich oil, is used all over the North- Eastern side of India. Sunflower oil is also being used commonly in Tamil Nadu and other South Eastern states and now being promoted as healthy due to their high polyunsaturated fatty acid contents. Lard is the most commonly used animal fat, especially in food industries, for the preparation of shortening as similar food items.

Since the health impact of thermally oxidized products of these representative fatty acids are the overall aim of our study, in the current chapter, we analyze the representative edible oils used in India for medium chain saturated (coconut oil), monounsaturated (mustard oil), polyunsaturated (sunflower oil) and long chain fatty acid rich animal fat (lard) as well as the thermal oxidation induced changes in these oils and fats. Physico-chemical alterations are studied together with the changes in structural and fatty acid composition.

3.2 Materials and Methods

3.2.1 Oils and fats used in the study

The different edible oils used include, medium chain saturated fatty acid rich coconut oil (CO), monounsaturated fatty acid containing mustard oil (MO) and polyunsaturated fatty acid rich sunflower oil (SO). The animal fat used in the present study is lard, which is of porcine in origin.

3.2.2 Thin later chromatography

The different edible oils were analyzed by TLC and chromatogram was prepared under UV illumination and iodine vapor as described in **Chapter 2.3.1**.

3.2.3 UPLC-Q-TOF-MS analysis

Ultra high pressure liquid chromatographic (UPLC) analysis was carried out using Acquity UPLC H class (Waters) system with a diode-array detector (DAD with C18 column, the details of which are provided in the **Chapter 2.3.2.**

3.2.4 GC-MS analysis

Gas chromatography-Mass spectrometric analysis of edible oils was conducted as per the protocols detailed in the **Chapter 2.3.3.**

3.2.5 Thiobarbituric acid reactive substances (TBARS) (Pegg, 2004)

The reaction based on the formation of lipid peroxides- thiobarbituric acid complex under acidic condition was estimated as the absorbance at 532 nm as described previously in **Chapter 2.3.4.**

3.2.6 Conjugated diene (CD) and conjugated triene (CT) (Pegg, 2004)

CD and CT are the initial lipid peroxidation products formed, which has characteristic UV absorbance maxima at 234 and 269 nm. The detailed protocol has been described in **Chapter 2.3.5.**

3.2.7 p- Anisidine value (Tompkins and Perkins, 1999)

The levels of aldehydes are indicated by p-Anisidine value and were detected by the condensation reaction of p-Anisidine with these aldehydes to form Schiff bases. The procedure is given in **Chapter 2.3.6.**

3.3 Results

3.3.1 Thin later chromatography

Thin layer chromatographic analysis of CO and TCO revealed similarity with respect to bands with Rf values 0.80, 0.72, and 0.50 when stained with iodine (Figure 3.2a). However, a new band with Rf value 0.54 was observed in iodine stained TCO (**Band iii**). In long wave UV analysis compound with Rf value 0.15 was found common to both CO and TCO was observed whereas two additional bands (Rf values 0.54 and 0.72) was noted in TCO. (Figure 3.2b). In MO and TMO, iodine staining observed three bands in common with respective Rf values of 0.78, 0.70, and 0.10. In TMO, iodine staining identified a unique band (**Band iii**) at Rf value 0.62 (Figure 3.2c). Similarly under long wave UV, MO and TMO had a common band at Rf value 0.19; however, TMO showed two additional bands (**Band i and iii**) with Rf values 0.62 and 0.10 (Figure 3.2d).

Sunflower oil and TSO had showed five common bands with Rf values 0.76, 0.70, 0.64, 0.53, and 0.13; whereas SO possessed two additional bands (**Band i and ii**) with Rf values 0.96 and 0.89 (Figure 3.3a). There was no significant bands were formed under long wave UV (Figure 3.3b). In LD and TLD, there were three common bands with respective Rf values of 0.85, 0.76 and 0.08; in addition, TLD had a unique band at Rf value 0.67 under iodine staining (Figure 3.3c). In the long wave UV, LD and TLD showed three common fluorescent bands with Rf values 0.76, 0.16, and 0.08, respectively. In addition, TLD showed two additional bands (**Band iii and v**) with Rf value 0.67 and 0.85 under iodine staining (Figure 3.3d).

3.3.2 FTIR spectroscopic analysis of fresh and thermally oxidized oils

FTIR spectra of CO and TCO showed striking differences, especially in the region around 458, 583, 1111, 1743, and 3500 cm^{-1}. Respective transmittance of CO at these regions was 0.940, 0.959, 0.737, 0.513 and 0.995. In TCO, the transmittance at 458, 583, 1111, 1743, and 3500 cm^{-1} was 0.912, 0.933, 0.762, 0.596, and 0.977 (Figure 3.4a).

In thermally oxidized mustard oil (TMO), the FTIR spectral differences were mainly around 3500, 3008, 1744, 1377, 1236, 967, 721, and 455 cm^{-1}. The transmittance of MO at these regions was 0.991, 0.944, 0.626, 0.912, 0.864, 0.727, 0.821, and 0.948, respectively. The transmittance at the same regions in TMO was 0.985, 0.966, 0.664, 0.899, 0.857, 0.737, 0.859, and 0.933, respectively (Figure 3.4b).

In SO and TSO, the striking differences were observed in the regions 3500, 3009, 1744, 1237, 987, and 455 cm^{-1}. The transmission intensity at these respective regions of SO was 0.991, 0.946, 0.621, 0.843, 0.834, and 0.924. The transmittance at the same regions in TSO was 0.987, 0.960, 0.624, 0.835, 0.856, and 0.923 (Figure 3.5a).

FTIR spectra of LD and TLD showed distinct changes in the regions at 3500, 3008, 1377, 1234, 1161, 1161, 1099, 969, 585, and 461 cm^{-1}. In LD, the respective transmittance at these regions was 0.994, 0.949, 0.905, 0.862, 0.731, 0.823, 0.924, 0.945, and 0.943. In TLD, the transmittance at the same regions was changed to 0.970, 0.967, 0.885, 0.839, 0.717, 0.824, 0.897, 0.928, and 0.914, respectively (Figure 3.5b).

3.3.3 LC-Q-TOF-MS analysis

LC-MS analysis of CO identified distinct peaks with m/z ratio ranging from 240.9, 285.03, 386.83, and 431.09. In TCO, the peaks were mainly at 240.90, 285.03, 386.83, 431.09, 450.50 and 547.14 (Figure 3.6).

Mustard oil had m/z ratio 190.92, 241.09, 285.02, 290.89, 335.88, 362.87, 386.83 and 421.04. In TMO, the m/z ratios were 181.9, 206.99, 221.01, 276.11, 280.05, 305.00, 352.24, 375.05, 382.16, 409.87, and 485.10. The major peaks at 241.09 and 386.83 in MO were completely disappeared in TMO, producing new peaks at 181.9, 206.99 and 221.01 (Figure 3.7).

In SO, the main m/z ratio observed was 169.01 followed by 240.90, 191.05 and 387.11. However, in TSO the numbers of peaks were much larger, which includes m/z ratios 160.83, 191.05, 172.05, 215.02, 221.04, 239.07, 331.06, 342.11, 377.08, 387.11, and 401.12 (Figure 3.8).

In LD, the main m/z ratios were 180.2, 240.9, 285.03, 386.83, and 545.19. Whereas in TLD, the m/z ratios observed were mainly 190.92, 240.9, 285.03, 386.83, and 547.14. The m/z ratios 285.03 and 386.83 were considerably increased during thermal oxidation of lard (Figure 3.9).

3.3.4 Fatty acid composition and changes in edible oils during thermal oxidation

The coconut oil is rich in medium chain saturated fatty acids, especially lauric acid (52%). As shown in the Figure 3.10 a, other predominant fatty acids in CO were myristic acid and palmitic acid with 19.0 and 11.0 %. However, during thermal oxidation, the level of MCFAs such as lauric acid (48.5%), capric acid (4.5%) and caprylic acid (4.5) were reduced, with an increase in LCFA such as myristic (20.1%) and palmitic acid (13.8%).

In Mustard oil, the predominant fatty acid was erucic acid (47.0%), oleic acid (22.0%) and linoleic acid (14.2). The percentage composition was changed to 42.6% erucic acid, 23.5% oleic acid and 12.9% linoleic acid after thermal oxidation (Figure 3.10 b).

Sunflower oil had higher level of linoleic acid (63.5%) followed by oleic acid (27.5%) and the total saturated fatty acid content was 8.0%. Upon thermal oxidation, the linoleic acid concentration was reduced to 53.7% and oleic acid content was elevated

to 29.8%. Total saturated fatty acid content was also increased to 13.2% of the total (Figure 3.10 c).

In lard, predominant fatty acids were linoleic acid (46.5%), palmitic acid (25.5%), stearic acid (13.0), and oleic acid (8.5%). Upon thermal oxidation, the composition was changed to linoleic acid (40.1%), palmitic acid (27.8%), stearic acid (13.6), and oleic acid (9.2%) (Figure 3.10 d).

3.3.5 Biochemical changes during thermal oxidation

3.3.5.1 Thiobarbituric acid reactive substances

Lipid peroxidation levels as indicated by TBARS were found to be 0.241±0.035 nmoles/kg in CO, which was increased to 0.861±0.091 nmoles/kg in TCO. Similarly, TBARS in MO was 0.365±0.094 nmoles/kg, which elevated to 1.210±0.115 nmoles/kg in TMO (Figure 3.11).

Lipid peroxidation level in sunflower oil was 0.299±0.08 nmoles/kg and it elevated during thermal oxidation (TSO) to 1.01±0.09 nmoles/kg. In Lard, the level was 0.229±0.05 nmoles/kg and in TLD it elevated to 1.12 ±0.08 nmoles/kg (Figure 3.11).

3.3.5.2 Conjugated dienes

Conjugated diene level in fresh CO was found to be 0.181±0.005 mmol/kg, which increased considerably to 0.278±0.012 in TCO. In MO, the CD level was 0.109±0.007 mmol/kg, which increased to 0.359±0.020 mmol/kg in TMO (Figure 3.11).

Sunflower oil had a CD level of 0.183±0.01 mmol/kg and which was increased to 0.422±0.07 mmol/kg in TSO. In LD, the conjugated diene content was 0.202±0.03 mmol/kg. It was increased to 0.344±0.06 mmol/kg in TLD (Figure 3.11).

3.3.5.3 Conjugated triene

CT is another lipid peroxidation marker, which was found to be 0.095±0.012 mmol/kg in CO, elevated to 0.185±0.027 during thermal oxidation. In MO, the conjugated triene level was 0.158±0.041 mmol/kg and increased in TMO to a level of 0.537±0.068 mmol/kg (Figure 3.11).

In fresh SO, the CT level was 0.131±0.01 mmol/kg and which increased to 0.234±0.09 mmol/kg in TSO. The CT level of LD was 0.122±0.04 mmol/kg and it increased upon thermal oxidation to 0.155±0.03 mmol/kg (Figure 3.11).

3.3.5.4 p- Anisidine value

In fresh CO, p- Anisidine value was 0.365±0.065 AnV, which increased to 0.685±0.084 AnV in TCO. Similar increase was also noticed in MO during thermal oxidation, where from 1.619±0.314 AnV of fresh MO, it was elevated to 4.320±0.201 AnV in TMO (Figure 3.11).

Sunflower oil had a p- Anisidine of 1.449± 0.182 AnV which was elevated in thermally oxidized sunflower oil to 4.329±0.408 AnV (Figure 3.11). The lard had a p-Anisidine of 1.084± 0.212 AnV which was elevated to 2.881±0.308 AnV in TLD.

3.3.5.5 Cholesterol levels

Though other edible oils did not show the presence of cholesterol, LD had significant amounts of raw cholesterol in it. The cholesterol level of fresh LD was found to be 89.6±5.27 mg/dL and it came down to a level of 67.3±7.11 mg/dL in TLD.

Discussion

The present study observes that the edible oils undergo severe oxidative damages, especially oxidative reactions. The increased level of lipid oxidation structures such as conjugated diene, conjugated triene, thiobarbituric acid reactive substances and p-anisidine values, indicate the oxidative insults in edible oils. The degree of oxidative modifications increases with the unsaturation of oil; hence, rendering the polyunsaturated fat rich oil most susceptible to the thermal oxidation.

Compared to the unsaturated fatty acid rich edible oils, medium chain saturated fat rich CO is found to have lesser level of oxidation products. However, there observed an increase in lipid peroxidation products to a moderate level. FTIR spectra of TCO also supported these results as the reduced transmittance in the 3500 cm^{-1} region, which indicate the aldehyde or hydroperoxide groups, and increased accumulation of aldehyde groups. This is in accordance with the previous studies conducted by Moros *et al.* (2009) and Zahir *et al.* (2017). The aldehyde or peroxide content may be generated as a result of thermal oxidation of unsaturated fatty acids such as oleic acid

and linoleic acid present in coconut oil at 4-6%. Stretches at 1111, 1154, and 1254 cm^{-1} indicate saturated acyl groups (Guillén and Cabo, 1997), in our study they remained unaltered both during thermal oxidation thus indicating the intact saturated fat content. Vilela *et al.* (2015) identified the region at 3008 cm-1 as an indicative of cis- bonds, which is absent in CO or TCO due to their reduced total unsaturation. The spectral data from LC-MS as well as the changes in fatty acid profile indicate a considerable change induced by thermal oxidation in coconut oil. The increased m/z values of thermally oxidized coconut oil possibly indicate the formation of triglyceride polymerization products formed from medium chain saturated fatty acids. The considerable reduction in the MCFA content, as indicated by fatty acid profile also supports this assumption.

The different unsaturated fatty acid rich edible oils have increased oxidative modifications, as indicated by the lipid peroxidation indices of conjugated diene, triene and TBARS. This observation is supported by the reduced transmittance at 3500 cm^{-1} in TMO, which indicates increased hydroperoxide or aldehyde generation. Further, Ali *et al.* (2017) described the region at 3008 cm^{-1} as the possible cis- to trans- conversion, hence increased transmittance in TMO at this region possibly indicates the cis- to trans- conversion occurred during thermal oxidation. Region 1744 cm^{-1} is indicative of carbonyl compounds, which is found to be reduced in MO than TMO. Since the increased in carbonyl content found in MO may be the presence of iso-thiocynates, which got decreased during thermal oxidation. Further, the increased formation of minor peaks observed in LC-MS analysis of TMO also indicates the possible oxidation of larger fatty acids at their unsaturation points to compounds with lesser m/z ratio.

Similar observations have been made in TSO and TLD at the regions 3500, 3008, and 1744 cm^{-1} indicated the increased hydroperoxides and cis- to trans- conversion occurred during thermal oxidation. Further, the regions at 1160 and 1237 cm^{-1} indicate saturated acyl groups (Guillén and Cabo, 1997). All the unsaturated fatty acids rich edible oils upon thermal oxidation (TMO, TSO and TLD) have shown reduced transmittance at these regions, which may due to the conversion of unsaturated acyl group to saturated form. This assumption has been well supported by the fatty acid composition analysis, where a reduction in the unsaturated fatty acid

content with concomitant increase in saturation has been observed in mustard oil, sunflower oil as well as lard.

Further, previous reports have indicated that thermally oxidized vegetable oils contain higher levels of cyclic fatty acid monomers (CFAM), especially in unsaturated edible oils (Romero *et al.*, 2006). It is thus expected that lipid oxidation in the mustard oil, sunflower oil and lard may have resulted in the formation of CFAM. The CFAMs formed in the thermally oxidized are known to be unhealthy, they inhibit the pancreatic lipase activity in animals (Lamboni *et al.*, 1998). On the contrary, studies have reported that a diet rich in medium chain saturated and monounsaturated fatty acids enhance the enzymatic activity of lipases (Kris-Etherton *et al.*, 1999; Wang *et al.*, 2015).

Altogether, the thermal oxidation induced changes in the edible oils vary with their triglyceride composition. Medium chain saturated fatty acid rich oils have suffered less from oxidative modifications, however, higher levels of polymerization products are accumulated in the oil. In contrast, unsaturated fatty acid rich edible oils have higher level of primary and secondary lipid peroxidation products including peroxides and aldehydes.

Accumulation of lipid oxidation products are often recognized as a risk factor for many of the degenerative diseases including non-alcoholic fatty liver (Konishi *et al.*, 2006; Poli *et al.*, 1987) and colorectal cancers (Skrzydlewska *et al.*, 2005). Studies have come up with the observations that deep fried foods especially using thermally oxidized edible oils increase the risk for various metabolic and lifestyle associated diseases (Gadiraju *et al.*, 2015). In animal models consumption of thermally oxidized edible oils have also shown to induce hypertension (Jaarin *et al.*, 2011), dyslipidemia and oxidative stress (Adam *et al.*, 2008; Liu *et al.*, 2014), impaired glycerolipid metabolism as well as gut microbiota (Zhou *et al.*, 2016).

Though these studies have indicated the possible toxic effects of thermally oxidized oils, the overall aim of our study is to analyze the effect of thermally oxidized oil consumption on insulin resistance, and subsequent changes in the extent of hepatosteatosis and colon carcinogenesis. It is expected that, due to the higher levels of polymerization or oxidation products in thermally oxidized oils, they may induce deleterious changes. However, further independent investigations on each of the

edible oil using animal models are necessary to understand the actual effects of these thermally oxidized edible oils on the extent of hepatosteatosis and colon carcinogenesis.

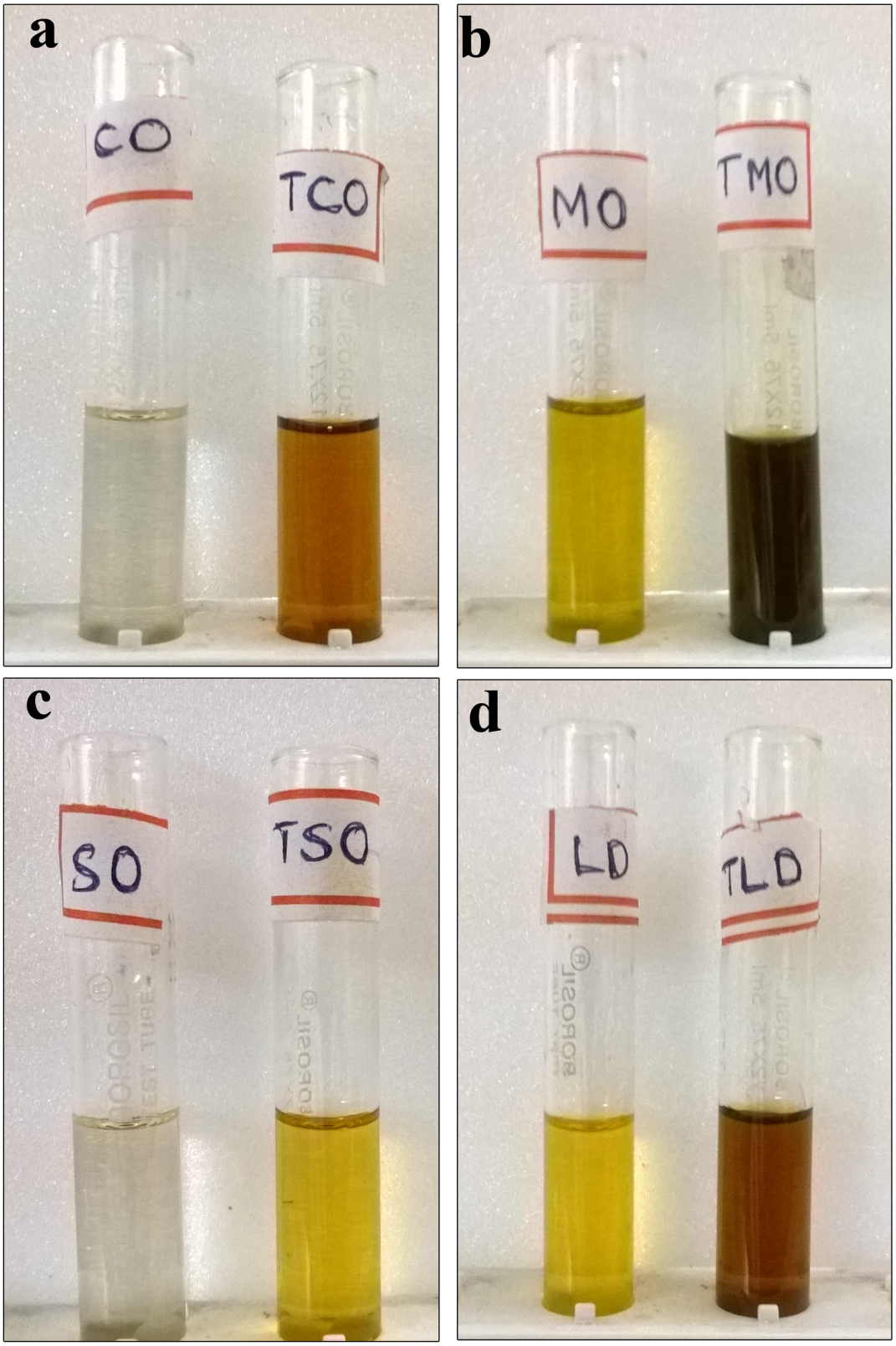


Figure 3.1 The changes in physical appearance of coconut oil (a), mustard oil (b), sunflower oil (c) and lard (d), during thermal oxidation at l 60°C for 6 hours. ***Abbreviations***: CO- Fresh coconut oil; TCO- Thermaly oxidized coconut oil; MO- Fresh mustard oil; TMO- Thermally oxidized mustard oil

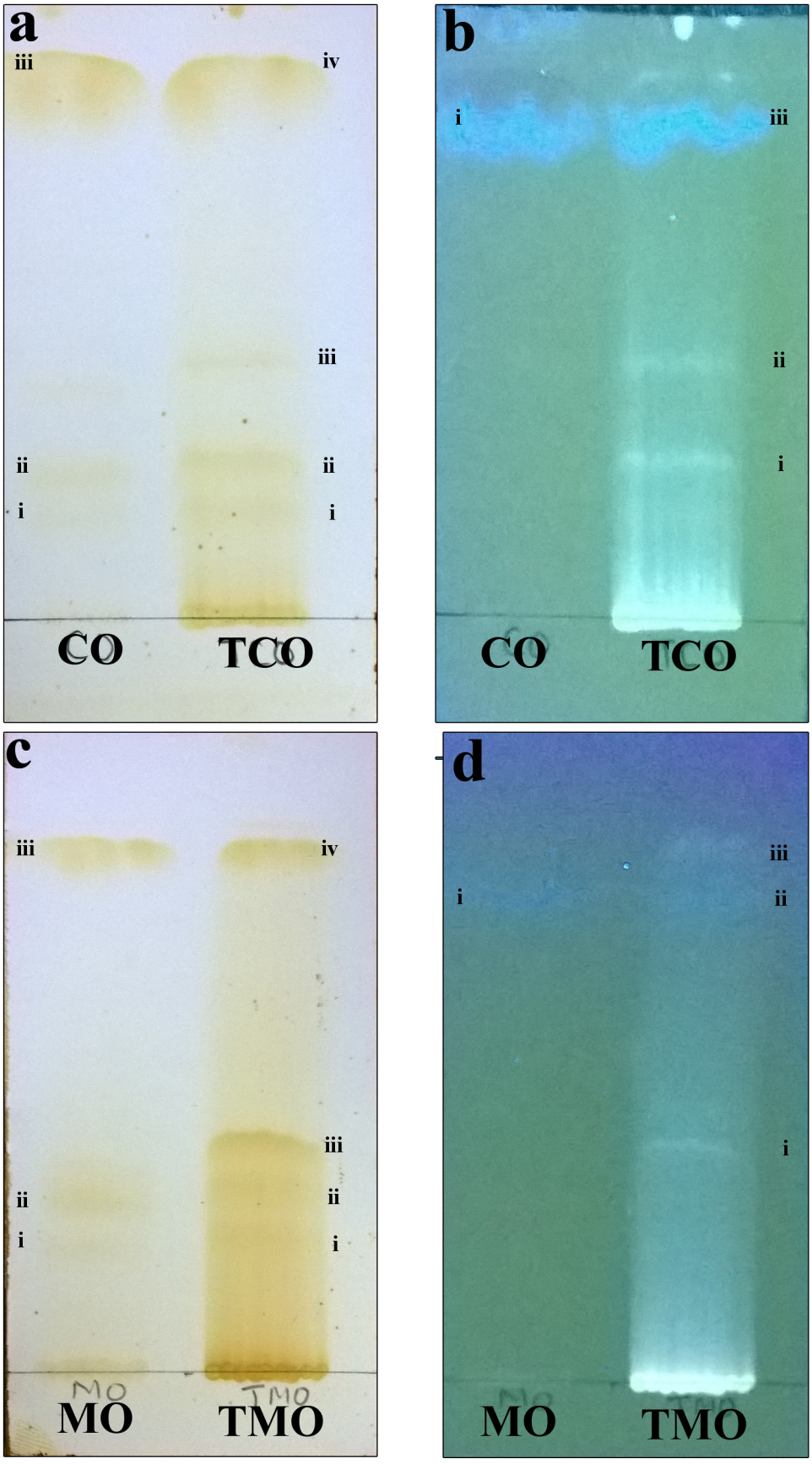


Figure 3.2 Thin layer chromatographic profile of coconut oil (CO), mustard oil (MO) and their thermally oxidized forms (TCO and TMO) visualized under iodine vapour exposure (a & c) and long wave U.V. (360 nm) (b & d).

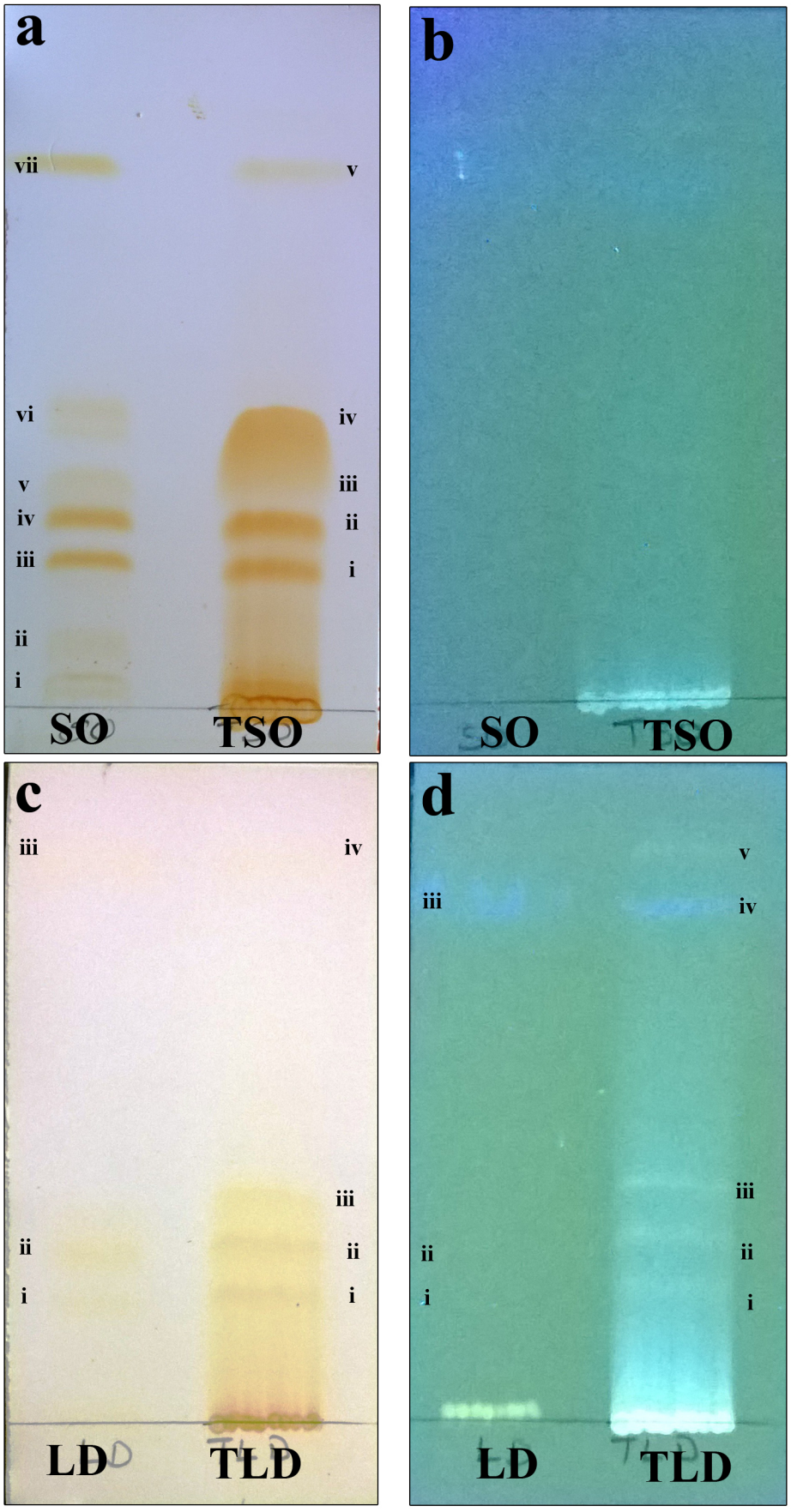


Figure 3.3 Thin layer chromatographic profile of sunflower oil (SO), lard (LD) and their thermally oxidized forms (TSO and TLD) visualized under iodine vapour exposure (a & c) and long wave U.V. (360 nm) (b & d).

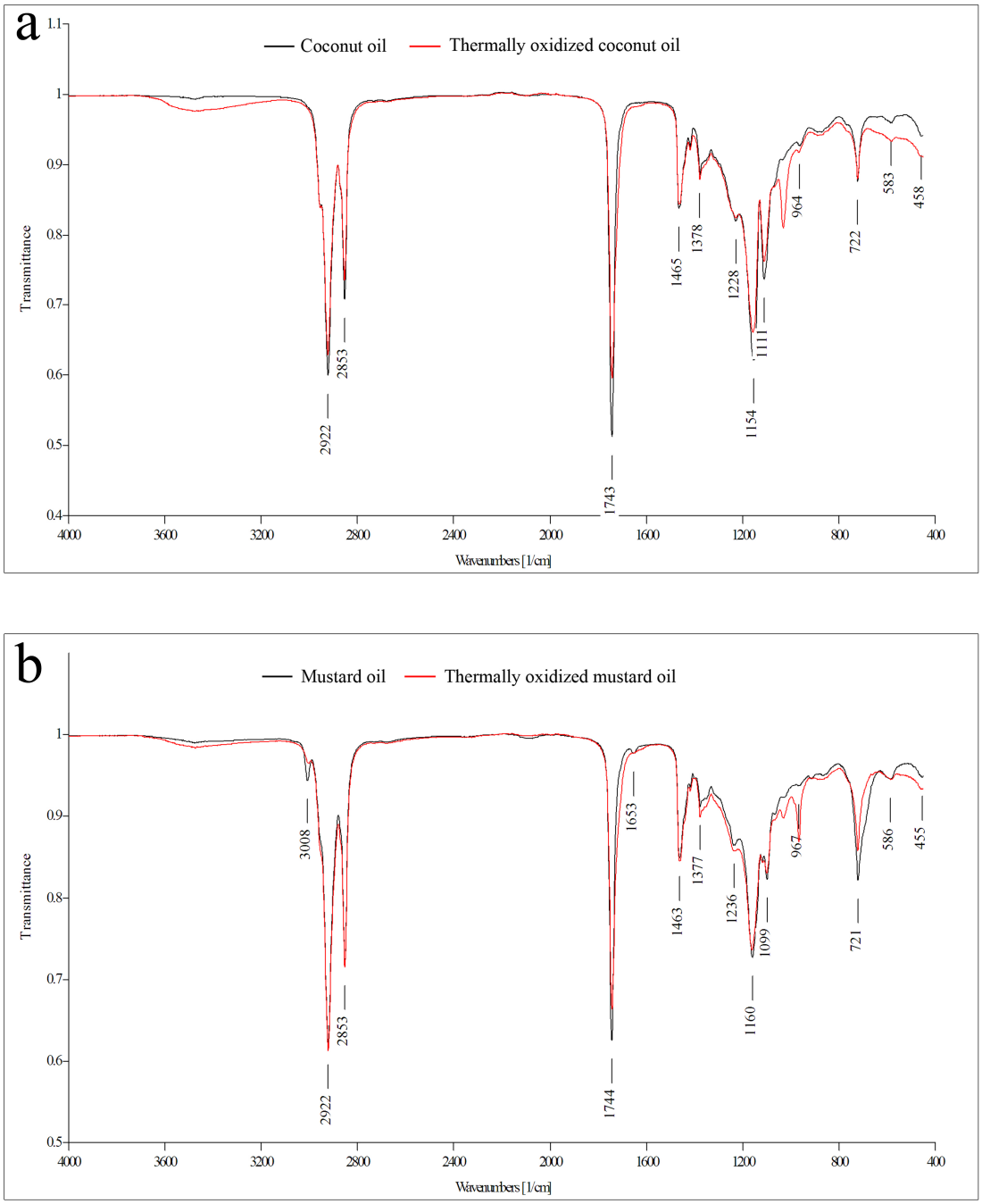


Figure 3.4 Fourier Transmission Infrared spectroscopic analysis of fresh as well as thermally oxidized coconut oil (a) and mustard oil (b). The black line spectra indicate fresh oil and the red line spectra indicate thermally oxidized oils. The FTIR spectra were analysed in Attenuated total reflectance mode in PerkinElmer Spectrum Two TM (Massachusetts, USA). The wavenumber (cm-1) was plotted on X- axis against the transmittance on Y-axis.

Regions at 3008 cm-1 indicate cis- bonds, 1744 cm-1 represent carbonyl groups, and 1161-1234 cm-1 indicates the saturated acyl groups.

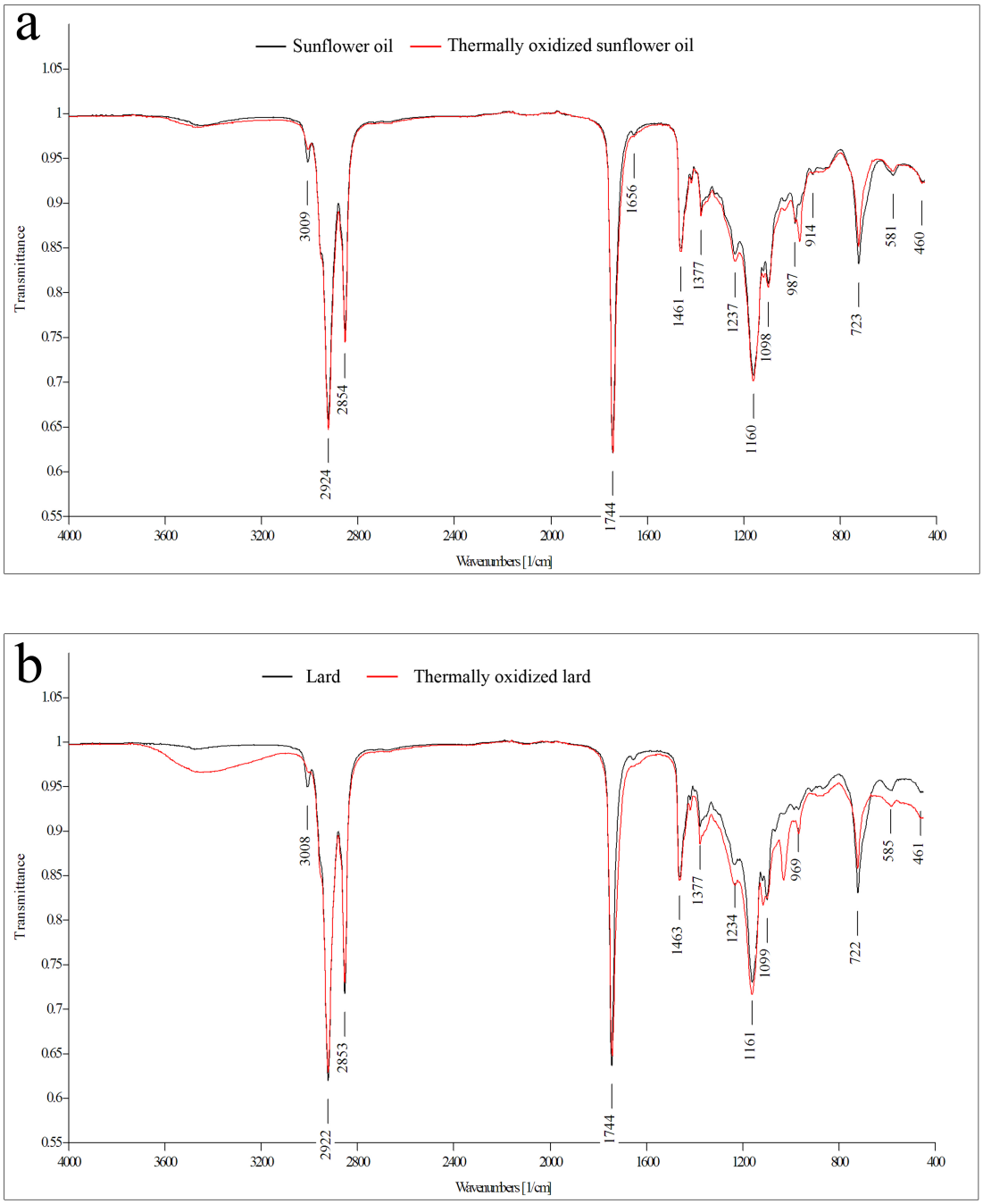


Figure 3.5 Fourier Transmission Infrared spectroscopic analysis of fresh as well as thermally oxidized sunflower oil (a) and lard (b). The black line spectra indicate fresh oil and the red line spectra indicate thermally oxidized oils. The FTIR spectra were analysed in Attenuated total reflectance mode in PerkinElmer Spectrum Two™ (Massachusetts, USA). The wavenumber (cm^{-1}) was plotted on X- axis against the transmittance on Y-axis.
Regions at 3008 cm^{-1} indicate cis- bonds, 1744 cm^{-1} represent carbonyl groups, and 1161-1234 cm^{-1} indicates the saturated acyl groups.

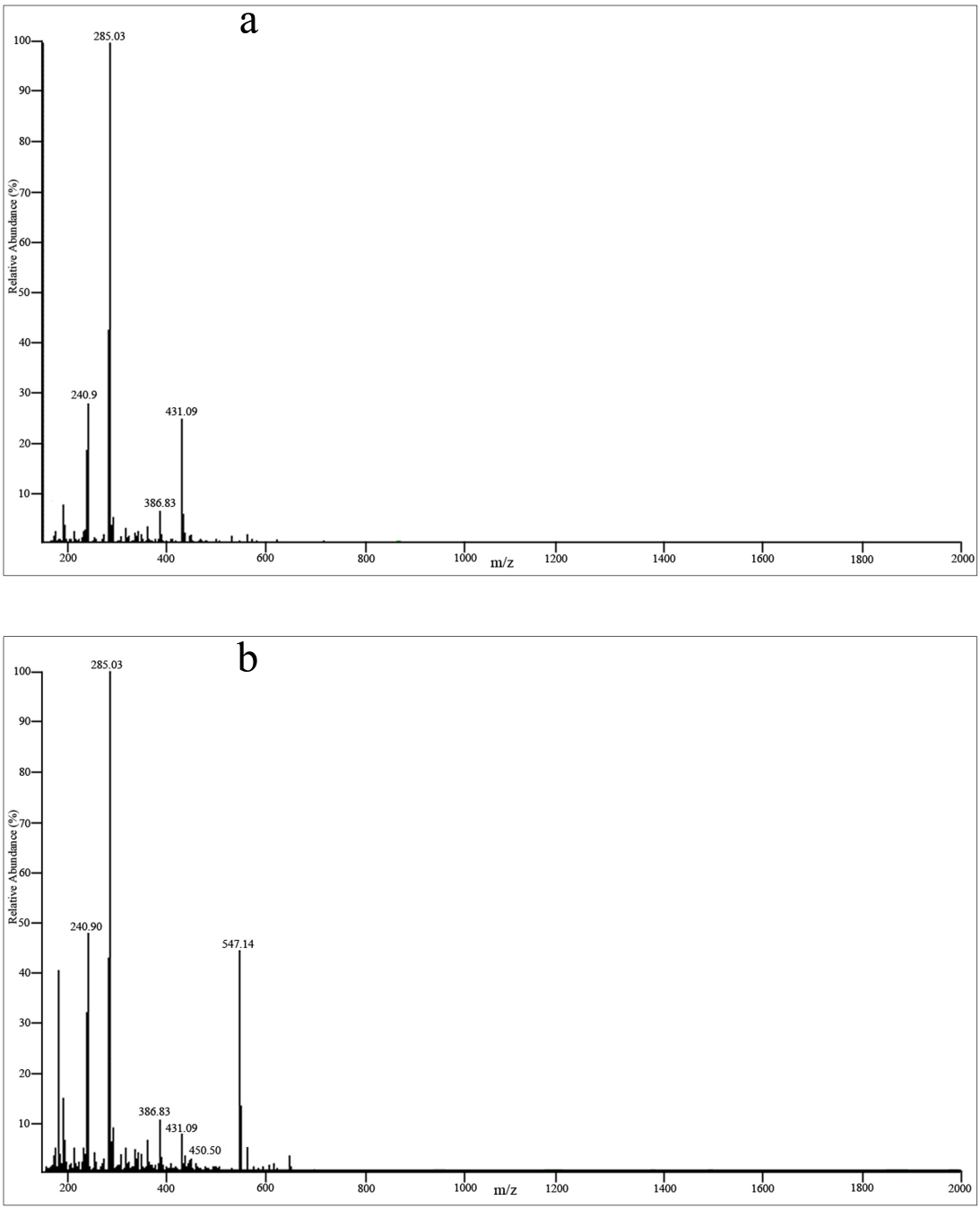


Figure 3.6 LC-Q-TOF- Mass spectrometry analysis of fresh coconut oil (a) as well as thermally oxidized coconut oil (b) in Acquity UPLC H class (Waters) system with a diode-array detector (DAD). For the analysis, a C18 column with dimensions 50 mm x 2.1 mm x 1.7 µm (Waters, USA) was used under Gradient elution was conducted using the mobile phase acetonitrile (A) and methanol (B) (flow rate 0.5 mL/ min)

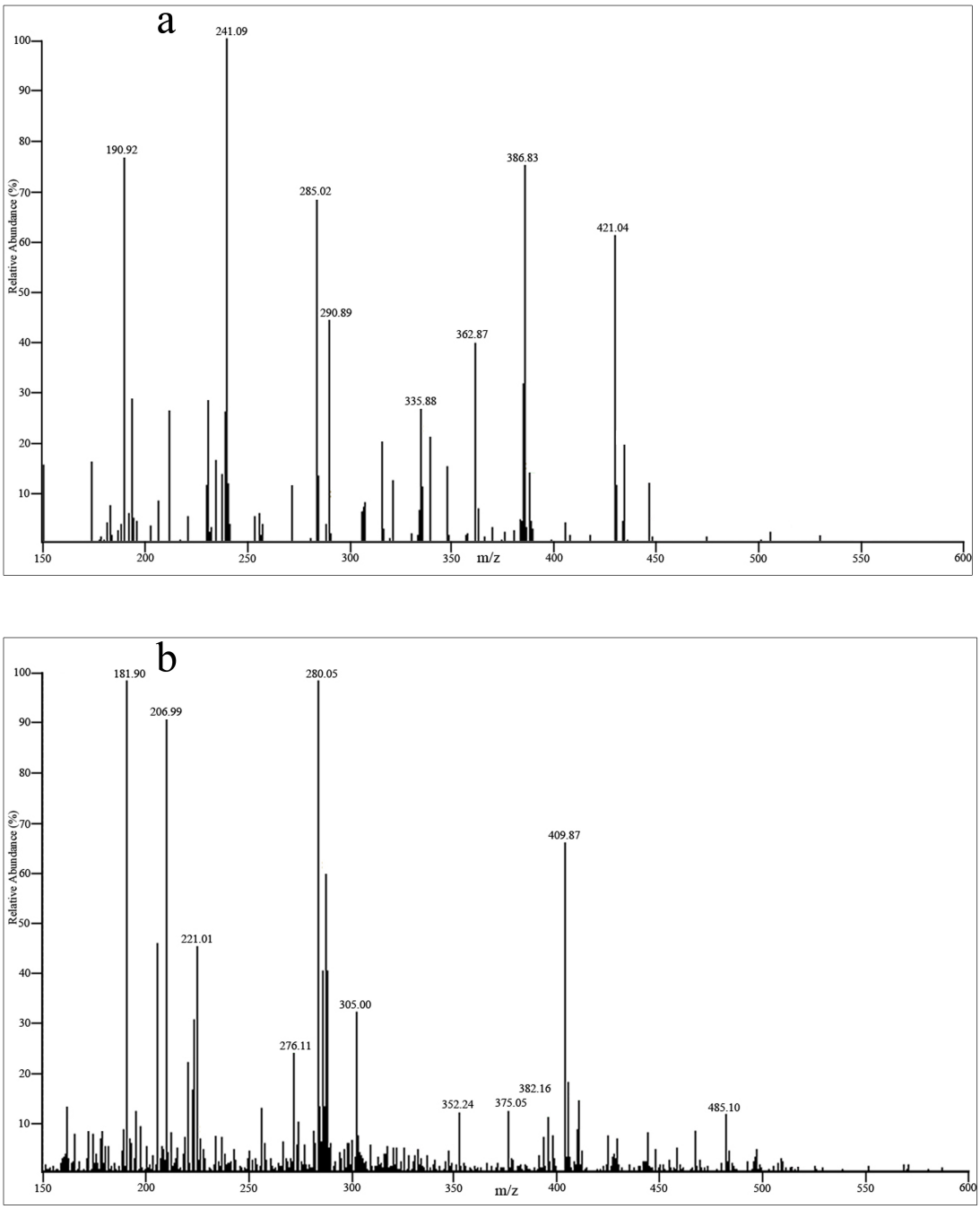


Figure 3.7 LC-Q-TOF- Mass spectrometry analysis of fresh mustard oil (a) as well as thermally oxidized mustard oil (b) in Acquity UPLC H class (Waters) system with a diode-array detector (DAD). For the analysis, a C18 column with dimensions 50 mm x 2.1 mm x 1.7 µm (Waters, USA) was used under Gradient elution was conducted using the mobile phase acetonitrile (A) and methanol (B) (flow rate 0.5 mL/ min)

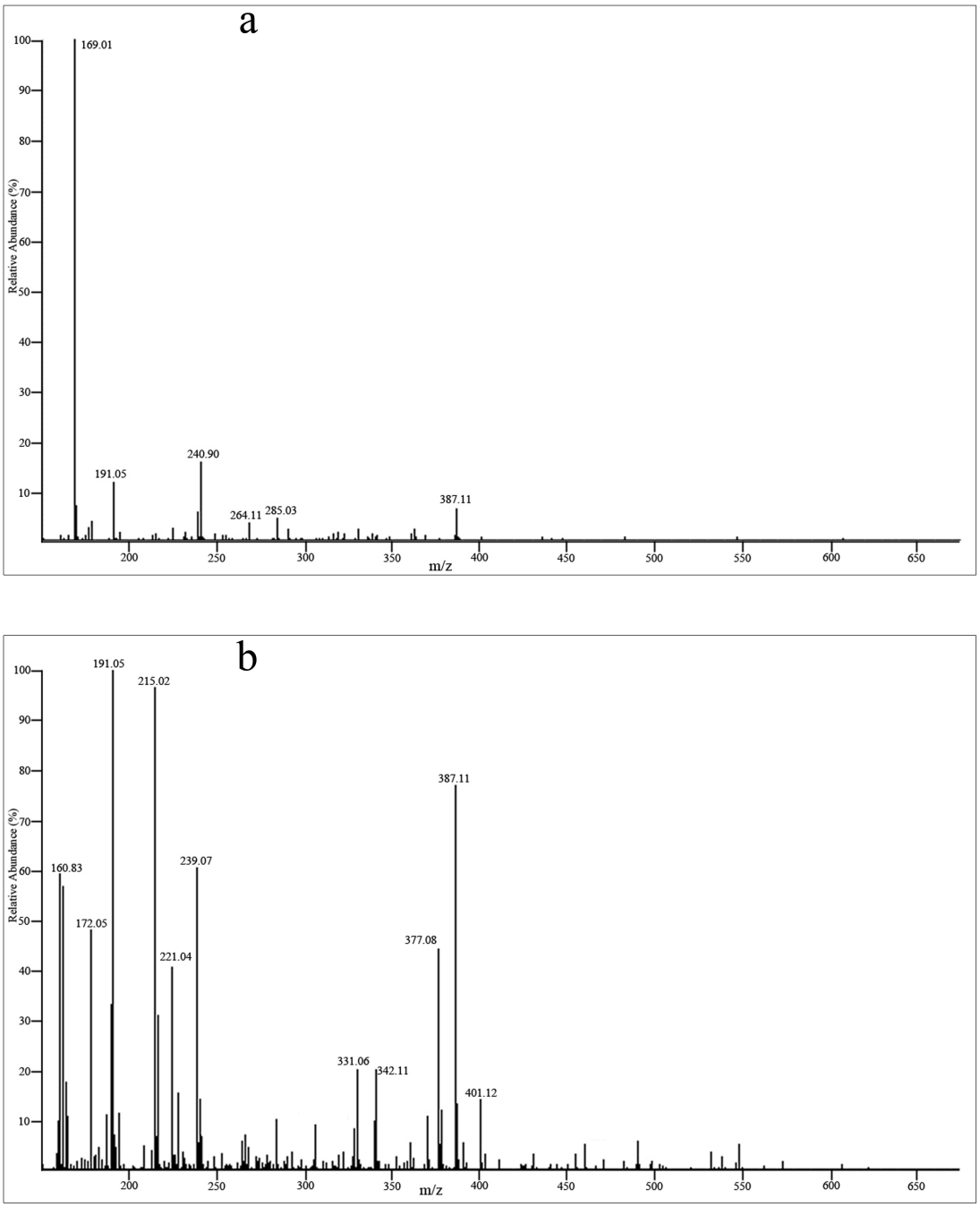


Figure 3.8 LC-Q-TOF- Mass spectrometry analysis of fresh sunflower oil (a) as well as thermally oxidized sunflower oil (b) in Acquity UPLC H class (Waters) system with a diode-array detector (DAD). For the analysis, a C18 column with dimensions 50 mm x 2.1 mm x 1.7 μm (Waters, USA) was used under Gradient elution was conducted using the mobile phase acetonitrile (A) and methanol (B) (flow rate 0.5 mL/min)

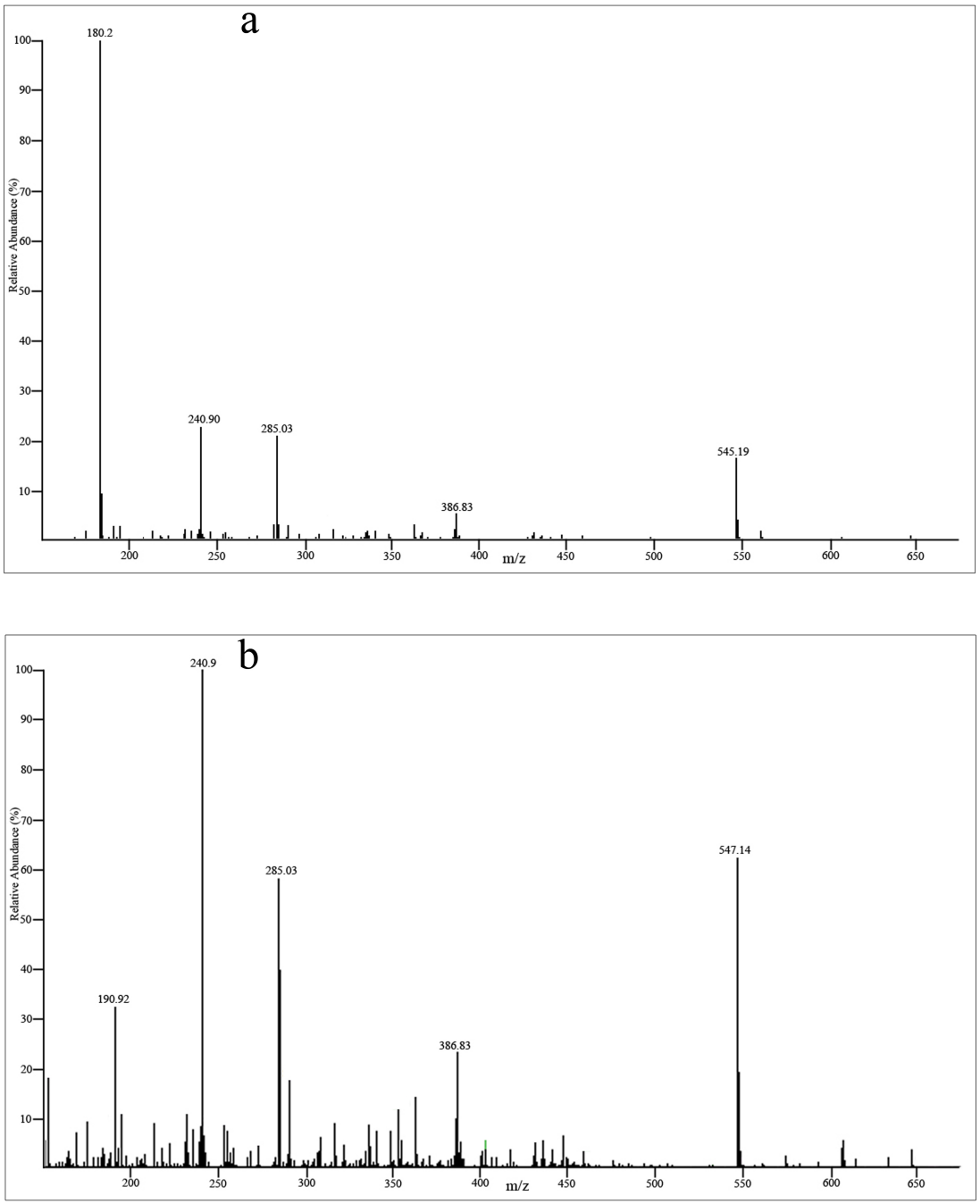


Figure 3.9 LC-Q-TOF- Mass spectrometry analysis of fresh lard (a) as well as thermally oxidized lard (b) in Acquity UPLC H class (Waters) system with a diode-array detector (DAD). For the analysis, a C18 column with dimensions 50 mm x 2.1 mm x 1.7 µm (Waters, USA) was used under Gradient elution was conducted using the mobile phase acetonitrile (A) and methanol (B) (flow rate 0.5 mL/ min)

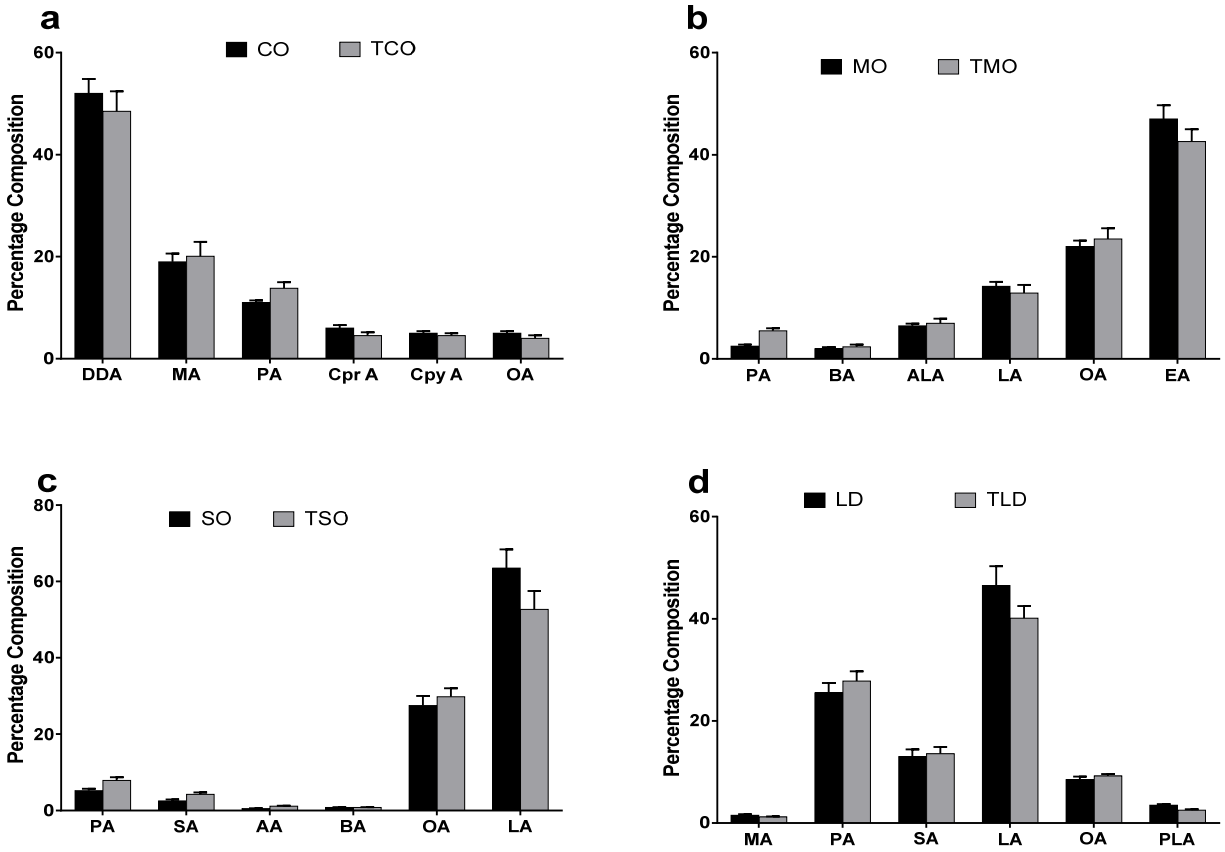


Figure 3.10 Changes in the fatty acid compositions of coconut (a), mustard (b), sunflower (c) and lard (d) oils upon thermal oxidation.

Abbreviations:

Cpr A- Capric acid (C10:0); **Cpy A**- Caprylic acid (C8:0); **DDA**- Lauric acid (C12:0); **MA**- Myristic acid (C14:0); **PA**- Palmitic acid (C16:0); **SA**- Stearic acid (C 18:0); **OA**- Oleic acid (C18:1); **ALA**- α-Linolenic acid (C18:3; n=3); **LA**- Linoleic acid (C18:2); **PLA**- Palmitoleic acid (C16:1); **EA**- Erucic acid (C22:1): **BA**- Behenic acid (C22:0)

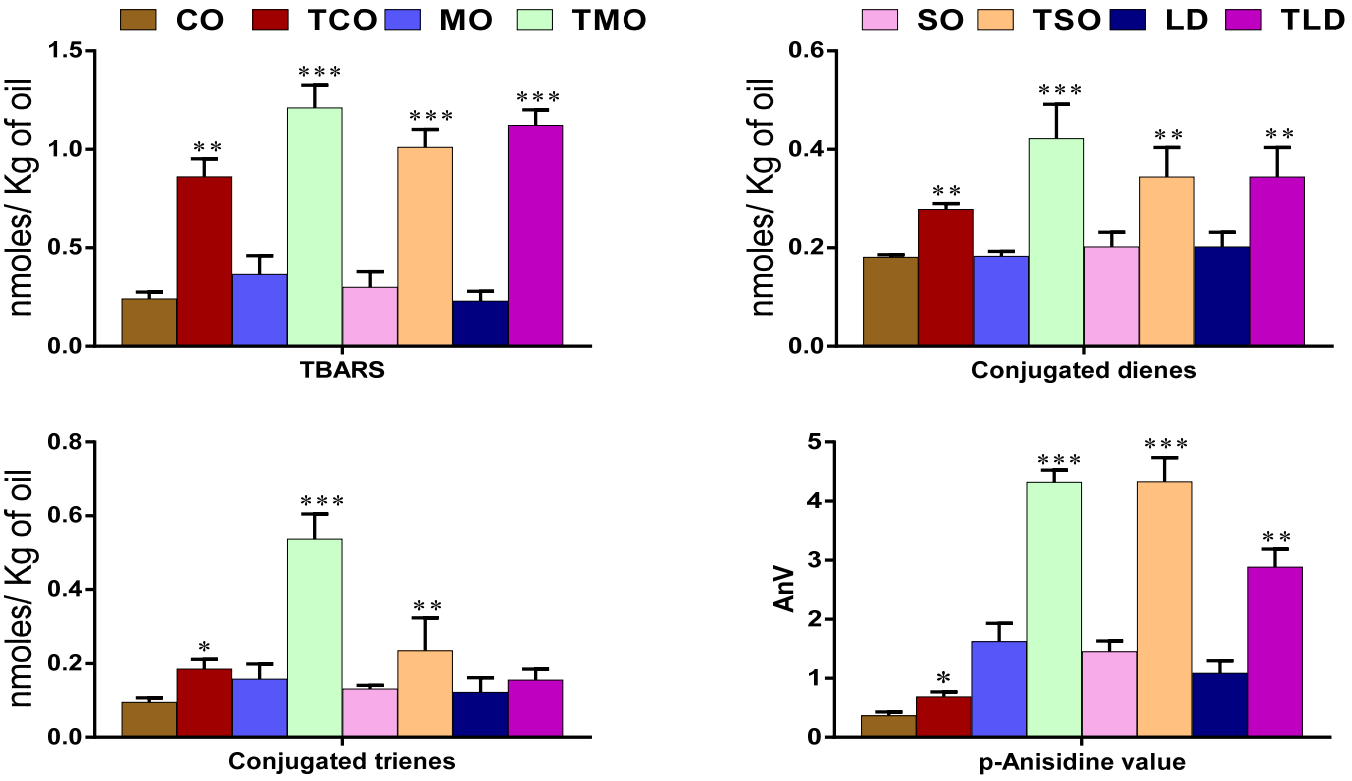


Figure 3.11 Changes in the lipid peroxidation indices of different edible oils after thermal oxidation (* indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Chapter 4

Effect of thermally oxidized coconut oil on HFr- induced metabolic dysregulation

4. 1 Introduction

Medium-chain triglycerides (MCT) are rich sources of medium chain fatty acids (MCFA), which contain 8- 12 carbons and are mostly saturated. The difference in the size and structure usually leads to changes in water solubility, digestion, absorption and transport of these fatty acids (Papamandjaris *et al.*, 1998). The MCT are easily hydrolyzed by various lipases in the gastrointestinal tract of humans, hence MCT rich coconut oils are easily digestible and absorbable (Jorgensen *et al.*, 2001; Pham *et al.*, 1998). The hydrolysis of MCTs generates 2- monoacyl glycerol or 1- monoacyl glycerol. Since coconut oils are rich in lauric acid, this yields sufficient quantity of mono lauryl glycerol which subsequently generates monolaurin compounds (Bragdon and Karmen, 1960). MCFA are not significantly incorporated into triglycerides since the enzyme acyl CoA synthetase is specific to long chains. Instead, they are readily absorbed through intestinal villi and enter the circulation through the hepatic portal system (Bach and Babayan, 1982; Bragdon and Karmen, 1960; McDonald *et al.*, 1980; Porsgaard and Hoy, 2000).

Mitochondrial beta-oxidation is the major catabolic pathway of lipids. MCFA directly transported across the mitochondrial membrane (Garlid *et al.*, 1996), producing acetyl-CoA for the citric acid cycle. Two of the enzymes, medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD) are responsible for the β- oxidation of lauric acid (Christensen *et al.*, 1989). Further, The acetyl-CoA formed by the β- oxidation can undergo subsequent modifications to yield ketone bodies such as acetoacetate, beta-hydroxybutyric acid and acetone (Nehlig, 2004; Nonaka *et al.*, 2016). These ketone bodies are later transported to various other organs especially to brain, muscles and heart where they form an energy source (Owen, 2005). Increased ketosis always raises health concern in conditions such as diabetes; however, limited studies are available on the effect of MCT diet on ketoacidosis. Studies by Nakamura *et al.* (1994) showed that MCT diet induces the ketosis in experimentally induced diabetic conditions, however, which still remains in the acceptable levels. The increased utilization of ketone bodies as an energy source or the shuttling of acetyl Co-A to cholesterol synthesis or gluconeogenesis could be the possible reason. MCT feeding for a long-term induces de novo fatty acid synthesis in the liver while down-regulates cholesterol biosynthesis by inhibiting HMG-CoA reductase activity (Hill *et al.*, 1990).

Apart from the beta oxidation, Cytochrome P450 4A11 dependent Omega (ω) oxidation is also possible (Hoch *et al.*, 2000; Uehara *et al.*, 2016). Together with this, CYP 4A1 (lauric acid hydroxylase) and CYP 450 2E1 are the other enzymes that catalyze the hydroxylation of lauric acid in liver (Amet *et al.*, 1994; Hardwick *et al.*, 1987). Desaturation of lauric acid to 12:1n-3 compound is also reported, which is believed to be the initial steps in the biosynthesis of alpha linoleic acid (Legrand *et al.*, 2002).

Coconut oil is the generally consumed form of edible oil in south India. Coconut oil possesses several health benefits, it includes neuroprotection (Nafar and Mearow, 2014) and improvement of lipid metabolism in pre-menopausal women (Feranil *et al.*, 2011). At the same time, reports indicate an increased incidence of cardiovascular diseases when saturated fat intake is high indicating its possible health hazard (Sakata and Shimokawa, 2013; Siri-Tarino *et al.*, 2010). Also, coconut oil as a fatty acid source in high fructose diet induces hyperglycemia and oxidative stress in murine model (Shawky *et al.*, 2014) and lead to hepatosteatosis (Narayanankutty *et al.*, 2016). Though CO these health benefits, due to its high saturated fat content, higher risk for cardiovascular diseases are expected (Wallstrom *et al.*, 2012). Limited studies are available on the effects of thermally oxidized coconut oil on health. Srinivasan and Pugalendi (2000) had shown that ingestion of thermally oxidized coconut oil induce oxidative stress and lipid profile changes. However, there are no available literatures on thermally oxidized coconut oil.

Considering the results from previous chapter, the thermally oxidized coconut oil contain higher amount of triglyceride polymers, which may prove deleterious. The present chapter thus evaluates the effects of long-term consumption of the fresh or thermally oxidized coconut oil along with a high fructose diet (60%) on insulin resistance and subsequent changes associated with hepatosteatosis and colon mucosal redox status in male Wistar rats.

4.2 Materials and methods

4.2.1 Animals

Male Wistar rats weighing approximately 150-160 g on the average were supplied by the Kerala Veterinary and Animal Science University. The animals were fed a

commercial diet from the Sai Durga feeds (Bangalore, India). The rats were randomly assigned into three groups of six rats each, on the basis of their weights using the Spiral method which resulted in almost equal total initial weights of groups. The rats were maintained at standard conditions and fed water *ad libitum*. The experimental procedure followed the established guidelines for the care and handling of laboratory animals by Institutional animal ethical committee and CPCSEA, Government of India (Approval No. ACRC/IAEC/15/06-(2)).

4.2.2 Preparation of the thermally oxidized oil

Fresh CO was purchased from local market the oil was then divided into two equal portions; one portion was stored at 15^{o} C to be used in the fresh oil containing diets later on in the experiment, while the other portion was heated at 160^{o} C for 48 h (González-Muñoz *et al.*, 2003). For heat treatment, these oils were put in 1 L glass beakers which were placed on a heating block set at the intended temperature. The thermally oxidized oil was stored at -20^{o} C in order to prevent further oxidation during storage before being included in the respective diets. The extent of lipid peroxidation in the oil was estimated as TBARS, CD and *p*-anisidine value (Commission, 1999).

4.2.3 Diets and experimental procedure

Experimental diets were prepared as per previously described method. (Narayanankutty *et al.*, 2016; Prakash *et al.*, 2014; Singh *et al.*, 2015). The composition of diet and grouping of animals were as described in Chapter 2.2.1.

4.2.4 Estimation of glucose tolerance

The animals fasted overnight before oral glucose tolerance test. Glucose tolerance was measured using the method of Narayanankutty *et al.* (2016). Briefly, rats were orally administered with 2 g/kg glucose following this blood glucose levels were measured using glucometer (One touch select, UK) at 0, 30, 60, 90 and 120 min intervals. The rate of glucose clearance and area under the curve were also calculated as described in Chapter 2.4.3.

4.2.5 Biochemical analysis

Various serum parameters such as total cholesterol, HDLc, triglycerides, ALT, AST, ALP, blood glucose were determined using commercially available kits Agappe

diagnostics (Chennai, India) as per the manufacturer's instructions. Lactate dehydrogenase and gamma-glutamyl transferase activities were determined using commercially available kits (Span Diagnostics Ltd., Mumbai). Details have been given in Chapter 2. Serum IL-6 was estimated using ELISA kit as per the manufacturer's instructions (Peprotech, Germany).

4.2.6 Analysis of hepatic redox status

Liver tissue was excised and washed in ice-cold saline. A small portion of each was fixed in 10% buffered formalin for histopathological studies. The liver homogenate was prepared (10% w/ v) in Tris HCl buffer (0.1 M, pH 7.4) and used for the estimation of lipid peroxidation (Ohkawa *et al.*, 1979).

The homogenates were prepared by centrifuging at 10000 rpm for 15 min at 4^{o}C. The supernatant was collected and used for GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), catalase (Beers and Sizer, 1952) and GR (Carlberg and Mannervik, 1975) assays. The detailed description on the methodology has been given in Chapter 2.9 and its subdivisions.

4.2.7 Analysis of colonic redox balance

Colon was excised and washed in ice-cold saline. A small portion of each was fixed in 10% buffered formalin for histopathological analysis. Colonic epithelium was collected and the homogenate was prepared (10% w/ v) in Tris HCl buffer (0.1 M, pH 7.4). Estimation of lipid peroxidation (Ohkawa *et al.*, 1979) and protein carbonyls (Levine *et al.*, 1990) was done in the tissue homogenate. The tissue supernatant was prepared by centrifuging at 5000 rpm for 60 min at 4^{o}C, collected and used for the assays of GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), GPx (Kinoshita *et al.*, 1996) and GR (Carlberg and Mannervik, 1975). The detailed description on the methodology has been incorporated in Chapter 2.9 and its subdivisions.

4.2.8 Measurement of polyol pathway enzymes and ODC activity

Polyol pathway enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH) activities were measured by spectrophotometric procedures as described by Jang *et al.* (2010) which has been described in Chapter 2.8. Ornithine decarboxylase activity was

determined according to the methods of Ngo *et al.* (1987) as described in Chapter 2.7.3.

4.2.9 Histopathological analysis

A portion of the formalin-fixed liver and colon tissues were embedded in wax following dehydration with series of alcohol. Serial sections of the tissues were then taken in a microtome at a thickness of 4 μm and stained with hematoxylin and eosin. Histopathological examination was carried out by a pathologist who was blind to the plan of this study. Scoring of hepatosteatosis was carried out according to a standard method (Brunt *et al.*, 1999). Changes in the colon epithelial histology were examined for accessing the incidence of colon carcinogenesis. The detailed outline of procedures used is given in Chapter 2.10.

4.2.10 Real-time quantitative PCR analysis

Total RNA was isolated from the colon epithelial tissues using TriZol reagent and cDNA was synthesized (Chapter 2.11.1 and 2.11.2). Gene expression in the colon epithelial tissue was analyzed by qPCR followed by ΔΔCT method. The details of primer and PCR cycling are given in Chapter 2.11.3.

4.2.11 Statistical analysis

The values are represented as mean ±standard deviation. Statistical analysis of the data was done by one way ANOVA followed by Tukey- Kramer multiple comparisons test using Graph Pad software (La Jolla, California, USA) (details are given in Chapter 2.12).

4.3 Results

4.3.1 Hyperglycemia and insulin resistance

The animals fed with a diet containing high fructose and different edible oils had a gradual increase in the blood glucose level in comparison with the normal (reference group) rats. Normal animals had an initial blood glucose level of 81.12±12.46 mg/dL, which gradually increased to 103.69 ± 5.72 mg/dL over a period of 30 weeks (net increase of 21.18±3.21 mg/dL). In the same experimental period, the changes in blood glucose level of CO fed animals was from initial 88.53±6.52 mg/dL to a final

210.3±7.68 mg/dL (net increase of 114.01±6.45mmg/dL). However, the increase in TCO fed group was more profound with a net increase of 145.13±10.24 mg/dL in blood glucose (initial 88.72±6.69 and final 225.90±14.0 mg/dL).

Insulin level in the normal animal was 45.71 ± 10.07 pmols/L, which was elevated to 66.66 ± 2.04 pmols/L, in high fructose-fed animals (Table 4.1). In CO-containing diet-fed animals, the plasma fasting insulin level was found to be 64.19 ± 3.59 pmols/L and it was comparatively lesser in TCO group of animals (62.38 ± 1.36 pmols/L).

HOMA2 %B or percentage of pancreatic beta cell function was found to be 63.8 ± 13.5% in reference diet fed animals and reduced significantly to 22.0 ± 4.1% in HFr group (Table 4.1). In CO-containing diet-fed animals, the HOMA2 %B was reduced further to 21. 0 ± 2.0% and in TCO group it was only 18.1 ± 1.6%.

HOMA2 %S or the % insulin sensitivity in normal animals fed on a reference diet was 116.9 ± 27.2% which was considerably reduced to 66.1 ± 0.5 % in HFr group of rats (Table 4.1). Compared to HFr, the HOMA2 %S was marginally higher in CO and TCO containing diet fed animals (68.9 ± 3.7 and 68.7 ± 1.9 %).

HOMA2 IR or the insulin resistance of normal animals was 0.89 ± 0.19 which was elevated to 1.51 ± 0.01 in HFr feeding (Table 4.1). Fresh CO and TCO containing diet fed rats had a HOMA-IR index of 1.45 ± 0.08 and 1.46 ± 0.04, which is comparatively lesser than the HFr group.

4.3.2 Oral glucose tolerance

Before sacrifice, oral glucose tolerance was assessed in these rats to evaluate the level of insulin resistance. All animals were given an oral gavage of 2g/ kg glucose after an overnight fasting and blood glucose was estimated at different time intervals (before glucose administration, 30, 60, 90 and 120 mins after glucose administration). In normal animals, from an initial level 100.12±1.24 mg/dL, blood glucose was elevated to 204.69 ± 2.54 mg/dL within 30 min (Figure 4.1). The level returned to normal within 120 min (100.23±3.41 mg/dL), with a glucose clearance rate of 52.23 ± 2.39 mg/dL/h. In the case of CO fed animals, the blood glucose was elevated to 329.24 ± 12.67 from the initial level 205.26 ± 6.18 mg/dL. Even after 120 min, the levels failed to return to their basal glucose levels (251.07 ± 8.59 mg/dL). Blood glucose level in

TCO group was also found to be elevated within 30 min of glucose administration (2g/Kg) to 335.57±5.61 mg/dL, which was returned to a level of 272.97 ± 8.41 mg/dL at 120 min time.

4.3.3 Effects on serum and hepatic lipid profile

In normal diet fed animals, the serum total cholesterol was found to be 61.73±6.83 mg/dL, it was elevated to 89.10±7.60 mg/dL in HFr group (Figure 4.2a). In the CO and TCO containing diet-fed animals, serum TC level was further elevated (94.61±5.98 and 95.9±5.82 mg/dL).

Serum triglyceride level of the normal animal was 84.5±16.9 mg/dL and it was further elevated to 159.4±11.6 mg/dL in HFr group of animals. In CO-containing diet-fed animals, the TG level was 172.1±13.3 mg/dL and in TCO it was 165.5±11.6 mg/dL (Figure 4.2a).

HDLc levels of reference (normal diet fed) and CO fed groups were more or less the same (28.06±3.94 and 28.17±6.05 mg /dL). In HFr group, it was 25.9±1.8 mg/dL and it was 26.91±5.82 mg/dL in TCO containing diet fed group (Figure 4.2a).

The LDLc level in normal diet fed rats was 16.8±3.2 mg/dL. An increase in the LDLc level was observed in the HFr group (28.9±3.7 mg/dL), CO (32.0±6.2 mg/dL) and TCO (32.4±2.0 mg/dL) containing diet fed groups (Figure 4.2a).

4.3.3 Effects on hepatic lipid profile

Total cholesterol of the liver tissue was significantly elevated in CO and TCO containing diet fed animals compared to reference diet fed group. The TC levels in the normal rats were 133.0±7.3 mg/g of tissue. In HFr group of animals, the liver TC was 199.1 ± 21.6 mg/g tissue (Figure 4.2b). Among the oil fed animals, the CO fed animals had the lower TC levels (247.3 ± 19.1 mg/g tissue). Animals fed with a diet containing TCO were shown to have elevated liver cholesterol levels (275.1±12.2 mg/g of tissue).

Hepatic triglyceride level in normal diet fed animals was 164.89±15.63 mg/g tissue. In HFr group of animals, the TC level was 262.5±31.6 mg/g tissue. In CO and TCO groups, an increased level of 320.4±23.1 and 302.8±14.1 mg/g of tissue was observed (Figure 4.2b).

Hepatic HDLc levels showed a reduction from 30.32±1.62 mg/g in normal rats to 23.2±3.0 and 22.6±2.3 mg/g in CO and TCO group of rats. In HFr group of animals, it was estimated to be 23.1±2.8 mg/g tissue (Figure 4.2b).

4.3.4 Effect on hepatic antioxidants and oxidative stress

In the present study, animals in the reference diet have a GSH level of 5.91 ± 0.24 nmoles/ mg protein, which was reduced to 5.18 ± 0.33 nmoles/ mg protein in HFr group. In fresh CO-containing diet-fed animals, the level was moderately reduced to 5.22 ± 0.40 nmoles/ mg protein (Figure 4.3b); whereas a significant reduction was noticed in TCO containing diet fed animals (4.19 ± 0.22 nmoles/ mg protein).

Catalase activity was 73.85 ± 8.90 U/ mg protein in normal animals fed on reference diet. High fructose feeding reduced the catalase activity to 54.47 ± 10.60 U/ mg protein. The extent of reduction was more significant in CO and TCO, where the catalase activity was found to be 48.16 ± 7.86 and 37.40 ± 3.51 U/ mg protein (Figure 4.3a).

Superoxide dismutase activity in the liver tissue of normal untreated rats was 6.90 ± 0.28 U/ mg protein, which was reduced to 4.11 ± 0.28 U/ mg protein in HFr group of animals. Animals fed on a modified diet containing CO and TCO as the fatty acid source had SOD activities of 4.28 ± 0.13 and 3.14 ± 0.55 U/ mg protein (Figure 4.3a).

Glutathione reductase activity in normal animals was 23.61 ± 3.26 U/ mg protein, which was shown as a mild reduction to 20.81 ± 2.7 U/ mg protein. However, the levels remained unaltered in CO or TCO containing diet fed animals (23.44 ± 2.53 and 23.94 ± 3.19 U/ mg protein) (Figure 4.3a).

Lipid peroxidation products as measured in terms of thiobarbituric acid reactive substances (TBARS) was significantly elevated in experimental diet fed animals. In normal animals, the serum and liver TBARS level were found to be 4.21 ± 0.87 and 4.07 ± 0.85 nmoles/ mg protein. In HFr fed animals, the serum and liver TBARS was elevated to 5.19 ± 0.29 and 5.34 ± 0.72 nmoles/ mg protein. In CO-containing diet-fed animals, the levels were 5.33 ± 0.93 and 5.49 ± 0.60 nmoles/ mg protein. The increase in serum and liver TBARS was most profound in TCO containing diet fed animals (6.64 ± 1.37 and 7.65 ± 0.78 nmoles/ mg protein) (Figure 4.3b).

4.3.5 Effect on polyol pathway enzymes and ornithine decarboxylase

In the normal rats, the AR activity was 1.42±0.41 U/mg protein, which was elevated to 3.28±0.38 U/mg protein in HFr group. In the CO and TCO containing diet fed rats, the respective levels were increased to 2.82±0.46 and 3.59±0.47 U/mg protein (Figure 4.4a).

SDH activity was 0.94±0.18 U/mg protein in the normal animals (reference diet) and it increased to 1.22±0.19 U/mg protein. In the CO and TCO containing diet fed rats, levels were increased to 1.11±0.21 and 1.46±0.29 U/mg protein (Figure 4.4a).

Ornithine decarboxylase activity was found to be 4.78±0.76 U/mg protein in normal rats, which was increased into 6.24±0.31 U/mg protein inHFr group. The CO-containing diet fed rats had an ODC activity of 5.69±0.52 U/mg protein). In TCO fed rats, the level was further raised to 6.42±0.71 U/mg protein (Figure 4.4a).

4.3.6 Liver function parameters

In normal animals, the AST activity is found to be 49.7±5.2 IU/L, which was increased to 66.3±4.9 IU/L in HFr group of rats. In fresh CO fed animals, the activity was elevated significantly to 73.7±6.1 IU/L and in TCO it was 113.5±11.5 IU/L (Figure 4.4b).

Serum ALT activity of the HFr group of animals (44.2±6.1 IU/L) was higher than that of the normal animals (32.6±2.8 IU/L). In CO-containing diet fed group, the activity was 47.6±3.9 IU/L and it was further elevated to 58.3±7.4 IU/L in TCO group of animals (Figure 4.4b).

Alkaline phosphatase activity was found to be 238.0±10.6 IU/L in normal animals, which was elevated to 251.5±25.4 IU/L. In CO group it was elevated to 277.0±7.3 IU/L and it was 282.3±2.8 IU/L in TCO containing diet fed animals (Figure 4.4b).

4.3.7 GGT and LDH activity and IL-6 levels

The GGT activity of the normal animals fed on a reference diet was 30.91 ± 4.85 IU, which was increased to 38.50 ± 8.10 IU in HFr group. In CO and TCO containing diet fed animals the GGT activity were 38.93 ± 5.33 and 47.83 ± 3.85 IU (Table 4.2).

Lactate dehydrogenase activity in the normal animals was 170.80 ± 13.55 IU and it was increased to 194.16 ± 9.70 IU in HFr group. In CO-containing diet-fed animals, the activity was 208.70 ± 13.86 IU and it was 232.33 ± 10.58 IU in TCO group of animals (Table 4.2).

As shown in the Figure 4.6, the IL-6 levels of Normal animals was 0.54± 0.06 pg/mL; in HFr diet fed animals, the level was 1.73± 0.17 pg/mL. In fresh CO containing diet fed animals, the serum IL-6 level was 0.66± 0.18 pg/mL and in TCO containing diet fed animals, the levels showed a significant ($p<0.01$) rise to 1.99± 0.55 pg/mL.

4.3.8 Effect on the colonic antioxidant status

Reduced glutathione levels in the colon mucosa of normal animals were 25.3 ± 3.2 nmoles/ mg protein, which was reduced to 14.60 ± 1.30 nmoles/ mg protein in HFr group. In fresh CO-containing diet-fed animals, the level was 16.2 ± 0.5 nmoles/ mg protein and in TCO containing diet fed animals it was 15.7 ± 1.3 nmoles/ mg protein (Figure 4.5a).

Superoxide dismutase activity in the colon mucosa of normal rats fed with reference diet was 7.0 ± 0.9 U/ mg protein, which was reduced in HFr group of animals to 4.90 ± 0.6 U/ mg protein. Animals fed on a modified diet containing CO and TCO had SOD activities of 4.4 ± 0.3 and 4.6 ± 0.6 U/ mg protein (Figure 4.5a).

Glutathione peroxidase activity in normal animals was 156.5 ± 7.2 U/ mg protein, which was shown a mild reduction to 144.70 ± 12.10 U/ mg protein. In the CO or TCO containing diet fed animals the activity was found to be 133.6 ± 4.0 and 127.1 ± 3.0 U/ mg protein (Figure 4.5b).

Thiobarbituric acid reactive substances (TBARS) level in the colon of normal rats was 46.7 ± 3.9 nmoles/ mg protein. In HFr fed animals, the serum and liver TBARS was elevated to 60.2 ± 7.7 nmoles/ mg protein. In CO and TCO containing diet-fed animals, the levels were 59.2 ± 3.39 and 69.9 ± 3.1 nmoles/ mg protein (Figure 4.5b).

4.3.9 Histopathological analysis

Histopathological analysis of the liver tissues of animals fed with normal diet and those fed on high fructose and CO (b) and TCO (d) fed animal are shown in Fig. 4.7. The reference diet (normal) fed animals showed normal hepatic architecture with the

normal central venous system, the portal triads, sinusoidal spaces and Kupffer cells. However, CO and TCO diet fed groups showed signs of hepatosteatosis, observed as microvesicles. TCO containing diet fed animals showed progressed hepatosteatosis than those fed with CO diet. Grading of hepatosteatosis in shown in Table 4.3, where it was clear that animals fed with TCO had a progressed NAFLD with macrovesicular steatosis, hepatocellular ballooning and lipogranulomas.

Histopathological analysis of colon tissue of untreated animals (Figure 4.8a) and those fed with fresh oil diet (Figure 4.8c) showed normal glands and villi, with mucous and serosa layer appearing normal. Whereas in animals fed with TCO (Figure 4.8d) containing diet had mild and diffused infiltration by lymphocytes, plasma cells and polymorphonuclear cells.

4.3.10 Gene expression profile using qPCR analysis

Quantitative real-time PCR was used to analyze the expression pattern of genes, where fold change of each gene than the Normal rats was represented. In HFr group, the IL-6 expression was increased by 25.24 ± 1.47 fold. However, in CO-containing diet fed animals the expression was increased by 10.06 ± 2.89 fold; whereas TCO group of animals had 28.84 ± 2.89 fold increases in IL-6 expression. Tumour necrosis factor α was increased by 16.68 ± 1.30 fold in HFr group of animals. However, it was only 6.45 ± 1.46 and 11.24 ± 1.97 fold in CO and TCO containing diet fed rats (Figure 4.9).

Expression of P53 gene was increased 1.88 ± 0.24 fold in HFr group, which was increased by 2.28 ± 0.20 and 1.16 ± 0.32 fold in CO and TCO containing diet fed animals. In HFr group, Wnt-1 gene expression was increased by 40.84 ± 1.89 fold, whereas in CO and TCO containing diet fed rats, it was increased by 13.09 ± 4.10 and 29.24 ± 2.82 folds (Figure 4.9). Peroxisome proliferator activator receptor-γ expression was increased by 45.55 ± 3.22 and 42.52 ± 0.55 fold in HFr and CO group of rats. However, in TCO containing diet fed rats, the expression was increased by 21.11 ± 0.53 fold. Protein kinase B (Akt) expression was increased by 2.94 ± 0.18 in HFr group, which was 1.06 ± 0.16 and 1.52 ± 0.20 fold in CO and TCO containing diet fed rats (Figure 4.9).

4.4 Discussion

As concluded in Chapter 3, deep frying of CO documented a mild increase in peroxide and aldehyde molecules. Copra oil also contains minor amounts of mono and polyunsaturated fatty acids (5-9% of Oleic and linoleic acids) accounting for the increased lipid peroxidation products, which is observed during its thermal oxidation.

Compared to reference diet (normal rat chow) fed animals, rats fed with fructose alone and fructose along with fresh or thermally oxidized CO containing diet shows increased levels of blood glucose and reduced glucose tolerance. Increase in blood glucose level and reduced glucose clearance rate are the markers of insulin resistance (Wilcox, 2005). Results show that thermally oxidized coconut oil diet significantly altered the HFr-induced hike in glycemic conditions and glucose metabolism than the fresh oil fed animals. The results have been in concordance with the HOMA indices, where a considerable reduction in the beta cell functioning and insulin sensitivity is observed in TCO containing diet fed animals. Together, increased HOMA IR values also confirm the development of insulin resistance in thermally oxidized oil fed animals. However, compared to HFr alone given group, MCFA containing CO has been shown to have an improved insulin resistance and sensitivity. Supporting our studies, MCFAs has been shown to ameliorate high fat diet induced insulin resistance (Wein *et al.*, 2009). Previously, Vessby *et al.* (2001) have reported that saturated fats intake increase insulin resistance than monounsaturated fats, however, in the study the saturated fats used are of long chain in nature.

Associated with this, an increase in TG and reduction in HDLc levels are seen in fructose along with fresh or thermally oxidized oil containing diet fed animals. According to Song *et al.* (2015), insulin resistance is manifested by dyslipidemia where increased levels of TG and low HDL are evident. Total cholesterol and LDL levels are also found to be higher among these groups. Therefore, it is assumed that insulin resistance and associated hyperlipidemia might have contributed to the progressive hepatosteatosis which is observed in high fructose-containing diet fed animals. Dyslipidemia is often known to be involved in several of the hepatic disorders. Hypertriglyceridemia is the major contributor to hepatic damages by the accumulation of triglycerides in the liver that leads to the vacuolation of hepatocytes and disrupting their normal functioning (Perry *et al.*, 2013). Here in this study,

animals fed with a diet containing fructose (60%) alone or in conjunction with the edible oils (fresh and fried) are shown to have higher triglyceride levels in comparison with normal diet fed animals, TCO being most damaging. It is expected that lipid oxidation products formed during the deep frying of these oils such as CFAM are known to inhibit the lipase activity(Lamboni *et al.*, 1998). This could also be a reason for the hypertriglyceridemia observed in this study. On the contrary, studies have reported that a diet rich in medium chain saturated and monounsaturated fatty acids reduce the triglyceride levels in the body by enhancing the enzymatic activity of lipases(Kris-Etherton *et al.*, 1999; Wang *et al.*, 2015). Thus it is also possible that the changes observed in these animals may be partially caused by high fructose intake in these animals (Briand *et al.*, 2012).

Prolonged insulin resistant conditions are also reported to have elevated activities of LDH (Zappacosta *et al.*, 1995) and GGT (Haghighi *et al.*, 2011). GGT and LDH are also indicators of oxidative damages to tissues(Ramos *et al.*, 2013). Thermally oxidized oil consumed groups have increased activities of LDH over HFr or CO diet, possibly indicating the oxidative damages. Results have already shown that oxidized oils have generated peroxides and aldehydes, which could induced oxidative stress and carbonyl adducts in the colonic epithelium of diabetic rats.

Corroborating with this assumption, higher levels of protein carbonyls and lipid peroxidative changes are noted in TCO group, which is in line with studies by Adam *et al.* (2008), where increased lipid peroxidation is noted in fried palm oil fed rats. Previous reports have indicated that thermally-oxidized vegetable oils contain higher levels of CFAM (Romero *et al.*, 2006). Here in this study, TCO contains more lipid hydroperoxides and aldehydes than their fresh counterparts. The oxidative and inflammatory insults by these oxidation molecules may be responsible for the observed increase in lipid peroxidation products in the serum and liver tissue of fried oil fed animals. Accumulation of these lipid oxidation products is often recognized as a risk factor for many of the liver diseases including non-alcoholic fatty liver (Konishi *et al.*, 2006; Poli *et al.*, 1987).

Providing further insight into this, reduced levels of GSH, which is actively involved in the elimination of toxic radicals including peroxides, are observed in TCO fed rats than HFr or CO group. Possibly, hyperglycemia associated activation of polyol

pathway, as observed in the TCO fed rats may be responsible for the observed reduction in GSH levels. Here, an increase in the aldose reductase activity is observed in fried oils fed animals. AR, which is a key enzyme involved in Polyol pathway, has significant role in maintaining GSH level. It converts excess blood glucose to sorbitol, which disturbs oxidative balance by exhausting NADPH (Bravi *et al.*, 1997). On the other hand, this depletion in NADPH affects GSH regeneration, as it is necessary for the restoration of GSH from GSSG by the action of glutathione reductase (Bravi *et al.*, 1997). Along with the reduced level of GSH, the diminished activity of hepatic antioxidant enzymes, comprising of superoxide dismutase and catalase, are observed in the TCO fed group, which are in line with the previous studies (Narasimhamurthy and Raina, 1999; Purushothama *et al.*, 2003). Reduction in the antioxidant enzyme activity is also thought to be involved in the progression of NAFLD (Koruk *et al.*, 2004). These observations are in corroboration with the increased incidence of microvesicles, hepatocellular ballooning and lipogranuloma seen in the TCO fed rats. These results thus indicate a comparatively higher degree of hepatic damage in the animals fed with high fructose and thermally oxidized edible oil possibly indicate the progression of steatosis to nonalcoholic steatohepatitis (NASH).

Further, the reduced SOD activity and GSH levels also observed in the colon epithelial tissues. Reduction in the detoxification systems of the body, as observed in thermally oxidized oil fed animals, can increase the accumulation of the peroxidation products and other free radicals, leading to increased modifications of macromolecules including lipids and protein, ultimately promoting inflammation (Tabak *et al.*, 2011). Increased oxidative damages often lead to the secretion of pro-inflammatory cytokines (Elmarakby and Sullivan, 2012). In our study, the increase in the level of inflammatory cytokines such as IL-6 and TNF-α over HFr alone fed group has been observed in thermally oxidized oil containing diet fed animals. Compared to other groups, CO containing diet fed animals had only a marginal increase; the phenolic content of coconut oil may be the protective agents against inflammation, which may have lost during thermal oxidation in TCO. The increase in inflammatory conditions of colon by TCO diet can be correlated with the comparatively lower expression of PPARγ than fresh oil fed groups. PPARγ is a gene that is highly expressed in the colon epithelial cells (Vidal-Puig *et al.*, 1997), the impaired

expression of which leads to chronic inflammation modulated by NF-KB (Dubuquoy *et al.*, 2006).

The chapter thus concludes that, prolonged intake of thermally oxidized coconut oil exacerbate HFr induced hyperglycemia and glucose intolerance than its fresh counterpart. Further, the thermally oxidized coconut oil exacerbates the hepatosteatosis condition possibly proceeding to the nonalcoholic steatohepatitis (NASH) level. The possible role of triglyceride polymers may be considered as key mediators and need to be further evaluated by isolating these molecules. The effect of thermally oxidized coconut oil in the colon epithelial cells has been limited to the altered redox imbalance. Hence, it can be concluded that the extensive progression of hepatosteatosis during thermally oxidized coconut oil feeding, may indicate the possible risk of development of liver cirrhosis and later progression in to the hepatocellular carcinoma in long-term.

Table 4.1 Changes in the blood glucose level, plasma insulin level and various HOMA indices in HFr diet alone fed animals and those fed with CO or TCO containing diet over a period of 30 weeks

Parameters	Normal	HFr	CO	TCO
HOMA2 %B	63.8± 13.5	22.0± 4.1***	21.0± 2.0***	18.1± 1.6***
HOMA2 %S	116.9± 27.2	66.1± 0.5***	68.9± 3.7***	68.7± 1.9***
HOMA2 IR	0.89± 0.19	1.51± 0.01***	1.45± 0.08***	1.46± 0.04***
Glucose (mg/dL)	103.69 ± 5.72	212.97± 21.0 **	210.3±7.68**	225.90±14.0 **
Insulin (pmols/L)	45.71± 10.07	66.66± 2.04***	64.19± 3.59**	62.38± 1.36**

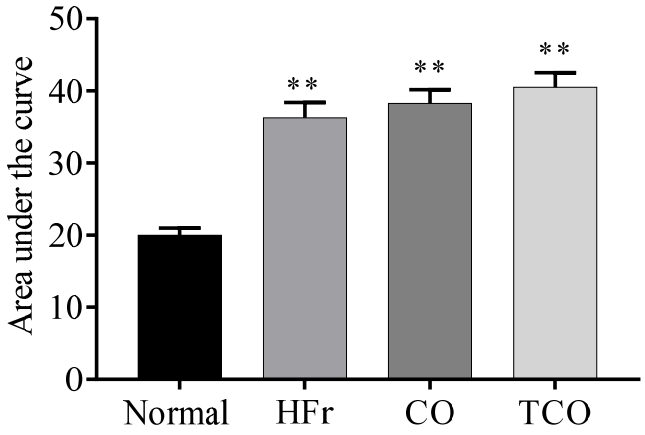


Figure 4.1 Change in the oral glucose tolerance as indicated by the area under the curve in HFr diet fed animals (30 weeks) and effect of CO or TCO supplementation

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

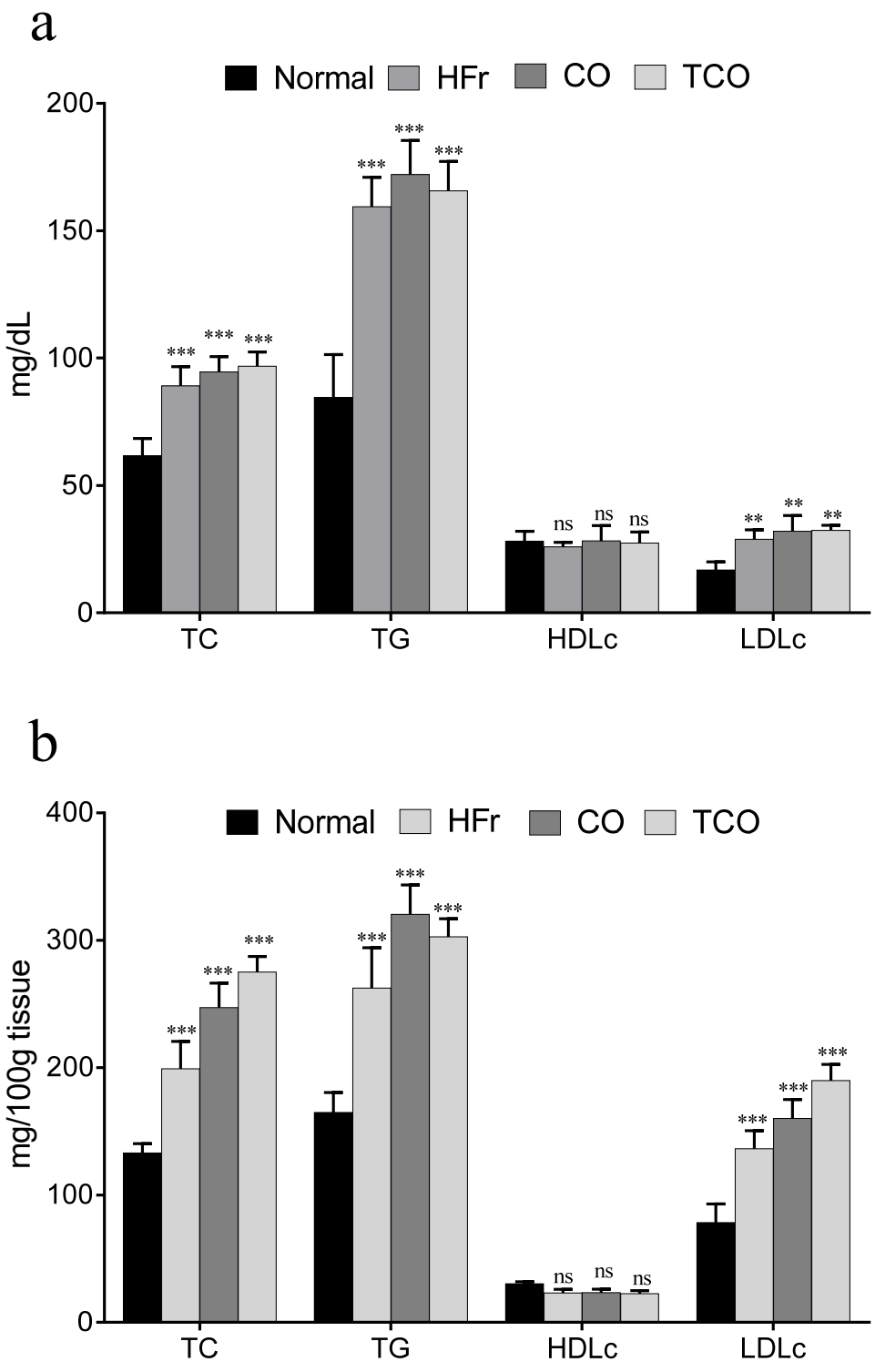


Figure 4.2: Effect of CO or TCO supplementation as well as HFr alone diet on serum (a) and hepatic (b) lipid profile of animals over a period of 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

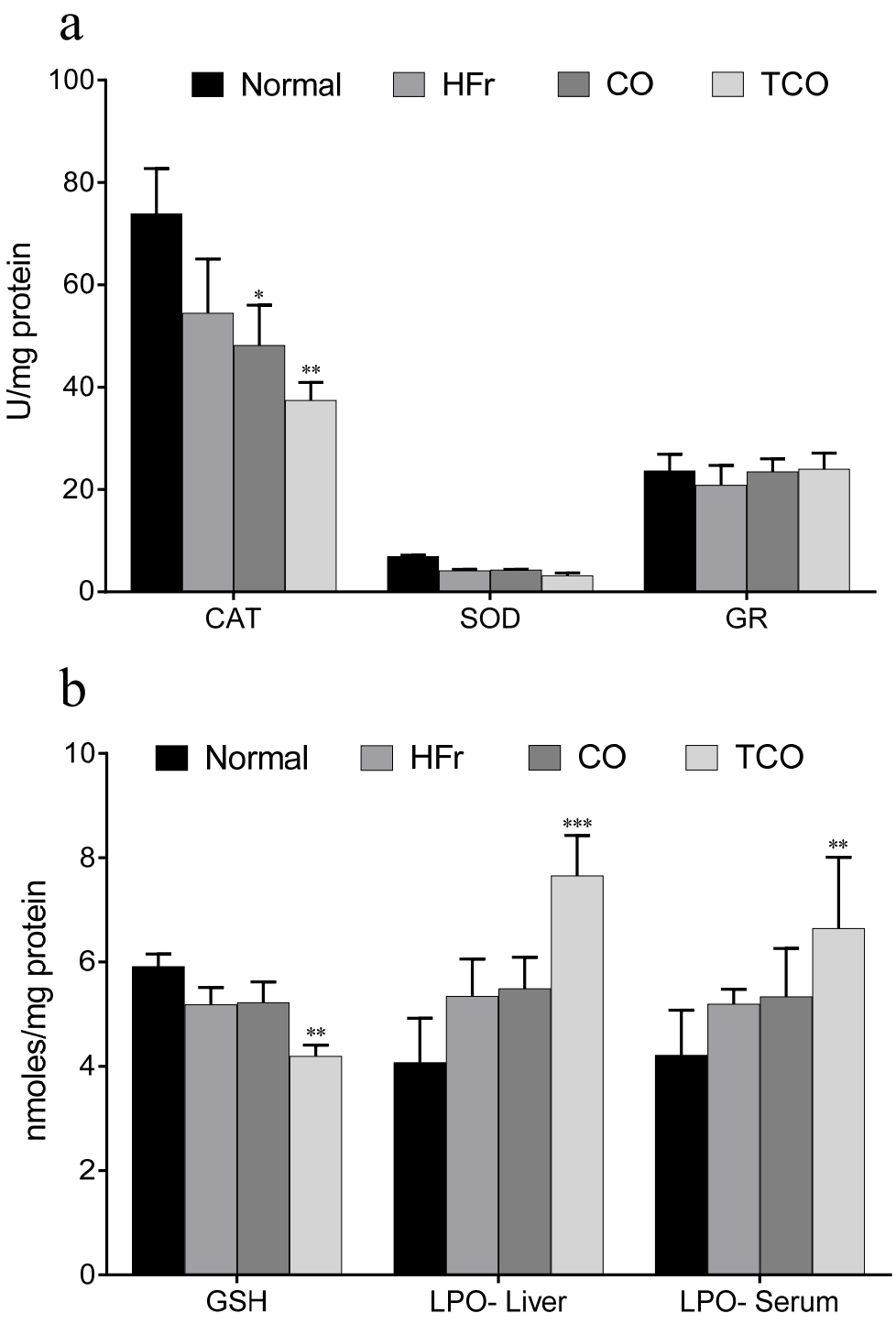


Figure 4.3: Effect of CO or TCO supplementation on HFr induced hepatic redox imbalance as indicated by the change in activities of catalase, superoxide dismutase and glutathione reductase (a) as well as the levels of GSH and TBARS (b)

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

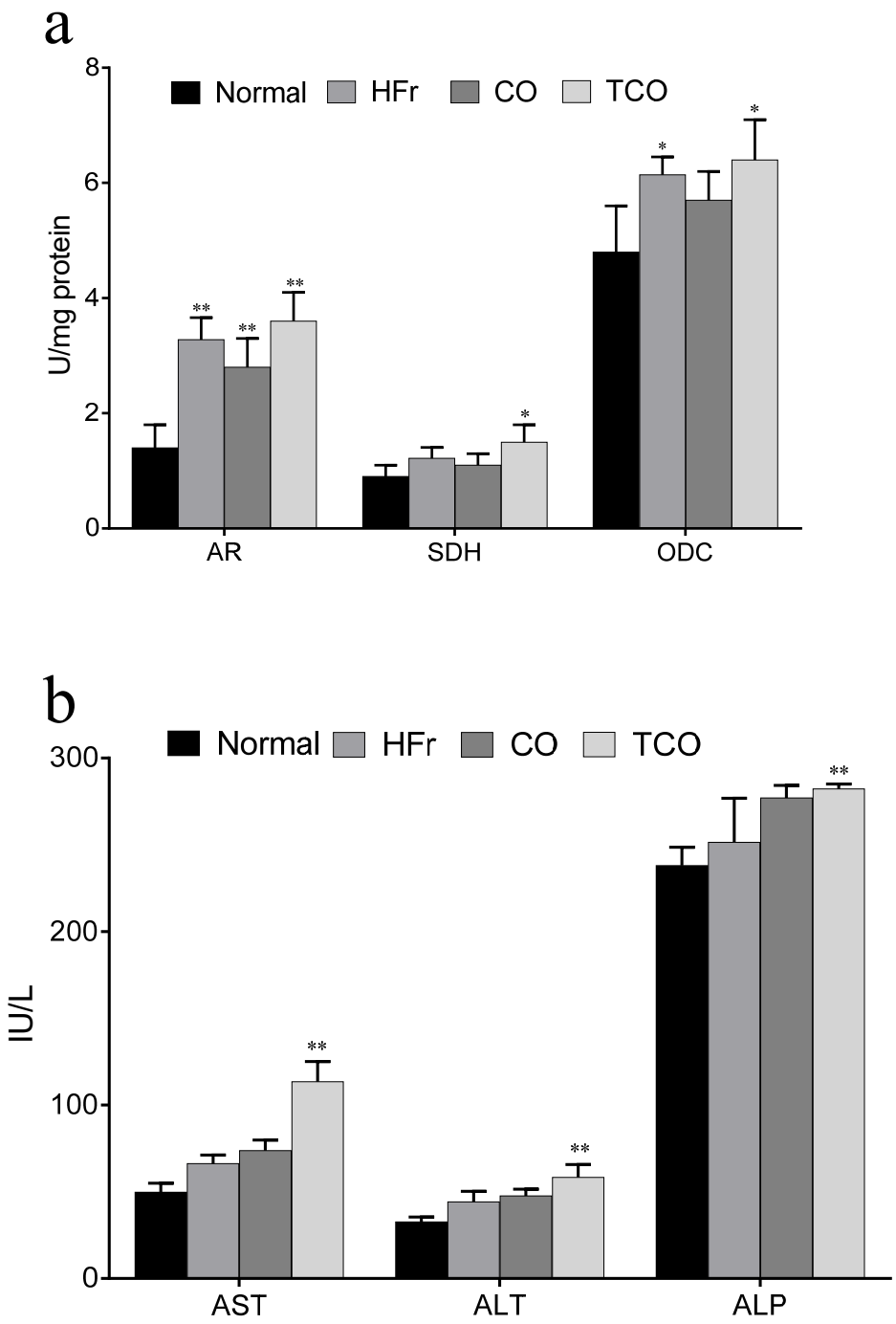


Figure 4.4: Changes in the activities of hepatic polyol pathway enzymes such as aldose reductase and sorbitol dehydrogenase (a) and liver function markers enzymes such as AST, ALT & ALP, in animals under different experimental diets after 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

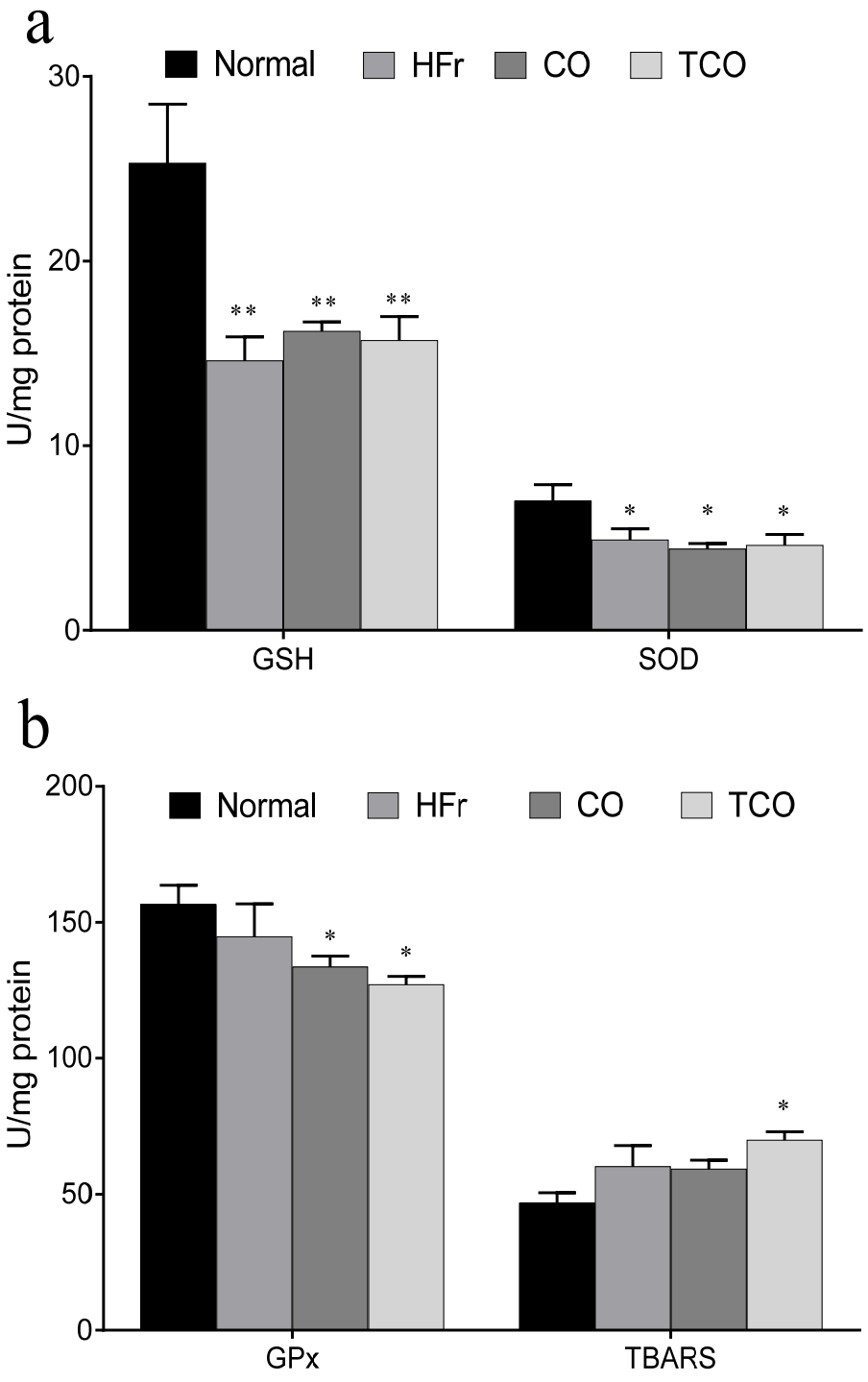


Figure 4.5 Changes in the redox parameters such as, GSH level and SOD activity (a) as well as of GPx activity and TBARS level (b) in the colon epithelial tissues of animals under different experimental diets for a period of 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Table 4.2 Changes in the activities of gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) enzymes (IU/L) in the serum of animals fed on HFr diet as well as those fed with CO or TCO containing diets

	Normal	HFr	CO	TCO
GGT	30.91± 4.85	38.50± 8.10	38.93± 5.33	47.83± 3.85*
LDH	170.80± 13.55	194.16± 9.70*	208.70± 13.86*	232.33± 10.58*

Table 4.3 Grades of hepatosteatosis in animals fed with HFr alone diet as well as CO or TCO supplemented groups

Characteristics	Normal	HFr	CO	TCO
Micro vesicular steatosis	0	1	1	2
Hepatocellular ballooning	0	1	1	2
Portal tract inflammation	0	1	1	1
Glycogenated nuclei	0	1	1	1
Lipogranuloma	0	0	0	1

(0- absence; 1- mild; 2-moderate; 3- severe)

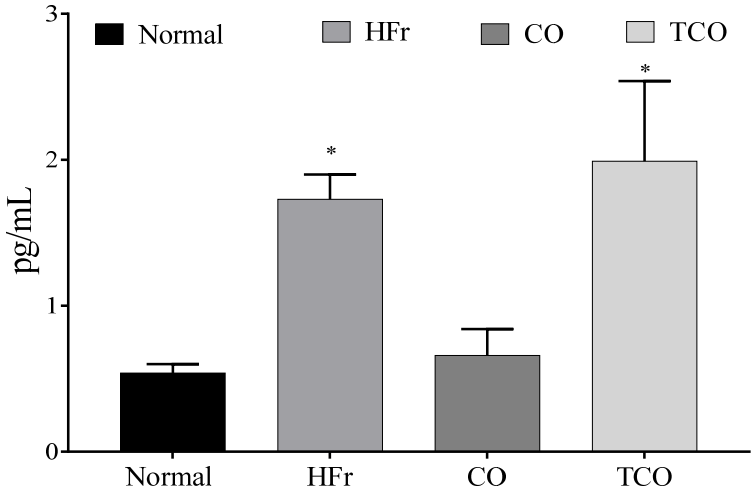
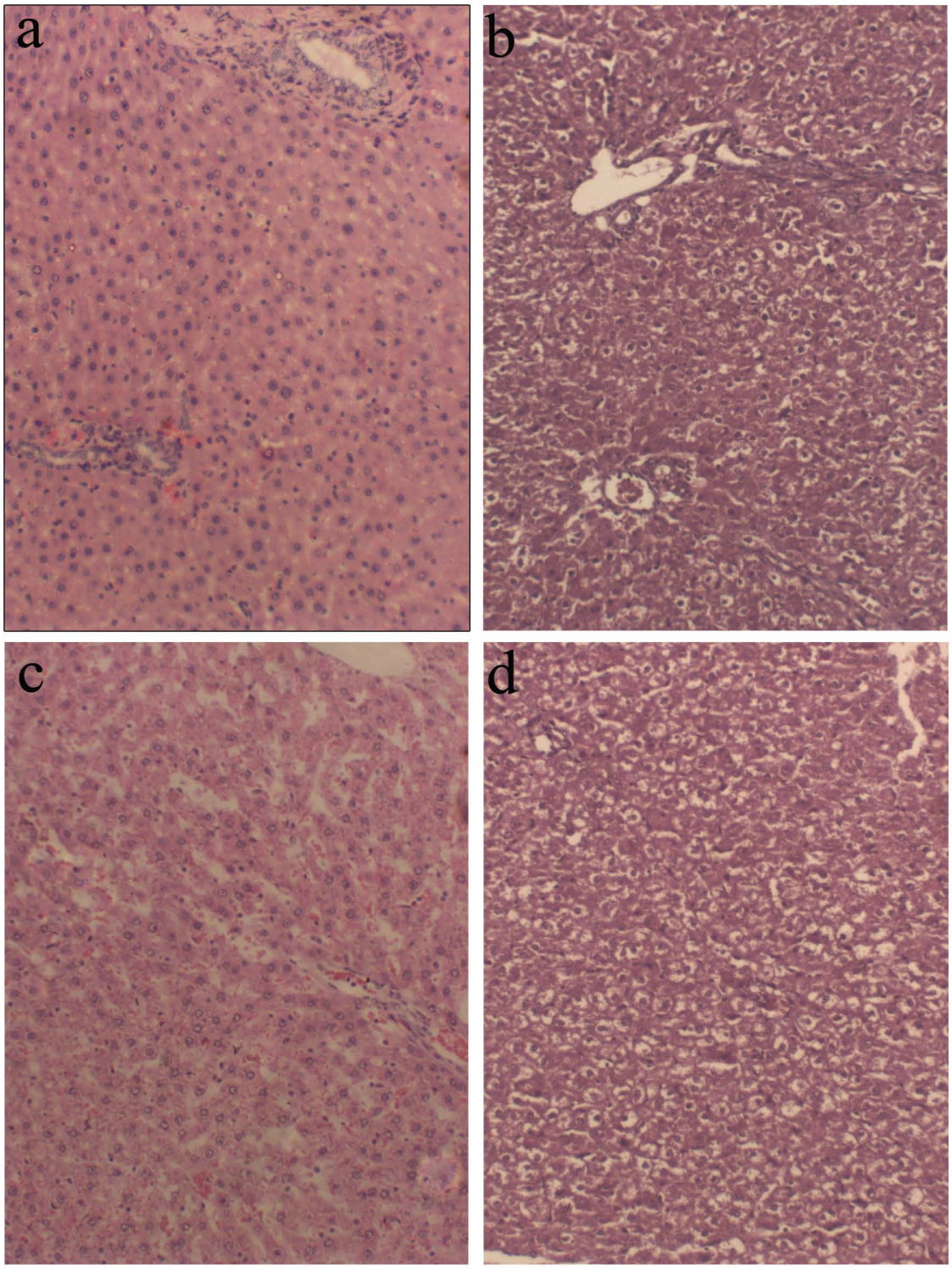
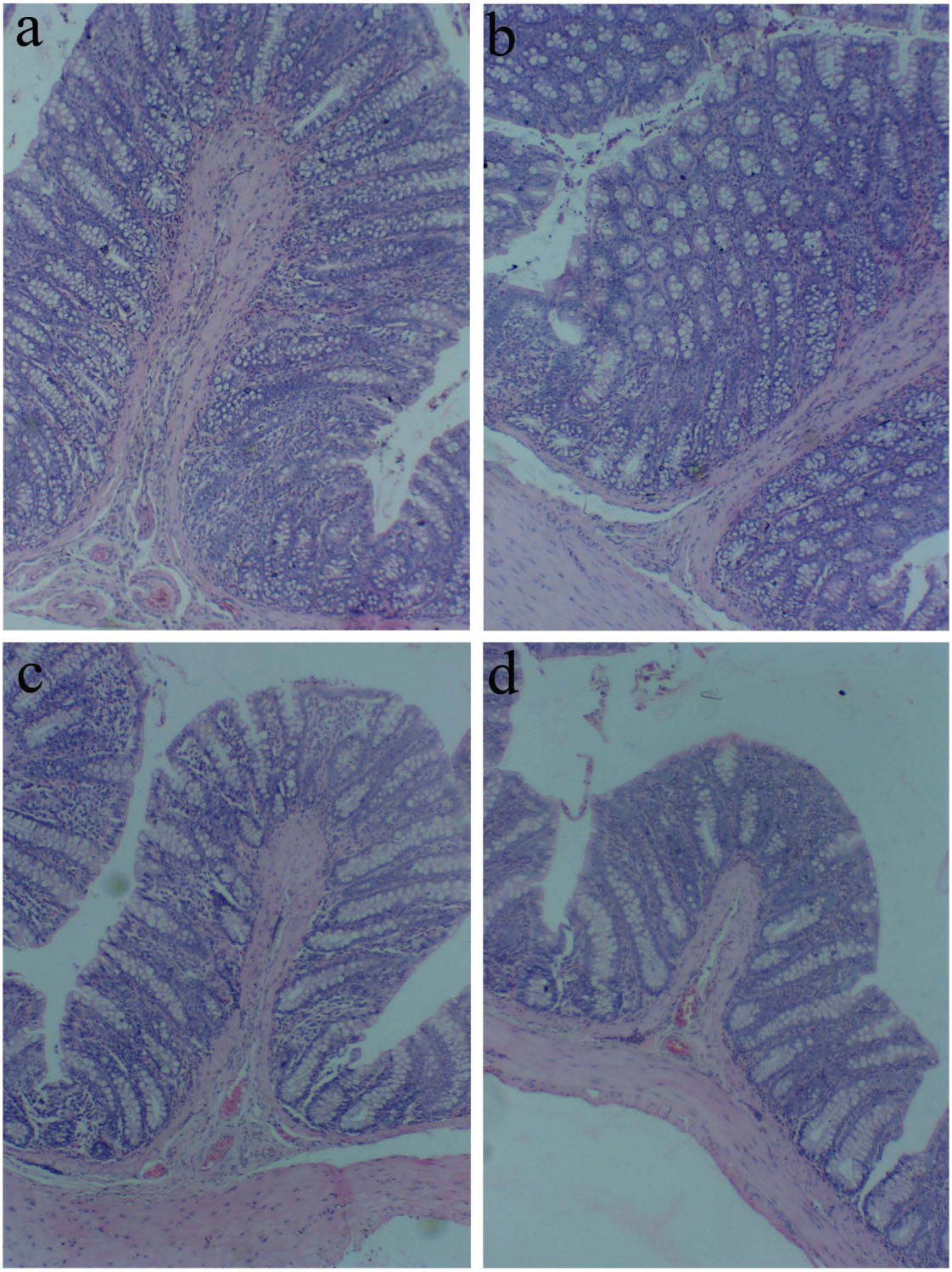


Figure 4.6 Change in the level of serum IL-6 in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)



Figrure 4. 7 Histomorphometry of the hematoxylin- eosin stained sections of liver tissues of reference diet (A), HFr (B), Coconut oil (C), and thermally oxidized coconut oil (D) containing diet fed animals. Photographs were taken under 200x magnification in Magnus INVI microscope (Chennai, India).



Figrure 4. 8 Photomicrography of the hematoxylin- eosin stained colon tissues of animals fed with reference diet (A), HFr alone diet (B), Coconut oil (C), and TCO (D) containing diet. Photographs were taken under 200x magnification in Magnus INVI microscope (Chennai, India).

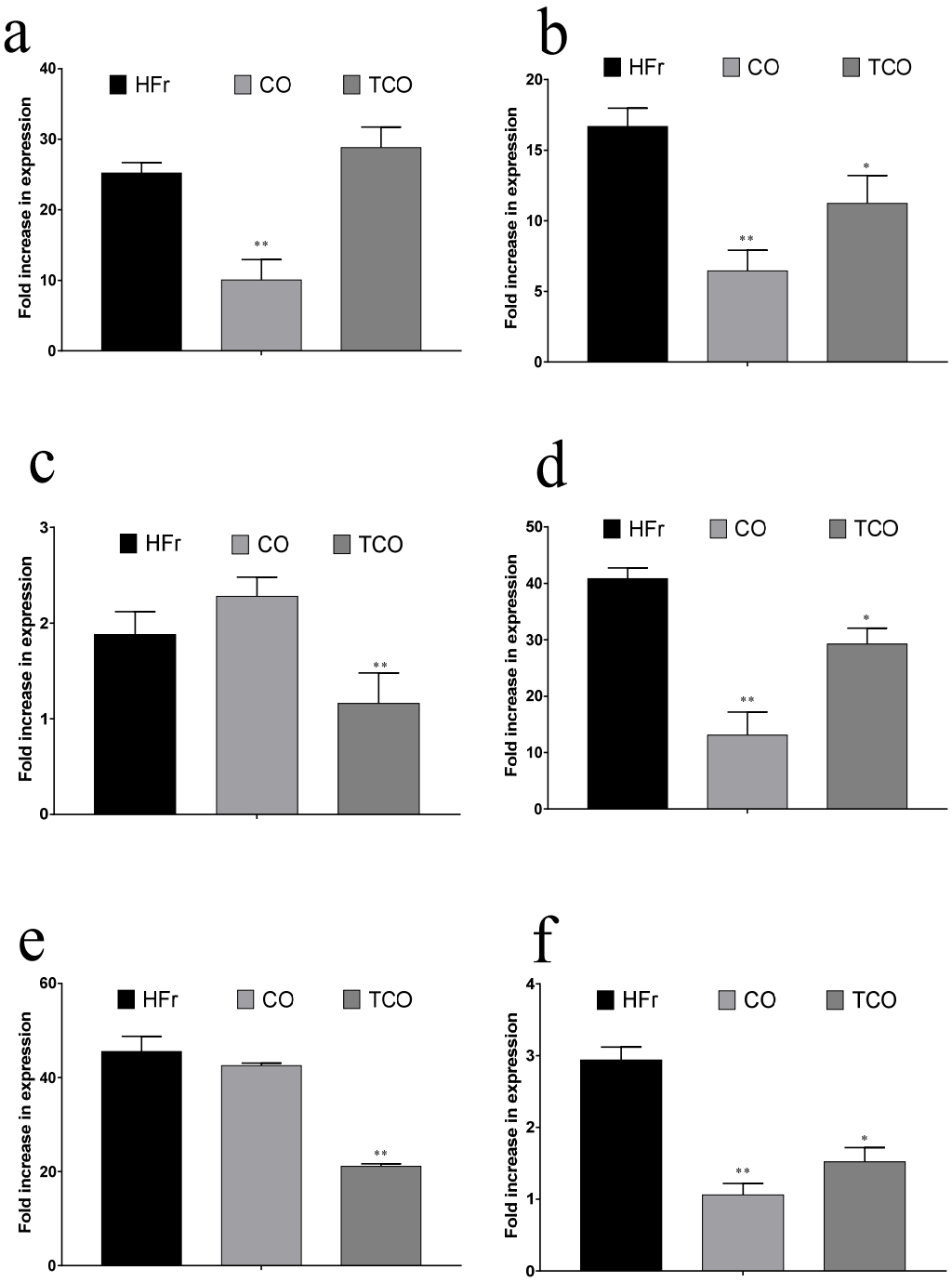


Figure 4.9 Changes in the expression of various genes in the colon tissues of rats analyzed by real time quantitative PCR: IL- 6 (a), TNF-α (b), P53 (c), Wnt-1 (d), PPARY (e) and Akt-1 (f).

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Chapter 5

Effect of thermally oxidized mustard oil on HFr-induced metabolic syndrome

5.1 Introduction

Mustard oil is one among the most common edible oil in India, which is rich in monounsaturated fats especially, erucic acid. Biological effects of dietary mustard oil have been identified. In acute myocardial infarction patients, mustard oil has shown to induce placebo effect and effectively modulates the lipid metabolism and reduce oxidative stress (Singh *et al.*, 1997). It has also been shown that mustard oil possess anti-mutagenic effect as indicated by reduction in chromosomal breaks (Choudhury *et al.*, 1997) and chemopreventive effects on colon tumors possibly due to its polyunsaturated fat content (Dwivedi *et al.*, 2003). Though the beneficial effects have been reported, because of the higher monounsaturated fatty acid content, these fats raise some health concerns also. It has been documented that dietary mustard oil can induce hyperalgesia (Jiang and Gebhart, 1998) and neurologic inflammation (Banvolgyi *et al.*, 2004). In addition, the MO has shown to enhance hepatic pre-neoplastic foci development in rats (Shukla and Arora, 2003). Consumption of mustard oil increases the serum triglycerides with a reduction in mitochondrial cardiolipin content (Sen and Gupta, 1980).

The biological effects of mustard oil are thought to be due to their higher monounsaturated fatty acid content. Compared to the polyunsaturated fatty acids (PUFA) having two or more double bonds, MUFA contains a single double bond. It can exist either in two forms, the naturally occurring cis- form or the trans- form. In the cis form, the hydrogen atoms are located on the same side of the unsaturation. The naturally occurring cis-form is widely present among the various edible oils of vegetable origin. Oleic acid (18:1) is the most commonly found MUFA followed by palmitoleic acid (16:1) and erucic acid (C22:1). Among the trans form of MUFA the common are elaidic acid (18:1) and brassic acid (trans C22:1). Mustard oil contains higher amount of erucic acid and oleic acid.

Like other fatty acids, MUFA are almost completely absorbed by the intestine and further they are oxidized for energy production, converted into other fatty acids, or incorporated into tissue lipids (Kohout *et al.*, 1971). Studies by Murphy *et al.* (2008) has shown that erucic acid is mainly taken up by the liver tissue followed by heart. In the liver tissue, the erucic acid is rapidly converted in to saturated fatty acids such as stearic acid; whereas in the heart tissue, the fatty acids are incorporated in to the

neutral lipids and used for the β-oxidation. However, erucic acid undergoes oxidation relatively at a slower rate both in heart and liver mitochondria (Christophersen and Bremer, 1972). Consumption of erucic acid rich diet reduced the oxygen uptake in heart and liver tissues of rats and the elevation in the levels of long-chain fatty acyl CoA has also been reported to be slow. Further studies by Christophersen and Bremer (1972) have shown that in addition to the reduced level of metabolism of erucic acid, elevated levels of erucylcarnitine further slows down the metabolism of other fatty acids such as palmitylcarnitine. This inhibition was uplifted by the presence of malate, suggested the inhibitory effect of erucylcarnitine on β-oxidation rather than TCA cycle. The inhibition of β-oxidation leads to the accumulation of lipids in the heart tissues, thereby inducing lipotoxicity, cardiac fibrosis and vacuolation (Gopalan *et al.*, 1974).

Compared to the fresh oils, there occur several oxidative modifications in thermally oxidized oils. A recent study has indicated that intake of oxidized mustard oil induces lipid accumulation and necrosis in the hepatic tissue of rabbits (Zeb and Rahman, 2017). Other than that no further information is available on the health impact of erucic acid rich oils, especially on hepatotoxicity and health of colon. Hence, the present chapter analyzes the effect of long term consumption of the fresh or thermally oxidized mustard oils along with a high fructose diet (60%) on insulin resistance and development of hepatosteatosis and changes in colon epithelia of male Wistar rats.

5.2 Materials and methods

5.2.1 Animals

Male Wistar rats weighing approximately 200-230 g on the average were supplied by the Kerala Veterinary and Animal Science University. The animals were fed a commercial diet from the Sai Durga feeds (Bangalore, India). The rats were randomly assigned into four groups of six rats each, on the basis of their weights using the Spiral method which resulted in almost equal total initial weights of groups. The rats were maintained at standard conditions and fed water *ad libitum*. The experimental procedure followed the established guidelines for the care and handling of laboratory animals by Institutional animal ethical committee and CPCSEA, Govt. of India (Approval No. ACRC/IAEC/15/06-(2)).

5.2.2 Preparation of the thermally oxidized oil

Fresh MO was purchased from local market and divided into two equal portions; one portion was stored at 4^{o} C to be used in the fresh oil containing diets later on in the experiment, while the other portion was heated at 160^{o} C for 48 h (González-Muñoz *et al.*, 2003). For heat treatment, these oils were put in 1 L glass beakers which were placed on a heating block set at the intended temperature. The thermally oxidized oils were stored at -20^{o} C in order to prevent further oxidation during storage before being included in the respective diets. The extent of lipid peroxidation in the oil was estimated by assaying TBARS, conjugated dienes and *p*-anisidine value (Commission, 1999).

5.2.3 Diets and experimental procedure

Experimental diets for inducing insulin resistance as based on the previous studies (Narayanankutty *et al.*, 2016; Prakash *et al.*, 2014; Singh *et al.*, 2015). The composition and experimental set up are described in Chapter 2.2.1.

5.2.3 Estimation of glucose tolerance

The animals were fasted overnight before oral glucose tolerance test. Glucose tolerance was measured using the method described by Narayanankutty *et al.* (2016). Briefly, rats were orally administered with 2 g/kg glucose following this blood glucose levels were measured using glucometer (One touch select, UK) at 0, 30, 60, 90 and 120 min intervals. The rate of glucose clearance and area under the curve were also calculated as described in Chapter 2.4.3.

5.2.4 Biochemical analysis

Various serum parameters such as total cholesterol, HDLc, triglycerides, ALT, AST, ALP, blood glucose were determined using commercially available kits Agappe diagnostics (Chennai, India) as per the manufacturer's instructions. Serum IL-6 was estimated by ELISA kit as per the manufacturer's instructions (Peprotech, Germany).

5.2.5 Analysis of oxidative stress and antioxidant activity

Liver tissue was excised and washed in ice cold saline. A small portion of each was fixed in 10% buffered formalin for histopathological studies. Liver homogenate was

prepared (10% w/ v) in Tris HCl buffer (0.1 M, pH 7.4) and used for the estimation of lipid peroxidation (Ohkawa *et al.*, 1979).

The homogenates were prepared by centrifuging at 10000 rpm for 15 min at 4^{o}C. The supernatant was collected and used for GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), catalase (Beers and Sizer, 1952) and GR (Carlberg and Mannervik, 1975) assays. The detailed description on the methodology has been given in Chapter 2.9 and its subdivisions.

5.2.6 Measurement of polyol pathway enzymes

Polyol pathway enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH) activities were measured by spectrophotometric procedures as described by Jang *et al.* (2010). The detailed procedure is described in the Chapter 2.8. Ornithine decarboxylase activity was determined according to the methods of Ngo *et al.* (1987), details of which are given in Chapter 2.7.3.

5.2.7 Histopathological analysis

A portion of the formalin fixed tissues (Liver and colon) were embedded in wax following dehydration with series of alcohol. Serial sections of the tissues were then taken in a microtome at a thickness of 4 μm and stained with hematoxylin and eosin. Histopathological examination was carried out by a pathologist who was blind to the plan of this study. Scoring of hepatosteatosis was carried out according to standard method (Brunt *et al.*, 1999). The detailed outline of procedures used is given in Chapter 2.10.

5.2.8 Real time quantitative PCR analysis

Total RNA was isolated using Trizol reagent (Chapter 2.11.1) and cDNA was synthesized using Verso cDNA synthesis kit (Chapter 2.11.2). Gene expression in the colon epithelial tissue of different experimental groups was analyzed by qPCR followed by ΔΔCT method. The details of primer and PCR cycling are given in Chapter 2.11.3. Data are represented as fold change of expression with respect to the untreated control group.

5.2.9 Statistical analysis

The values are represented as mean ±standard deviation. Statistical analysis of the data was done by one way ANOVA followed by Tukey- Kramer multiple comparisons test using Graph pad software (La Jolla, California, USA) (details are given in Chapter 2.12).

5.3 Results

5.3.1 Hyperglycemia and Insulin resistance and oral glucose tolerance

The animals fed with diet containing high fructose and different edible oils had a gradual increase in the blood glucose level in comparison with the normal (reference group) rats. Normal animals had an initial blood glucose level of 81.12±12.46 mg/dL, which gradually increased to 103.69 ± 5.72 over a period of 30 weeks (net increase of 21.18±3.21 mg/dL). In MO group of rats, from an initial level of 89.07±8.68, the blood glucose was raised to 211.43±12.80 mg/dL (net increase of 122.36mg/dL). In the TMO fed animals, blood glucose level had an increase from 89.62±7.08 to 233.98±8.47 mg/dL (net increase of 144.36mg/dL).

The fasting plasma insulin level (FPI) of normal animals was 45.71 ± 10.07 pmols/L and it was elevated in HFr fed animals to 66.66 ± 2.04 pmols/L. In fresh mustard oil fed animals, the FPI level was 71.43 ± 7.98, whereas it reduced in TMO containing diet fed animals to 68.47 ± 4.62 pmols/L.

HOMA indices were measured as the marker of insulin resistance and beta cell function. The percentage beta cell function (%B) was found to be 63.8 ± 13.5% in normal animals fed on reference diet. It was reduced to 22.0 ± 4.1% during high fructose consumption. In MO containing diet fed 18.5 ± 1.4animals, the %B was 23.1 ± 2.1 pmols/L and it further reduced to 18.5 ± 1.4 pmols/L in TMO containing diet fed animals (Table 5.1).

HOMA2 %S or percentage insulin sensitivity in normal animal was 116.9 ± 27.2% and it came down to 66.1 ± 0.5% in HFr group of animals. In MO containing diet fed animals, the %S was found to be 62.6 ± 7.4% and marginally reduced further in TMO group of animals (62.1 ± 4.6%) (Table 5.1).

Insulin resistance or HOMA-IR of normal animals was 0.89 ± 0.19 and it was elevated significantly to 1.51 ± 0.01 in HFr group of animals. In fresh (MO) and thermally oxidized (TMO) mustard oil containing diet fed animals, HOMA-IR was found to be 1.62 ± 0.20 and1.62 ± 0.12 (Table 5.1).

5.3.2 Oral glucose tolerance

In normal animals, from an initial level 100.12±1.24 mg/dL, blood glucose was elevated to 204.69 ± 2.54 mg/dL within 30 min (Figure 5.1). The level returned to normal within 120 min (19.96±1.0 AUC), with a glucose clearance rate of 52.23 ± 2.39 mg/dL/h. In HFr group, the OGT was 36.26±2.14 AUC. MO diet fed animals had reduced glucose tolerance (36.74 ± 1.84 AUC) than the TMO fed group (38.44 ± 1.92 AUC).

5.3.3a Effects on serum lipid profile

In normal diet fed animals, the serum total cholesterol was found to be 61.73±6.83 mg/dL and it was elevated to 89.1±7.6 mg/dL in HFR (Figure 5.2a). The TC level in MO and TMO containing diet fed animals (96.0 ± 5.8 and 96.3±6.0 mg/dL).

Triglycerides in normal diet fed rats were 84.46±16.92 mg/dL, and increased to 159.4 ± 11.6 mg/dL in HFr group. The TG level was increased to 189.7 ± 13.6 mg/dL and 195. 7 ± 13.2 mg/dL in MO and TMO containing diet fed groups (Figure 5.2a).

HDLc level of reference (normal diet fed) groups was 28.06±3.94 mg /dL which was reduced to 25.9 ± 1.8 mg/dL in HFr group. In MO and TMO containing diet fed groups the HDLc levels reduced to 26.9 ± 5.8 and 26.8 ± 4.2 mg/dL (Figure 5.2a).

5.3.3b Effects on hepatic lipid profile

The TC levels in the normal rats were 128.86±3.34 mg/g of tissue, which was significantly elevated in the HFr group to 199.1±21.6 mg/ g of tissue. In MO and TMO containing diet fed animals, the levels were 282.0±6.9 and 279.6±12.8 mg/g of tissue (Figure 5.2b).

Hepatic triglyceride level in normal diet fed animals was 164.89±15.63 mg/g tissue and elevated to 262.5±31.6 mg/ g of tissue in HFr group. The hepatic TG levels in

MO and TMO containing diet fed animals were 336.2±21.9 and 304.0±20.1 mg/g of tissue (Figure 5.2b).

The HDLc level in normal animal was 30.32±1.62 mg/g of tissue and reduced in HFr group to 23.1±2.8 mg/ g of tissue. In MO and TMO containing diet fed animals, the levels were 22.6±2.3 and 25.3±2.3 mg/g of tissue (Figure 5.2b).

5.3.4 Hepatic redox balance

Reduced glutathione levels in the untreated normal animal were 5.91 ± 0.24 nmoles/mg protein. It was reduced to 5.18 ± 0.33 nmoles/mg protein HFr diet fed animals. Animals fed with fresh or thermally oxidized mustard oils had 4.99 ± 0.22 and 4.28 ± 0.39 nmoles/mg protein (Figure 5.3b).

Catalase activity in the hepatic tissue of normal animals was found to be 73.85 ± 8.90 U/mg protein and it was reduced to 54.47 ± 10.60 U/mg protein in HFr group of rats. In MO containing diet fed animals, the catalase activity was 47.44 ± 3.84U/mg protein and it was further reduced to 31.94 ± 3.75 during dietary modification with TMO (Figure 5.3a).

Superoxide dismutase activity in normal animals fed with reference diet was 6.90 ± 0.28 U/mg protein (Figure 5.3a). In HFr group of animals, the activity was reduced to 4.11 ± 0.28 U/mg protein. In MO containing diet fed animals, the SOD activity was 4.87 ± 0.38 U/mg protein and it among the experimental group TMO had the least SOD activity (3.07 ± 0.18 U/mg protein).

The GR activity in normal and HFr groups were found to be 23.61±3.26 and 20.81 ± 3.9 U/mg protein. GR activity of MO group was reduced marginally to 20.87±0.82 U/mg protein; whereas in TMO containing diet fed animals, the GR activity was elevated to 27.46±0.9 U/mg protein (Figure 5.3a).

Serum and liver TBARS levels were found to be 4.21 ± 0.87 and 4.07 ± 0.85 nmoles/mg protein in reference diet fed animals. In HFr group, it was elevated to 5.19 ± 0.29 and 5.34 ± 0.72 nmoles/mg protein in serum and liver tissue. In MO containing diet fed animals, there was a respective increase in these parameters to 5.61 ± 1.15 and 6.49 ± 0.87 nmoles/mg protein. TMO containing diet fed animals had the highest

levels of lipid peroxidation products; in serum and hepatic tissue the levels were 6.77 ± 0.88 and 7.16 ± 0.94 nmoles/mg protein (Figure 5.3b).

5.3.5 Effect on polyol pathway enzymes and ornithine decarboxylase

In the normal rats the AR activity was 1.42±0.41 U/mg protein and it was elevated in HFr group of animals to 3.28±0.38 U/mg protein. In MO and TMO containing diet fed rats, there was a marginal increase in the activity (3.12±0.49 and 3.69±0.66 U/mg protein, respectively) (Figure 5.4a).

A similar increase in SDH activity of HFr group (1.22±0.19 U/mg protein) was noted from the normal animals (0.94±0.18 U/mg protein). The SDH activities in MO and TMO group of animals were 1.42±0.18 and 1.49±0.41 U/mg protein (Figure 5.4a).

Ornithine decarboxylase activity was found to be 4.78±0.76 U/mg protein in normal rats and it was increased to 6.14±0.31 U/mg protein in HFr group. The activity was elevated in the MO and TMO containing diet fed rats to 5.95±0.95 and 6.37±0.29 U/ mg protein (Figure 5.4a).

5.3.6 Liver function parameters

The liver function marker enzyme AST activity was found to be 49.7±5.2 IU/L in normal animals. In HFr group, AST activity was increased to 66.3±4.9 IU/L. In MO containing diet fed animals, it was elevated to 69.9±5.3 IU/L and in TMO containing diet fed animals it was 107.1±1.6 IU/L (Figure 5.4b).

Alanine transaminase activity in normal rats was 32.6±2.8 IU/L. and further increased in HFr group to 44.2±6.1 IU/L. In MO containing diet fed group, it was increased to 47.7±2.3 IU/L and further elevated to 64.0±3.6 IU/L in TMO containing diet fed rats.

Alkaline phosphatase activity of the normal group of rats was 238.0±10.6 IU/L, which was increased to 251.5±25.4 IU/L in HFr alone fed group of animal. The activity was further elevated to 263.4±4.0 in MO containing diet fed group. In TMO containing diet fed animals, ALP activity was 288.4±10.2 IU/L (Figure 5.4b).

5.3.7 GGT and LDH activity and IL-6 levels

The GGT activity of the normal animals fed on a reference diet was 30.91 ± 4.85 IU, which was elevated marginally to 38.50 ± 8.10 IU in HFr group. In MO containing

diet fed animals the activity was 38.29 ± 5.48 IU and it was significantly elevated to 47.56 ± 6.11 IU in TMO containing diet fed animals (Table 5.2).

Lactate dehydrogenase activity in the animals fed on a reference diet was 170.80 ± 13.55 IU and it was increased to 194.16 ± 9.70 IU in HFr group. In MO and TMO containing diet fed animals, the activities were 214.73 ± 7.75 IU and it was 235.82 ± 17.11 IU (Table 5.2).

Serum IL-6 level of normal animal was 0.54± 0.06 pg/mL (Figure 5.6), which was increased significantly to 1.73± 0.17 pg/mL by 30 weeks of HFr diet feeding. In fresh MO containing diet fed animals, the level was increased to 1.22± 0.14 pg/mL and a further elevated IL-6 level was noticed in TMO containing diet fed animals (4.97± 0.82 pg/mL).

5.3.8 Colon redox balance

As shown in Figure 5.5a, the reduced glutathione level of colon epithelial cells in the normal animal was 25.3 ± 3.2 nmoles/mg protein, which was reduced in HFr fed animals to 14.60 ± 1.30 nmoles/mg protein. In MO containing diet fed animals, there was 12.0 ± 0.7 nmoles/mg protein of GSH; and in TMO group it was 12.0 ± 1.2 nmoles/mg protein.

Superoxide dismutase activity in the normal animal fed on a reference diet was 7.0 ± 0.9 U/mg protein. In high fructose diet group a reduced SOD activity of 4.90 ± 0.6 U/mg protein was estimated. It was further reduced to 2.7 ± 0.2 and 2.9 ± 0.3 U/mg protein in MO and TMO containing diet fed animals (Figure 5.5a).

The glutathione peroxidase activity in the colon epithelial cells of normal rats was 156.5 ± 7.2 U/mg protein and it was reduced to 144.7 ± 12.10 U/mg protein in HFr diet fed animals. It was reduced further in MO group to 113.4 ± 5.4 U/mg protein and in TMO group it was 110.4 ± 4.5 U/mg protein (Figure 5.5b).

The lipid peroxidation products, TBARS, were estimated to be 46.7 ± 3.9 nmoles/mg protein in normal animals. In HFr group of rats, it was increased to 60.22 ± 7.70 nmoles/mg protein. In MO containing diet fed animals, 59.4 ± 2.9 nmoles/mg protein of TBARS was estimated and it was significantly increased to 79.4 ± 5.0 nmoles/mg protein, in TMO containing diet fed animals (Figure 5.5b).

5.3.9 Histopathological analysis

Histopathological analysis of the liver tissues of animals fed with normal diet a), Hfr group (b) and those fed on high fructose and MO (c) and TMO (d) fed animal are shown in Figure. 5.7 The reference diet (a) fed animals showed normal hepatic architecture with normal central venous system, the portal triads, sinusoidal spaces and Kupffer cells. However MO and TMO diet fed groups showed signs of hepatosteatosis, observed as microvesicles. TMO containing diet fed animals showed progressed hepatosteatosis than those fed with MO diet. Grading of hepatosteatosis in shown in the Table 5, where it was clear that animals fed with TMO had a progressed NAFLD with macrovesicular steatosis and hepatocellular ballooning.

Colon histology of untreated animals (a) and those fed with fresh oil (c) containing diet showed normal intestinal glands and villi, with mucous and serosa layer appearing normal. In HFr group and those fed with TMO containing diet (d) had mild and diffused infiltration of lymphocytes, plasma cells and polymorphonuclear cells indicating possible inflammatory changes (Figure 5.8).

5.3.10 Gene expression profile using qPCR analysis

IL-6, a pro-inflammatory cytokine expression was increased by 25.24 ± 1.47 fold in HFr group. In fresh MO containing diet fed animals, there was 9.85 ± 1.58. However, in TMO containing diet fed animals the expression was increased by 58.49 ± 2.17 fold. Tumor necrosis factor α expression in HFr group was increased by 16.68 ± 1.30 fold, which was increased by11.88 ± 0.82 and 17.39 ± 2.25 fold in MO and TMO containing diet fed animals.

Expression of P53 gene in HFr group was increased by 1.88 ± 0.24 fold. In MO and TMO containing diet fed animals, the increase was 1.27 ± 0.10 and 1.64 ± 0.12 fold. In HFr group, Wnt-1 gene expression was 40.84 ± 1.89 fold higher than the normal rats, whereas in MO and TMO group, it was increased by 11.88 ± 3.30 and 41.64 ± 1.82 fold. In HFr group of animals, expression of peroxisome proliferator activator receptor-γ (PPAR γ) was increased by 45.55 ± 3.22 fold. In MO and TMO containing diet fed animals, the increase was 35.51 ± 0.15 and 12.30 ± 1.96 fold. Protein kinase B (Akt) expression in HFr was increased by 2.94 ± 0.18 fold, which was 1.31 ± 0.12 and 3.58 ± 0.15 fold in MO and TMO groups.

5.4 Discussion

Natural edible oils are rich sources of medium and long chain saturated or unsaturated fatty acids. Heating of these oils, especially containing unsaturated fatty acids, generates fatty acid decomposition products mostly carbonyl molecules. These fatty acid derived molecules are known to induce toxic and inflammatory signaling in the body (Ramana *et al.*, 2013). In addition to these, studies reported the formation of triacylglycerol polymers and dimers (Bastida and Sánchez-Muniz, 2001) and also toxic cyclic fatty acid monomers (CFAM) in deep fried vegetable oils (Lamboni *et al.*, 1998). Our study has documented that deep frying of MO increases peroxide and aldehyde molecules (Chapter 3).

In this study, compared to reference diet (normal rat chow) fed animals, rats fed with fructose and fresh or thermally oxidized MO containing diet shows increased levels of blood glucose and reduced glucose tolerance. Consumption of the thermally oxidized or fresh oils along with high fructose results in hyperglycemia and development of insulin resistance. Associated with this, an increase in TG and reduction in HDLc levels are seen in fructose along with fresh or thermally oxidized oil containing diet fed animals. According to Song *et al.* (2015), insulin resistance is manifested by dyslipidemia where increased levels of TG and low HDL are evident. Total cholesterol and LDL levels are also found to be higher among these groups. Therefore, it is assumed that insulin resistance and associated hyperlipidemia might have contributed to the progressive hepatosteatosis which is observed comparatively high in fructose and TMO containing diet fed animals.

Dyslipidemia is often known to be involved in several of the hepatic disorders. Hypertriglyceridemia is the major contributor of hepatic damages by the accumulation of triglycerides in liver leads to the vacuolation of hepatocytes and disrupting their normal functioning (Perry *et al.*, 2013). Here in this study, animals fed with a diet containing fructose (60%) and the mustard oils (fresh and fried) shown to have higher triglyceride levels in comparison with normal or HFr alone diet fed animals. It is expected that lipid oxidation products formed during the deep frying of these oils such as CFAM are known to inhibit the lipase activity (Lamboni *et al.*, 1998). This probability should also be considered while explaining the hypertriglyceridemia observed in this study. On the contrary, studies have reported that a diet rich in

medium chain saturated and monounsaturated fatty acids reduces the triglyceride levels in body by enhancing the enzymatic activity of lipases (Kris-Etherton *et al.*, 1999; Wang *et al.*, 2015). Thus it is also possible that the changes observed in these animals may be partially caused by high fructose intake in these animals (Briand *et al.*, 2012).

Prolonged insulin resistance conditions are also reported to have elevated activities of LDH (Zappacosta *et al.*, 1995) and GGT (Haghighi *et al.*, 2011), which are the indicators of oxidative damages to tissues (Ramos *et al.*, 2013). In our study, thermally oxidized mustard oil consumed groups have increased levels of LDH, indicating a possibly higher oxidative damages compared to HFr alone or MO containing diet fed animals. Corroborating with this, higher levels of lipid peroxidative changes are noted in TMO group, which is in line with studies by Adam *et al.* (2008), where increased lipid peroxidation is noted in fried palm oil fed rats. Previous reports have indicated that thermally oxidized Sunflower and Peanut oils contain higher levels of CFAM (Romero *et al.*, 2006). Here in this study TMO contain more lipid hydroperoxides and aldehydes than their fresh counterparts. The oxidative and inflammatory insults by these oxidation molecules may be responsible for the observed increase in lipid peroxidation products in the serum and liver tissue of fried oil fed animals. Accumulation of these lipid oxidation products are often recognized as a risk factor for many of the liver diseases including non-alcoholic fatty liver (Konishi *et al.*, 2006; Poli *et al.*, 1987).

Providing further insight in to this, comparatively higher reduction in the level of GSH is observed in TMO fed rats. Intra cellular tripeptide GSH is actively involved in the elimination of toxic radicals including peroxides. Polyol pathway is an important determinant of GSH level especially AR, which is a key enzyme involved in Polyol pathway, has significant role in maintaining GSH level (Bravi *et al.*, 1997). Here in this study, an increase in the aldose reductase activity is observed in TMO fed animals. Possibly, hyperglycemia associated activation of polyol pathway, as observed in the TMO fed rats may be responsible for the observed reduction in GSH levels.

Along with the reduced level of GSH, the diminished activity of hepatic antioxidant enzymes, comprising of superoxide dismutase and catalase, are also observed in the

TMO groups, which are in line with the previous studies (Narasimhamurthy and Raina, 1999; Purushothama *et al.*, 2003). Reduction in the antioxidant enzyme activity is thought to be involved in the progression of NAFLD (Koruk *et al.*, 2004). These observations are in corroboration with the increased incidence of microvesicles and hepatocellular ballooning seen in the TMO fed rats. These results thus indicate a comparatively higher degree of hepatic damage in the animals fed with high fructose and thermally oxidized edible oil.

Similar increase in the TBARS level with a concomitant reduction in the SOD activity and GSH levels are also observed in the colon tissues of TMO containing diet fed animals. Antioxidants are molecules which protects the body from the oxidative damages caused by various free radicals and oxidative molecules (Powers and Jackson, 2008). Reduction in the detoxification systems of the body, as observed in thermally oxidized MO fed animals, can increase the accumulation of the peroxidation products and other free radicals, leading to increased modifications of macromolecules including lipids and protein, in these animals. Lipid carbonyls and its protein adducts are known to promote inflammation in the colon tissues (Tabak *et al.*, 2011). In comparison with fried coconut oil fed animals (Chapter 4), those who are fed with a diet containing thermally oxidized mustard oil had higher TBARS level in the colon epithelium. This might be due to the increased levels of oxidative modifications taken place in the MO during thermal oxidation due its high unsaturated fat content, such as erucic acid (C22:1) and alpha-linoleic acid (C18:3).

Increased oxidative damages often lead to the secretion of pro-inflammatory cytokines (Elmarakby and Sullivan, 2012). In our study, the increase in the level of inflammatory cytokines such as IL-6 and TNF-α has been to a greater extent in TMO groups. The increase in inflammatory conditions of colon in TMO can be correlated to the comparatively lower expression of PPARγ than fresh oil fed groups. PPARγ is a gene that is highly expressed in the colon epithelial cells (Vidal-Puig *et al.*, 1997), the impaired expression of which leads to chronic inflammation modulated by NF-KB (Dubuquoy *et al.*, 2006). Reduction in the PPARγ expression also leads to a subsequent reduction in the expression of another tumor suppressor gene, PTEN, which inhibits Akt activation in colon cells (Patel *et al.*, 2001). It is thus possible that the increase in Akt-1 & Wnt-1 expression in TMO containing diet fed animals may be

due to the reduced activation of PPARγ, and may have a key role as a pro-carcinogenic response over long term.

Previously, MCFA rich CO during thermal oxidation has been found to generate triglyceride polymerization products (Chapter 3). Further, the consumption of TCO exacerbated the HFr induced hepatosteatosis in the rats though it is less damaging to the colon epithelial tissues (Chapter 4). In contrast, TMO with its higher oxidative stress and chronic inflammation is more damaging to Colon tissues than liver tissues. Further, the elevated expression of proliferative genes and reduction in anti-proliferative genes in Colon tissues of animals fed TMO feeding, possibly indicate a pro-carcinogenic potential over time. The role of erucic acid oxidation products may be key in the toxic effects of MO; however further studies are necessary to ascertain this fact. In conclusion, the use of unsaturated fat containing edible oils may be cautioned for culinary purposes, where deep frying is involved.

Table 5.1 Changes in the blood glucose level, plasma insulin level and various HOMA indices in animals fed with HFr alone as well as CO or TCO containing diet over a period of 30 weeks

Parameters	Normal	HFr	MO	TMO
HOMA2 %B	63.8± 13.5	22.0± 4.1***	23.1± 2.1***	18.5± 1.4***
HOMA2 %S	116.9± 27.2	66.1± 0.5***	62.6± 7.4***	62.1± 4.6***
HOMA2 IR	0.89± 0.19	1.51± 0.01***	1.62± 0.20***	1.62± 0.12***
Glucose (mg/dL)	103.69 ± 5.72	212.97± 21.08**	211.43±12.8**	233.98±8.47**
Insulin (pg/L)	45.71± 10.07	66.66± 2.04**	71.43± 7.98***	68.47± 4.62**

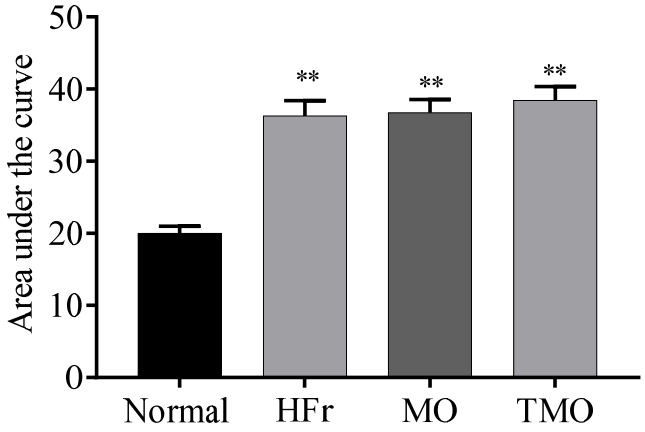


Figure 5.1 Effect of MO or TMO supplementation on HFr diet induced oral glucose intolerance

(* indicates significant difference at p<0.05; ** indicates p<0.01; *** indicates p<0.001)

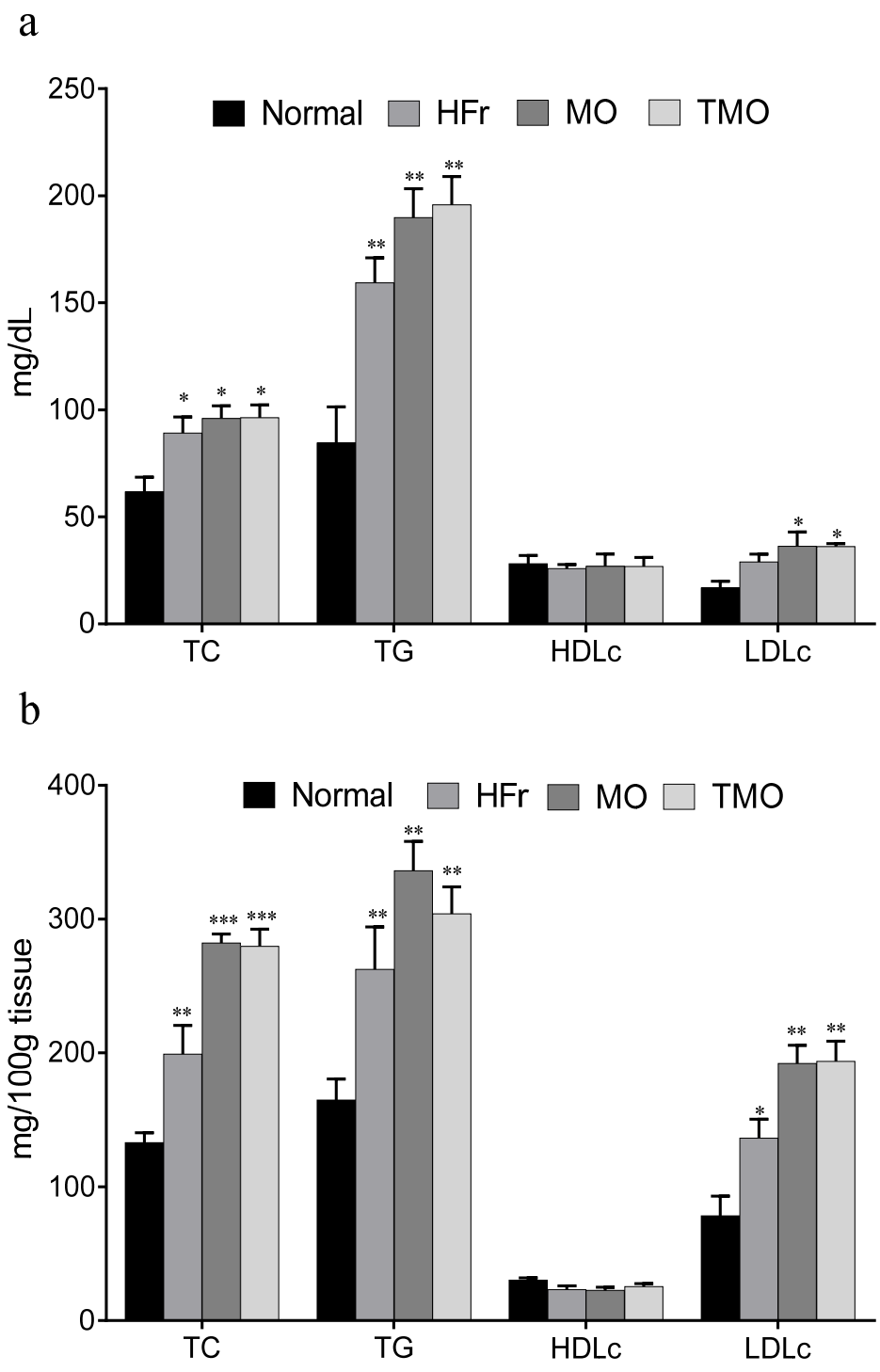


Figure 5.2: Serum (a) and hepatic (b) lipid profile of animals fed with the reference diet, HFr alone diet and those supplemented with MO or TMO for a period of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

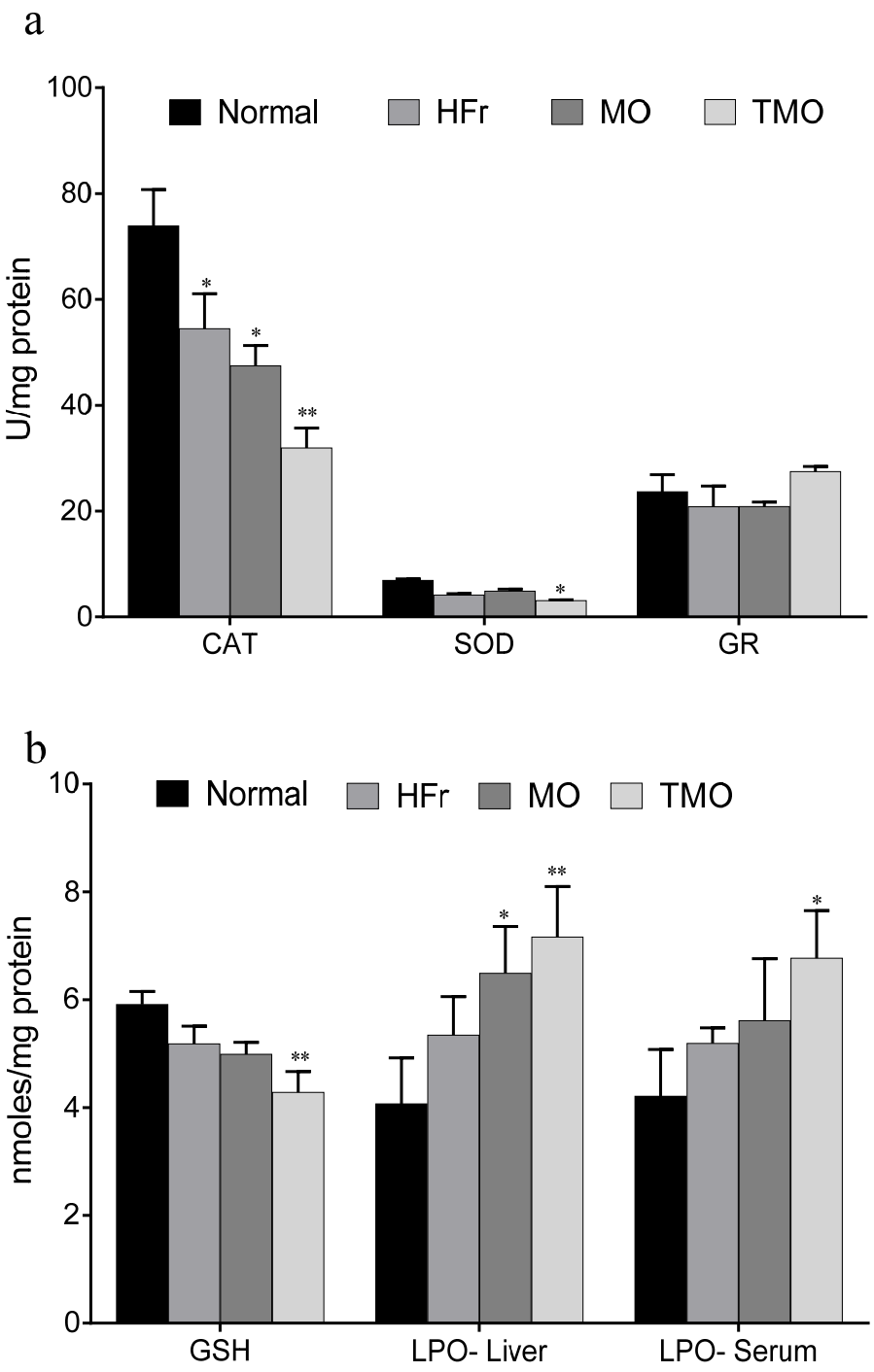


Figure 5.3: Effect of MO or TMO supplemented diet on HFr induced hepatic redox imbalance as indicated by the alterations in the activities of catalase, superoxide dismutase and glutathione reductase (a) activities as well as by the variation in the levels of hepatic GSH and TBARS

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

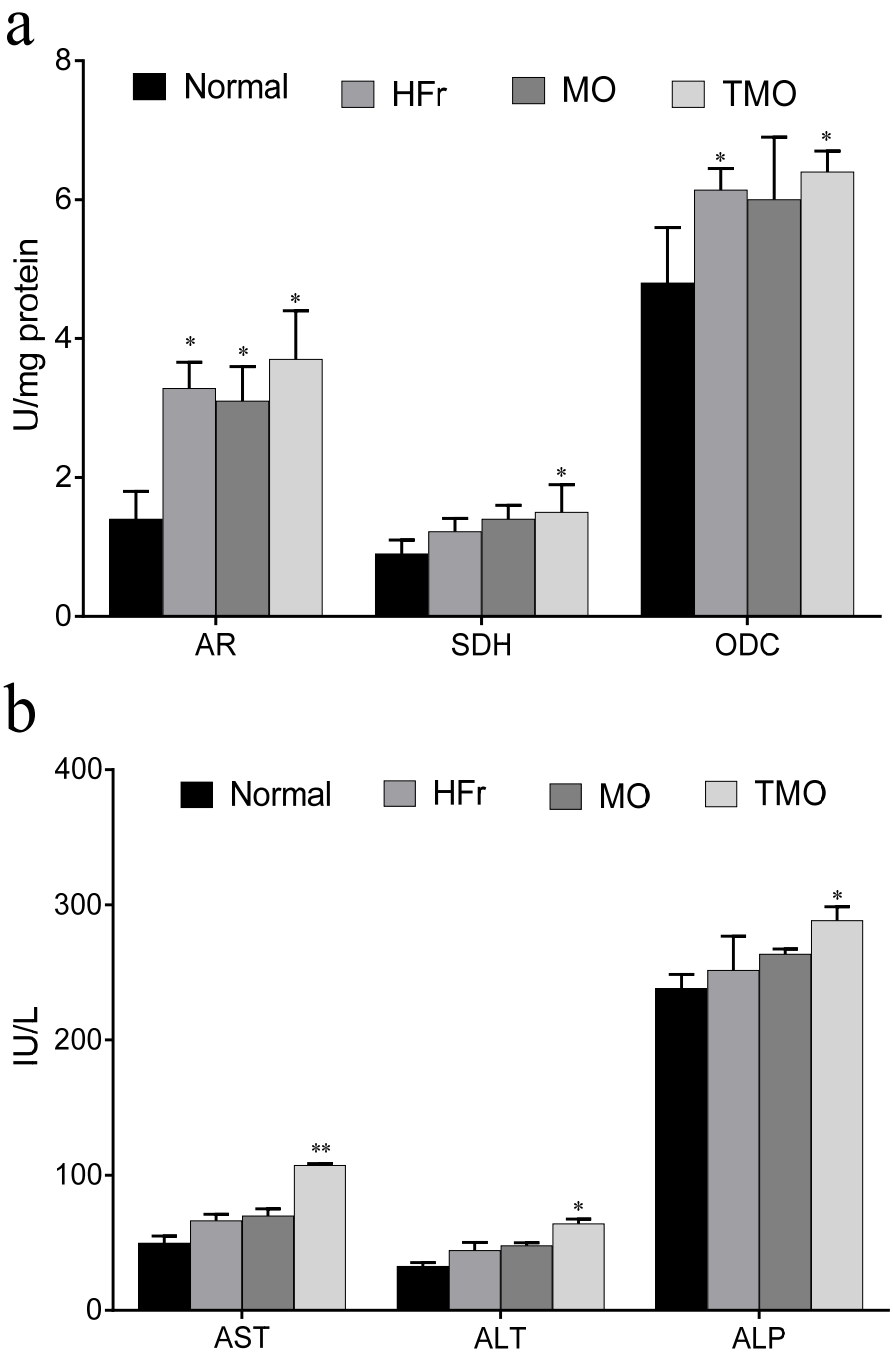


Figure 5.4: Changes in the activities of hepatic polyol pathway enzymes such as aldose reductase and sorbitol dehydrogenase (a) as well as liver function markers enzymes (AST, ALT & ALP) in animals under different experimental diets after 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

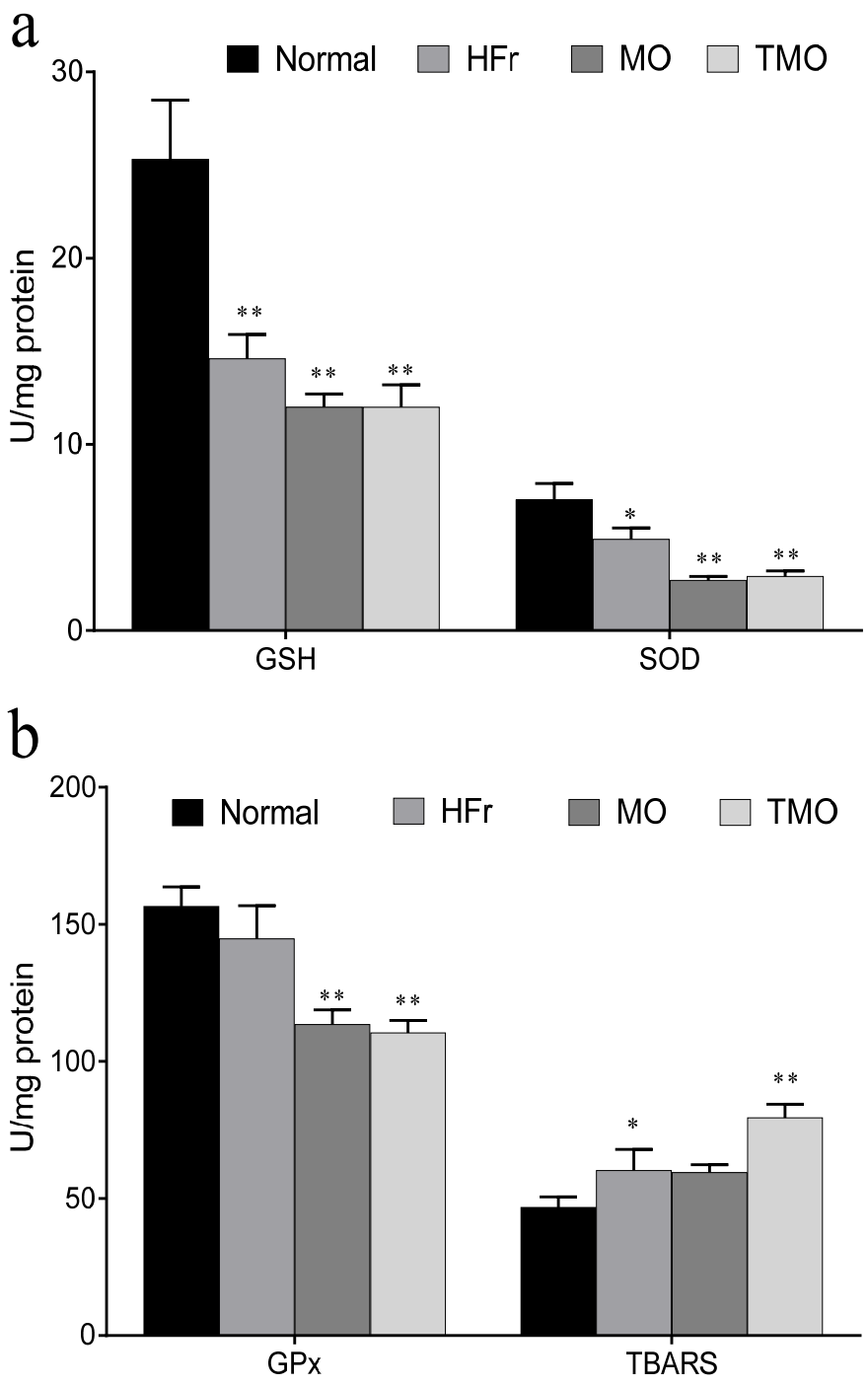


Figure 5.5 Changes in GSH and TBARS level and activities of SOD and GPx in the colon epithelial tissues of animals under different experimental diets over a period of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Table 5.2 Changes in the activities of gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) enzymes (IU/L) in the serum of animals fed on reference as well as different experimental diets

	Normal	HFr	MO	TMO
GGT	30.91± 4.85	38.50± 8.10	38.29± 5.48	47.56± 6.11*
LDH	170.80± 13.55	194.16± 9.70*	214.73± 7.75**	235.82± 17.11**

Table 5.3 Grade of hepatosteatosis in reference diet and different experimental diets containing MO or TMO

Characteristics	Normal	HFr	MO	TMO
Micro vesicular steatosis	0	1	1	2
Hepatocellular ballooning	0	1	1	2
Portal tract inflammation	0	1	1	1
Glycogenated nuclei	0	1	1	1
Lipogranuloma	0	0	0	0

(0- absence; 1- mild; 2-moderate; 3- severe)

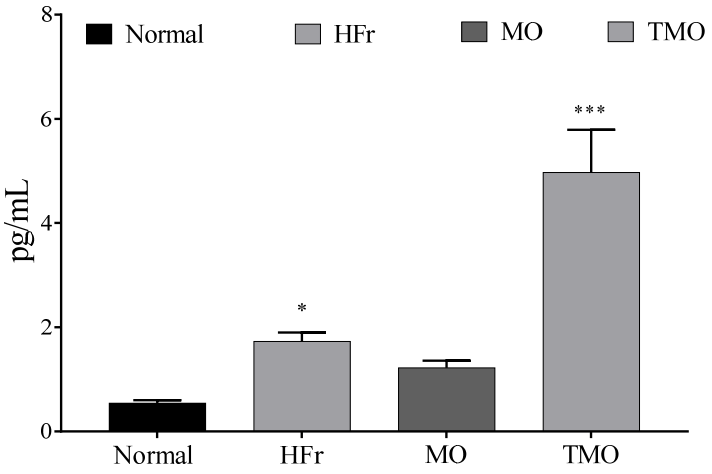
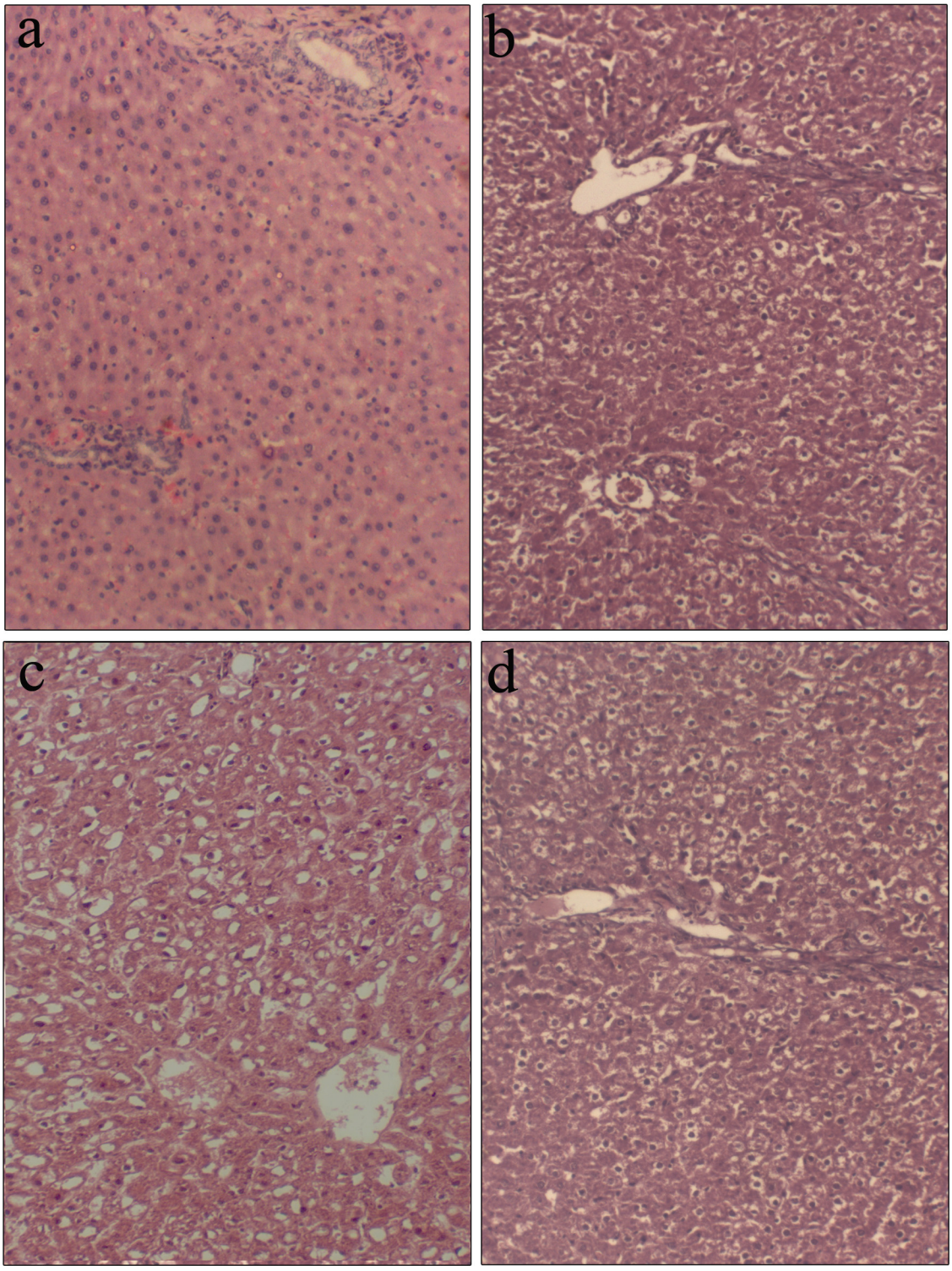
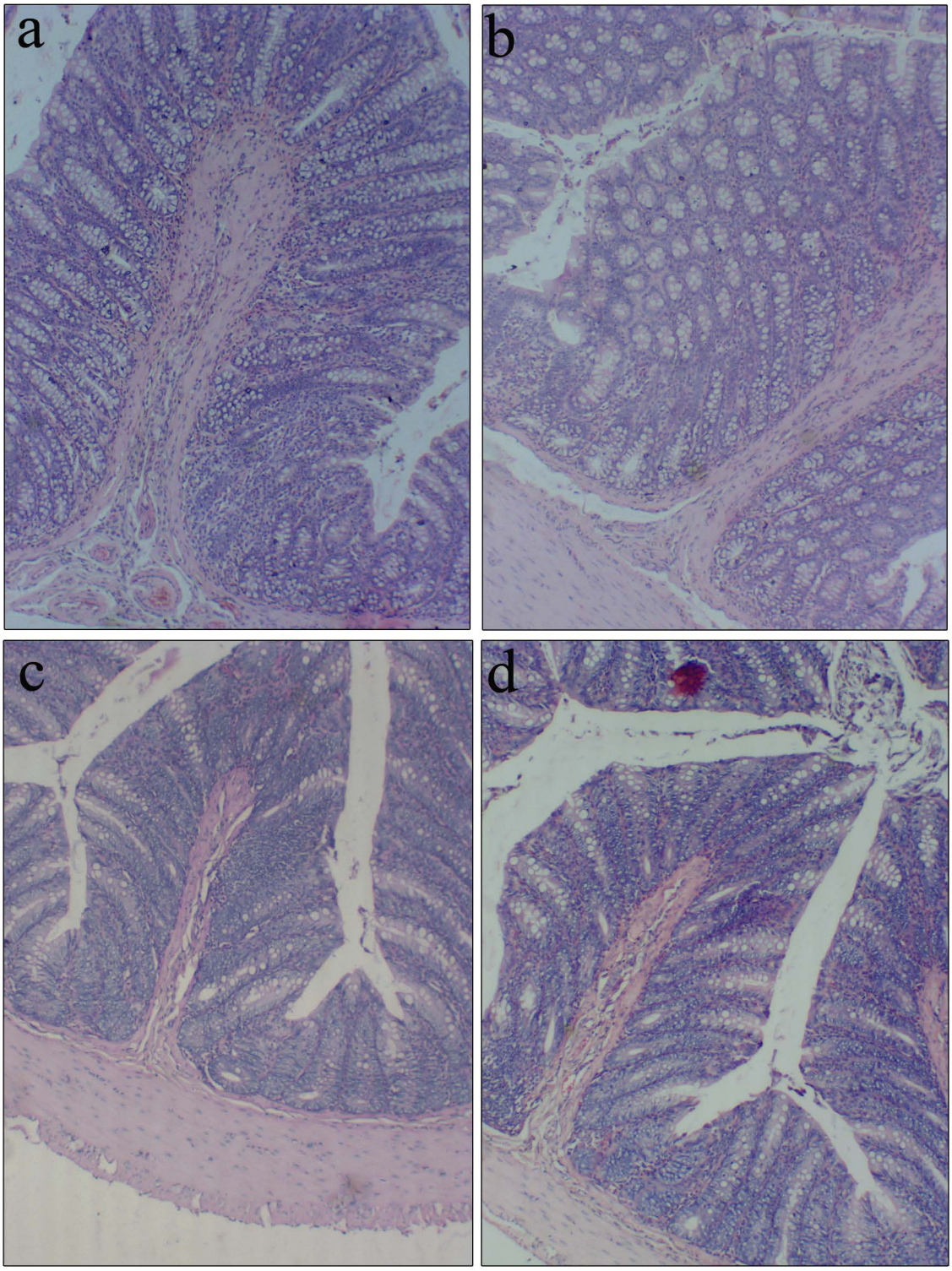


Figure 5.6 Change in the level of serum IL-6 (pg/ mL) in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)



Figrure 5. 7 Histomorphometry of hematoxylin- eosin stained hepatic tissues of animals fed with reference diet (A), HFr alone diet (B), mustard oil (C), and thermally oxidized mustard oil (D) containing diet. Photos were captured at 200x magnification in Magnus INVI inverted microscope (Chennai, India).



Figrure 5. 8 Changes in the colon epithelial histology of reference diet fed animals (A) and those consumed HFr alone diet (B), mustard oil (C), and TMO (D) containing diet. Images were captured at 200x magnification in Magnus INVI microscope (Chennai, India).

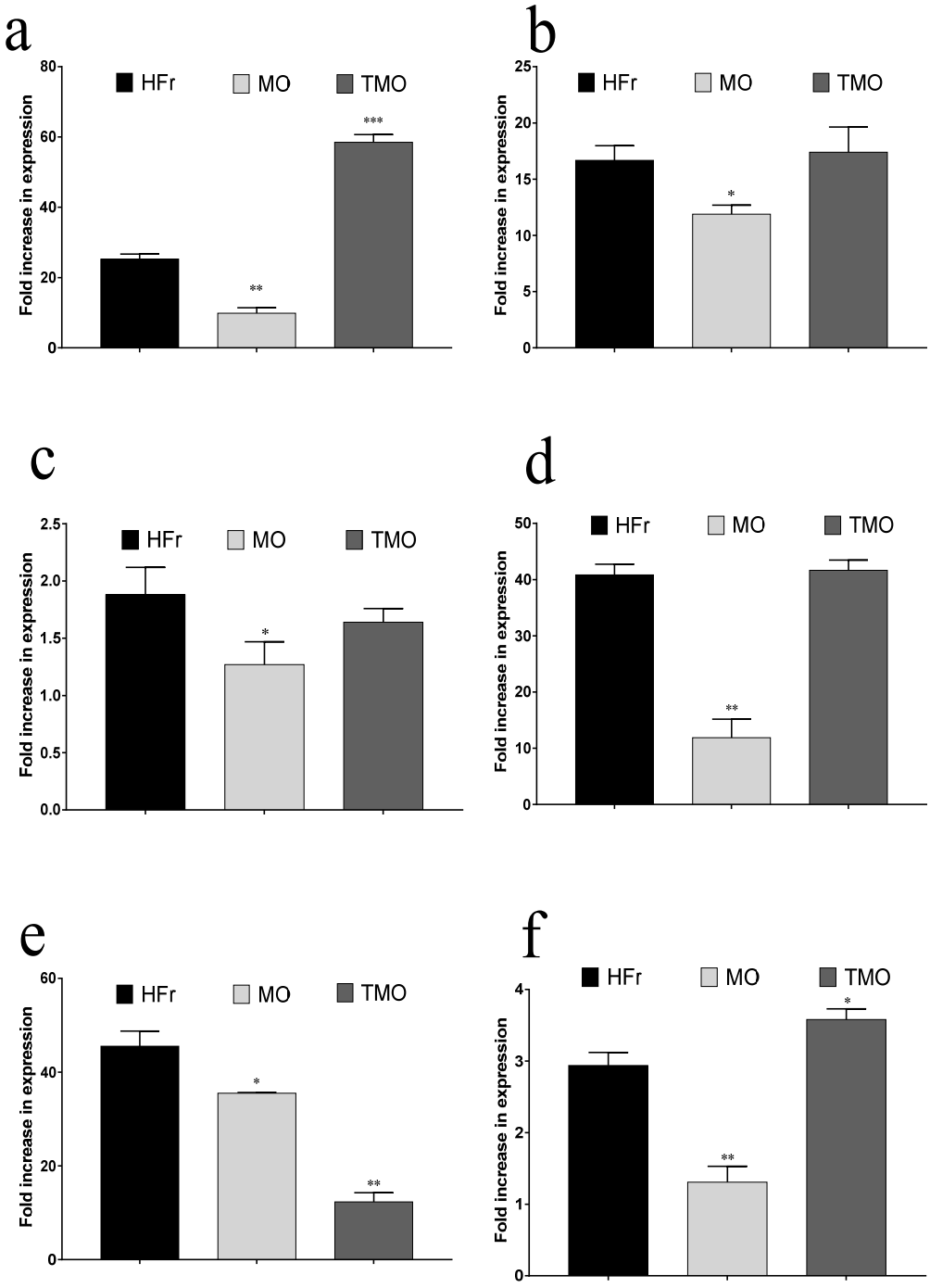


Figure 5.9 Changes in the gene expression pattern in the colon tissues of rat under different experimental diets. Gene expression was analyzed by real time quantitative PCR; IL- 6 (a), TNF-α (b), P53 (c), Wnt-1 (d), PPARY (e) and Akt-1 (f).

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Chapter 6

Effect of thermally oxidized Sunflower oil on HFr-induced metabolic syndrome

6.1 Introduction

Polyunsaturated fatty acids are the well-known class of dietary fats with two or more double bonds in their carbon chain. They include omega-3 and omega-6 class of fatty acids, which are named based on the position of their first double bond from the omega end. The primary sources of PUFA are the marine foods and also in various vegetable oils from canola, corn, soybean, walnuts and sunflower. The predominant form of omega 6 fatty acids in humans is linoleic acid (LA), which is an essential fatty acid. The common omega 3 fats include alpha linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Among these, LA and ALA are known as essential fatty acids, since the body can't synthesize them de novo. However, by the desaturase enzyme activities, LA and ALA can be converted to arachidonic acid, DHA or EPA.

Generally, PUFA area considered being healthy; however, the actual determinants of health are the ratio between omega 3 and 6 fatty acids in the body. Higher levels of omega 6 fatty acids are not recommended, since the increased n-6 PUFA may lead to the generation of its oxidation products, which are inflammatory in nature. In contrast, the biological roles of n-3 PUFA oxidation product still remain controversial. Studies have indicated that, 4- Hydroxy hexenal (4-HHE) is the common aldehyde derived from omega-3 fatty acid oxidations EPA and DHA. HHE has been shown to improve heme oxygenase 1 mRNA and protein levels in the vascular endothelial cells (Ishikado *et al.*, 2010), suggesting the possible involvement of Nrf2 /Keap 1 axis. However, studies by Lee *et al.* (2004) has reported that, HHE induces the expression of various pro-inflammatory cytokines and thereby initiating apoptotic events in the vascular endothelial cells. Further, increased levels of HHE in the intestinal tissues of rats fed with oxidized omega-3 fat have been noticed (Awada *et al.*, 2012). Similar results are also shared by Bradley *et al.* (2012) in several of the neurodegenerative disorders. Lee *et al.* (2004) has further extend studies to show the profound effect of HHE redox system challenged body. It is thus clear that lipid oxidation products of PUFA are being reported to be unhealthy.

Sunflower oil (SO) is one among the commonly consumed edible oil in various parts of India. About 63% of the total fatty acid content is formed by linoleic acid. Sunflower oil is shown to reduce hyperlipidemia in rats with fatty liver by improving

hepatic lipid metabolism, in comparison with palm oil (Go *et al.*, 2015). Similar observations also made by Trautwein *et al.* (1999) and Trautwein *et al.* (1997). However, recent studies have indicated that deep frying of the SO increases its peroxide contents as well as the free fatty acid and polymerized triglycerides (Carbonera *et al.*, 2014). Further, several animal model studies have also indicated that consumption of thermally oxidized sunflower oil (TSO) over a short period of time, increases the oxidative damage (Olivero David *et al.*, 2010; Wang *et al.*, 2016).

This chapter describes the effect of prolonged consumption of fresh or thermally oxidized sunflower oil along with a high fructose diet over a period of 30 weeks. Changes in insulin resistance, incidence and extent of hepatosteatosis as well as changes in the colon epithelium are analyzed.

6.2 Materials and methods

6.2.1 Oils, thermal oxidation and TLC analysis

Sunflower oil was purchased from the local market and kept under refrigerated conditions in order to avoid auto-oxidation. A portion of this oil was used for thermal oxidation according to the methods described by González-Muñoz *et al.* (2003). The thermally oxidized oils were kept at $^{-}20\ ^{o}C$ to avoid further oxidative changes. Thin layer chromatographic analysis was performed using hexane: diethyl ether (3:1 v/v) using readymade TLC plates (Merck, Bangalore, India).

6.2.2 Animals

Thirty six male wistar rats (140-160g) were purchased from the Kerala Veterinary and Animal Science University, Mannuthy, Thrissur. Experimental animals were fed on a non-purified rat chow from Sai Durga Feeds (Bangalore, India). The rats were maintained at standard conditions and fed water *ad libitum* for 2 weeks as part of acclimatization. All the experimental procedures had prior permission from institutional animal ethical committee, Amala Cancer Research Centre [Approval No. ACRC/IAEC/15/06-(2)].

6.2.3 Diets and experimental procedure

Experimental animals were divided into four groups, each containing six rats; Group 1 was kept control (rats fed on normal rat chow), group 2, 3 and 4 were fed with a

high fructose diet fed containing groundnut oil, fresh sunflower oil and thermally oxidized sunflower oil. Animals were maintained in their respective diets for a period of 30 weeks. All animals had free access to their respective food and water throughout the experimental period. The details of animals feed composition used in the experimental diet were as described in Chapter 2.2.1.

6.2.4 Biochemical analysis

Serum parameters such as blood glucose, total cholesterol, HDLc and triglycerides were determined using commercially available kits Agappe diagnostics (Chennai, India). Liver function parameters including ALT, AST and ALP was estimated using Span diagnostics kits (Bangalore, India) as per the manufacturer's instructions. Serum IL-6 was estimated using ELISA kit as per standard protocols (Peprotech, USA).

6.2.5 Estimation of polyol pathway enzymes

Polyol pathway enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH) was done as per the standard protocol described by Jang *et al.* (2010) with modifications. AR activity was assayed in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.4 mM of ammonium sulfate (0.4 mM), EDTA (0. mM), DL-glutaraldehyde (20 μM), and NADPH (25 μM). The reaction was initiated by the addition of 100 μL tissue sample, and rate was measured as the change in absorbance at 340 nm for 2 minutes. SDH activity was measured in100mM triethanolamine buffer (pH 7.4). The reaction was based on the oxidation of NADH (12mM) by SDH using fructose (1.1M) as substrate in the tissue sample at 340 nm for 2 minutes (Chapte 2.9). Ornithine decarboxylase activity was determined according to the methods of Ngo *et al.* (1987); the details of which has been incorporated in Chapter 2.7.3.

6.2.6 Analysis of oxidative stress and antioxidant activity

Liver homogenate was prepared (10% w/ v) in Tris HCl buffer (0.1 M, pH 7.4) and used for the estimation of lipid peroxidation (Ohkawa *et al.*, 1979) and protein carbonyls (Levine *et al.*, 1990). The homogenates were prepared by centrifuging at 10000 rpm for 15 min at 4^{o}C. The supernatant was collected and used for GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), catalase (Beers and Sizer,

1952) and GR (Carlberg and Mannervik, 1975) assays. The detailed description on the methodology has been given in Chapter 2.9 and its subdivisions.

6.2.7 Histopathological analysis

Briefly, the tissues were dehydrated in a series of alcohol, impregnated in wax and serial sections were taken. Tissues were then stained with hematoxylin and eosin, visualized under microscope (200X) and photographed. The detailed outline of procedures used is given in Chapter 2.10.

6.2.8 Real time quantitative PCR analysis

Total RNA was isolated from colon epithelial tissues of rats and cDNA was synthesized using commercial kits; the protocols are described in Chapter 2.11.1 and 2.11.2. Expression of various genes in the colon epithelial tissues of experimental rats was analyzed by qPCR by ΔΔCT method. The primer sequences and PCR cycling are given in Chapter 2.11.3.

6.2.9 Statistical analysis

The results of the study were represented as mean ±standard deviation. Statistical analysis of the results was done by one way ANOVA followed by post-hoc test (Tukey- Kramer multiple comparisons test) using Graph pad software (La Jolla, California, USA) (details are given in Chapter 2.12).

6.3 Results

6.3.1 Insulin resistance and oral glucose tolerance

The animals fed with diet containing high fructose and different edible oils had a gradual increase in the blood glucose level in comparison with the normal (reference group) rats. Normal animals had an initial blood glucose level of 81.8 ± 12.5 mg/dL, which gradually increased to 95.1 ± 5.7 over a period of 30 weeks (16.3%). In high fructose diet fed animals the initial and final blood glucose levels were found to be 88.8 ± 6.5 and 216.0±17.2 mg/dL (143.2%). In SO fed groups, there was an increase from 84.7 ± 5.8 mg/dL to 224.6 ± 17.2 (165.2%). In the thermally oxidized SO containing diet fed rats, the change was more profound from an initial level of 78.7 ± 9.5 mg/dL, blood glucose was elevated to 253.0 ± 9.2 mg/dL (221.5%).

Insulin levels in the untreated animals was 45.71 ± 10.07 pmol/L, which was elevated in high fructose fed rats to 66.66 ± 2.04 pmol/L. In SO containing diet fed group, the level was 69.62 ± 7.78 pmol/ L, whereas in TSO group, it was 80.48 ± 16.40 pmol/L (Table 6.1).

HOMA %B, which is an index of beta cell functioning was found to be 63.8 ± 13.5 % in reference diet fed normal animals, which was reduced significantly to 22.0 ± 4.1 % in HFr group. In SO containing diet fed rats, the beta cell functioning was 21.2 ± 4.3%, which was further reduced to 18.7 ± 2.9% in TSO containing diet fed animals (Table 6.1).

Insulin sensitivity or HOMA %S was found to be 116.9 ± 27.2% in normal rats. High fructose feeding reduced the sensitivity to 66.1 ± 0.5%. In SO fed animals, HOMA %S was 63.0 ± 7.2%, which was considerably reduced to 52.2 ± 10.6% in TSO containing diet fed animals (Table 6.1).

Insulin resistance as measured by HOMA- IR was 0.89 ± 0.19 in normal rats, which was elevated significantly by high fructose feeding (1.51 ± 0.01). In SO containing diet fed animals HOMA-IR was 1.60 ± 0.18 and which was further elevated to 1.98 ± 0.41 in TSO group of rats (Table 6.1).

In the normal animals glucose tolerance level was found to be 19.96±1.96 AUC. In the high fructose fed animals, glucose tolerance was reduced to 38.28±4.71 AUC (Figure 6.1). In SO containing diet fed animals, glucose tolerance was slightly improved (36.74±2.20 AUC). However, TSO containing diet fed rats, the tolerance was highly disturbed (40.50±3.60 AUC).

6.3.2 Effects on serum and hepatic lipid profile

In the normal diet fed animals, the serum total cholesterol was found to be 66.7 ± 7.4 mg/dL. High fructose fed animals showed a percentage increase of 30.2 (Table 1). In the SO and TSO containing diet fed animals, the TC levels was increased by 33.8 and 51.2%, respectively. Similarly, the serum triglyceride level in the HFr group was 97.3% higher than the normal diet fed rats (81.6 ± 10.1mg/dL). Animals fed with SO and TSO containing diets had a respective increase of 122.0 and 134.5% in their TG levels. In contrast, HDLc levels of animals in HFr, SO and TSO containing diets were reduced by 26.5, 18.1 and 25.6% than the normal animals (37.3 ± 6.2) (Figure 6.2a).

As shown in (Figure 6.2b), hepatic total cholesterol was also showed a significant increase ($P<0.01$) in all the groups compared to normal rats. In HFr, SO and TSO containing diets, the TC was increased from the normal level (133.0 ± 7.3) by 46.4, 54.4 and 82.9%, respectively. Normal diet fed rats had a TG level of 155.37 ± 15.5mg/100g tissue. High fructose containing diet fed animals showed an increase of 55.6% ($P<0.01$). In the SO and TSO fed animals, more profound increase was observed (86.9 and 114.9%). In the case of hepatic HDLc levels, only HFr and TSO containing diet fed animals had a significant decrease ($P<0.05$), where they showed a 20.4 and 22.2% reduction. In SO fed rats, though the reduction was not statistically significant, there observed 15.1% reduction.

6.3.3 Effect on hepatic antioxidants and oxidative stress

In the present study, animals in the reference diet has a GSH level of 8.51 ± 0.34 nmoles/ mg protein, which was reduced to 6.17 ± 0.25 nmoles/ mg protein in HFr group. In fresh SO containing diet fed animals, the level was moderately reduced to 6.80 ± 0.17 nmoles/ mg protein; whereas a significant reduction was noticed in TSO containing diet fed animals (4.83 ± 0.24 nmoles/ mg protein) (Figure 6.3b).

Catalase activity was 87.53 ± 8.73 U/ mg protein in normal animals fed on reference diet. High fructose feeding reduced the catalase activity to 51.95 ± 8.38 U/ mg protein. The extent of reduction was more significant in SO and TSO, where the catalase activity was found to be 57.66 ± 9.30 and 38.97 ± 3.41 U/ mg protein (Figure 6.3a).

Superoxide dismutase activity in the liver tissue of normal untreated rats was 6.90 ± 0.28 U/ mg protein, which was reduced to 4.28 ± 0.13 U/ mg protein in HFr group of animals. Animals fed on modified diet containing SO and TSO as fatty acid source had SOD activities of 4.87 ± 0.38 and 3.14 ± 0.55 U/ mg protein (Figure 6.3a).

Glutathione reductase activity in normal animals was 23.73 ± 3.2 U/ mg protein, which was shown a mild reduction to 22.44 ± 2.7 U/ mg protein. However, the levels remained unaltered in SO or TSO containing diet fed animals (20.87 ± 0.8 and 23.35 ± 3.2 U/ mg protein) (Figure 6.3a).

Lipid peroxidation products as measured in terms of thiobarbituric acid reactive substances (TBARS) was significantly elevated in experimental diet fed animals. In

normal animals, the serum and liver TBARS level was found to be 3.84 ± 0.32 and 4.1 ± 0.9 nmoles/ mg protein. In HFr fed animals, the serum and liver TBARS was elevated to 4.94 ± 0.26 and 5.50 ± 0.6 nmoles/ mg protein. In SO containing diet fed animals, the levels were 5.23 ± 0.68 and 6.5 ± 0.9 nmoles/ mg protein. The increase in serum and liver TBARS was most profound in SO containing diet fed animals (5.75 ± 0.50 and 7.7 ± 0.8 nmoles/ mg protein) (Figure 6.3b).

6.3.4 Polyol pathway and ornithine decarboxylase activity

As shown in Figure 6.4 a, fructose feeding for 4 weeks induced hyperglycemia and associated activation in polyol pathway. In the normal rats the AR and SDH activities were found to be 1.59±0.35 and 0.84±0.11 U/mg protein. In the high fructose fed rats, the respective levels were increased to 3.47±0.57 and 1.34±0.27 U/mg protein. In SO fed rats, there was a marginal decrease in the activities of AR and SDH, with values 3.38±0.29 and 1.26±0.19 U/mg protein respectively. However, TSO fed animals showed the highest activities for AR and SDH, with values 4.01±0.44 and 1.55±0.61 U/mg protein.

Ornithine decarboxylase activity was found to be 5.43±0.29 in normal rats, which slightly increased in fructose fed rats (6.07±0.15). In SO fed rats, the level was same as that of the normal (5.27±0.21 U/mg protein). Whereas in the TSO fed rats, there was an augmentation in ODC activity to 7.34±0.39 U/ mg protein (Figure 6.4a).

6.3.5 Liver function parameters

Aspartate transaminase activity in normal rats fed with standard diet had an AST activity of 94.4 ± 9.8 IU/L. In HFr group of rats, the activity was increased to 134.5 ± 6.4 IU/L. In MO and TMO containing diet fed rats, the AST activities were found to be 143.5 ± 11.7 and 191.0 ± 16.3 IU/L (Figure 6.4b).

Alanine transaminase activity of normal rats was 35.8 ± 4.4 IU/L, which was increased to 46.8 ± 6.0 IU/L. Whereas in SO containing diet fed animals, it was slightly reduced than the HFr group (45.4 ± 3.2 IU/L). TSO group had the highest ALT activity than the other experimental groups (55.3 ± 4.3 IU/L) (Figure 6.4b).

Alkaline phosphatase activity of the normal animal was 196.4 ± 7.4 IU/L, which was increased in HFr feeding to 210.4 ± 12.6 IU/L. In SO containing diet fed animals, it

was found to be 230.8 ± 9.4 IU/L and further elevated to 261.2 ± 10.5 IU/L in TSO containing diet fed animals (Figure 6.4b).

6.3.6 GGT and LDH activity and IL-6 levels

Gamma glutamyl transferase activity in the normal animal was 28.65 ± 2.71 IU/L. In high fructose fed group (HFr), the GGT activity was elevated to 37.30 ± 8.10 IU/L. In SO containing diet fed animals, GGT activity was 38.42 ± 4.70IU/L and it was further elevated to 49.61 ± 6.20 IU/L in TSO containing diet fed animals (Table 6.2).

Lactate dehydrogenase activity in the normal animals was 166.9 ± 21.5 IU/L and it increased to 189.6 ± 9.7 IU/L in HFr group. In SO containing diet fed animals, the activity was 199.2 ± 14.1 IU/L and further elevated to 227.8 ± 7.8 IU/L in TSO containing diet fed group of rats (Table 6.2).

As shown in Figure 6.6, compared to the normal animals (0.59±0.07 pg/mL), serum IL-6 level of HFr diet fed animals showed a four-fold increase (1.97±0.21 pg/mL). In SO containing diet fed animals, the IL-6 level was 0.42± 0.04 pg/mL; on contrary, TSO containing diet fed animals has a serum IL-6 level of 3.78± 0.15 pg/mL.

6.3.7 Effect on colonic antioxidant status

Reduced glutathione levels in the colon mucosa of normal animals was 25.3 ± 3.2 nmoles/ mg protein, which was reduced to 16.18 ± 0.5 nmoles/ mg protein in HFr group. In fresh SO containing diet fed animals, the level was 22.01 ± 2.4 nmoles/ mg protein and in TSO containing diet fed animals it was 11.99 ± 0.74 nmoles/ mg protein (Figure 6.5a).

Superoxide dismutase activity in the colon mucosa of normal rats fed with reference diet was 6.25 ± 0.6 U/ mg protein, which was reduced in HFr group of animals to 4.40 ± 0.3 U/ mg protein. Animals fed on modified diet containing SO and TSO had SOD activities of 4.56 ± 0.6 and 2.67 ± 0.23 U/ mg protein (Figure 6.5a).

Glutathione peroxidase activity in normal animals was 153.67 ± 8.2 U/ mg protein, which was shown a mild reduction to 133.65 ± 4.0 U/ mg protein. However, the activity remained unaltered in SO or TSO containing diet fed animals (127.06 ± 3.0 and 113.42 ± 5.42 U/ mg protein) (Figure 6.5b).

Thiobarbituric acid reactive substances (TBARS) level in the colon of normal rats was 43.87 ± 3.3 nmoles/ mg protein. In HFr fed animals, the serum and liver TBARS was elevated to 57.28 ± 4.9 nmoles/ mg protein. In SO and TSO containing diet fed animals, they were 49.55 ± 5.5 and 64.05 ± 5.78 nmoles/ mg protein (Figure 6.5b).

6.3.8 Histopathological analysis

Histopathological analysis of the liver tissues of animals fed with normal diet (Figure 6.8 a) and those fed on high fructose (b), SO (c), TSO (d). The normal animals had liver tissue architecture with normal central venous system, the portal triads, sinusoidal spaces and Kupffer cells. However SO and TSO diet fed groups showed signs of hepatosteatosis, observed as microvesicles. TSO containing diet fed animals showed progressed hepatosteatosis than those fed with SO diet.

Histopathological evaluations of colon tissues observed normal intestinal glands and villi with normal looking mucosa and serosa layers in untreated group (a) as well as SO containing diet fed animals (c). In HFr group (b) and animals fed with TSO containing diet (d) had mild and diffused infiltration of inflammatory cells (Figure 6.8).

6.3.9 Gene expression profile using qPCR analysis

Expression of IL-6, a pro-inflammatory cytokine was increased by 24.08 ± 1.48 in HFr group, whereas it was increased only by 8.11 ± 0.81 in SO containing diet fed rats. However, consumption of thermally oxidized counterpart (TSO) containing diet, there was an increase of 54.19 ± 3.55 fold in the IL-6 expression. Tumor necrosis factor α expression was increased by 16.11 ± 1.42 fold in HFr group, which was increased by 7.57 ± 1.08 and 20.82 ± 1.99 fold in SO and TSO containing diet fed animals.

Expression of anti-proliferative gene P53 was increased 1.84 ± 0.23 fold in HFr group. The increase in expression of p53 gene in SO and TSO containing diet fed rats were 4.53 ± 0.25 and 2.99 ± 0.11 folds, respectively. Expression of Wnt-1 gene was increased by 42.81 ± 2.19 fold in HFr group, whereas in SO and TSO containing diet fed rats, it was increased by 13.74 ± 3.95 and 56.10 ± 2.13 fold. Peroxisome proliferator activator receptor-γ expression in HFr and SO group was increased by

42.22 ± 3.61 and 43.11 ± 0.12 fold, however in TSO containing diet fed rats, the expression was increased only by 19.84 ± 0.82 fold. Protein kinase B (Akt) is an important gene in insulin signaling, whose expression was increased by 2.85 ± 0.14 in HFr group. In SO and TSO containing diet fed rats, the expression was increased by 1.64 ± 0.17 and 1.89 ± 0.16 fold.

6.4 Discussion

The physicochemical as well as biochemical changes in sunflower oil generating lipid carbonyls and aldehydes during thermal oxidation have been described in Chapter 3. Studies by Ammouche *et al.* (2002) also reported similar oxidative modifications as increase in its peroxide value and free fatty acids content. Studies have also observed elevated levels of triacylglycerol polymers, dimers and cis- to trans- conversion in thermally oxidized sunflower oil (Bastida and Sánchez-Muniz, 2001). Recently, Romero *et al.* (2006) and Barriuso *et al.* (2015) has reported the formation of cyclic fatty acid monomers (CFAMs) and oxysterols during the thermal oxidation of sunflower oil; these deleterious changes make the oil unsuitable for edible purpose.

Compared to reference (normal group), HFr alone and SO containing diet fed animals, TSO containing diet fed animals show increased levels of fasting blood glucose as well as reduced glucose tolerance. In addition TSO containing diet fed have documented significantly increased insulin resistance and reduced beta cell function as indicated by the HOMA IR and HOMA B indices. Previous study by Simopoulos (1994) has indicated that dietary linoleic acid as well as trans fats can impair insulin sensitivity. Further, Alkazemi *et al.* (2008) has indicated a positive correlation between the oxysterols, hyperinsulinemia and insulin resistance in body. It is thus possible that the oxysterols and linoleic acid oxidation products together with the trans-fats in TSO may have contributed to the exacerbations of high fructose induced insulin resistance.

Increase in the activities of gamma glutamyl transferase and lactate dehydrogenase has been observed in animals fed with a diet containing thermally oxidized edible oils. It has been previously reported that GGT activity increase in response to insulin resistance (Bonnet *et al.*, 2011). Further, reports have also indicated that increased LDH expression and activity reduce beta cell functioning and increase insulin resistance (Ainscow *et al.*, 2000; Wu *et al.*, 2016). Prolonged diabetic conditions are

reported to have elevated levels of LDH (Zappacosta *et al.*, 1995) and GGT (Haghighi *et al.*, 2011). GGT and LDH are also indicators of oxidative damages to tissues (Ramos *et al.*, 2013), therefore it can be presumed that thermally oxidized oils consumption may have increased the oxidative damages in colon tissues of rats.

Associated with this an increase in TG level and reduction in HDLc levels are seen in the liver tissues of HFr alone and SO diet fed animals, however with higher degree in TSO group animals. According to Song *et al.* (2015), insulin resistance is also manifested by dyslipidemia condition, wherein increased level of triglycerides and reduced HDLc are obvious. Under dyslipidemic conditions, triglycerides tend to accumulate in liver and thereby leading to vacuolation and disruption of the normal physiological functioning (Perry *et al.*, 2013). Deursen *et al.* (2008) reported that sugars such as glucose and fructose increases *hepatic lipase* (HL) expression via elevation of upstream stimulatory factors (USF), USF1 and USF2, which is responsible for the development of the dyslipidemic conditions. As already established, due to its phospholipase activity, HL is associated with reduced plasma HDL_2 levels, thus contributing towards chronic dyslipidemia (Deeb *et al.*, 2003). Further, hepatic expression of lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC-1) following a high sugar exposure is shown to be driven by the activation of the hexosamine biosynthesis pathway (HBP) and HBP-related metabolites (Hirahatake *et al.*, 2011). It is thus possible that the observed increase in triglycerides in this study may be partially contributed by the fructose/glucose exposure; however, exacerbation of this condition by TSO could be due to its oxidation products.

Together with hyperglycemia and dyslipidemia, increased levels of lipid peroxidation products are noted in TSO group comparatively in high proportion than the other group of animals. In several of the degenerative disorders, including non-alcoholic fatty liver, increased lipid oxidation products are recognized as a risk factor (Konishi *et al.*, 2006; Poli *et al.*, 1987). Corroborating with these observations, a diminished activities of hepatic antioxidant enzymes including catalase, superoxide dismutase and glutathione peroxidase are observed in TSO fed animals. Koruk *et al.* (2004) have reported that reduced activities of antioxidant enzymes often accelerate the progression of NAFLD. Together with this, a reduced level of GSH in the TSO fed rats is also observed. The observed reduction in GSH levels may due to the increase in

the polyol pathway enzymes such as aldose reductase in TSO fed rats. Aldose reductase is a rate limiting enzyme in polyol pathway; under hyperglycemic conditions it converts excess blood glucose to sorbitol on expense of NADPH (Bravi *et al.*, 1997). On the other hand, this depletion in NADPH affects GSH regeneration, as it is necessary for GSH from GSSG by the action of glutathione reductase (Bravi *et al.*, 1997). Additionally, thermally oxidized oils are shown to reduce the hepatic isocitrate dehydrogenase activities, which is essential for the generation of NADPH required for GSH biosynthesis (Koh *et al.*, 2004).

The observed hepatic dyslipidemia and redox imbalance in the TSO containing diet fed group may have subsequently induced hepatotoxicity, which is manifested by the increase in the hepatic function marker enzyme activities such as AST, ALT and ALP. Justifying the increased activities of liver function marker enzymes, hepatic histology showed an increased frequency of microvesicles and higher degree of hepatocellular ballooning in the TSO containing diet fed rats.

In connection with these, diminished enzymatic as well as non-enzymatic antioxidant systems have been also observed in the colon tissues of TSO containing diet fed animals. A concomitant increase in the levels of lipid peroxidation products is also documented. This supports the assumption that TSO consumption worsens the HFr mediated oxidative stress conditions in the colon epithelial tissues. Together with the diminished endogenous antioxidant defense, higher levels of lipid peroxides detected in the TSO as a result of thermal oxidation might have contributed towards the increased levels of lipid peroxidation products and associated oxidative stress.

In addition to the oxidative damages, higher expression of pro-inflammatory cytokines such as IL-6 and TNF-α has also been observed in the colon epithelial tissues of thermally oxidized oil fed animals. Since SO is rich in linoleic acid, heat induced oxidation of linoleic acid may be expected in both the oils. Previous reports have been shown that thermal oxidation of linoleic acid generates 9- and 13-hydroxyoctadecadienoic acids (9-HODE and 13-HODE) (Patwardhan *et al.*, 2010). Numerous studies have indicated the pro-inflammatory potentials of both 9- and 13-HODE in different tissues (Dandona *et al.*, 2010; Mabalirajan *et al.*, 2013; Spindler *et al.*, 1997). Further, clinical studies have reported a reduced expression of 13-HODE dehydrogenase (an enzyme involved in the detoxification of HODE to 13-

oxooctadecadienoic acid) in colorectal cancers (Silverman *et al.*, 1996). Increased expression of cell survival or proliferative genes such as Wnt-1 and Akt, concomitantly reduced PPARγ expression possibly indicate a pro-carcinogenic potential of these thermally oxidized edible oils.

Altogether the study suggests that thermally oxidized PUFA rich edible oils together with high sugar diet induce lipotoxicity in liver upon long term feeding. Further, the increased oxidative stress, chronic inflammation and elevated expression of proliferative/ cell survival genes in colon together indicate a possible pro-carcinogenic potential of thermally oxidized sunflower oil in colon epithelial tissues over long-term consumption. Hence, in comparison with, MCFA rich Coconut oil (Chapter 4) and MUFA rich mustard oil (Chapter 5), TSO has been shown to be deleterious to both hepatic and colon tissues. The toxic insults to colon cells are significantly higher than the TCO and TMO. The higher levels of linoleic acid oxidation products and the phytosterol oxidation products may be the driving molecules. Hence, the chapter concludes that increased intake of diet containing Thermally oxidized polyunsaturated rich edible oils along with high sugar may increase the risk of both hepatic and colorectal cancers.

Table 6.1 Effect of SO and TSO on High fructose induced alterations in the blood glucose level, plasma insulin level and various HOMA indices in rats over a period of 30 weeks

Parameters	Normal	HFr	SO	TSO
HOMA2 %B	63.8± 13.5	22.0± 4.1***	21.2± 4.3***	18.7± 2.9***
HOMA2 %S	116.9± 27.2	66.1± 0.5***	63.0± 7.2***	52.2± 10.6***
HOMA2 IR	0.89± 0.19	1.51± 0.01**	1.60± 0.18**	1.98± 0.41***
Glucose (mg/dL)	95.1± 5.7	216.0± 17.2***	224.6± 17.2***	253.0± 9.2***
Insulin (pg/L)	45.71± 10.07	66.66± 2.04**	69.62± 7.78**	80.48± 16.40***

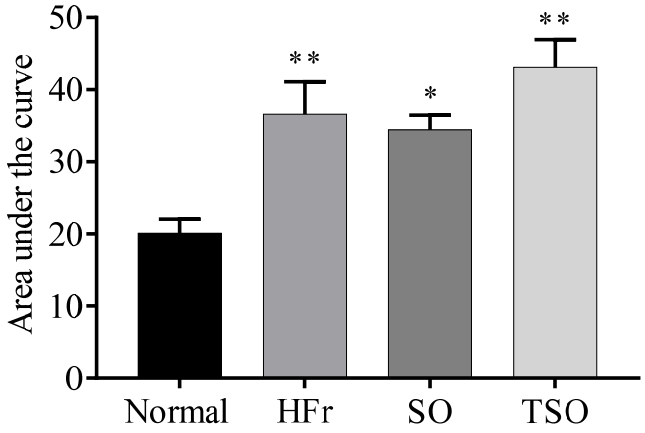


Figure 6.1 Change in the oral glucose tolerance in animals fed with different experimental diets in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

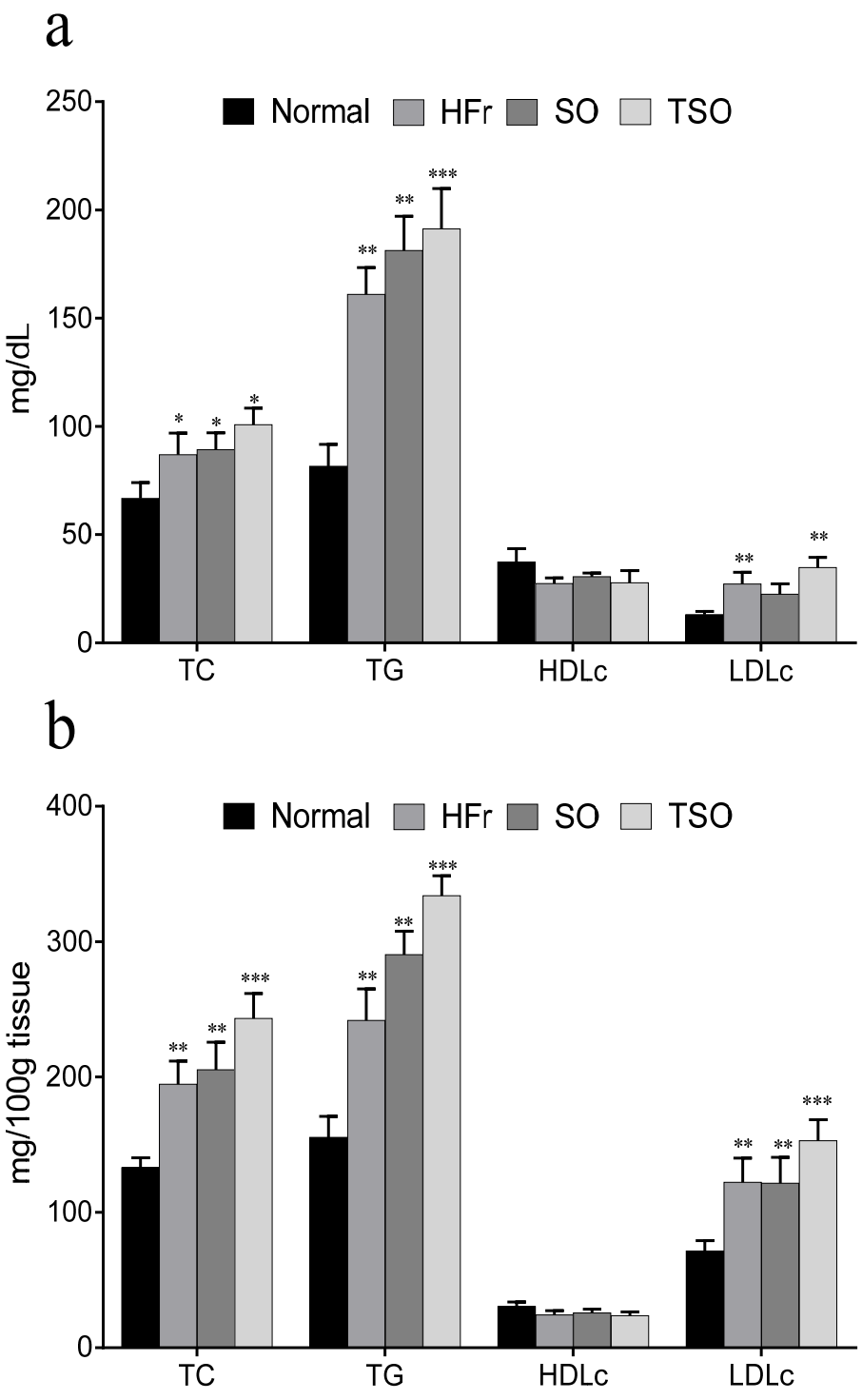


Figure 6.2: Serum (a) and hepatic (b) lipid profile of animals fed with the reference diet as well as different modified diets for a period of 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

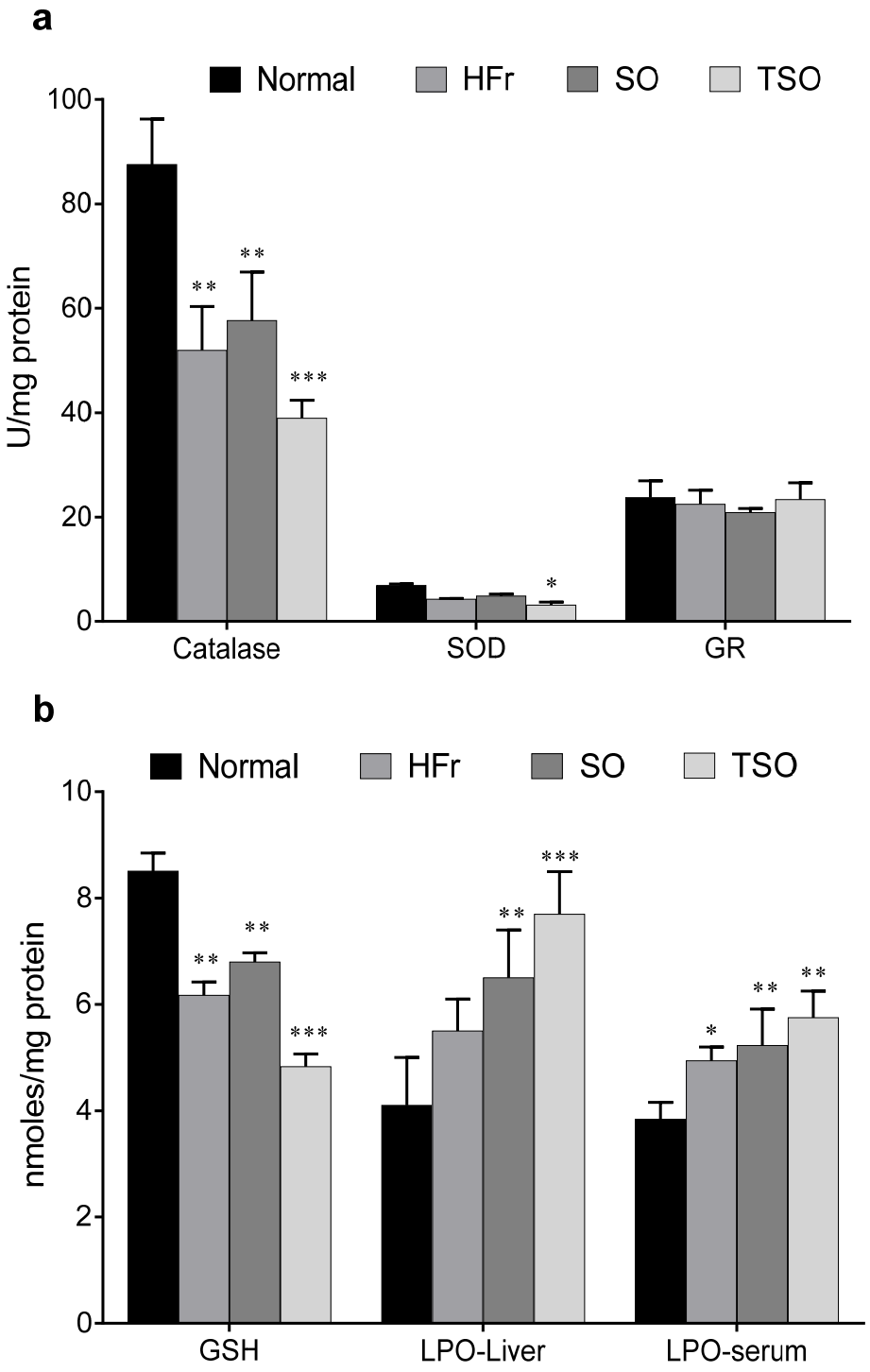


Figure 6.3: Changes in the hepatic redox status parameters such as catalase, superoxide dismutase and glutathione reductase (a) as well as GSH, serum and liver TBARs in animals fed with reference diet and modified experimental diets.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

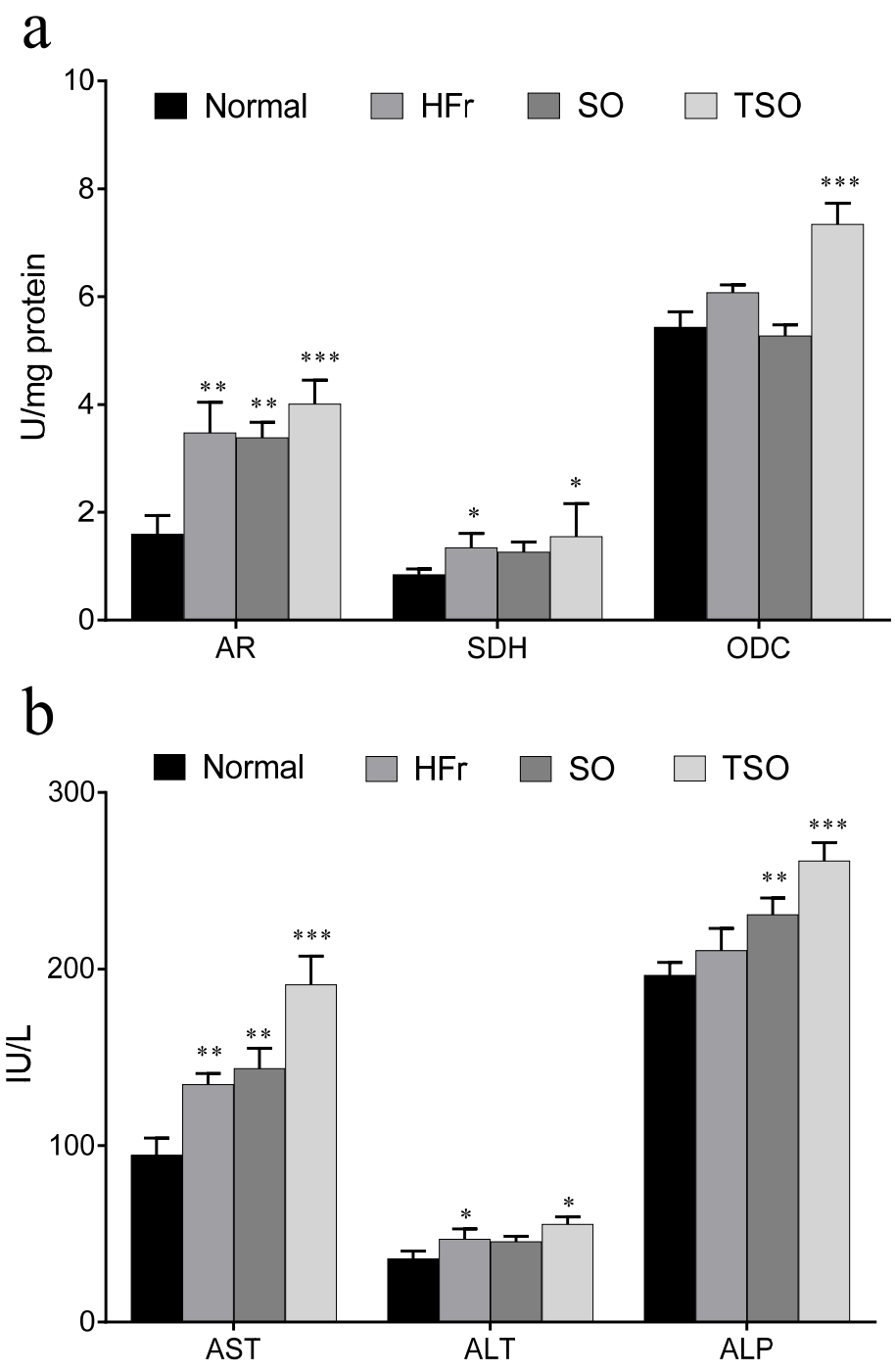


Figure 6.4: Changes in the activities of hepatic polyol pathway enzymes such as aldose reductase and sorbitol dehydrogenase (a) and liver function markers enzymes such as AST, ALT & ALP, in animals under different experimental diets after 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

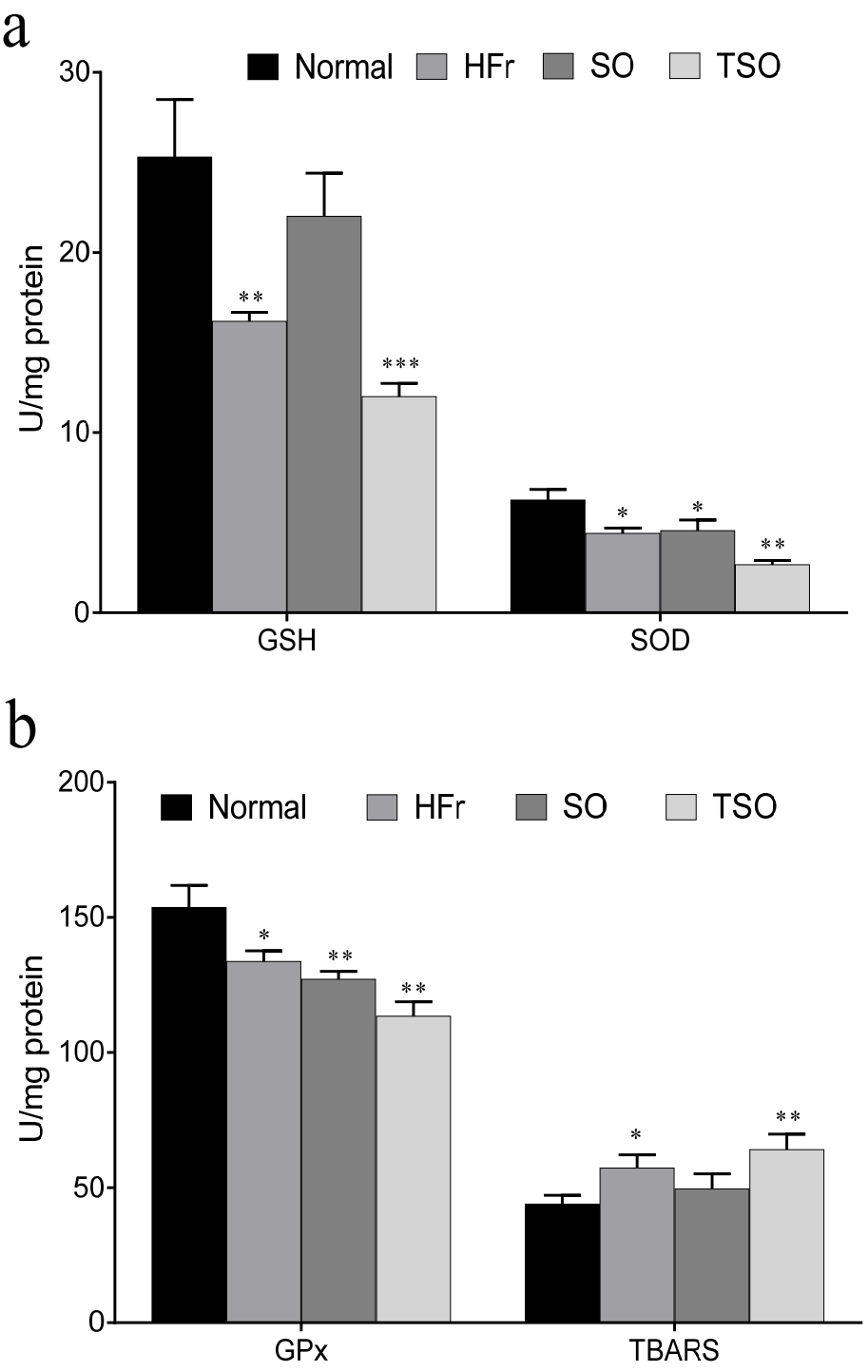


Figure 6.5 Changes in the redox parameters such as, GSH level and SOD activity (a) as well as of GPx activity and TBARS level (b), colon epithelial tissues of animals under different experimental diets for a period of 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Table 6.2 Effect of SO or TSO supplementation on the HFr diet induced alterations in gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) enzyme activities in rats expressed as IU/L.

	Normal	HFr	SO	TSO
GGT	28.65± 2.71	37.30± 8.10	38.42± 4.70*	49.61± 6.20**
LDH	166.9± 21.5	189.6± 9.7**	199.2± 14.1**	227.8± 7.8***

Table 6.3 Changes in the extent of hepatosteatosis induced by HFr diet by the supplementation of SO or TSO

Characteristics	Normal	HFr	SO	TSO
Micro vesicular steatosis	0	1	1	2
Hepatocellular ballooning	0	1	1	2
Portal tract inflammation	0	1	1	1
Glycogenated nuclei	0	1	1	1
Lipogranuloma	0	0	0	0

(0- absence; 1- mild; 2-moderate; 3- severe)

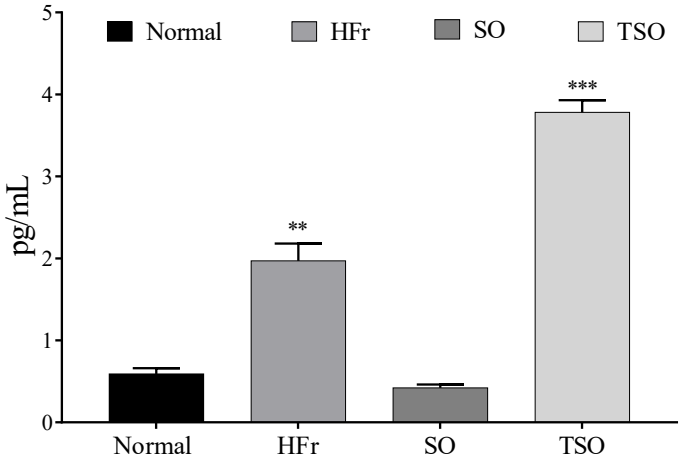
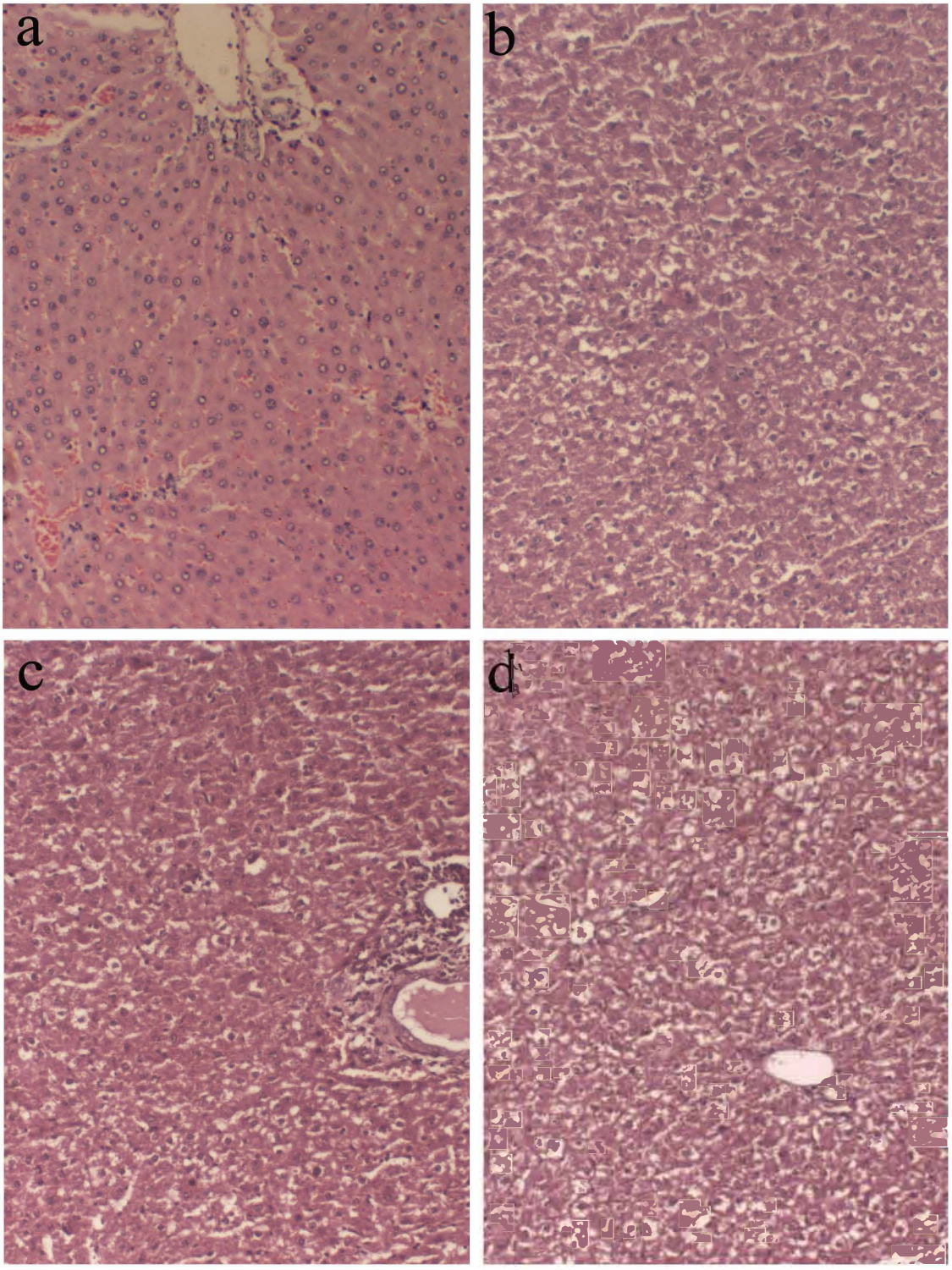
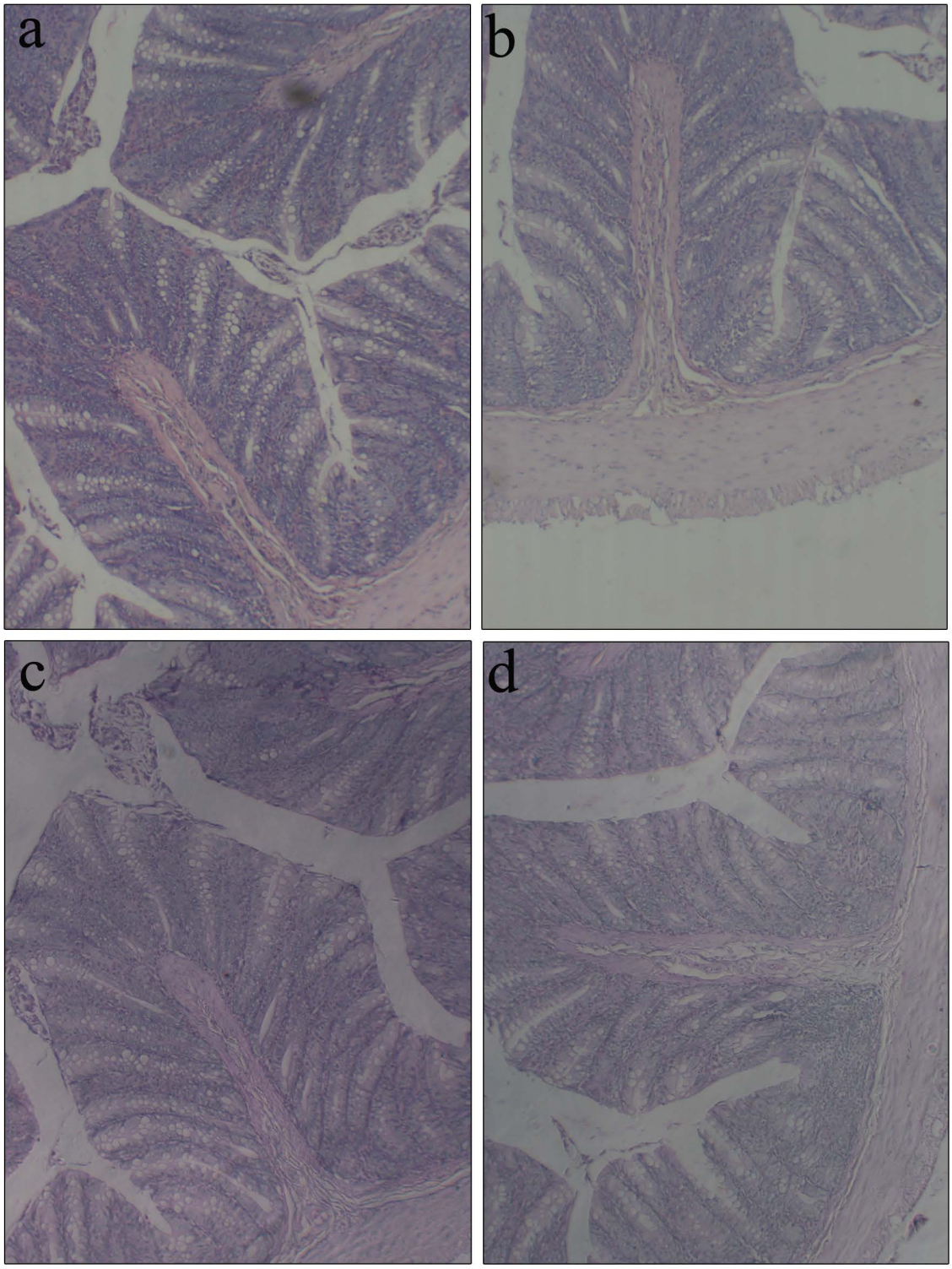


Figure 6.6 Change in the level of serum IL-6 (pg/ mL) in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)



Figrure 6. 7 Photomicrography of the hematoxylin- eosin stained sections of liver tissues of reference diet (A), HFr (B), sunflower oil (C), and thermally oxidized sunflower oil (D) containing diet fed animals. Photographs were taken under 200x magnification in Magnus INVI microscope (Chennai, India).



Figrure 6. 8 Photomicrography of the hematoxylin- eosin stained sections of colon tissues of reference diet (A), HFr (B), sunflower oil (C), and TSO (D) containing diet fed animals. Photographs were taken under 200 x magnifications in Magnus INVI microscope (Chennai, India).

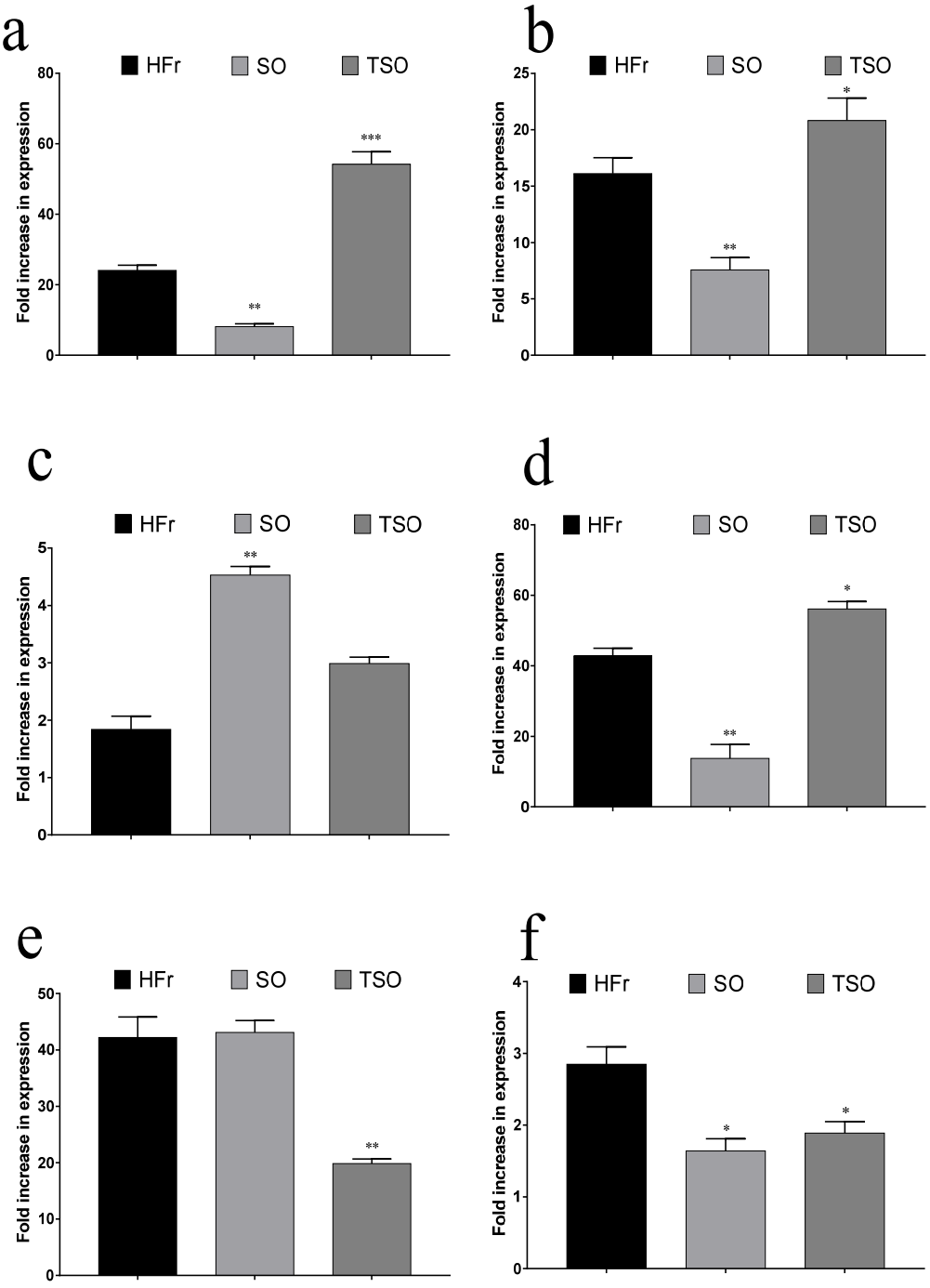


Figure 6.9 Quantitative real-time PCR analysis of IL- 6 (a), TNF-α (b), P53 (c), Wnt-1 (d), PPARY (e) and Akt-1 (f) expression in the colon tissues of rats.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Chapter 7

Effect of thermally oxidized Lard (Animal fat) on HFr-induced metabolic Changes

7.1 Introduction

Previous chapters have described the health impact of saturated and unsaturated edible oils of plant origin. Apart from these, oils and fats of animal origin have also been used for edible purpose. Compared to the edible oils and fats of vegetable in origin, these animals' fats contain cholesterol. Saturated animals fats especially of red meet have been shown to contribute to hepatosteatosis and colon carcinogenesis (Alexander *et al.*, 2015; Freedman *et al.*, 2010). Possibly the iron present in red meat triggers the oxidation of cholesterol and thereby results also leads to the formation of cholesterol oxidation products such as oxysterols (Brandsch *et al.*, 2002). The cholesterol may also get oxidized under various cooking conditions (Ferguson, 2010; Sugimura, 2000). Studies have indicated the ill effects of cholesterol oxidation products in hepatosteatosis as well as colorectal cancers (Hur *et al.*, 2014; Rossin *et al.*, 2017). These cholesterol oxidation products has been shown to exacerbate the colorectal carcinogenesis by modulating the TGF-β pathway (Biasi *et al.*, 2008).

Lard is an animal fat which is rich in monounsaturated fatty acids, often used in cooking as well as baking purposes. In addition to these triglycerides, it contains considerable amounts of cholesterol also as it is of animal origin, which makes its biological effects different from other plant derived PUFAs. Lard has shown to accentuate the obesity associate changes including insulin resistance and accumulation of visceral as well as subcutaneous fat mass, compared to the hydrogenated vegetable oils (Kubant *et al.*, 2015). Lard is often used as a component in high fat diet induced hepatosteatosis (Apolzan and Harris, 2012; Pranprawit *et al.*, 2013; Vendel Nielsen *et al.*, 2013) by inducing mitochondrial dysfunction (Lionetti *et al.*, 2014). In addition to the effects of hepatic tissue, high fat diets that contain lard as primary source of fat, leads to thyroid dysfunction, which was not reversible even after removal from the modified diet for 6 weeks (Shao *et al.*, 2014). Similarly, neurological problems including impairment of hippocampal dependent place recognition memory are noted in short term feeding of lard and sucrose (Beilharz *et al.*, 2014).

Several studies have been conducted on the physiological effects of lard alone or along with a sugar diet for short periods, however, no logical studies on the effect of long term consumption of thermally oxidized lard and high sugar diet is not yet

available. This chapter describes the effect of consumption of LD/TLD together with a high fructose diet over a period of 30 weeks, on insulin resistance, hepatosteatosis and colon epithelial changes.

7.2 Materials and methods

7.2.1 Oils and thermal oxidation procedure

Lard was obtained from the local market and maintained under low temperature. About 500mL of the oil was used for thermal oxidation according to the method described by González-Muñoz *et al.* (2003). After the oxidation process, the oils were stored under refrigeration to avoid further oxidative modifications.

7.2.2 Animals

Twenty four male Wistar rats (140-160g) were procured from the Small Animal Breeding Station (SABS) under Kerala Veterinary and Animal Science University, Mannuthy, Thrissur. Animals were acclimatized and maintained in the animal house facility of Amala Cancer Research Centre, they were fed on a non-purified rat chow from Sai Durga Feeds (Bangalore, India). All the experimental procedures had prior permission from institutional animal ethical committee, Amala Cancer Research Centre (Approval No. ACRC/IAEC/15/06-(2)).

7.2.3 Diets and experimental procedure

As shown in the table 2.1, the experimental diet was composed of 60% fructose, 20% protein and 10% vitamin and mineral mixture. The other 10% was formed of either groundnut oil (reference and HFr diet), LD or TLD as fat source.

The animals were grouped into four, each containing six rats (1) untreated normal rats (reference group), (2) high fructose diet fed group, (3) fresh lard fed group and (4) thermally oxidized lard containing diet fed group. Animals were continued their respective diets over a period of 30 weeks. The details of groups and feed composition are described in Chapter 2.2.1.

7.2.4 Estimation of glucose tolerance

Oral glucose tolerance of the animals under the experiment was carried out before sacrifice (Narayanankutty *et al.*, 2016). Briefly, the animals were starved overnight,

and then orally administered with 2 g/kg glucose. Blood glucose levels were monitored at regular intervals using glucometer (One touch select, UK). A graph was plotted taking blood glucose levels against time interval. The change in the glucose tolerance was represented as the area under the curve as described in Chapter 2.4.3.

7.2.5 Biochemical analysis

Serum parameters such as blood glucose, total cholesterol, HDLc and triglycerides were determined using commercially available kits Agappe diagnostics (Chennai, India). Liver function parameters including ALT, AST and ALP was estimated using Span diagnostics kits (Bangalore, India) as per the manufacturer's instructions. Inflammatory cytokine, IL-6, was estimated using commercially available ELISA Kits (PeptroTech, Hamburg, Germany).

7.2.6 Analysis of oxidative stress and antioxidant activity

Liver tissue was excised and washed in ice cold saline. A small portion of each was fixed in 10% buffered formalin for histopathological studies. Liver homogenate was prepared (10% w/ v) in Tris HCl buffer (0.1 M, pH 7.4) and used for the estimation of lipid peroxidation (Ohkawa *et al.*, 1979) and protein carbonyls (Levine *et al.*, 1990).

The homogenates were prepared by centrifuging at 10000 rpm for 15 min at 4^{o}C. The supernatant was collected and used for GSH (Moron *et al.*, 1979), SOD (McCord and Fridovich, 1969), catalase (Beers and Sizer, 1952) and GR (Carlberg and Mannervik, 1975) assays. The detailed description on the methodology has been given in Chapter 2.9 and its subdivisions.

7.2.7 Measurement of polyol pathway enzymes

Polyol pathway enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH) activities were measured by spectrophotometric procedures as described by Jang *et al.* (2010), which is detailed in the Chapter 2.8. ODC activity was determined as described in Chapter 2.7.3, according to the methods of Ngo *et al.* (1987).

7.2.8 Histopathological analysis

Histological studies on the tissues were carried out using hematoxylin and eosin staining. The stained slides were observed under 20x magnification of microscope

(Magnus, INVI) and photographed. Histopathological examination and scoring of steatosis was done as per the standard method (Brunt *et al.*, 1999) by an expert who is unknown to the results of the present study. The detailed outline of procedures used is given in Chapter 2.10.

7.2.9 Real time quantitative PCR analysis

Total RNA isolated from the colon epithelial tissue was used for the synthesis of cDNA, the details of which are described in Chapter 2.11.1 and 2.11.2. Gene expression in the colon tissue of various experimental groups was analyzed by qPCR according to the ΔΔCT method and data were expressed as fold change in expression compared to that of untreated normal animals. Details of RNA isolation, cDNA synthesis, sequences of primer used and PCR cycling were given in Chapter 2.11.

7.2.10 Statistical analysis

The results of the study were represented as mean ±standard deviation with the help of Microsoft office excel 2010 (New Mexico, USA). Statistical analysis was done by one way ANOVA following Tukey- Kramer multiple comparisons test using Graph pad software (La Jolla, California, USA) (details are given in Chapter 2.12).

7.3 Results

7.3.1 Insulin resistance and glucose tolerance

The animals fed with diet containing high fructose and different edible oils had a gradual increase in the blood glucose level in comparison with the normal (reference group) rats. Normal animals had an initial blood glucose level of 81.8 ± 12.5 mg/dL, which gradually increased to 95.1 ± 5.7 over a period of 30 weeks (16.3%). In high fructose diet fed animals the initial and final blood glucose levels were found to be 88.8 ± 6.5 and 216.0±17.2 mg/dL (143.2%). In LD fed groups, there was an increase from 89.6 ± 7.1mg/dL to 259.7 ± 9.5 (189.8%). In the TLD containing diet fed rats, the change was more profound from an initial level of 79.2 ± 10.3 mg/dL, blood glucose was elevated to 287.0 ± 16.9 mg/dL (262.4%).

In normal animals, fasting insulin level was found to be 45.71 ± 10.07 pmols/L, which was elevated to 66.66 ± 2.04 pmols/L, in HFr alone fed group. In LD containing diet

fed animals, the FPI level was 64.35 ± 4.67 pmols/L and further elevated to 71.43 ± 4.51 pmols/L in TLD containing diet fed animals (Table 7.1).

HOMA index of beta cell function (HOMA-%B) of normal animal was 63.8 ± 13.5% and reduced to 22.0 ± 4.1 during high fructose feeding. In LD containing diet fed animals, it was reduced to a level of 15.1 ± 1.5% and in TLD group, it was 14.1 ± 1.8% (Table 7.1).

Insulin sensitivity among the normal animals fed on a reference diet was found to be 116.9 ± 27.2% and came down to a level of 66.1 ± 0.5% in HFr diet feeding. In LD containing diet fed animals, it was reduced again to 62.2 ± 3.9% and a further decline in insulin sensitivity was observed in TLD containing diet fed animals (50.4 ± 5.2%).

HOMA-IR or insulin resistance of normal untreated animals was 0.89 ± 0.19 and it increased to 1.51 ± 0.01 in HFr group. In LD containing diet fed animals, it was elevated to 1.61 ± 0.10. Compared to others, a profound increase to 2.00 ± 0.21 has been observed in TLD containing diet fed animals (Table 7.1).

At the end of the experimental period, glucose tolerance level in the normal animal was found to be 19.96±1.96 AUC. In the high fructose fed animals, glucose tolerance was reduced to 38.28±4.71 AUC (Fig. 7.1). In LD containing diet fed animals also glucose tolerance was disturbed (38.4±2.84 AUC). However, TLD containing diet fed rats showed slight improvement in glucose tolerance than LD (36.7±1.94 AUC).

7.3.2 Serum and hepatic lipid profile

In the normal diet fed animals, the serum total cholesterol was found to be 66.7 ± 7.4 mg/dL. High fructose fed animals showed a percentage increase of 30.2 (Figure 7.2a). In the LD and TLD containing diet fed animals, the TC levels was increased by 75.96 and 91.5%, respectively. Similarly, the serum triglyceride level in the HFr group was 97.3% higher than the normal diet fed rats (81.6 ± 10.1mg/dL). Animals fed with LD and TLD containing diets had a respective increase of 122.9 and 166.3% in their TG levels. In contrast, HDLc levels of animals in HFr, LD and TLD containing diets were reduced by 26.5, 30.4 and 31.9% than the normal animals (37.3 ± 6.2).

As shown in Figure 7. 2b, hepatic total cholesterol was also showed a significant increase ($P<0.01$) in all the groups compared to normal rats. In HFr, LD and TLD

containing diets, the TC was increased from the normal level (133.0 ± 7.3) by 46.4, 78.4 and 102.0%, respectively. Normal diet fed rats had a TG level of 155.37 ± 15.5mg/100g tissue. High fructose containing diet fed animals showed an increase of 55.6% ($P<0.01$). In the LD and TLD fed animals, more profound increase was observed (96.4 and 106.8%). In the case of hepatic HDLc levels, only HFr, LD and TLD containing diet fed animals had a significant decrease ($P<0.05$), where they showed a 20.4, 20.2 and 22.2% reduction.

7.3.3 Hepatic antioxidants and oxidative stress

The hepatic GSH content of normal animals fed on the reference diet was 8.51 ± 0.34 nmoles/mg protein and it was reduced to 6.17 ± 0.25 nmoles/mg protein in the HFr group. In LD containing diet fed animals, the level was 6.27 ± 0.61 nmoles/mg protein and reduced significantly to 5.32 ± 0.96 nmoles/mg protein in TLD group (Figure 7.3b).

The catalase activity in the hepatic tissue of animals in the normal group was 87.53 ± 8.73 U/mg protein. In HFr group, it was significantly reduced to 51.95 ± 8.38 U/mg protein. In LD and TLD containing diet fed rats, the hepatic catalase activity was 52.57 ± 6.29 and 42.98 ± 5.41 U/mg protein (Figure 7.3a).

Superoxide dismutase activity of the HFr group of animals (4.28 ± 0.13 U/mg protein) was significantly lower than the normal animals (6.90 ± 0.28 U/mg protein). In LD and TLD containing diet fed animals, the activities were 3.07 ± 0.18 and 4.01 ± 0.25 U/mg protein (Figure 7.3a).

The glutathione reductase activity of the normal animals was 23.73 ± 3.2 U/mg protein and it remained similar in all the groups. In HFr, LD and TLD group of rats, the GR activity was 22.44 ± 2.7, 26.98 ± 1.1 and 24.36 ± 1.6 U/mg protein, respectively (Figure 7.3a).

The thiobarbituric acid reactive substance levels in normal animals were 3.84 ± 0.32 and 4.1 ± 0.9 nmoles/mg protein in the serum and hepatic tissue. It was increased to 4.94 ± 0.26 and 5.5 ± 0.6 nmoles/mg protein in HFr group. In LD containing diet fed animals, the TBARS level was 4.96 ± 0.93 and 4.8 ± 0.4 nmoles/mg protein in the serum and liver. It was further elevated to 6.35 ± 0.88 and 7.2 ± 0.9 nmoles/mg protein in the serum and liver of TLD containing diet fed animals (Figure 7.3b).

7.3.4 Polyol pathway enzymes and ornithine decarboxylase activity

As shown in Figure 7.4 a, fructose feeding for 4 weeks induced hyperglycemia and associated activation in polyol pathway. In the normal rats the AR and SDH activities were found to be 1.59±0.35 and 0.84±0.11 U/mg protein. In the high fructose fed rats, the respective levels were increased to 3.47±0.57 and 1.34±0.27 U/mg protein. In LD fed rats, there was a marginal decrease in the activities of AR and SDH, with values 3.45±0.36 and 1.48±0.31 U/mg protein respectively. However, TLD fed animals showed the highest activities for AR and SDH, with values 3.97±0.59 and 1.58±0.32 U/mg protein.

Ornithine decarboxylase activity was found to be 5.43±0.29 in normal rats (Figure 3 b), which slightly increased in fructose fed rats (6.07±0.15). In LD fed rats, the level was same as that of the normal (6.17±1.2 U/mg protein). Whereas in the TLD fed rats, there was an augmentation in ODC activity was observed (6.03±0.48 U/ mg protein).

7.3.5 Liver function parameters

The AST activity in the normal untreated animals was 94.4 ± 9.8 IU/L, which was increased to 134.5 ± 6.4 upon 30 weeks of fructose feeding. In the LD group of rats, the AST activity was 131.0 ± 12.3 IU/L and in TLD containing diet fed rats an elevated activity of 170.5 ± 14.6 IU/L was observed (Figure 7.4 b).

Similar increase was observed in ALT activity also, where the animals fed with HFr, LD and TLD containing diet had 46.8 ± 6.0, 59.9 ± 2.3 and 60.8 ± 1.5 IU/L compared to the normal diet fed animals (35.8 ± 4.4 IU/L). Alkaline phosphatase activity of the normal rats was 196.4 ± 7.4 IU/L. The activities were increased in HFr, LD and TLD containing diet fed rats respectively to 210.4 ± 12.6, 225.5 ± 6.2, and 231.1 ± 10.2 IU/L (Figure 7.4 b).

7.3.6 GGT and LDH activity and IL-6 levels

In the reference diet fed normal animals, GGT activity was 28.65 ± 2.71 IU/L. It was elevated to 37.30 ± 8.10 IU/L in HFr group. GGT activity in LD and TLD containing diet fed animals was 44.39 ± 5.10 and 48.70 ± 2.10 IU/L (Table 7.2).

Lactate dehydrogenase (LDH) activity in the HFr group (189.6 ± 9.7 IU/L) was higher than that of the normal animals (166.9 ± 21.5 IU/L). In LD and TLD containing diet fed rats, the LDH activity was further elevated to 204.7 ± 17.1 and 229.60 ± 18.1 IU/L (Table 7.2).

The serum IL-6 level of normal animals was 0.59± 0.07 pg/mL and the same in HFr diet fed animals was 1.97± 0.21 pg/mL. In fresh lard fed animals, the serum IL-6 level was near normal (0.54± 0.08 pg/mL), whereas in TLD containing diet fed animals, the same was 2.5± 0.04 pg/mL (Figure 7.6).

7.3.7 Effect on colonic antioxidant status

Reduced glutathione levels in the colon mucosa of normal animals was 25.3 ± 3.2 nmoles/ mg protein, which was reduced to 16.18 ± 0.5 nmoles/ mg protein in HFr group. In fresh LD containing diet fed animals, the level was 19.61 ± 0.9 nmoles/ mg protein and in TLD groups it was 14.33 ± 2.0 nmoles/ mg protein (Figure 7.5.a).

Superoxide dismutase activity in the colon mucosa of normal rats fed with reference diet was 6.25 ± 0.6 U/ mg protein, which was reduced in HFr group of animals to 4.40 ± 0.3 U/ mg protein. Animals fed on modified diet containing LD and TLD had SOD activities of 5.08 ± 0.3 and 4.86 ± 0.4 U/ mg protein (Figure 7.5.a).

Glutathione peroxidase activity in normal animals was 153.67 ± 8.2 U/ mg protein, which was shown a mild reduction to 133.65 ± 4.0 U/ mg protein. However, the activity remained unaltered in LD or TLD containing diet fed animals (138.77 ± 9.0 and 110.40 ± 4.5 U/ mg protein) (Figure 7.5.b).

Thiobarbituric acid reactive substances (TBARS) level in the colon of normal rats was 43.87 ± 3.3 nmoles/ mg protein. In HFr fed animals, the serum and liver TBARS was elevated to 57.28 ± 4.9 nmoles/ mg protein. In LD and TLD diet fed animals, the levels were 55.95 ± 3.1 and 62.94 ± 6.3 nmoles/ mg protein (Figure 7.5.b).

7.3.8 Histopathological analysis

Histopathological analysis of the liver tissues of animals fed with normal diet (Figure 7.7 a) and those fed on high fructose (b), LD (c), TLD (d). The normal animals had liver tissue architecture with normal central venous system, the portal triads, sinusoidal spaces and Kupffer cells. However LD and TLD diet fed groups showed

signs of hepatosteatosis, observed as macrovesicles. TLD containing diet fed animals showed progressed hepatosteatosis than those fed with LD diet.

In normal animals (a), the colon mucosal and serosa layers found to be normal and unchanged intestinal glands. However, in HFr group (b), and those animals fed with a diet containing LD (c) or TLD (d), mild infiltration of inflammatory cells had been observed (Figure 7.8).

7.3.9 Gene expression profile using qPCR analysis

Interleukin- 6 expression was increased in HFr group of rats by 24.08 ± 1.48 fold. In LD and TLD containing diet fed animals, the expression was increased by 20.82 ± 2.46 and 43.71 ± 7.08 folds. Expression of another pro-inflammatory cytokine, TNF-α, was increased by 16.11 ± 1.42 fold in HFr group. It was increased by 16.91 ± 1.22 and 36.25 ± 1.20 fold in LD and TLD containing diet fed animals.

Expression of P53 gene was increased by 1.84 ± 0.23 fold in HFr group, whereas in LD and TLD containing diet fed rats the p53 expression was increased by 1.92 ± 0.19 and 3.20 ± 0.44 fold. Expression of Wnt-1 gene in HFr group was increased by 42.81 ± 2.19 fold. In LD and TLD containing diet fed rats, the expression was increased by 16.11 ± 2.84 and 46.21 ± 1.93 folds. In HFr group, the expression of PPAR-γ was increased by 42.22 ± 3.61. The increase in expression of the PPAR-γ gene in LD and TLD containing diet fed groups was 32.45 ± 0.28 and 28.44 ± 1.46 folds. Another proto-oncogene, protein kinase B (Akt), expression was increased by 2.85 ± 0.14 in HFr group, which was 1.06 ± 0.15 and 5.06 ± 0.25 folds in LD and TLD fed group of rats.

7.4 Discussion

Thermal oxidation Lard has been shown to increase in the peroxide and aldehyde contents as described in Chapter 3. The increase in the aldehydes is comparatively higher than the plant derived saturated and monounsaturated oils. Possibly, the oxidation and hydrolysis reactions act at the unsaturated fatty acids leading to the breakage as well as polymerization of triglycerides. Further, oxidation of linoleic acid (which is present in comparatively lesser quantity than that of SO), the predominant fatty acid in lard, can generate various toxic aldehydes and other byproducts. In

addition, cholesterol rich lard may also undergo oxidative modifications generating large quantities of oxysterols (Hur *et al.*, 2007). Supporting our results, Monika *et al.* (2017) has also shown the formation of oxysterols during thermal processing of cholesterol containing meat. Therefore, it is possible that oxidized cholesterol content may be high in TLD which make it unique from the other vegetable oils studied.

Consumption of TLD has been shown to exacerbate the hyperglycemia and glucose intolerance induced by high fructose diet over time, when compared to its fresh form (Lard). Further, increased insulin resistance, as indicated by HOMA-IR indices, has also been observed in animals fed with TLD. Studies by Alkazemi *et al.* (2008) has previously indicated a positive correlation between the oxysterols formation and insulin levels in body. Moreover PUFA content is lower in the LD than that of SO; it is thus possible that, together with the high fructose diet, the oxysterols present in TLD may have contributed to the significantly higher insulin resistance and associated hyperglycemia observed.

Increase in the activities of gamma glutamyl transferase and lactate dehydrogenase has been observed in animals fed with a diet containing thermally oxidized lard. It has been previously reported that GGT activity increase in response to insulin resistance (Bonnet *et al.*, 2011). Further, reports have also indicated that increased LDH expression and activity reduce beta cell functioning and increase insulin resistance (Ainscow *et al.*, 2000; Wu *et al.*, 2016). GGT and LDH are also indicators of oxidative damages to tissues (Ramos *et al.*, 2013), therefore it can be presumed that TLD containing diet fed animals may have increased the oxidative damages.

Increased hyperglycemic conditions in the body eventually lead to increased lipogenesis in the liver. As in the case of TLD fed rats, significantly higher levels of triglycerides and cholesterol accumulation is noted in this study. Though fresh LD fed rats also had dyslipidemic conditions, it was advanced to a greater extent in the TLD fed rats. Dyslipidemia is also confirmed in terms of the reduced levels HDLc in the serum and liver tissues of the animals. Similar results have been obtained in previous studies conducted with other edible oils (Aruna *et al.*, 2005a; Aruna *et al.*, 2004; Aruna *et al.*, 2005b; Lamboni and Perkins, 1996). This increase in the total cholesterol and triglyceride levels can be due to the endogenous cholesterol and free fatty acid content in lard. Partially the increase in TG and TC may be contributed by

the CFAMs, which have shown to alter the activity of hepatic lipid metabolizing enzymes (Lamboni *et al.*, 1998). Further, CFAMs has been previously shown to down regulate the protein and mRNA expression of Carnitine palmitoyl transferase (CPT), an enzyme involved in the transport and mitochondrial oxidation of fatty acids. In addition, Flickinger *et al.* (1997) has indicated that CFAMs diminish the activity of calcium dependent ATPase activity, whose inhibition is often correlated to hepatic lipid accumulation and hepatosteatosis (de Oliveira *et al.*, 2011).

The level of lipid peroxidative changes are noted in TLD group animals, which is in line with studies by Adam *et al.* (2008), where increased lipid peroxidation in fried palm oil fed rats. Elevated accumulation of these lipid oxidation products are often recognized as a risk factor for many of the liver diseases including non-alcoholic fatty liver (Konishi *et al.*, 2006; Poli *et al.*, 1987). Providing further insight in to this increased lipid peroxidation product accumulation, hepatic antioxidant defense system comprising of the superoxide dismutase and catalase is shown to be diminished in activity in the TLD groups. Reduction in the antioxidants enzyme activities is also suggested to be involved in the progression of NAFLD (Koruk *et al.*, 2004). These observations are in corroboration with the increased incidence of microvesicles and higher degree of hepatocellular ballooning in the liver tissues of TLD fed rats. These results thus indicate a comparatively higher degree of hepatic damage in the animals fed with high fructose and thermally oxidized lard containing diet.

In connection with these, reduced level of enzymatic as well as non-enzymatic antioxidants has also been observed in the colon tissue of TLD containing diet fed animals. Enhanced polyol pathway enzyme activities during hyperglycemia is a factor that contribute to the oxidative stress in these animals (Narayanankutty *et al.*, 2017). The increased levels of lipid peroxidation products formed in the edible oil during thermal oxidation may have also contributed towards the increased oxidative stress in TLD containing diet fed animals. Among the thermally oxidized vegetable oils studied, TCO has shown significant deleterious effect to Hepatic tissue that even projected lipogranuloma. TLD has more or less equivalent hepato toxic potential however no animals in this group develop lipogranuloma.

Along with the oxidative damages, higher expression of pro-inflammatory cytokines such as IL-6 and TNF-α has also been observed in the colon epithelial tissues of TLD

fed animals. The pro-oxidant and inflammatory potential are higher in magnitude that previously described TSO (Chapter 6). Since both the edible oils are rich in linoleic acid, heat induced oxidation of linoleic acid may be expected in both the oils generating the pro-inflammatory 9- and 13-hydroxyoctadecadienoic acids, however, with a lesser amount in TLD (9-HODE and 13-HODE) (Dandona *et al.*, 2010; Mabalirajan *et al.*, 2013; Patwardhan *et al.*, 2010; Spindler *et al.*, 1997). Additionally, the cholesterol oxidation products in TLD may also contribute to the inflammation and oxidative damages. Heat induced alterations in the cholesterol leads to the formation of oxysterols which includes α, β-epoxycholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, and 7-ketocholesterol (Orczewska-Dudek *et al.*, 2012). These cholesterol oxidation products have been found to induce toxicity in colon epithelial cells under in vitro conditions (J. *et al.*, 2003). They also has significant roles in inflammation and carcinogenesis in colorectal tissues (Rossin *et al.*, 2017). Increased expression of cell survival or proliferative genes such as Wnt-1 and Akt, concomitantly reduced PPARγ expression possibly indicate a pro-carcinogenic potential of these thermally oxidized lard. Possibly oxidized PUFA and oxysterols might have contributed to these pathological changes.

The study thus concludes that consumption of animal based fats is unhealthy; the deleterious effect could be due to the large amount of oxysterols and toxic fatty aldehydes derived carbonyls generated in the oil during thermal oxidation. Increased hepatic damage as observed in the TLD group is a clear indication of the ability of thermally oxidized lard in inducing metabolic disorders of liver over their fresh counterparts. However, though dyslipidemia and redox imbalance is elevated in TLD, the extent of NAFLD incidence has been higher in TCO containing diet fed animals (Chapter 4). Further, increased oxidative stress, chronic inflammation and elevated expression of proliferative/ cell survival genes in colon of TLD groups of rats together indicate a possible pro-carcinogenic potential over long-term consumption. Compared to other edible oils of vegetable origin (Chapter 4, 5& 6), TLD contains higher amounts of cholesterol oxidation products or oxysterols. The increased deleterious effects of TLD may be contributed by these oxysterols.

Table 7.1 Variation in the levels of blood glucose and plasma insulin as well as HOMA indices in rat under HFr diet and LD/TLD supplementation.

Parameters	Normal	HFr	LD	TLD
HOMA2 %B	63.8± 13.5	22.0± 4.1***	15.1± 1.5***	14.1± 1.8***
HOMA2 %S	116.9± 27.2	66.1± 0.5***	62.2± 3.9***	50.4± 5.2***
HOMA2 IR	0.89± 0.19	1.51± 0.01**	1.61± 0.10**	2.00± 0.21***
Glucose (mg/dL)	95.1± 5.7	216.0±17.2***	259.7± 9.5 ***	287.0 ± 16.9 ***
Insulin (pg/L)	45.71± 10.07	66.66± 2.04**	64.35± 4.67**	71.43± 4.51***

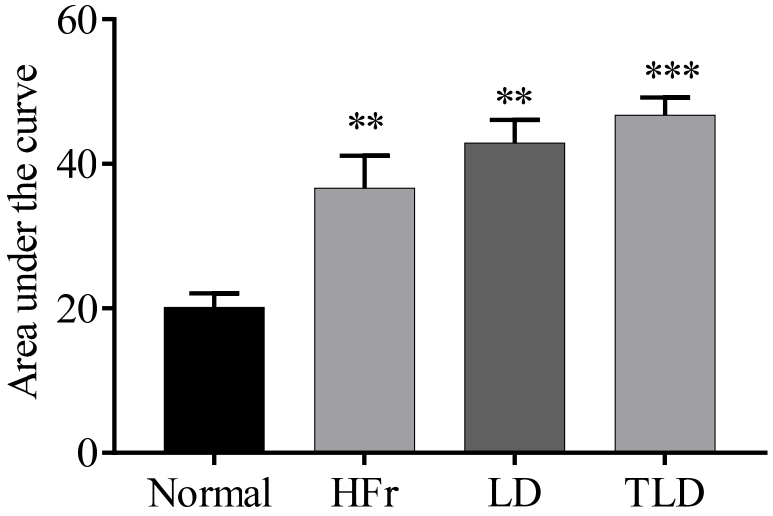


Figure 7.1 Change in the oral glucose tolerance as indicated by the change in area under the curve in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at p<0.05; ** indicates p<0.01; *** indicates p<0.001)

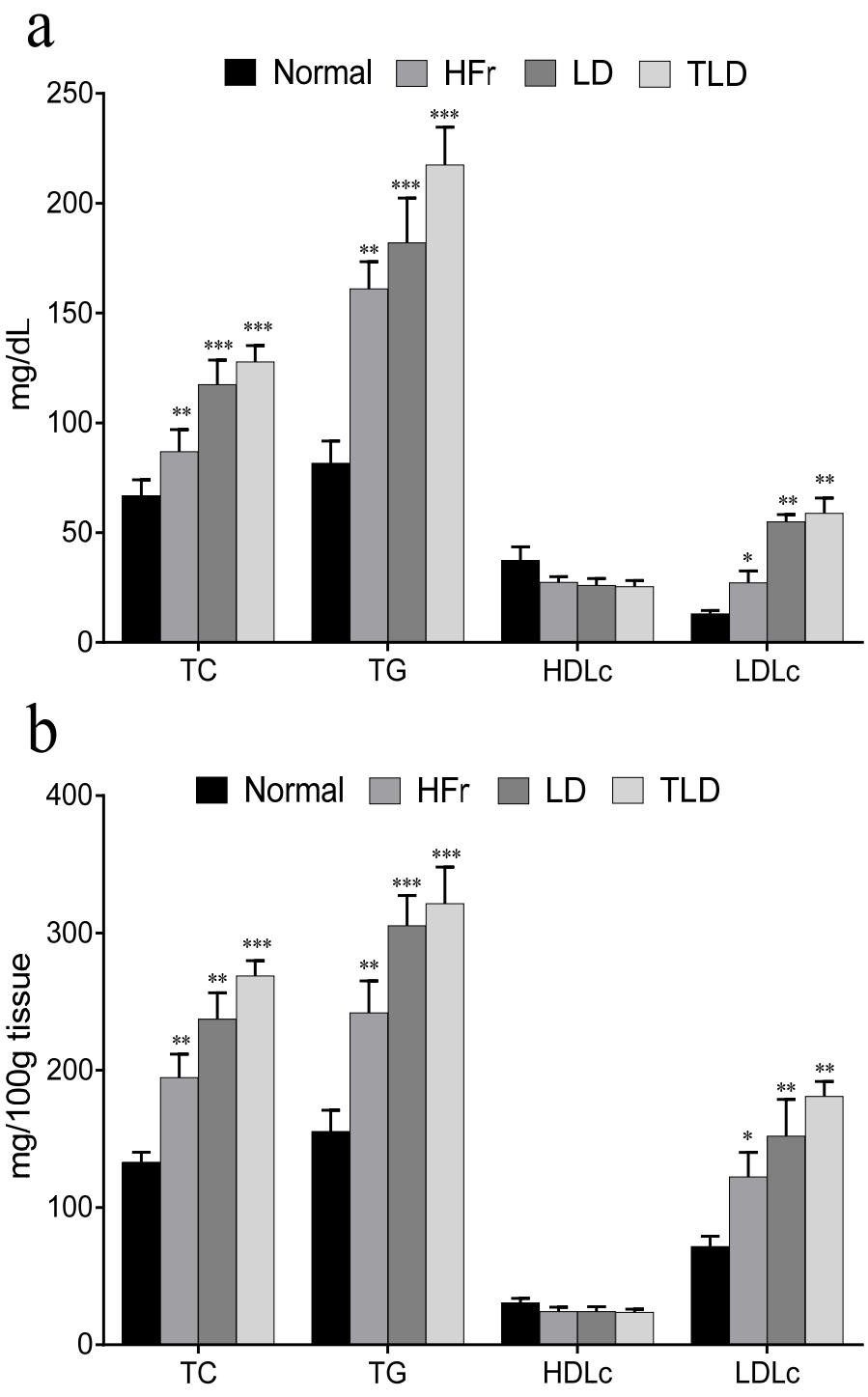


Figure 7.2: The serum (a) and liver (b) lipid profile of animals in the different experimental groups fed on a reference diet (normal) and modified experimental diet containing lard or thermally oxidized lard as fatty acid source for 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

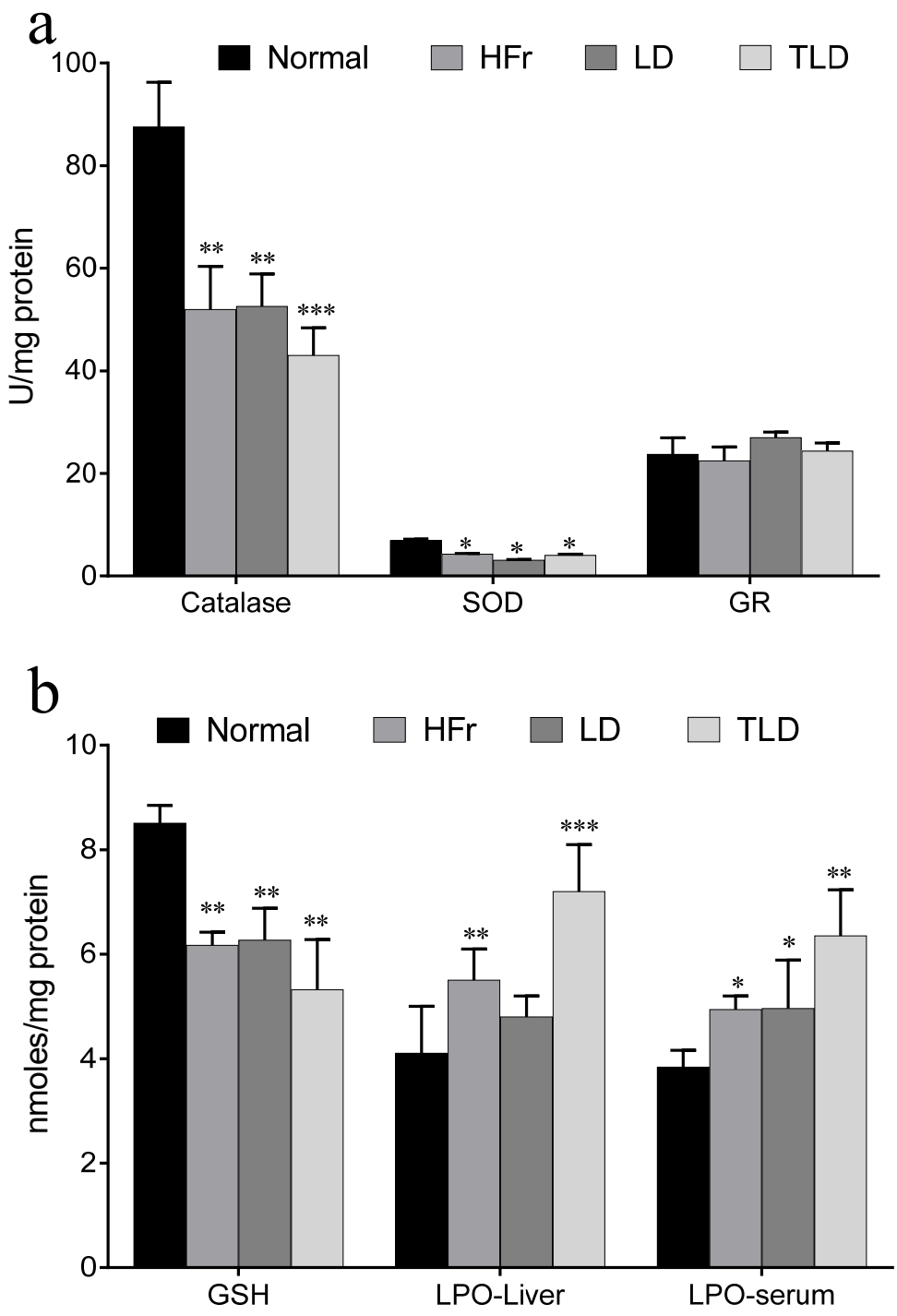


Figure 7.3: Changes in the hepatic redox status parameters such as catalase, superoxide dismutase and glutathione reductase (a) as well as GSH, serum and liver TBARs in animals fed with reference diet and modified experimental diets.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

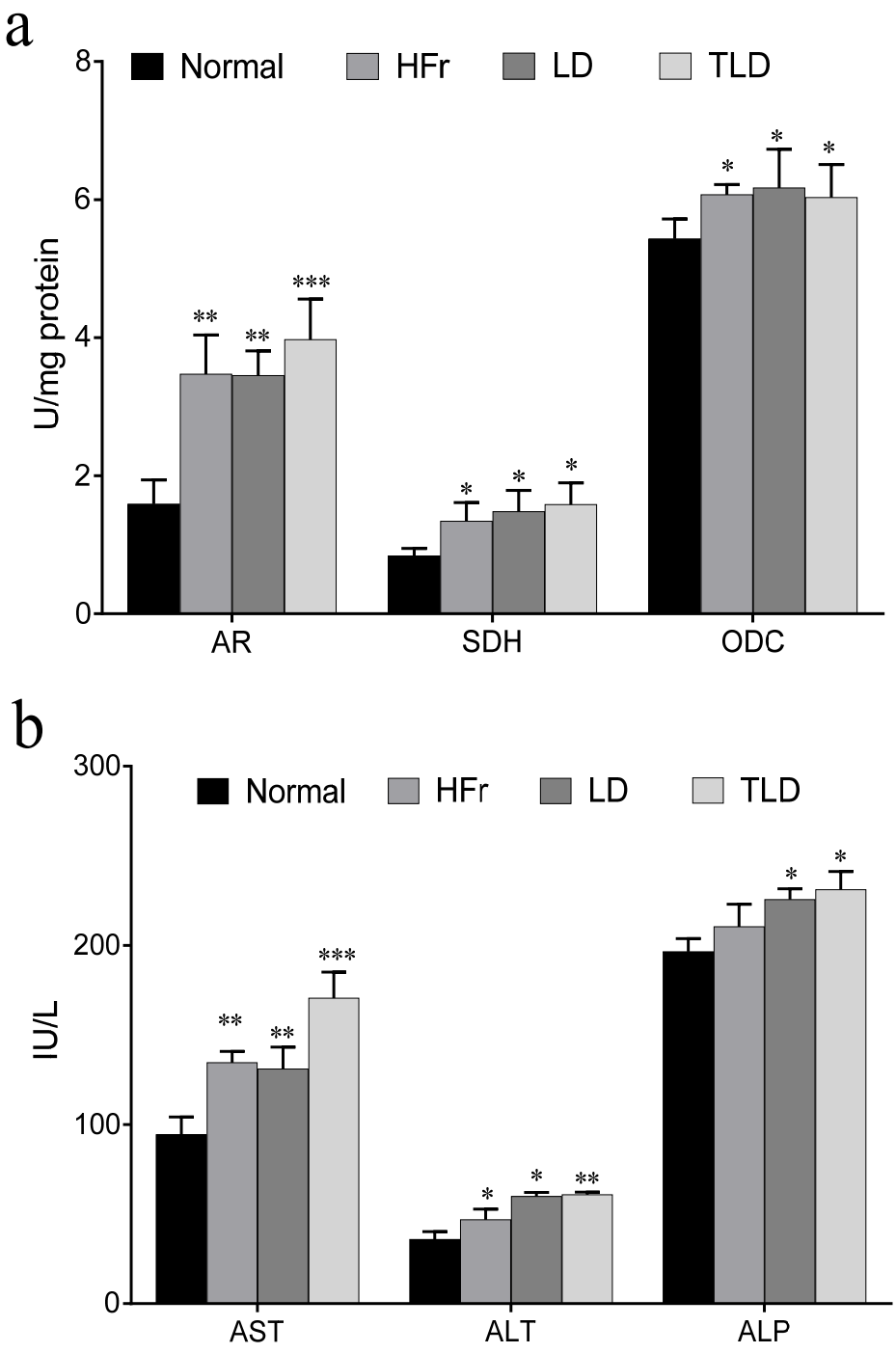


Figure 7.4: Changes in the activities of hepatic polyol pathway enzymes such as aldose reductase and sorbitol dehydrogenase (a) and liver function markers enzymes such as AST, ALT & ALP, in animals under different experimental diets after 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

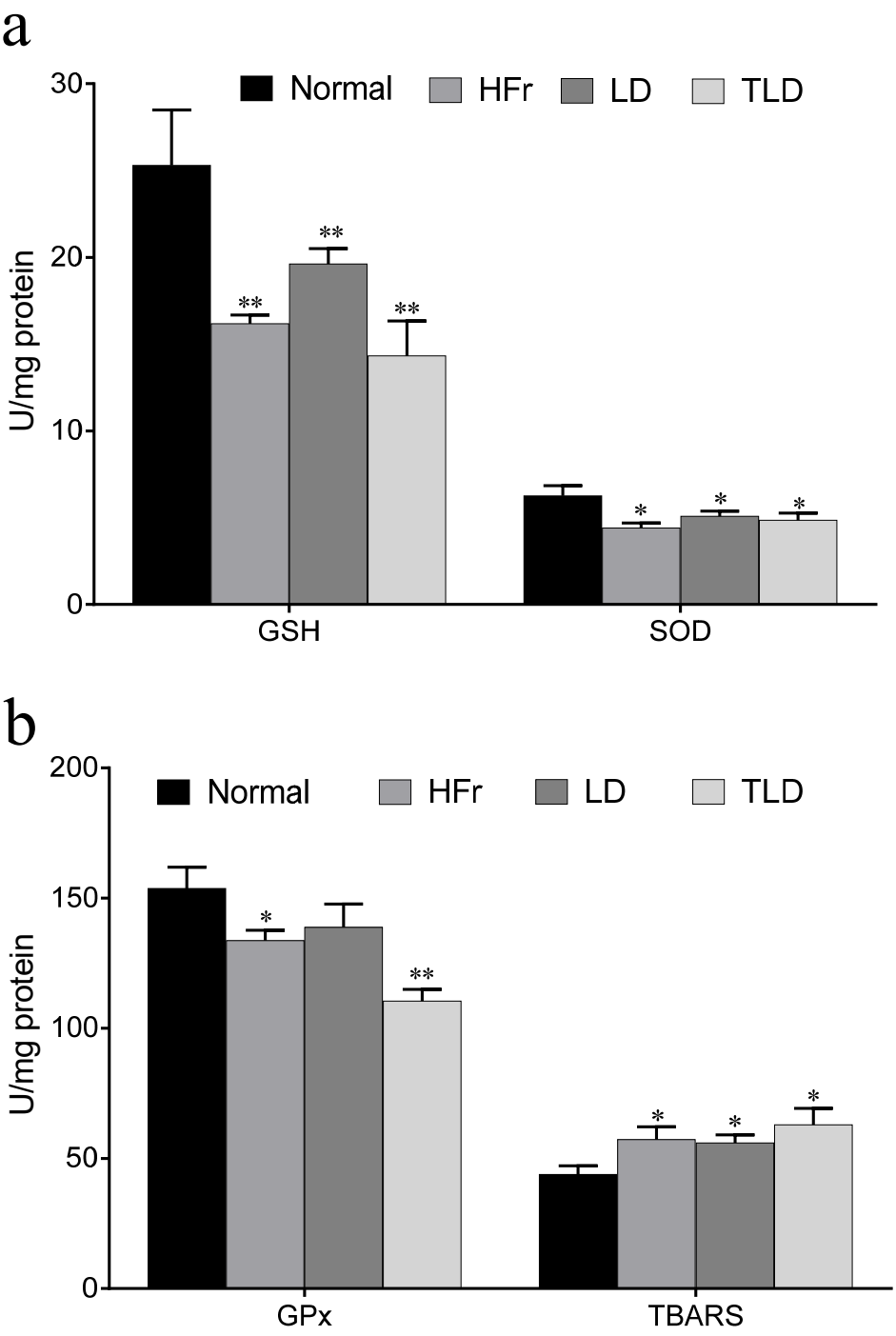


Figure 7.5 Changes in the redox parameters such as, GSH level and SOD activity (a) as well as of GPx activity and TBARS level (b), colon epithelial tissues of animals under different experimental diets for a period of 30 weeks.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Table 7.2 Changes in the activities of gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) in animals fed with HFr diet as well as LD or TLD supplementation.

	Normal	HFr	LD	TLD
GGT	28.65± 2.71	37.30± 8.10	44.39± 5.10**	48.70± 2.10***
LDH	166.9± 21.5	189.6± 9.7*	204.7± 17.1**	229.60± 18.1***

Table 7.3 Grades of hepatosteatosis in HFr diet fed animals and the effect of LD or TLD on the extent of hepatosteatosis.

Characteristics	Normal	HFr	LD	TLD
Micro vesicular steatosis	0	1	1	2
Hepatocellular ballooning	0	1	1	2
Portal tract inflammation	0	1	1	1
Glycogenated nuclei	0	1	1	1
Lipogranuloma	0	0	0	0

(0- absence; 1- mild; 2-moderate; 3- severe)

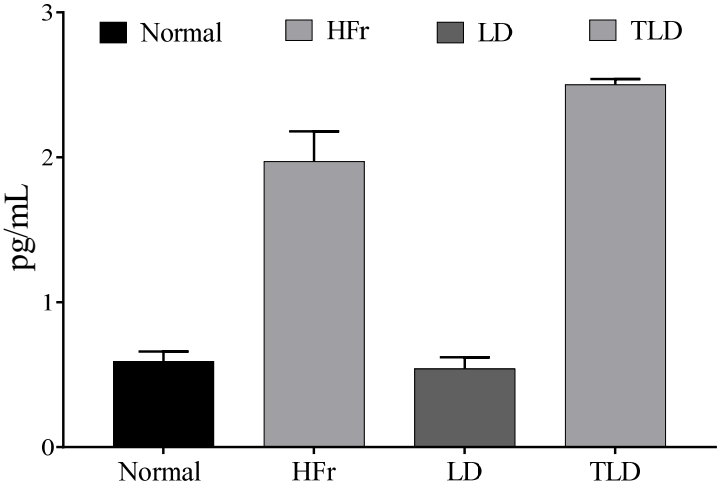
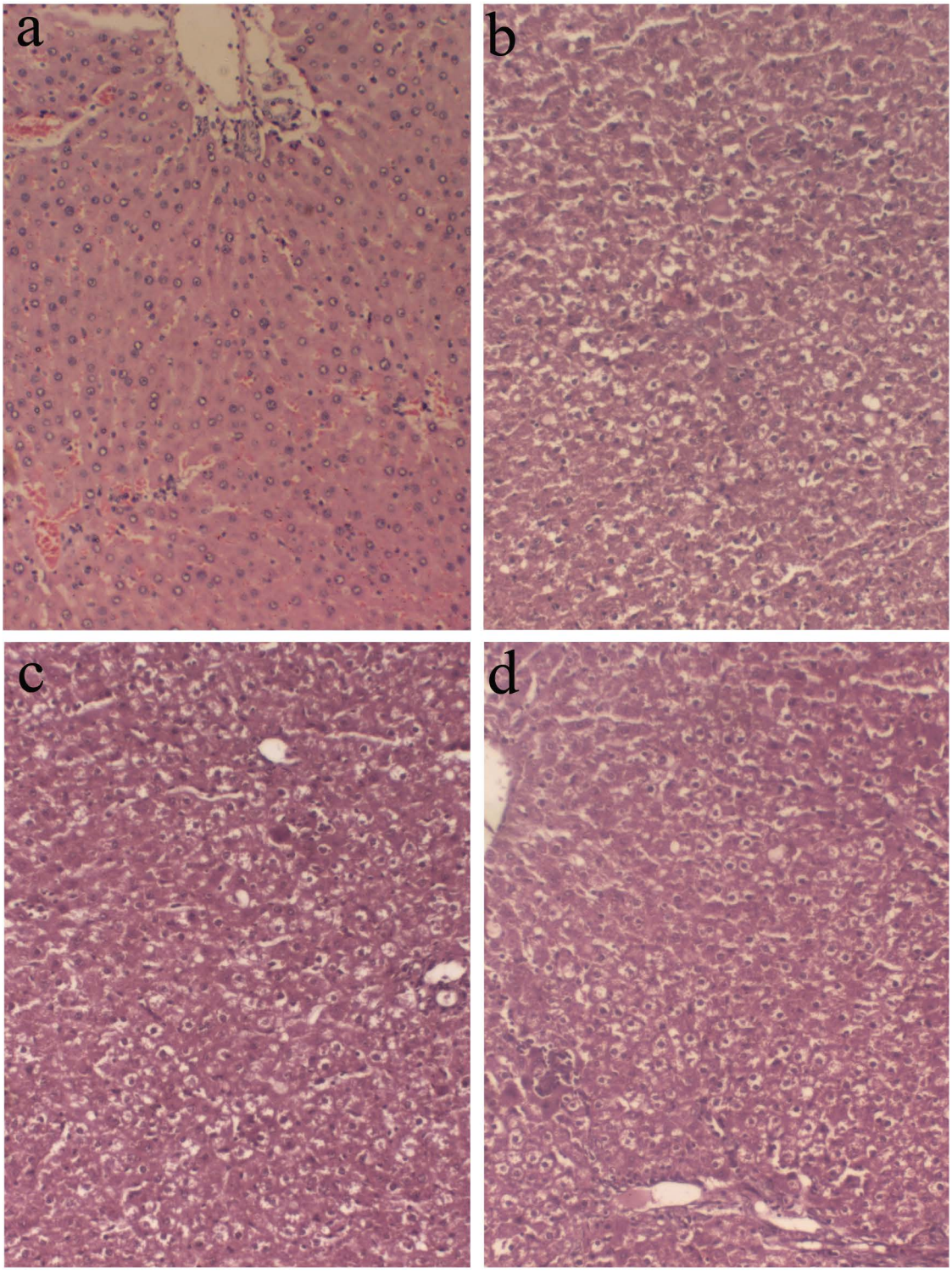
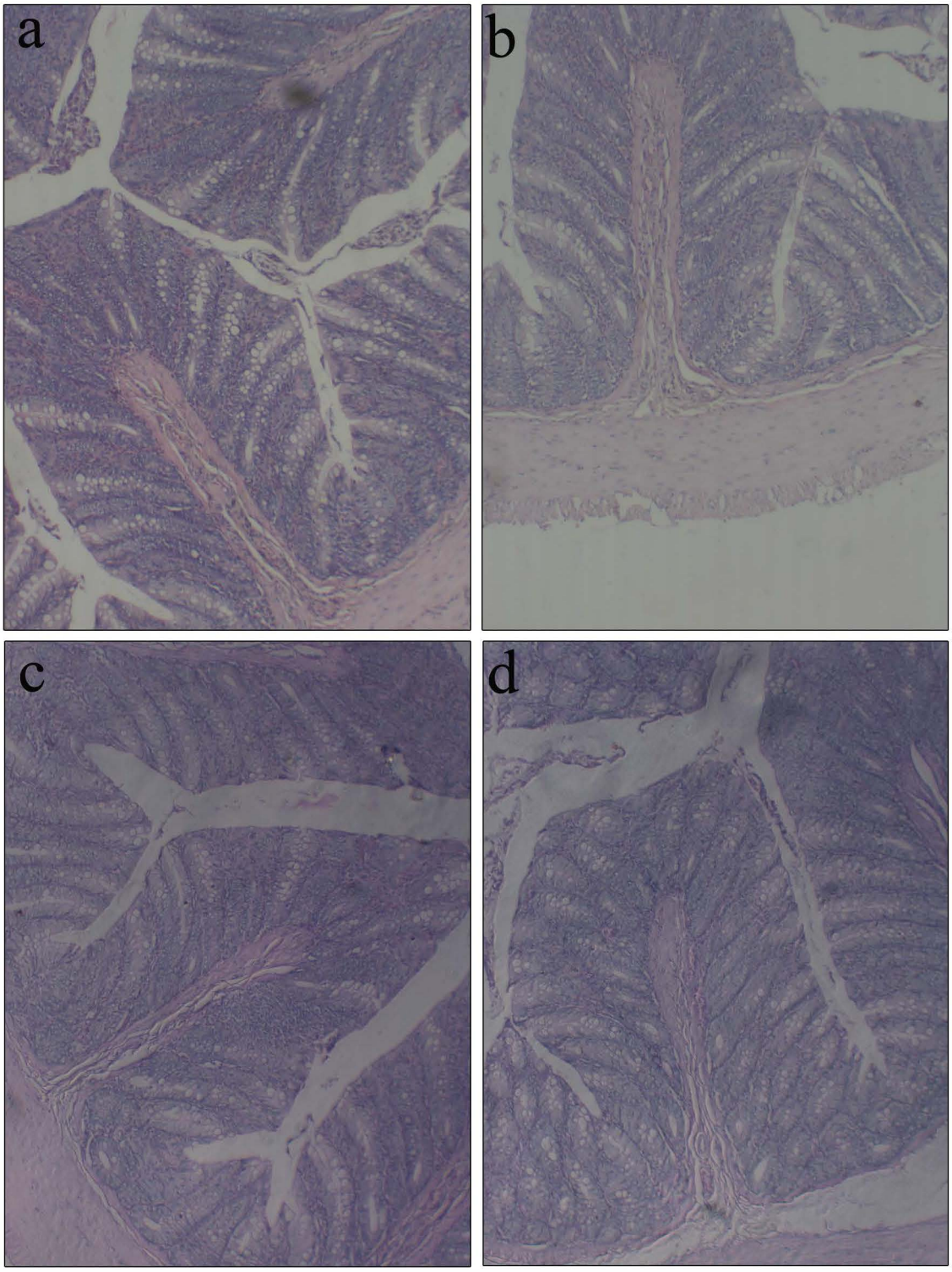


Figure 7.6 Change in the level of serum IL-6 in different experimental diet fed animals at the end of 30 weeks

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)



Figrure 7. 7 Hepatic histological changes of reference diet (Normal) animals (A) induced by feeding HFr alone diet (B), lard (C), and thermally oxidized lard (D) containing diets. Photographs were taken under 200x magnification in Magnus INVI microscope (Chennai, India).



Figrure 7. 8 Histomorphometry of the H&E stained colon tissues of animals fed with reference diet (A), HFr alone diet (B), lard (C), or TLD (D) containing. Photos were taken in Magnus INVI microscope (Chennai, India) at 200x magnification

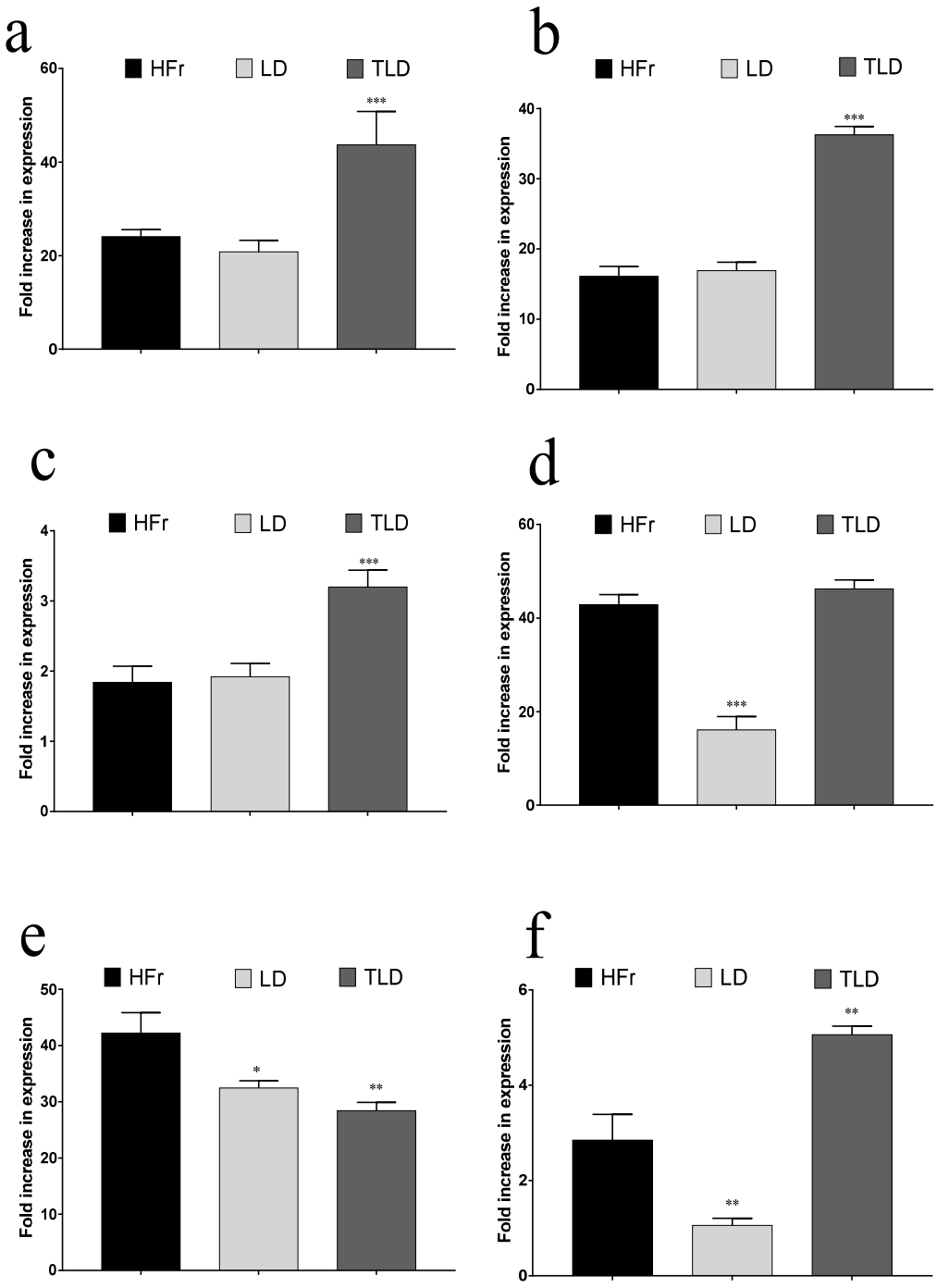


Figure 7.9 Expression of various genes such as IL- 6 (a), TNF-α (b), P53 (c), Wnt-1 (d), PPARY (e) and Akt-1 (f) in the colon epithelial tissues of experimental rats fed with reference and modified diets. The data represented fold changed in expression with respect to the untreated reference diet fed animals.

(* indicates significant difference at $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$)

Chapter 8
Summary and Conclusion

Conclusion

Edible oils are the essential sources of fatty acids and various other bioactive components such as polyphenols, tocopherol and other micronutrients. Intake of these oils at a normal level is necessary to maintain body health. The biological effects of oils vary with the changes in their physicochemical composition and thus different edible oils produce differential effects. Since most culinary processes involve heating and frying, the oils tend to undergo severe oxidative modifications such as hydrolysis, oxidation and polymerization, which make them unhealthy for consumption.

The study has identified the ill effects of consuming a high fructose- high-fat diet over prolonged periods. High fructose alone diet is found to induce insulin resistance dyslipidemia hepatotoxicity redox imbalance and inflammatory changes specifically in colon tissues. All the edible oils incorporated in the fructose diet in its thermally oxidized form exacerbated these deleterious effects, however, varied depending on the fatty acid composition and structure. In general, unsaturated fatty acid rich oils, especially polyunsaturated fatty acid rich SO as well as animal fat lard are found to exert a more deleterious effect. With respect to the insulin resistance, coconut oil and it's thermally oxidized form is found to be least toxic, owing to their medium chain saturated fat content. However, the hepatotoxic effect of TCO has been found to be predominant over other thermally oxidized edible oils and there observed a higher extent of NAFLD development. Since NAFLD raises the overall risk for the development of hepatocellular carcinoma, consumption of thermally oxidized saturated fat containing diet may be cautioned in these aspects.

Further, the HFr induced redox imbalance in colon tissue has been worsened by the incorporation of TSO and TLD into the diet fed than that of TMO and TCO. Consequently increased inflammatory insults are also evident in TSO and TLD fed group animals. The possible involvement of cholesterol oxidation products in TLD and plant sterol oxidation products in TSO is expected to be the actual driving molecules of oxidative imbalance and chronic inflammation. Together with this, upregulated expression of cytoprotective/ proliferative genes in the TLD and TSO containing diet fed animals possibly indicate a neoplastic conversion in the colon tissues. This possibility is found high with TLD. Therefore, it is assumed that the

consumption of unsaturated fat containing edible oil that contains cholesterol oxidation products possibly increase the risk for colorectal carcinogenesis.

The actual molecules behind these deleterious effects are not identified; however, the physicochemical analysis together with biochemical estimations has been helpful to make certain assumptions. The MCFA rich coconut oil tends to undergo triglyceride polymerization than oxidation. However, unsaturated fatty acid-rich edible oils have been shown to undergo oxidation, generating lipid carbonyls and aldehydes. Further, the formation of phytosterol oxidation products in TSO, as well as the formation of oxysterols in TLD, is possible during thermal oxidation.

Overall the study has identified two major setbacks over the consumption of edible oils, especially in their thermally oxidized forms. Since high fructose induces insulin resistance and dyslipidemia, a condition similar to that of Type 2 diabetes, the present study cautions that the continued use of thermally oxidized oils by diabetic individuals may increase the chance of fatty liver disease and colorectal cancer. Further, the dietary pattern of high fructose as well as high thermally oxidized edible oils and fats, used in the present study, may be equated to the current fast food habits and westernized diet that may be responsible for the current increase in the incidence of various degenerative disorders, especially NAFLD and colorectal cancer.

Chapter 9
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Chapter 10

List of publications

LIST OF PUBLICATIONS

1. Sheela DL, **Narayanankutty A.**, Nazeem PA, Raghavamenon AC, Muthangaparambil SR. (2019) Lauric acid induce cell death in colon cancer cells mediated by the epidermal growth factor receptor downregulation: An in silico and in vitro study. ***Human and Experimental Toxicology***. 38(7):753-761

2. Illam S.P., Hussain A, Elizabeth A, **Narayanankutty A.**, Raghavamenon AC (2019) Natural combination of phenolic glycosides from fruits resists pro-oxidant insults to colon cells and enhances intrinsic antioxidant status in mice. ***Toxicology Reports***. 18;6:703-711.

3. **Narayanankutty A.**, Anil A, Illam SP, Kandiyil SP, Raghavamenon AC. (2018) Non-polar lipid carbonyls of thermally oxidized coconut oil induce hepatotoxicity mediated by redox imbalance. ***Prostaglandins Leukotrienes and Essential Fatty Acids***. 138:45-51.

4. **Narayanankutty A.**, Illam S.P., and Raghavamenon A.C. (2018) Health impacts of different edible oils prepared from coconut (Cocos nucifera): A comprehensive review. ***Trends in Food Science & Technology***. 80: 1-7.

5. **Narayanankutty A**., Gopinath M.K., Vakayil M., Ramavarma S.K., Babu T.D. and Raghavamenon A.C. (2018), Non-enzymatic conversion of primary oxidation products of Docosahexaenoic acid into less toxic acid molecules. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.** 203: 222-228.

6. **Narayanankutty A.**, Manalil J.J., Suseela I.M., Ramavarmma S.K., Mathew S.E., Illam S.P., Babu T.D., Kuzhivelil B.T., and Raghavamenon A.C. (2017) Deep fried edible oils disturbs hepatic redox equilibrium and heightens lipotoxicity and hepatosteatosis in rats. ***Human and Experimental Toxicology*** 36 (9): 919-930. (DOI 10.1177/0960327116674530).

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Appendix

Urkund Analysis Result

Analysed Document:	01 Introduction and Review of Literature - Arunaksharan N.pdf (D40621723)
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Analysed Document: 02 Materials and Methods - Arunaksharan N.pdf (D40621724)
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https://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/l13_c_met_lipids.pdf
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http://www.ilexmedical.com/files/PDF/Cholesterol_ARC_CHEM.pdf
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Analysed Document: 03 Results and Discussion - Arunaksharan N.pdf (D40621725)
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