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Hepatoprotective Effect of Silymarin on Fructose Induced Nonalcoholic Fatty Liver Disease in Male Albino *Wistar* Rats.

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This is to clarify that thesis prepared by Tewodros Mengesha entitled "*Hepatoprotective Effect of Silymarin on Fructose Induced Nonalcoholic Fatty Liver Disease in Male Albino Wistar Rats"* is submitted in partial fulfillment of the Requirement for the Degree of Master of Sciences in Medical Biochemistry complies with the regulations of the university and meets the accepted standards with respect to the originality and quality.

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Abstract

Background: Nonalcoholic fatty liver disease is one of the most common causes of chronic liver disease in the Western world, and it‟s likely to parallel the increasing prevalence of type 2 diabetes, obesity, and other components of metabolic syndrome. There is also growing evidence in both animal models and human studies suggesting that high dietary intake of fructose is an important nutritional factor in the development of metabolic syndrome and its associated complications of NAFLD. However, an optimal treatment for NAFLD has not been established. Silymarin is gaining increasing recognition due to its beneficial effects in the control and prevention of alcoholic liver disease, acute and chronic viral hepatitis and toxin-induced liver diseases.

Objective: The current study aim to investigate the hepatoprotective effect of silymarin on fructose induced nonalcoholic fatty liver disease in rats.

Methodology: Thirty male *Wistar* rats were randomly divided into five groups: normal control group that consumed tap water; Silymarin control group that consumed tap water + silymarin (400 mg/kg/day), fructose control group that consumed 20% fructose solution; Treatment group that consumed 20% fructose solution + Silymarin (200 mg/kg/day) and treatment group that consumed 20% fructose solution + silymarin (400 mg/kg/day). The food and liquid intake, body weight, liver triglyceride, serum lipid profile, lipid peroxidation, antioxidant level and morphological as well as histopathological changes of liver were investigated. The data were analyzed using one way analysis of variance (ANOVA) followed by tukey multiple comparison test and statistical significance was determined at $p < 0.05$.

Result. This study showed that the fructose consumed model group had a significantly high values in the stage of steatosis grade, liver weight, hepatic triglyceride, serum triglyceride, total cholesterol, low density lipoprotein cholesterol, alanine amino transferase, aspartate aminotransferase and hepatic malondialdehyde concentration as compared to the normal control and contrariwise a significantly low values of reduced glutathione, plasma total antioxidant capacity and food consumption. The altered

parameters in the fructose model group had been partially ameliorated when treated with silymarin.

Conclusion: The rats fed with fructose developed dyslipidemia, oxidative stress and mild steatosis in liver, which are the characteristics feature of nonalcoholic fatty liver disease. When treated with silymarin showed amelioration in oxidative stress, dyslipidemia and steatosis of liver in some extent but could not reach the normal control values.

Key Words: Nonalcoholic Fatty Liver Disease, Silymarin; Lipid Peroxidation; Dyslipidemia; Total Antioxidant Status; Reduced Glutathione.

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List of Abbreviation/Acronyms

ACC: Acetyl Co-A Carboxylase

AGE: Advanced Glycation End Product

ALT: Alanine Aminotransferase

ANOVA: Analysis Of Variance

AST: Aspartate Aminotransferase

ATP: Adenosine Triphosphate

AUC: Area under the Curve

CK-18: Cytokeratin 18

CPT1: Carnitine Palmitoyltransferase-1

CRN: Center for Research Network

CRP: C-reactive protein

CT: Computed Tomography

CYP4A: Cytochrome P450A

DNL: De Novo Hepatic Lipogenesis

DTNB: 5-5'-dithiobis-2-nitrobenzoic acid

EDTA: Ethylene Diamine Tetra-Acetic Acid

ER: Endoplasmic Reticulum

FA: Fatty Acid

FABP: FA Binding Protein

FAS: FA Synthase

FAT/CD36: FA Translocase

FATP: Fatty Acid Transporter Protein

FFA: Free Fatty Acids

GSH: Reduced Glutathione

GSH-Px: Glutathione Peroxidase

HCC: Hepatocellular Carcinoma

HDL-C: High Density Lipoprotein Cholesterol

HE: Hematoxylin and Eosin

HFCS: High Fructose Corn Syrup

HFD: High Fat Diets

HCHF: High Carbohydrate High Fat

HMG-CoA: 3-Hydroxy-3-Methylglutaryl

IFCC: International Federation for Clinical Chemistry

IR: Insulin Resistance

LCFAs: Long Chain Fatty Acids

LD: Liver Disease

LDL: Low Density Lipoprotein

MetS: Metabolic Syndrome

MAPK: Mitogen Activated Protein Kinase

MDA: Malondialdehyde

MDH: Malate Dehydrogenase

MRI: Magnetic Resonance Imaging

NADH: Reduced Nicotinamide Adenine Dinucotide

NAFLD: Nonalcoholic Fatty Liver Disease

NAS: Nonalcoholic fatty liver disease Activity Score

NASH: Non-Alcoholic Steatohepatitis

NFϰB: Nuclear Factor Kappa B

PI3K: Phosphatidylinositol 3-Kinase

PPARα: Peroxisome Proliferator Activated Receptor Alpha

ROS: Reactive Oxygen Species

SPSS: Statistical Package for Social Science

TAC: Total Antioxidant Capacity

TAG: Triacylglycerol

TCA: Tri Chloro Acetic Acid

TG: Triglyceride

TBARS: Thiobarbituric Acid Reactive Substance

TNF-α: Tumor Necrosis Factor α

US: Ultrasonography

VLDL: Very Low Density Lipoprotein

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CHAPTER ONE

1. Introduction

1.1. Liver

The liver is an important organ that has a key role in the maintenance of homeostasis. The liver is responsible for multiple metabolic functions and physiological processes such as bile production, energy generation, vitamin storage, and the metabolism of carbohydrates, proteins, and lipids. After intestinal absorption is completed, the blood is then transported to the liver via the portal vein, which carries multiple toxic substances including ethanol (Et-OH), drugs, pharmaceuticals, and toxins. As a result, the liver is susceptible to toxicity and damage. Many people have been afflicted with some type of liver lesion. Examples of liver lesions include fatty liver, non-alcoholic steatosis, hepatitis A, B, C, cirrhosis, and hepatocellular carcinoma (Vargas-Mendoza *et al*., 2014).

1.2. Role of Liver in Lipid Metabolism

The liver plays a major role in lipid metabolism, importing free Fatty acids (FFAs) and manufacturing, storing, and exporting lipids; derangements in any of these processes can lead to the development of nonalcoholic fatty liver disease (NAFLD). Fatty acids are involved in many important cellular events, such as synthesis of cellular membranes, energy storage, and intracellular signaling pathways. However; chronically elevated FFAs can disturb diverse metabolic pathways and induce insulin resistance (IR) in many organs (Nguyen *et al*., 2008).

Hepatic fat accumulation has been strongly associated with IR. IR in the peripheral adipose tissue enhances lipolysis and increases the delivery of adipose derived FFAs to the liver. In particular, obesity increases tumor necrosis factor α (TNF-α) production in adipocytes, facilitates adipocyte IR, and increases lipolysis rate. Thus, the circulating pool of FFAs is increased in obese individuals and accounts for the majority of liver lipids in NAFLD (Berlanga *et al*., 2014).

1.3. Non-Alcoholic Fatty Liver Disease

The definition of nonalcoholic fatty liver disease (NAFLD) requires that: (a) there is evidence of hepaticsteatosis, either by imaging or by histology and (b) there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders (Chalasani *et al*., 2012).

Since first described by Ludwig in 1980, non-alcoholic fatty liver disease (NAFLD) has progressed from a poorly understood liver disease to one with more well defined boundaries. NAFLD is one of the most common causes of chronic liver disease in the Western world, and its prevalence is likely to parallel the increasing prevalence of diabetes, obesity, and other components of metabolic syndrome (Lam and Younossi, 2009). NAFLD has a wide spectrum of liver damage, which ranges from simple steatosis or intracellular triglyceride accumulation to inflammation and then to NASH, fibrosis and cirrhosis (Pettinelli *et al*., 2011).

The histological characteristic of NAFLD is accumulation of macrovesicular lipid similar to liver disease due to chronic consumption of alcohol. Fatty liver and steatohepatitis are two histological conditions for this disease. Liver histology in this disease is indistinguishable from alcoholic hepatitis and includes balloon degeneration, hepatocytes necrosis, and fibrosis. Pathogenesis of NASH is not well-understood but often the presence of two damages from which the first damage leads to accumulation of lipids in liver and steatosis and the second damage to inflammation and fibrosis is an accepted mechanism (Day and James, 1998).

The histological changes occurring in NAFLD typically predominantly affect the liver parenchyma, where they are mainly present in perivenular regions (acinar zone 3). However, there is an increasing recognition that portal and periportal lesions can also be seen as part of the spectrum of fatty liver disease (Hubscher, 2006). Resistance to insulin is likely the reason for the first damage in most patients, while oxidative stress and lipid peroxidation or damage by inflammatory cytokines are considered as responsible for the second damage (Hajiaghamohammadi *et al*., 2012).

NAFLD is now thought of as the hepatic manifestation of the metabolic syndrome, and is by now regarded as one of the most common liver diseases worldwide. It is estimated that about 20% of the general adult population of most Westernized countries have hepatic steatosis and that ~2% – 3% of adults even suffer from non-alcoholic steatohepatitis (Kanuri and Bergheim, 2013).

1.4. Fructose Induces NAFLD

There is growing evidence in both animal models and human studies suggesting that high dietary intake of fructose is an important nutritional factor in the development of metabolic syndrome and its associated complications. It was shown that fructose over consumption in human‟s leads to dyslipidemia and ectopic lipid deposition; along with increased hepatic insulin resistance (IR) (Seneff *et al*., 2011). There is considerable evidence that a high fructose intake can indeed produce adverse metabolic alterations, the most prominent ones being an increase in plasma triglycerides, hepatic insulin resistance, and hepatic steatosis. These effects are consistently observed in rodents fed a high fructose diet and are generally concomitant with an increased body mass (Tilg and Moschen, 2010).

1.5. Hepatoprotective Effect of Silymarin

Silymarin is extracted from the dried seeds of milk thistle plant, where it is present in higher concentrations than in other parts of the plant. The active components was first isolated and chemically characterized during 1968-1974. Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin with an empirical formula $C_{25}H_{22}O_{10}$. Its active constituents are collectively known as silymarin (Pradhan & Girish, 2006). Silymarin has been used for centuries to treat liver, spleen and gallbladder disorders. One of the important issues about plant *S. marianum* is that it may be accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (Solhi *et al*., 2014).

Hepatic protective effect of silymarin in general could be explained in four points: activity against lipid peroxidation as a result of free radical scavenging and the ability to

increase the cellular content of glutathione, efficacy to increase the membrane stability and to regulate membrane permeability in the presence of xenobiotic damage, ability to regulate nuclear expression by means of a steroid-like effect and inhibition of transformation of stellate hepatocytes into myofibroblasts which induce deposition of collagen fibers leading to the liver injury progression (Abenavoli *et al*., 2011).

1.6. Literature Review

Fatty liver is roughly divided into alcoholic and nonalcoholic fatty liver diseases. Nonalcoholic fatty liver disease (NAFLD) includes nonalcoholic fatty liver (NAFL) without hepatocellular injury and fibrosis and nonalcoholic steatohepatitis (NASH) accompanied by liver inflammation and hepatocyte injury with a risk of hepatic cirrhosis and hepatocellular carcinoma (Kumamoto *et al*., 2013).

1.6.1. Morphological Change of Liver in NAFLD

Non-alcoholic fatty liver disease (NAFLD) is histologically indistinguishable from the liver damage resulting from alcohol abuse. Liver biopsy features include steatosis, mixed inflammatory cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory‟s hyaline and fibrosis. The presence of these histologic features, alone or in combination leads to a wide spectrum of non-alcoholic fatty liver disease. Steatosis, predominantly as large droplets or macrovesicular fat, as well as necroinfammatory injury, Mallory‟s hyaline (MH) and fibrosis is typically concentrated in acinar zone 3. The presence of fibrosis is a concerning histologic finding because it suggests a more advanced and severe liver injury (Angulo and Lindor, 2002). Portal inflammation, ductular reaction and periportal fibrosis can also be seen as part of the morphological spectrum of NAFLD, particularly in the pediatric population (Hubscher, 2006).

In NASH, not only steatosis but also intralobular inflammation and hepatocellular ballooning are present, and this is usually accompanied by fibrosis. Intralobular inflammation in NASH is typically mild and of a mixed type and includes a small number of lymphocytes, macrophages, and neutrophils. Neutrophils tend to infiltrate in the area of marked steatosis and around MH. Portal inflammation is usually mild;

however, relatively intense chronic inflammation may be present in the portal area (Takahashi *et al*., 2012).

The histological severity of NAFLD is determined by the nonalcoholic fatty liver disease activity score (NAS) and fibrosis Score, developed and validated by the CRN (center for research network). The activity score ranges from 0 to 8 and fibrosis Score from 0 to 4. A NAS of 0-2 is not NASH and a score of ≥5 is usually NASH (Oh *et al*., 2008).

1.6.2. Pathophysiology of NAFLD

The mechanisms associated with the accumulation of triacylglycerol (TAGs) in the liver and the subsequent hepatocellular damage is multifactorial and not fully understood. The first metabolic abnormality that leads to liver steatosis, involving a lipotoxic reaction with a component of oxidative stress, includes nutritional factors and changes in liver lipid metabolism, which is mainly the result of insulin resistance (IR) (Pettinelli *et al*., 2011).

A fatty liver is the result of the accumulation of various lipids. Several mechanisms may lead to a fatty liver: (1) increased free fatty acids supply due to increased lipolysis from both visceral/subcutaneous adipose tissue and/or increased intake of dietary fat; (2) decreased free fatty acid oxidation; (3) increased *de novo* hepatic lipogenesis (DNL) and (4) decreased hepatic very low density lipoprotein triglyceride secretion (Tilg and Moschen, 2010). Tracer studies on patients with hepatic steatosis suggest that DNL may contribute as much as 30% of the fatty acids present in liver triacylglycerol. Ingested fatty acids are delivered to the liver either directly via spillover into the plasma non-esterified fatty acids (NEFA) pool or via uptake of chylomicron remnants. Together these pathways contribute around 15% of the fatty acids in liver triglyceride in obese patients with NAFLD (Bellentani *et al*., 2010).

1.6.3. Hepatic Fatty Acid (FA) Uptake

One of the sources for hepatic FAs is free fatty acid (FFA) recruitment from the blood stream. FFAs are derived from lipolysis in adipocytes, which usually occurs in the fasting state, promoted by catecholamines, natriuretic peptides, and glucagon, and are usually repressed by insulin (Arner, 2005). However, the IR state (obesity, metabolic syndrome)

goes along with increased adipocyte lipolysis, leading to abundant FFAs in the plasma pool independently from the nutritional status (Delarue and Magnan, 2007). FFAs are then taken up by the hepatocytes in a facilitated fashion rather than by passive processes. FA uptake into the liver contributes to the steady balance of hepatic triglycerides (TGs), as well as the pathogenesis of NAFLD. The rate of FA uptake from plasma into cells depends on the FA concentration in the plasma and the hepatocellular capacity for FA uptake, which also depends on the number and activity of transporter proteins on the sinusoidal plasma membrane of the hepatocyte (Berk, 2008).

The main plasma membrane transporters for FFAs are FA transporter protein (FATP), caveolins, FA translocase (FAT/CD36), and FA-binding protein (FABP) (Berlanga *et al*, 2014)

1.6.4. *De Novo* Lipogenesis

The process in which the liver synthesizes endogenous FAs is called *de novo* lipogenesis. This includes *de novo* synthesis of FAs through a complex cytosolic polymerization in which glucose is converted to acetyl-CoA by glycolysis and the oxidation of pyruvate. Acetyl-CoA carboxylase (ACC1) then converts acetyl-CoA into malonyl-CoA. Finally, FA synthase (FAS) catalyzes the formation of palmitic acid from malonyl-CoA and acetyl-CoA. Depending on the metabolic state, FAs are then processed to TGs and stored or rapidly metabolized.

Dietary fats are packed in chylomicrons and hydrolyzed; releasing FAs of which approximately 20% are delivered to the liver. In the fasting state, a decline of insulin levels stimulates adipocyte TG hydrolase, thereby releasing FFAs that are transported to the liver. In the liver, FFAs derived from peripheral tissue, endogenous synthesis, or diet, can be used for: 1) energy and ketone body production via mitochondrial β-oxidation; 2) esterified and stored as TGs in lipid droplets; or 3) packaged with Apo-lipoprotein B into VLDL that is secreted into the circulation. In NAFLD patients, enhanced acquisition of FAs through uptake and *de novo* lipogenesis are not compensated by FA oxidation or production of VLDL particles (Berlanga *et al*, 2014).

1.6.5. Inhibition of Fatty Acid Oxidation

Oxidation of FAs occurs within the mitochondria, peroxisomes, and the ER. It facilitates the degradation of activated FAs to acetyl-CoA. FAs are activated by acyl-CoA-synthetase to acyl-CoA in the cytosol, which is crucial for enabling FAs to cross membranes and enter organelles. Short-chain and medium-chain FAs pass the mitochondrial membrane without activation. However, activated long chain fatty acids (LCFAs) are shuttled across the membrane via carnitine palmitoyl transferase-1(CPT-1). Malonyl-CoA, an early intermediate of *de novo* lipogenesis, is an inhibitor of CPT-1 (Nassir and Ibdah, 2014).

In the fed state, FA oxidation is inhibited and *de novo* lipogenesis promoted, allowing for storage and distribution of lipids. In general, short, medium, and long chain FAs are oxidized within mitochondria (β-oxidation), while toxic; very-long-chain FAs are oxidized within peroxisomes. In diabetes or FA overload, cytochrome P450 (CYP4A) dependent ω-oxidation of LCFAs occurs in the ER and induces reactive oxygen species (ROS) and lipid peroxidation. During the process of β-oxidation, electrons are indirectly donated to the electron transport chain to drive ATP synthesis. Acetyl-CoA can be further processed via the tricarboxylic acid cycle, or in the case of FA abundance, be converted into ketone bodies. Peroxisome proliferator activated receptor alpha (PPAR-α) and insulin signaling are again involved in the regulation of FA oxidation and the formation of ketone bodies via transcriptional regulation of mitochondrial 3-hydroxy-3-methylglutaryl synthase (HMG-CoA) (Berlanga *et al*, 2014).

1.6.6. Two Hit Hypothesis of NAFLD

The first hit is characterized by hepatic triglyceride accumulation contributing to steatosis; therefore, steatotic liver appears to be more vulnerable to the „„second hit"" of adipokine induced liver injury, oxidative and endoplasmic reticulum stresses, mitochondrial dysfunction, and hepatic apoptosis, which subsequently promote the transition from simple steatosis to steatohepatitis (Ganzetti *et al*., 2015). Here insulin resistance (IR) appears to exert a central role in both the first and second hits. Insulin is an anabolic hormone that regulates glucose metabolism, gene expression,

energy homeostasis and enzymatic functions. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the ras mitogen activated protein kinase (MAPK) pathway are the two most important pathways that are involved in insulin mediated functions (Ganzetti *et al*., 2015).

1.6.7. Progression of NAFLD

The earliest stage of NAFLD is hepatic steatosis characterized by the deposition of cytoplasmic triglycerides as macro and/or microvesicular lipid vacuoles in more than 5% of hepatocytes. Hepatic steatosis is often self-limited; however, it can progress to NASH distinguished from simple steatosis by the presence of hepatocellular injury, inflammatory infiltrate and/or collagen deposition. Fibrosis usually originates in the perisinusoidal regions of zone 3 and may also be present in the periportal area. Up to now, it is not clear what causes the progression of steatosis to NASH or if steatosis and NASH are distinct disorders (Kanuri and Bergheim, 2013).

1.6.8. Epidemiology

Obtaining epidemiological data for NAFLD is difficult due to differences in diagnosis and reporting practices as well as lack of specific case defining criteria. Most of the studies in the general population are based on liver ultrasound or liver chemistries, with liver biopsy mostly restricted to subjects at high risk for more aggressive liver disease. Although the worldwide prevalence has not yet been determined, it has been quoted as 10-24% in various populations. Although these estimates may reflect referral bias, NAFLD is estimated to be the most common liver disease in the Western world, and its prevalence is likely increasing. It affects all racial and ethnic groups and has no age or sex predilection. 2.6% of children are affected and this figure increases to 22.5% to 52.8% in the obese child population (Sass *et al*., 2005).

The prevalence of NAFLD is 80-90% in obese adults, 30-50% in patients with diabetes and up to 90% in patients with hyperlipidemia. Moreover, pediatric NAFLD increased from about 3% a decade ago to 5% in 2010, with a male to female ratio of 2:1 (Bellentani *et al*., 2010). The incidence has reached to 9.6% of children overall and in up to 38% of obese children in 2014 (Manti *et al*., 2014).

In the United States it has been estimated that steatosis affects over two-thirds of the obese population, whereas NASH is found in 19% of these obese individuals. Further, about one-third of the U.S. populations suffering from type 2 diabetes mellitus have NAFLD. It is likely that the increasing prevalence of NAFLD in the United States and other developed countries parallels the surge of obesity and diabetes that has become evident among all age groups (Sass *et al*., 2005). There is no any data that showed the prevalence of NAFLD in Africa particularly Ethiopia.

1.6.9. Nutrition as a Risk Factors for NAFLD

One of the most commonly used methods to induce progressive NAFLD in laboratory mice is through dietary modification. Many variant diets have been used for this purpose. Key factors influencing the extent to which each diet promotes NAFLD, NASH, and HCC include the types and amounts of fat, carbohydrate, and cholesterol, as well as the presence or absence of critical nutrients, gender, strain background, and composition of the gut microbiome. Interactions between each of these factors ultimately determine the phenotypic outcome of feeding a modified diet. Given that dietary factors play a large role in human NAFLD, these models provide a relevant system in which to generate and test hypotheses about disease induction and progression (Riordan and Nadeau, 2014).

1.6.10. High Fat Diet

Fat is the most energy dense macronutrient in human nutrition thereby increasing the odds to develop obesity when consumed in excessive amounts. Accordingly, diets rich in fat (HFD), e.g., 30%-75% of total calories derived from saturated fatty acids (± unsaturated fatty acids) have been proposed to be a useful tool to induced metabolic alterations and NAFLD. Indeed, depending on the duration of the feeding time and combination of fatty acids, rodents fed a high fat diet (HFD) display obesity, impaired glucose tolerance, dyslipidemia, increased expression of regulators of lipogenesis (e.g., SREBP1c and liver X-receptor) and expression of proinflammatory cytokine (Kanuri and Bergheim, 2013).

1.6.11. Sugar Rich Diet

Results of several epidemiological and some small clinical studies conducted in different countries (e.g., US, Japan, Israel and Germany) suggest that a shift in dietary patterns towards a sugar rich diet may also be risk factor for the development of NAFLD in humans. Diets with elevated carbohydrate content (e.g., high-sucrose or high-fructose diets) have also been used to induce the development of NAFLD in mouse models (Kanuri and Bergheim, 2013).

Fructose is a highly lipogenic sugar present in processed foods and beverages in large amounts throughout the world. Fructose can be found in its monosaccharide form or can be bound to glucose with a disaccharide bond in sucrose. The primary dietary sources of fructose are high-fructose corn syrup and sucrose (cane or beet sugar) because both are commonly used to sweeten beverages and processed foods. Since its introduction in 1967, the use of high-fructose corn syrup (HFCS) has increased relative to sucrose because it is less expensive, transports easily, and stabilizes the texture of some processed foods better than sucrose (Vos and Lavine, 2013).

1.6.11.1. High Fructose Corn Syrup

High-fructose corn syrup (HFCS) is a fructose-glucose liquid sweetener alternative to sucrose (common table sugar) first introduced to the food and beverage industry in the 1970s. It is not meaningfully different in composition or metabolism from other fructose-glucose sweeteners like sucrose, honey, and fruit juice concentrates. HFCS was widely embraced by food formulators, and its use grew between the mid-1970s and mid-1990s, principally as a replacement for sucrose. This was primarily because of its sweetness comparable with that of sucrose, improved stability and functionality, and ease of use (White, 2008). Calorically sweetened beverage intake has also been related to the risk of nonalcoholic fatty liver disease, and, in men, gout. Calorically sweetened beverages contribute to obesity through their caloric load, and the intake of beverages does not produce a corresponding reduction in the intake of other food, suggesting that beverage calories are “add-on” calories. The increase in plasma triglyceride concentrations by

sugar-sweetened beverages can be attributed to fructose rather than glucose in sugar (Bray, 2013).

1.6.11.2. Mechanisms Responsible For Fructose-Induced Metabolic Alterations

In some subjects, fructose absorption is quantitatively limited, and some malabsorption occurs when large amounts of fructose are ingested. This can cause abdominal discomfort and diarrhea, and production of volatile fatty acids from colonic fructose fermentation. Fructose absorbed from the gut into the portal vein is nearly completely metabolized in the liver through metabolic pathways distinct from those of glucose; furthermore, the initial steps of its metabolism are insulin-independent, and hence, fructose is largely metabolized without requiring insulin secretion and without increasing plasma glucose (Tappy *et al.*, 2010).

Fructokinase and aldolase B are not inhibited by ADP and citrate and hence are not regulated by the cellular energy status. In that, fructose differs from glucose, because the ADP and citrate concentrations exert a negative feedback control on the initial steps of glycolysis. As a consequence of this absence of feedback inhibition, virtually all the fructose ingested with a meal is rapidly converted into hepatic triose-phosphates. These substrates are subsequently oxidized within the liver cells or converted into glucose and lactate to be released into the bloodstream, or converted into hepatic glycogen. A small, but significant amount of triosephosphate is also converted into triacylglycerol in liver cells through the process of *de novo* lipogenesis (TG). Although quantitatively far less important than the other pathways of fructose disposal, *de novo* lipogenesis appears to be closely associated with the adverse metabolic effects of fructose (Mayes, 1993).

The adverse effects of fructose on glucose metabolism are closely linked to alterations of lipid metabolism. With impaired insulin signaling in the liver, there is decreased glycogen synthesis, and increased glycogenolysis and gluconeogenesis. As a compensatory response, insulin secretion increases. It has been suggested that the increased insulin secretion is a direct response to increased FFA levels rather than increased glucose production (Stanhopea and Havel, 2008).

1.6.12. Biomarkers in NAFLD

Due to the important limitations of the currently available noninvasive tests, several investigators have tried to identify potential novel biomarkers based on the current knowledge of the pathophysiologic mechanisms involved in disease progression in NAFLD. An ideal biomarker should be simple, reproducible, inexpensive, readily available, and accurate for a particular disease process. In the case of NAFLD, this biomarker should serve for distinguishing NASH from NAFL and/or determine the extent of liver fibrosis present. It should also be useful for monitoring disease progression over time, response to different therapeutic interventions, and prognosis. Finally, identification of such a biomarker may also help as a new tool in the development of effective new therapeutics as well as to identify the specific population that may obtain the best benefit from these treatments (Wieckowska *et al*., 2007). These below are some potential biomarkers under investigation for NAFLD.

1.6.12.1. C - Reactive Protein (CRP)

C-reactive protein (CRP), an inflammatory marker, has mixed results in NAFLD. Several studies showed no predictive value. However, recently, it was showed that a significant elevation of high sensitivity assays for CRP in cases of NASH compared with cases of simple steatosis. High sensitivity CRP was also significantly elevated in those with advanced fibrosis compared with mild fibrosis. Reverse transcriptase polymerase chain reaction also showed intrahepatic mRNA expression of CRP was increased in patients with NASH compared to simple steatosis (Oh *et al*., 2008).

1.6.12.2. Oxidative Stress By-Products

Markers of oxidative stress including lipid peroxidation byproducts may also be useful biomarkers of NAFLD. However these substances are relatively volatile and not always easily measured in serum. Malondialdehyde, thiobarbituric acid reactive substances (TBARS) and oxidized low density lipoprotein (LDL) have all been measured as markers of oxidative stress in patients with NASH but with some conflicting results (Fitzpatrick and Dhawan, 2014).

1.6.12.3. Cytokeratin 18 (CK-18)

Quantification of circulating levels of apoptotic markers accurately predicts the presence of NASH, supporting the potential usefulness of these markers in clinical practice for noninvasive diagnosis of NASH (Tamimi *et al*., 2011). CK-18 fragments were markedly increased in patients with NASH as compared to not NASH and borderline diagnosis. Moreover, the odds of having fibrosis on liver biopsy increased with increasing plasma CK-18 fragment levels ($P < 0.001$). On multivariable regression analysis, CK-18 fragments remained an independent predictor of NASH after adjusting for variables associated with CK-18 fragments or NASH on the univariable analysis (fibrosis, ALT, AST, age, biopsy length). Determination of CK-18 fragments in the blood predicts histological NASH and severity of disease in a large, diverse population of patients with biopsy-proven NAFLD, supporting the potential usefulness of this test in clinical practice (Feldstein *et al*., 2009).

1.6.13. Diagnosis

1.6.13.1. Symptoms and Signs

Most patients with NAFLD have no symptoms or signs of liver disease at the time of diagnosis. A high proportion of patients with cryptogenic cirrhosis share many of the clinical and demographic features of patients with NAFLD (Angulo and Lindor, 2002).Vague right upper quadrant abdominal pain, fatigue, and malaise are the most common of these nondescript symptoms. Rarely, pruritus, anorexia, and nausea may develop (Sass *et al*., 2005). Physical examination does not provide any findings that reliably indicate the presence of NAFLD or NASH. Because insulin resistance is present in most patients with NAFLD, the associated examination abnormalities of centripetal obesity, hypertension may be seen in association with NAFLD. Hepatomegaly is said to be present in up to 75% of patients, although it can be difficult to identify on physical examination in patients with marked abdominal obesity. Lipodystrophys are associated with NAFLD and NASH. An unusual distribution of body fat may suggest a diagnosis of lipodystrophy, but subtle lipodystrophic phenotypes can be difficult to identify (Neuschwander-Tetri, 2005).

1.6.13.2. Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

Although 80% of patients with steatosis may have aminotransferases in the normal range, in some patients with NAFLD, the diagnosis is suspected in the presence of mildly elevated aminotransferases. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) fluctuate, with two-thirds of patients with NASH having normal levels at any point in time. Alkaline phosphatase may also be mildly elevated. Aminotransferases greater than two times normal are predictive of septel and bridging fibrosis across different populations. Hyperbilirubinemia and a low albumin, however, indicate a state of advanced liver disease and are not otherwise found in NAFLD (Oh *et al*., 2008).

1.6.13.3. Imaging Studies

Several noninvasive imaging techniques, including ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI), can identify hepatic steatosis and have been advocated as diagnostic tests for NAFLD. Of these, US are the least expensive. The sonographic findings of diffuse fatty change include a diffuse bright liver, increased liver echo texture compared with the kidneys, vascular blurring, and deep attenuation. Fatty infiltration of the liver produces a low-density hepatic parenchyma on CT scanning. In a direct comparison of CT with US, US were found to be more sensitive in detecting fatty change. However, when fatty change is patchy or focal, CT scan and MRI are superior to US (Sass *et al*., 2005).

1.6.13.4. Liver Biopsy

A liver biopsy is a procedure that involves taking a small piece of liver tissue with a biopsy needle for examination with a microscope for signs of damage or disease. Examination of a liver biopsy by an experienced pathologist is an essential diagnostic complement. However, given the lack of effective medical treatment for patients with NAFLD, there has been a reasonable hesitation to perform liver biopsy with the simple purpose of confirming the diagnosis. Nevertheless, liver biopsy remains not only the best diagnostic tool for confirming NAFLD, but also the most sensitive and specific means of providing important prognostic information. Some histologic features have been

recognized as useful in determining the risk of progression to more advanced liver disease (Angulo and Moschen, 2002).

1.6.14. Pharmacologic Therapy

Currently, there are no approved therapies for NAFLD. There are many proposed agents being evaluated currently, each targeting a different step in the pathogenesis of development of hepatic steatosis or progression to steatohepatitis. The proper dosing, duration of treatment, safety, and tolerability of these treatments remain under investigation. Here below is some list of proposed drugs which has been tried so far.

1.6.14.1. Insulin Sensitizers

Agents that improve insulin sensitivity are currently undergoing extensive evaluation with regard to safety and ability to improve histological features of fibrosis and inflammation.

1.6.14.1.1. Thiazolidinediones

Thiazolidinediones (TZDs) are a class of oral antidiabetic drugs that induce a nuclear transcription factor peroxisome proliferator activated receptor-γ (PPAR-γ). PPAR-γ is predominantly expressed in adipose tissue and leads to decreased hepatic fat lipolysis and improves glycemic control with insulin sensitivity. TZDs also increase plasma adiponectin levels, activate AMP-activated protein kinase and induce fatty acid stimulation. A lot of human and animal studies have investigated the effect of TZDs on liver enzymes and histology to date. In rat models, pioglitazone and rosiglitazone prevented activation of hepatic stellate cells in vitro and improved hepatic steatosis and fibrosis in vivo (Ozturk and Kadayifci, 2014).

1.6.14.1.2. Metformin

Metformin is in the class of medication called the biguanides and is a widely used oral medication for the treatment of type 2 diabetes. The insulin sensitizing effect of metformin is attributed to its ability to activate the adenosine monophosphate activated protein kinase (AMPK) pathway which switches cells from anabolic to catabolic pathways. This results in inhibition of gluconeogenesis and lipogenesis in favor of fatty

acid β-oxidation and fatty acid and glucose uptake in the liver tissue and peripheral skeletal muscle. Metformin has also been shown in ob/ob mice, an animal model of hepatic steatosis, to reverse hepatomegaly, steatosis, and aminotransferase abnormalities (Le and Loomba, 2012).

1.6.14.1.3. Exedin-4 (Nateglinide)

Exedin-4 (nateglinide) improves insulin sensitivity by stimulating the release of insulin from the pancreas and stimulating growth of pancreatic beta cells. Studies on ob⁄ob mice have demonstrated a significant improvement in hepatic steatosis and IR. A small pilot study, which investigated the use of nateglinide in five diabetic patients with NASH, found a significant improvement in biochemical and histological markers of disease (Oh *et al*., 2008).

1.6.14.2. Antioxidants

Reactive oxygen species (ROS) generated by microsomal and peroxisomal free fatty acid oxidation play an important part in progression of simple steatosis to steatohepatitis and progressive hepatic fibrosis. They bind with intracellular organelles, disrupt cell signalling, contribute to membrane peroxidation, elicit cytokine release and can activate hepatic stellate cells (Chitturi, 2008).

1.6.14.2.1. Vitamin E

The antioxidant properties of vitamin E results from its ease in donating hydrogen from its hydroxyl group to neutralize free radicals and thereby to prevent lipid peroxidation. Vitamin E supplementation is a representative antioxidant drug treatment that has become the standard treatment for NASH. Administration of vitamin E improves non-alcoholic fatty liver disease activity scores (NAS) for clinical and histological activity within two years, but increases insulin resistance and plasma triglyceride levels. However, the recovery of fibrosis progression has not been demonstrated. Antioxidants do not affect body weight, waist circumference, and cholesterol metabolism (Takaki *et al*., 2014).

1.6.14.2.2. Betaine

Betaine is a metabolite of choline that assists with the synthesis of S-adenosyl methionine. Betaine improved hepatic steatosis and may protect against worsening steatosis. High dose betaine supplementation failed to reduce S-adenosyl homocysteine and did not positively affect any of the second hit mechanisms postulated to contribute to NASH that was studied. Although betaine has been proven effective in treating hepatic steatosis in several animal models, translating novel therapeutic options noted in animal studies to humans with NASH will prove challenging (Abdelmalek *et al*., 2009).

1.6.14.2.3. Pentoxifylline

The anti-TNF-α agent pentoxifylline has been considered for treatment of NASH. Pentoxifylline is a non-selective phosphodiesterase inhibitor reported to decrease TNF-α gene transcription as well as affecting multiple steps in the cytokine/chemokine pathway by direct or indirect inhibition of TNF-α (Li *et al*., 2011).

1.6.14.3. Anti-Lipidemic Drugs

In addition to IR, alterations in lipid metabolism may be an antecedent for development of steatosis and NASH. Targeting the mechanisms of lipid production and accumulation in the liver has generated several potential therapies for NASH.

1.6.14.3.1. Hydroxymethylglutaryl-CoA Reductase Inhibitors (Statins)

Statins reduce cholesterol production and hence serum cholesterol by competitively inhibiting hepatic 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase. They are commonly used in patients with vascular disease and T2DM to prevent further vascular events. The use of statins in patients with chronic liver disease has raised concerns about their potential to cause hepatotoxicity. However, it is now established that statin use in patients with compensated liver disease is essentially safe (Mehta, 2010).

1.6.14.3.2. Clofibrate

In contrast to statins, this group of drugs does not inhibit cholesterol biosynthesis. However, these drugs stimulate β-oxidation of fatty acids mainly in peroxisomes and

partly in mitochondria. Therefore, this group of drugs is known to lower plasma levels of fatty acid and triacylglycerol. Clofibrate was the first such drug, developed in Japan in the1960s (Ozawa and Ozawa, 2002). However, an open label, one year pilot study failed to show an improvement in lipid profile, aminotransferases and histological grade of steatosis (Duvnjak *et al*., 2007).

1.6.14.3.3. Probucol

Probucol is an anti-hyperlipidemic agent with powerful antioxidant activity that prevents lipid oxidation. This drug has shown promising results based on its significant reductions in aminotransferase levels and improvements in liver histology among a small observational study of 8 patients. However, probucol concomitantly decreased HDL-cholesterol levels, which are causes of concern in patients with coronary artery disease (Federico *et al*., 2014).

The therapeutic arena for NAFLD continues to develop and focuses on improving IR and decreasing inflammatory microenvironments to prevent or slow the development of NASH. There are no leading drug candidates at this point, although there are several promising concepts in drug development. Currently, therapy should address the features of the metabolic syndrome, including diabetes, obesity and dyslipidemia.

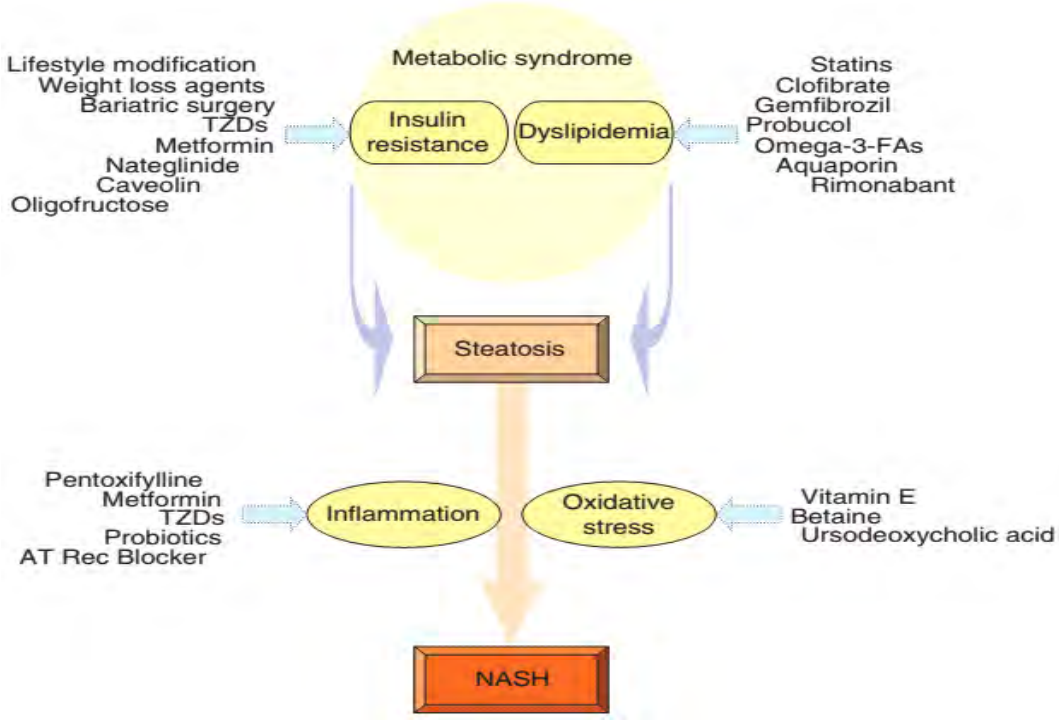

Figure 1: Therapeutic agents under investigation for nonalcoholic steatohepatitis

Source: (Oh *et al*., 2008).

1.6.14.4. Silymarin

Milk thistle is a natural compound that is present in species derived from *Silybum marianum* that belongs to the Aster family (Asteraceae or Compositae). The mature plant has large brilliant purple flowers and abundant thorns. The plant grows in places with sufficient sun exposure (Voinovich *et al*., 2009). The plant contains at least seven flavolignans and the flavonoid taxifolin. The most important flavolignans present include silybin, silydianin, and silychristin. Silybin represents between 50% and 70% of the extract from *Silybum marianum*. Silymarin has been used worldwide for many years as a complementary alternative medicine because of the beneficial effects associated with the treatment of hepatic diseases (Abenavoli *et al*., 2010).

Silybin is the major compound of silymarin and limiting factors such as low solubility in water, low bioavailability, and poor intestinal absorption reduce its efficacy (Abenavoli *et*

al., 2010). The level of silymarin absorption is between 20% and 50% (Voinovich *et al*., 2009). New soluble silybin-derived bio compounds silybin-bis-hemisuccinate, β-cyclodextrin complex, silybin-N-methyl-glucamine, silybin-11-O-phosphate, and silybin-phosphatidylcholine have been designed (Vargas-Mendoza *et al*., 2014).

The crude drug contains 15-30% lipids (triglycerides, linoleic, oleic and palmitic acid), about 30% proteins, sugars (arabinose, rhamnose, xylose, glucose); tocopherol (0.038%), sterols (0.063%) with cholesterol, compsterol and stigmasterol, and flavonoids including quercetin, taxifolin, eriodictyol and chrysoeriol. However, the constituents responsible for the activity are flavanolignans initially isolated as a mixture of addition products of a coniferyl alcohol, phenylproponoid alcohol, 2, 3-dihydroflavonol and taxifolin. This mixture, known as silymarin, represents 1.5-3% of the dry drug weight and consists of Silybin (approximately 50% to 60%), isosilibyn (about 5%), silychristin (about 20%) and silydianin (about 10%), as well as silimonin, isosilychristin, isosilibinin (Wu *et al*., 2009).

1.6.15. Clinical Trials of Different Drugs for the Treatment of NAFLD

Studies had been done on different drugs which have been believed to have a mechanism to ameliorate or treat the patient with NAFLD and thus drugs have showed different outcomes. For example, A study done on a randomized, placebo controlled trial of pioglitazone in non-diabetic subjects with Nonalcoholic Steatohepatitis suggests that pioglitazone therapy over a 12-month period resulted improvements in metabolic and histologic parameters, most notably in liver injury and fibrosis (Aithal *et al*., 2008). In another study a randomized clinical trial study was done on Silymarin to evaluate the efficacy of silymarin, a known herbal drug, in the treatment of NASH. According to the study, the patients who had taken silymarin experienced more notable fall in hepatic enzymes (Solhi *et al*., 2014).

In other study in contrary a drug like metformin which is insulin sensitizer had shown no contribution to the patient with NAFLD. In this randomized controlled trial study, Metformin was used to treat patients with non-alcoholic fatty liver disease. According to the study, the result suggests that treatment with metformin for 6 months was no better than placebo in terms of improvement in liver histology in patients with NAFLD.

Nevertheless, the use of metformin could still be beneficial in this group as it is associated with a reduction in serum levels of lipids and glucose (Haukeland *et al*., 2009).

Some studies also tried to compare the effects of different drugs which have been thought to have the potential to treat NAFLD. A study done to assess the effects of metformin, pioglitazone, and silymarin on treatment of NAFLD showed that all drugs are beneficial in improving biochemical indices in patients with NAFLD. Changes in AST and ALT in silymarin group were demonstrated more improvement than the other groups and the average difference between changes were significant between silymarin and metformin group (Hajiaghamohammadi *et al*., 2012).

A study done on an open label, prospective randomized study to compare the therapeutic effects of 70 mg silymarin three times daily and 400 IU vitamin E per day in nonalcoholic fatty liver disease at the end of the 12-week treatment period showed that there was a significant decrease in the serum AST and ALT levels in both treatment groups. The decrease in AST level in the *S. marianum* group as compared to the vitamin E group was significant ($P < 0.007$). In this study no side effects were reported in both cases. In general *S. marianum* and vitamin E treatment appears to be significantly effective in biochemical improvement and decreasing the ALT and AST levels in patients with NAFLD (Hajiani and Jalal, 2009).

Some drugs had been tried to alleviate the dyslipidemic profile of patients with NAFLD in other studies. A study done on lipid targets during statin treatment in dyslipidemic patients affected by nonalcoholic fatty liver disease indicated that statin treatment was effective in patients with dyslipidemia and NAFLD (Maroni *et al*., 2011).

1.6.16. Drug Trials on Experimental Models of Rats

In the table below, the effect and mechanism of action of drugs had been seen on the experimental models of rodents and different outcomes have been observed.

Table 1: Effect of different drugs on NAFLD induced rodents

NAFLD Rodent model	Drugs Used	Effect	Reference
High fat-induced fatty liver in rats	Pioglitazone	Attenuate insulin resistance and biochemical and histological injury in high fat-induced fatty liver in rats	XU *et al*., 2006
Dietary methionine and choline deficient (MCD) mice	Polaprezinic	Attenuates fibrosis in NASH by reducing inflammation and lipid peroxidation	Sugino *et al*., 2008
Dietary Rat Model of Nonalcoholic Steatohepatitis	Tranilast	Ameliorates the rat model by targeting TGF-β this represents a new mode of therapy for NASH	Uno *et al*., 2008
NAFLD in streptozotocin (STZ)-induced type 2 diabetic mice	L-carnitine (LC)	Ameliorated fatty liver in type 2 diabetic mice by increasing fatty acid oxidation and decreasing the LC/ALC ratio in the liver	Xia *et al*., 2011
Murine Models of Nonalcoholic Fatty Liver Disease	Ursodeoxycholyl Lysophosphatidyl ethanolamide	Improves hepatic injury at different stages of NAFLD. By concurrently lowering hepatic lipid overloading, susceptibility of hepatocytes toward inflammatory stimuli	Pathil *et al*., 2012
LDLR-/- mice after a high-fat diet	LPSF/GQ-02	Improving the hepatic architecture, decreasing fat accumulation, reducing the amount of collagen, decreasing inflammation	AK *et al*., 2015

Table 2: Effect of Silymarin and Silybin on damaged liver in Rodents

Liver damaged Rodent	Drugs Used	Effect Of The Drug	Reference
Palmitate-Induced Lipotoxicity in HepG2 Cells	Silymarin	Effective agent in protecting hepatocytes from saturated fatty acids induced cell death	Song *et al*., 2007
CCl_4-induced damage of liver fibrosis in rats	Silymarin	Decreased the elevation of AST, ALT, and ALP in serum, and also reversed the altered expressions of smooth muscle actin in liver tissue.	Tsai *et al*., 2008
NASH induced by the MCD diet in Rats	Silybin-Phospholipid Complex	Effective in preventing severe oxidative stress, preserving hepatic mitochondrial bioenergetics, conferring anti-inflammatory and antifibrotic effects	Serviddio *et al*., 2010
High-fat-induced fatty liver in rats	Silybin	Mitochondrial membrane stabilization, oxidative stress inhibition, as well as improved insulin resistance	Yao *et al*., 2011
Dietary rat model of NASH	Silymarin	Increased the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) and decreased tumor TNF-α mRNA expression	Kim *et al*., 2012
Liver of rats with NAFLD	Silibinin	Prevents visceral obesity by reducing visceral fat, enhanced lipolysis and inhibited gluconeogenesis by down-regulating associated genes such as Forkhead box O1, phosphoenol pyruvate carboxykinase and glucose-6-phosphatase	Yao *et al*., 2013
Hepatic regeneration after partial hepatectomy on Rats	Silymarin	Antiproliferative, anti-inflammatory and antioxidant effects, but with no contribution in proliferative regeneration of the liver-which has very important metabolic functions after partial hepatectomy.	Wu *et al*., 2015

Pharmacologic therapy of NAFLD has so far focused on the two arms of the pathogenesis of the disease: insulin resistance and oxidative stress. Similarly, a number of hepatoprotective drugs including vitamin E, ursodeoxycholic acid, betaine, lecithin, beta-carotene, and selenium have shown marginal benefit in improving liver enzymes and reversing inflammation but still remain experimental.

1.7. Statement of the Problem

Fatty liver disease (FLD) is a growing public health problem worldwide. Global prevalence of NAFLD based on meta-analysis study is about 25.24% with highest prevalence in the Middle East and South America and lowest in Africa. Metabolic comorbidities associated with NAFLD included obesity 51.34%, type 2 diabetes 22.51%, hyperlipidemia 69.16%, hypertension 39.34% and metabolic syndrome 42.54% (Younossi *et al*., 2015). The prevalence of NAFLD is approximately 30% in developed countries and nearly 10% in developing nations (Qiu and Chen, 2015).

Different studies revealed high fructose and high fat diets consumption has been associated with their harsh role for development of NAFLD. Excessive fructose intake has been linked to an increased prevalence of metabolic diseases and growing evidence suggests that it may also contribute to the development and severity of NAFLD by exacerbating fat deposition, inflammation, and possibly fibrosis (Longato, 2013).

Furthermore, several groups of drugs have been suggested according to the pathomechanisms of liver injury in NASH; including antioxidants, carnitine, and insulin sensitizers . Nevertheless, while some agents show modest improvements in liver function test (LFTs) and even histologic parameters, the agents mentioned above are generally used to modify risk factor profiles rather than as primary therapy for NASH (Solhi *et al*., 2014). Only a limited number of studies are available regarding pharmacological interventions for NAFLD. As per our knowledge there was no research done on silymarins effect on fructose induced NAFLD in rats. The most researched herbal treatment for liver diseases is *Silybium marinum* or milk thistle therefore in this study we investigated the hepatoprotective effect of silymarin prepared from this plant on fructose induced NAFLD.

1.8. Significance of the Study

In humans, higher amounts of fructose in the diet are associated with metabolic syndrome, obesity and NAFLD. Fructose promotes protein fructosylation and the formation of reactive oxygen species in the liver. These days the consumption of sugar in Ethiopia is also rapidly increasing due to an increase in different soft drink suppliers in different forms and due to life style changes which is related to western countries. So this study showed the negative consequences of fructose in liver.

Dietary factors play a large role in human NAFLD, this experiment may provide a relevant system to generate and test hypotheses about fructose‟s drastic potential for NAFLD induction. In over all, the treatment and effect of silymarin would be assessed since different literature survey brings to light the different kinds of medicinal and hepatoprotective activities of silymarin. Despite its usage as an agent, there is no information regarding effect of silymarin on fructose induced NAFLD in rats. Hence; this study was evaluated the effect of silymarin on fructose induced NAFLD in rats. In addition, this study would serve as a baseline data for researchers who are interested to conduct further research.

1.9. Hypothesis of the Study

The hepatoprotective effect of silymarin on fructose induced NAFLD rats was assessed. The study also investigated the effect of silymarin in serum hepatic enzymes, lipid profile and liver TG, antioxidant status, lipid peroxidation as well as histological profile of liver.

The null hypothesis (HO) hypothesizes that silymarin has no protective effect on fructose induced NAFLD rats.

The alternative hypothesis (HA) hypothesizes that silymarin has protective effect on fructose induced NAFLD rats.

1.10. Objectives

1.10.1. General Objective

To investigate the hepatoprotective effect of silymarin on fructose induced NAFLD in male Albino *Wistar* rats.

1.10.2. Specific Objectives

- To find out fructose induced changes in chow consumption, Growth, liver morphological changes and the effect of silymarin administration.
- To evaluate the fructose induced biochemical changes in serum, liver and its variation due to silymarin administration.
- To measure fructose induced alteration in the antioxidants level and restoration by silymarin.
- To observe the liver histopathological variation induced by fructose intake and alteration by silymarin administration.

CHAPTER TWO

2. Materials and Methods

2.1. The Study Area

The study was conducted at Black Lion Collage of Health Science School of medicine at Department of Biochemistry Master of Science laboratory, Department of Pharmaceutics and Social study laboratory and Department of Microbiology and Immunology laboratory.

2.2. Ethics Statement

The experiment was performed after the protocol was approved by Department of Biochemistry Research And Ethical Review Committee meeting number DRERC: 06/15 with protocol number of M.Sc. thesis 9/15 in accordance with the code of ethics of animal experiments which comply with national and international scientific and ethical guidelines.

2.3. Drugs/Chemicals

Silymarin was purchased from micro labs India, Most of the reagents were purchased from the Research Lab Fine Chem Industries. The kits for total antioxidant capacity, reduced glutathione and lipid peroxidation parameters were purchased from Himedia India. However, some specific chemicals obtained from England or other countries have been mentioned accordingly. All the chemicals used in the experiments were of analytical grade.

2.4. Study Animals

Thirty male *Wistar* Albino rats weighing 151-170 g were obtained from the Animal Experiment Center of Ethiopian Public Health Institute and from College of Health Science Department of pharmacology. Animals were maintained at 23 ± 1°C room temperature and 12/12 h dark/light rhythm. The rats were acclimatized for two weeks. Female rats were excluded from the study because of their cyclic hormonal variations.

2.5. Preparation of Fructose and Silymarin

The fructose solution was prepared accordingly Mamikutty *et al*. (2015) by dissolving 20g pure fructose (fructose crystalline, Kibbutz Maanit, Israel) in 100 ml of tap water (20% w/v). Silymarin was dissolved in distilled water. The maximum amount of silymarin solution given to the rats was decided by their weight using 20 ml/kg as a reference volume based on the OECD‟s (organization of economic corporation and development‟s) guidelines, (2000). According to the guide for the lower dose (200 mg/kg) 10 mg/ml solution and for the higher dose (400 mg/kg) 20 mg/ml of silymarin was prepared. The dose was established based on the lethal dose (LD-50) according to Radko and Cybulski (2007).

2.6. Treatment Protocol and Animal Grouping

These experimental rats were randomly divided into five groups consisting of 6 rats in each group. The experimental animals in different groups were as follows:

- ✓ **Normal Control Group (NC)** the negative controls group took chow and water only.
- ✓ **Fructose Control Group (FC)** positive controls group took 20% fructose solution as well as chow.
- ✓ **Silymarin Control Group (SC)** treatment control group took chow and water along 400 mg/kg treatment of silymarin.
- ✓ **Fructose + Silymarin (200mg/Kg) Group (FTH)** the treatment group took chow and 20% fructose solution as well as 200 mg/kg silymarin.
- ✓ **Fructose + Silymarin (400mg/Kg Body Weight) Group (FFH)** the treatment group took chow and 20% fructose solution along 400 mg/kg silymarin.

2.7. Measurement of Body Weight, Liver weight, Food and Water Intake

Food intake and liquid intake were measured and recorded. Body weight for each rat was measured weekly. After scarification, rat liver was isolated, weighed and recorded.

2.8. Biochemical Studies

2.8.1. Tissue Preparation

At the end of the experiment day, the rats were fasted overnight, anesthetized using diethyl ether and the blood was collected through cardiac puncture. After that the rats were sacrificed by cervical dislocation and liver was isolated. Liver were minced with sharp scissors in the proportion of 1:10 (w/v) ice cold phosphate buffer saline (0.1 M; PH 7.4) and homogenized (RZR-2100, Germany). Then the homogenates were centrifuged for 20 min at 4,000xg at 4ºC. Aliquots of homogenates were used for the determination of hepatic malondialdehyde (MDA) and reduced glutathione (GSH).

2.8.2. Measurement of Lipid profiles

Serum lipid profiles were measured with conventional laboratory methods using an auto analyzer (Mindrey BS-200 Full Chemistry Analyzer, China).

Test Principles

A. Total Cholesterol

The assay is based on the enzyme driven reaction that quantifies both cholesterol esters and free cholesterol. Cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase into the ketone cholest-4-en-3-one plus hydrogen peroxide. One of the reaction byproducts, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a color. The reaction sequence is as follows:

Cholesteryl Ester + H_2O $\xrightarrow{\text{Cholesteryl Ester Hydrolase}}$ Cholesterol + Fatty acid

Cholesterol + O_2 $\xrightarrow{\text{Cholesterol Oxidase}}$ Cholest-4-en-3-one + H_2O_2

2 H_2O_2 + 4-aminophenazone + phenol $\xrightarrow{\text{Peroxidase}}$ 4-(p-benzoquinonemonoimino)-phenazone + 4 H_2O

Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration

B. Triglycerides

Triglycerides are hydrolyzed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to Glycerol-3-Phosphate (G-3-P) which is oxidized by the enzyme glycerol-3-Phosphate oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-aminoantipyrine and 4-Chlorophenol in the presence of enzyme peroxidase (POD) to produce Quinoneimine dye compound. Absorbance is measured at 500 nm. The reaction sequence is as follows:

Triglycerides + $3H_2O$ $\xrightarrow{\text{Lipase}}$ Glycerol + Fatty acid

Glycerol + ATP $\xrightarrow{\text{Glycerokinase}}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O2 $\xrightarrow{\text{Glycerophosphate oxidase}}$ Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + $2H_2O$ + HCl

Absorbance is measured at 500 nm. The color intensity is proportional to triglyceride concentration

C. High-Density Lipoprotein (HDL-C) Cholesterol

HDL is measured directly in serum. The basic principle of the method is as follows. The apoB containing lipoproteins in the specimen are reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL-C is detected under the assay conditions. The method uses sulfated alpha-cyclodextrin in the presence of Mg^{+2}, which forms complexes with apoB containing lipoproteins, and polyethylene glycol coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement. The reactions are as follows:

1. ApoB containing lipoproteins + α-Cyclodextrin + Mg^{+2} + Dextran SO_4 ⟶ Soluble non-reactive complexes with apoB-containing lipoproteins

2. HDL-cholesteryl esters $\xrightarrow{\text{PEG-CE}}$ HDL-unesterified cholesterol + Fatty Acid

3. Unesterified chol + O_2 $\xrightarrow{\text{PEG-Cholesterol Oxidase}}$ Cholestenone + H_2O_2

4. H_2O_2 + 5-aminophenazone + N-ethyl-N-(3-methylphenyl)-N-succinyl ethylene diamine + H_2O + H^+ $\xrightarrow{\text{peroxidase}}$ Quinoneimine dye + H_2O

Absorbance is measured at 600 nm.

D. LDL-Cholesterol

The LDL-Cholesterol test is a two reagent homogenous system. The assay is comprised of two distinct phases. In phase one a unique detergent solubilizes cholesterol from non-LDL- lipoprotein particles. This cholesterol is consumed by cholesterol esterase, cholesterol oxidase, peroxidase and 4-aminoantipyrine to generate a colorless end product.

In phase two a second detergent in reagent 2 releases cholesterol from the LDL-lipoproteins. This cholesterol reacts with cholesterol esterase (che), cholesterol oxidase (cho) and a chromogen system to yield a blue color complex which can be measured bichromatically at 540/660nm. The resulting increase in absorbance is directly proportional to the LDL-C concentration in the sample.

Reaction phase 1

- ✓ HDL-C,VLDL-C, LDL-C, Chylomicrons $\xrightarrow{\text{Che And Cho}}$ Cholest-4-en-3-one + Fatty acids + H_2O_2
- ✓ H_2O_2-4-AAP $\xrightarrow{\text{Peroxidase}}$ LDL-C + Colorless end product

Reaction phase 2

- ✓ LDL-C $\xrightarrow{\text{Che And Cho}}$ Cholest-4-en-3-one + Fatty acids + H_2O_2
- ✓ H2O2 + DSBmT + 4-AAP $\xrightarrow{\text{Peroxidase}}$ Blue color complex

Absorbance is measured bichromatically at 540/660nm.

2.8.3. Extraction of Total Lipid And Assay of Liver Triglyceride

Reagents for the Assay

The hepatic triglyceride was measured according to the method described previously by Danno *et al*, 1992. In this method, reagents listed below were used.

- ➢ Chloroform (Research-Lab Fine Chem Industries, India)
- ➢ Methanol (Research-Lab Fine Chem Industries, India)
- ➢ tert-butyl alcohol (Schuchardt Munchen, Germany)
- ➢ Triton X-100 (Research-Lab Fine Chem Industries, India)
- ➢ Enzymatic triglyceride reagent kit (Human, Germany)
- ➢ Benzene (Research-Lab Fine Chem Industries, India)
- ➢ Diethyl ether (Research-Lab Fine Chem Industries, India)

Procedure for Total Lipid Extraction

To prepare lipid extracts from liver tissues, 0.5 g (wet weight) each of rat liver tissues were homogenized with 10 ml of the chloroform/methyl alcohol mixture (2/1 by volume), and then centrifuged at 2000 rpm for 20 minute and this extraction was done according to Folch *et al.* (1957). Briefly, the crude extract was mixed thoroughly with 0.2 its volume of normal saline and the mixture was allowed to separate into two phases,

without interfacial fluff by centrifugation at 2400 rpm for 20 minute. Then as much of the upper phase as possible was removed by siphoning, and removal of its solutes was completed by rinsing the interface three times with small amounts of pure solvents upper phase in such a way as not to disturb the lower phase. Finally, the lower phase and remaining rinsing fluid were made into one phase by the addition of methanol, and the resulting solution was diluted to any desired final volume by the addition of 2:1 chloroform-methanol mixture.

Principle and Procedure of Triglyceride Determination

After total lipid extraction, simple enzymatic determination of tissue triglyceride was done according to Danno *et al*. (1992). Briefly, evaporate the extraction on a centrifugal concentrator and then redissolved the residue in a small amount of benzene. Transfer this new mixture to a 15 ml falcon tube and diluted to the mark with more benzene. Thereafter, aliquots of the known working standards and of the liver lipid extracts in benzene were transferred into test tubes. Then the solvents were evaporated with a centrifugal concentrator, and redissolved the triglyceride standards and liver samples in 30 µL of tert-butyl alcohol and 20 µL of the Triton X-100/methyl alcohol mixture. These redissolved materials were mixed carefully. To each test tube, 1.0 ml of enzymatic reagent was added and mixed carefully again. Then the standards, samples, and appropriate blanks were incubated for 18 min at 37 ^{0}c, and then measured the absorbance at 505 nm vs. a reagent blank on Solar CM 2203 Spectro-fluorometer.

Thereafter, the triglyceride was determined after enzymatic hydrolysis by lipases. Indicator was quinoneimine formed from hydrogen peroxide, 4-amino-antipyrine and 4-chlorophenol under the catalytic influence of peroxidase. The amount of tissue triglyceride was calculated from the standard curve generated. The curve was drawn with the standard concentration of (0.03125-2 mg/ml) as shown below.

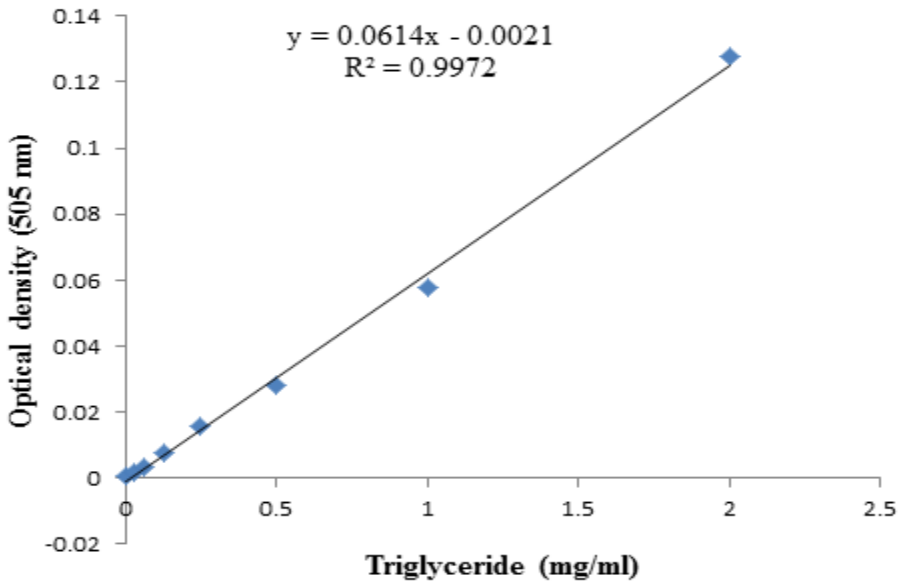

Figure 2: Standard curve for Triglyceride

2.8.4. Determination of Lipid Peroxidation

Lipid peroxide content was estimated according to the method of Ohkawa *et al*. (1979).

Principle of the Assay

Acetic acid detaches the lipid and protein of the tissue. The protein in the reaction mixture was dissolved by the addition of sodium dodecyl sulphate. 2-thiobarbituric acid (TBA) reacts with lipid peroxide, hydroperoxide and oxygen labile double bond to form the color products with absorption maxima at 532 nm.

Reagent Preparation

- 0.1 M phosphate buffer saline (pH= 7.4)
- Sodium dodecyl sulphate (SDS) 8% (Research Lab Fine CHEM Industries, India)
- Acetic acid (20%)(Fisher scientific, UK)
- 2-Thiobarbituric acid (0.8%) 800 mg of TBA (Hemedia, India) was suspended in 20 ml distilled water; the pH was adjusted to 7.0 by 0.1 N NaOH. This TBA was dissolved and volume was adjusted to 100 ml with distilled water.

- Butanol/pyridine mixture (15:1 v/v) (Research Lab Fine CHEM Industries, India)

Procedure of the Assay

0.2 ml of tissue homogenate was mixed with 1.0 ml of 20% acetic acid. Subsequently, 0.2 ml of 8% aqueous SDS was mixed in the above reaction mixture, the pH of the mixture was adjusted at 4.0 using concentrated NaOH solution. After adjusting the pH of the reaction mixture, 1.5 ml of 0.8% TBA solution and sufficient amount of distilled water were added to a final volume of 4.0 ml. Then the reaction mixture was incubated in a boiling water bath for one hour. After cooling, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1 v/v) were added and mixed. The reaction mixture was then centrifuged at 10,000 x g for 15 minutes. The organic phase obtained after centrifugation was used for measuring the absorbance at 532 nm in Solar CM 2203 Spectro-fluorometer.

The extinction coefficient of malondialdehyde (1.56×10^5) was used to calculate the amount of lipid peroxide in the samples and results were expressed as nmol of MDA/g tissue weight.

X= O.D x DF/E x wet Weight tissue (g)

Where X= nmol of MDA/g tissue

E= Extinction Coefficient of Malondialdehyde ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$)

DF=Dilution Factor=10/3

2.8.5. Measurement of Plasma Total Antioxidant Capacity

A direct measurement method for total antioxidant capacity using a new generation, more stable 2,2‟-Azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) diammonium salt (ABTS) radical cation was used to determine the total antioxidant capacity as previously described by Erel. (2004).

Assay Principle of the Novel Measurement Method

The reduced ABTS molecule is oxidized to $ABTSS^{+}$ using hydrogen peroxide alone in acidic medium (the acetate buffer 30 Mm, pH 3.6). In the acetate buffer solution, the concentrate (deep green) $ABTS^{+}$ molecules stay more stable for a long time. While it is diluted with a more concentrated acetate buffer solution at high pH values (the acetate buffer 0.4 M, pH 5.8), the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related with the TAC of the sample. The reaction rate is calibrated with Ascorbic acid standard for TAC measurement assays, and the assay results are expressed in mmol Ascorbic acid equivalent/L.

Reagent Preparation

Reagent 1

1. The 0.4 M acetate buffer solution (pH 5.8) was obtained as follows: 32.8 g of CH3COONa was dissolved in 1000 ml of deionized water (final concentration: 0.4 M).
2. Reagent-grade glacial acetic acid (22.8 ml) was diluted to 1000 ml with deionized water (final concentration: 0.4 M).
3. The sodium acetate solution (940 ml) was mixed with 60 ml of the acetic acid solution under a pH meter; the pH of the acetic acid–sodium acetate buffer was 5.8.
4. The buffer solution was stable for at least 6 months at $4\ ^{0}c$.

Reagent 2

1. The 30 mM acetate buffer solution, pH 3.6, was prepared as follows: 2.46 g of CH3COONa was dissolved in 1000 ml of deionized water (final concentration: 30 mM).
2. Reagent-grade glacial acetic acid (1.705 ml) was diluted to 1000 ml with deionized water (final concentration: 30 mM).

3. The sodium acetate solution (75 ml) was mixed with 925 ml of the acetic acid solution under a pH meter; the pH of the acetic acid–sodium acetate buffer was 3.6.
4. Then 200 micro liter of commercial H_2O_2 solution (30%, Sigma) was diluted to 1000 ml of the buffer solution (final concentration, 2 mM).
5. Then 50 mg ABTS (Sigma lot-030M8213V) was dissolved in 9.11 ml of prepared solution (final concentration: 10 mM).
6. After 1 hour of incubation at room temperature the characteristic deep green color of $ABTS^+$ was appeared. The colored reagent was stable for at least 6 months at 4 ^{0}c.
7. The standard Ascorbic acid was prepared in acetate buffer (30 mM) solution and mixed just like the sample and run on micro plate with respect to the sample in duplicate.

Procedure of the Assay

Prepared reagents and solutions for the experiment were brought to room temperature before the experiment had begun. This procedure was done according to the modified micro plate assay (improved method of total antioxidant assay) for total antioxidant capacity as described before by Gupta *et al*. (2009).

First 200 µL of reagent 1 was added to the micro plate reader followed by 5 µL of plasma sample using the multi-Chanel dispenser and mix carefully. Thereafter, the first absorbance had been read on automated micro plate reader (Labtek LT-4000, India) and immediately reagent 2 was added and mixed. Thereafter, it was incubated for 5 minute and the last absorbance was read at 600 nm. The calibration type was linear and ascorbic acid was used as a standard between 200-1000 µM concentration ranges.

The total antioxidant capacity of the sample was calculated from the standard curve drawn for ascorbic acid and the assay results were expressed in mmol of Ascorbic acid equivalent/L of sample.

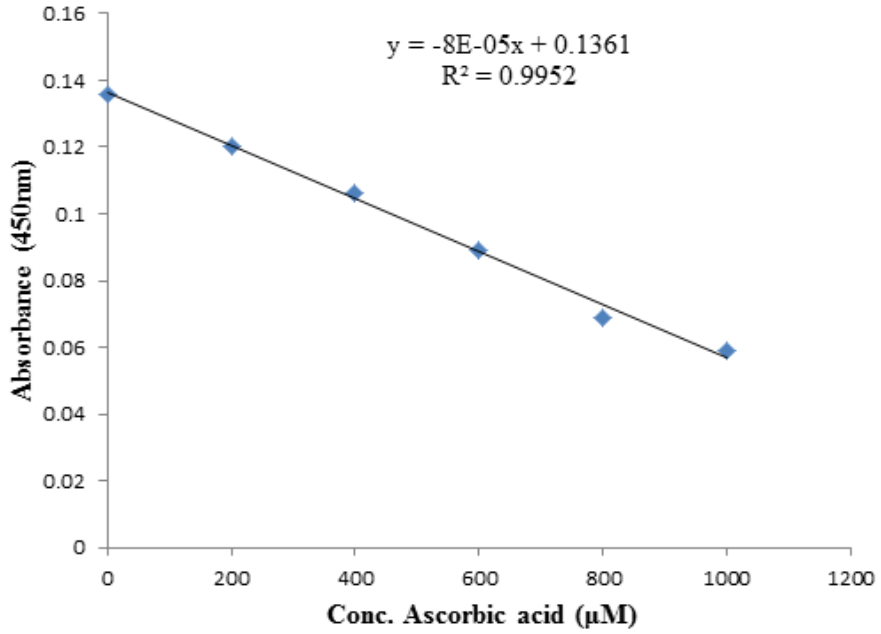

Figure 3: Standard curve for Ascorbic acid

2.8.6. Estimation of Free Sulfhydryl Group (GSH)

Free sulfhydryl group was estimated by the method of Ellman, (1959) as modified by Sedlak and Lindsay, (1968).

Principle

5-5'-dithiobis-2-nitrobenzoic acid (DTNB) is reduced by -SH groups of glutathione (GSH) in alkaline medium to produce one mole of 2-nitro-5-mercaptobenzoic acid per mole of -SH group. Since the anion (2-nitro-5-mercaptobenzoic acid) has an intense yellow color, it can be used to measure –SH group at 412 nm.

Reagent Preparation

- Standard solution preparation: A standard solution of 1 mM of GSH (Himedia, India) was prepared by dissolving 15.4 mg GSH in 50 ml of 0.1 N HCL.
- 0.1 M Hydrochloric Acid (B.D.H. Chemical Ltd Poole England)
- 10% trichloro acetic acid (TCA) (LABORT FINE CHEM PVT, India)

- ❖ 0.2 M Phosphate buffer, pH 7.6 was prepared by dissolving 0.43 g monobasic and 4.53 dibasic phosphate in 100ml of distilled water.
- ❖ 1 mM DTNB (Himedia, India) was prepared by dissolving 19.8 mg DTNB in 50 ml of 0.2 M Phosphate buffer.

Procedure of the Assay

1 ml tissue homogenate was deproteinized by adding 1 ml of 10% TCA and centrifuged (Heraus, Germany) at 5000xg for 15 minutes. 0.2 ml aliquot from clear supernatant was added to the test tube. Thereafter, 2.3 ml of phosphate buffer and 0.5 ml DTNB were added to the test tube with proper mixing. The absorbance was read at 412 nm within 5 minutes of the addition of DTNB on the tube by Solar CM 2203 Spectro-fluorometer.

A calibration curve with different concentrations of GSH (6.66-66.66 µM) was drawn. The values were plotted and Free -SH (GSH reduced) in the samples were calculated using the standard curve and the results were expressed as µmol/g tissue.

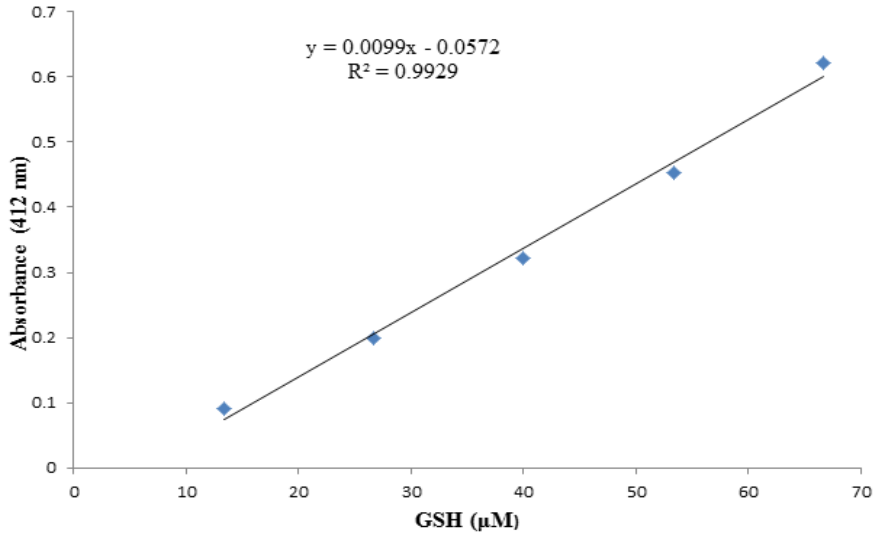

Figure 4: Standard curve for reduced glutathione

2.8.7. Determination of Liver Function Test

At the end of the experimental period, the rats were anesthetized using diethyl ether and Blood was drawn from heart by cardiac puncture and collected into serum separator test tubes (Improvacuter, Guangzhou, China) for serum and ethylene diamine tetra-acetic acid

tubes (EDTA tube) (Improvacuter, Guangzhou, China) for plasma. Plasma samples were inverted gently several times and kept for no longer than 30 min until centrifugation at 1000xg for 10 minute at 4 ^{0}c. After 30-40 minute of stay at room temperature, serum sample were centrifuged at 2000xg at 4^{0}c for 15min. The sample was stored at -80 ^{0}c till analysis. Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) were measured with routine laboratory methods using an auto analyzer (Mindrey, BS-200 Full chemistry Analyzer, China).

A. Determination of Alanine Aminotransferase (ALT)

Principle

Alanine aminotransferase (ALT) catalyzes the transfer of the amino group from alanine to α-Ketoglutarate with the formation of glutamate and pyruvate. The latter is reduced to lactate by lactate dehydrogenase (LDH) in the presence of reduced nicotinamide adenine dinucotide (NADH). The rate of change of the absorbance difference between 340 nm and 405 nm is due to the conversion of NADH to NAD+ and is directly proportional to the amount of ALT present in the sample (Henry RJ., *et al*. 1960; IFCC Export panel on enzymes, 1986).

The method is linear up to 800 U/L. The series of reactions involved in the assay system was as follows:

The method developed for use on the Analyzers is a modification of the IFCC-recommended procedure.

L-Alanine + α-Ketoglutarate $\xrightarrow{\text{ALT}}$ L-Glutamate + Pyruvate

Pyruvarte + NADH + H^+ $\xrightarrow{\text{LDH}}$ Lactate + NAD^+

Reagent Composition and Preparation

Reagent A: Tris 150 mmol/L, L-alanine 750 mmol/L, lactate dehydrogenase >1350 U/L, pH 7.3

Reagent B: NADH 1.9mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, Sodium azide 9.5 g/L. Mix 4ml of reagent A to 1ml of reagent B.

Procedure

Samples and reagents were brought to room temperature and mixed prior to analysis. All 30 samples at a time were placed in fully automated clinical chemistry analyzer. The analyzer was programed for appropriate wavelength, temperature and volume of serum (50 μL) and 1.0 ml reagents. The analyzer took appropriate volume of reagent and sample, mix and incubates at 37^0c for one minute. Based on the intensity of color compound formed, the automated analyzer read the absorbance at 340 nm kinetically at one, two, three minutes and determined the concentration (activity) of ALT in the sample by calculating the mean of the results to obtain the average change in absorbance per minute (ΔA/min).

B. Determination of Aspartate Aminotransferase (AST)

Principle

Aspartate aminotransferase (AST) catalyzes the reaction of L-aspartate and α-Ketoglutarate into oxaloacetate and L-glutamate. Oxaloacetate is converted to malate and NADH is oxidized to NAD^+ by the catalyst Malate dehydrogenase (MDH).
The reaction is monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from oxidation of NADH to NAD^+ proportional to the activity of AST present in the sample.
The method is linear up to 800 U/L.

L-Aspartate + α-Ketoglutarate $\xrightarrow{\text{AST}}$ L-Glutamate + Oxaloacetate

Oxaloacetate + NADH + H^+ $\xrightarrow{\text{MDH}}$ Malate + NAD^+

Reagent Composition and Preparation

Reagent A: Tris 121 mmol/L, L-aspartate 362 mmol/L, malate dehydrogenase > 460, lactate dehydrogenase > 660 U/L, Sodium hydroxide 255 mmol/L, P^H 7.8.
Reagent B: NADH 1.9 mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, Sodium azide 9.5 g/L. Mix 4ml of reagent A to 1ml of reagent B.

Procedure

Samples and reagents were brought to room temperature and mixed prior to analysis. All 30 samples at a time were placed in fully automated clinical chemistry analyzer. The analyzer was programed for appropriate wavelength, temperature and volume of serum (50 µL) and 1.0 ml reagents. The analyzer took appropriate volume of reagent and sample, mix and incubated at 37^0c for one minute. Based on the intensity of color compound formed, the automated analyzer read the absorbance at 340 nm kinetically at one, two, three minutes and determined the concentration (activity) of AST in the sample by calculating the mean of the results to obtain the average change in absorbance per minute (ΔA/min).

2.9. Histopathological Examination

Liver tissues were cut and fixed with 4% paraformaldehyde. The tissue slices were embedded in paraffin. Tissue sections of 5µm were stained with Hematoxylin and eosin (HE) and histology result were read by a single independent pathologist, blinded to experimental design and treatment groups using light microscope (LEICA DM750). Steatosis, fibrosis, and disease activity score were semi-quantitatively evaluated according to the standard criteria of grading and staging for NAFLD. Steatosis (the percentage of liver cells containing fat) was scored 0 with less than 5% of the cells containing fat; 1 with 5% to 33% of cells containing fat; 2 with 33% to 66% of the cells containing fat; 3 with > 66% of the cells containing fat. The scoring system comprised 14 histological features, 4 of which were evaluated semi-quantitatively: the grading for steatosis, lobular inflammation, hepatocellular ballooning and fibrosis. Another nine features were recorded as present or absent (Kleiner *et al*., 2005).

2.10. Statistical Analysis

Data were analyzed using Statistical Package for Social Science (SPSS) software (V-16.00). Data were compared using one way ANOVA followed by post-hoc Tukey‟s test to determine significance difference between groups. Frequency data (pathologic grading of fatty liver) were analyzed. A p-values < 0.05 were considered statistically significant.

CHAPTER THREE

3. RESULT

3.1. Effect of Silymarin on Food and Liquid Intake

The fructose control group consumed less standard chow (67.69 g/day) than the normal control group (114.36 g/day) as well as silymarin control group (108.05 g/day) ($p < 0.001$). Silymarin either 200 mg/kg (70.07 g/day) or 400 mg/kg (70.52 g/day) were brought slight increase in chow consumption as compared to fructose model group however it was not statistically significant. The fructose solution and tap water intake did not show any significance difference among groups unlike their food consumption. The fructose control group had better fructose solution intake (179.04 ml//day) than the normal control groups (171.27 ml/day).

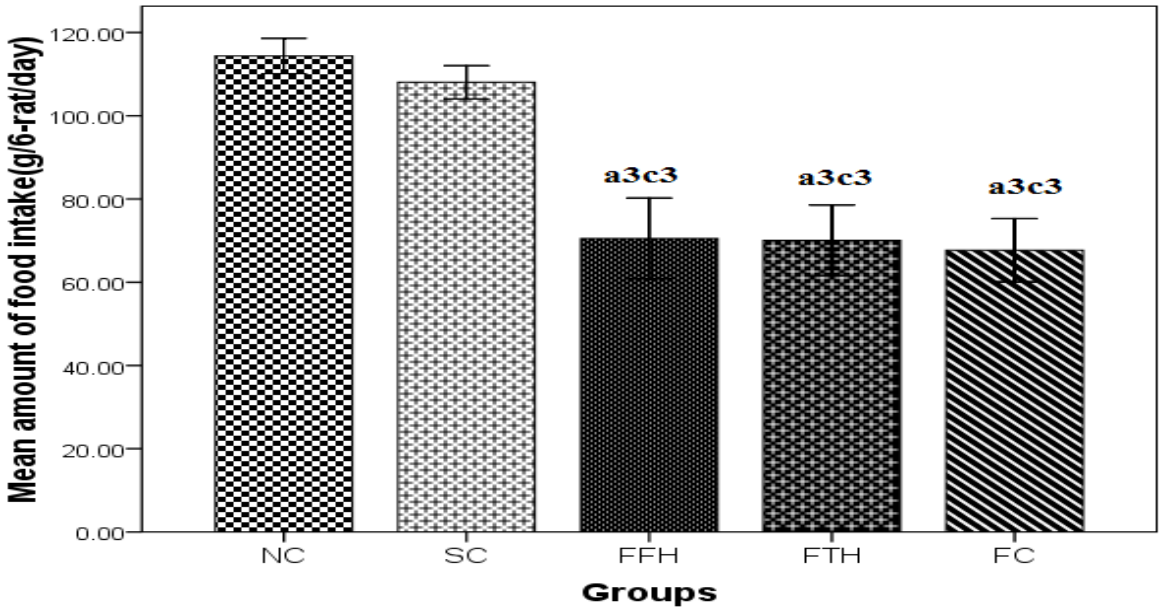

Figure 5: Effect of silymarin on food intake

NC= normal control, SC= silymarin control, FFH= fructose + 400 mg/kg silymarin, FTH= fructose + 200 mg/kg silymarin, FC= fructose control. Values are mean ± SD; n=6 for each group. a = compared with normal control, c = compared with silymarin Subscript numbers with letters indicate significant level: 3 = very highly significant ($p < 0.001$).

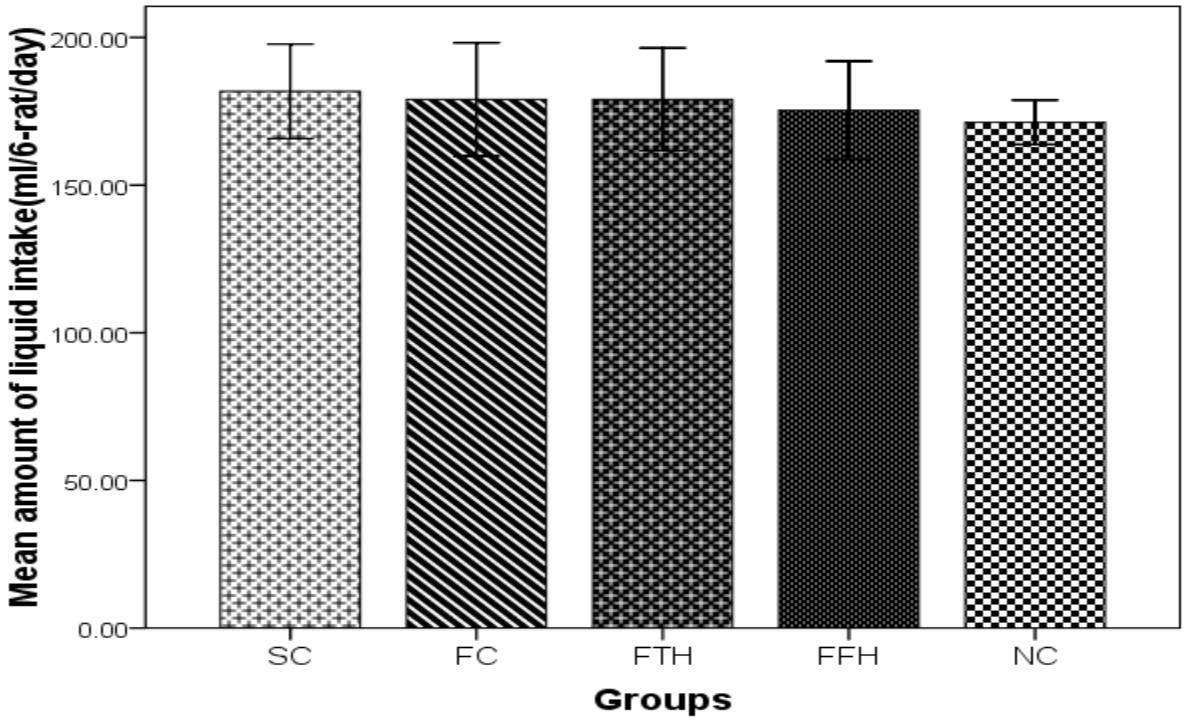

Figure 6: Effect of silymarin on liquid intake

NC= normal control, SC= silymarin control, FFH= fructose + 400 mg/kg silymarin, FTH= fructose + 200 mg/kg silymarin, FC= fructose control. Values are mean ± SD; n=6 for each group.

3.2. Effect of silymarin on Body Weight Gain, Liver weight and Liver weight to body weight ratio

The body weight gain and body mass index didn‟t bring any significance difference but the liver weight was significantly increased ($p < 0.05$) in the fructose model group as compared to normal control. However, didn‟t bring any significance change in liver weight to body weight ratio.

Table 3: Effect of silymarin on body weight gain, BMI, liver weight and LW/BW ratio

Group	Weight measurement			
	Liver weight(g)	Liver weight/body weight(g/g)	Body mass index(kg/m^2)	Weight Gain(g)
Normal Control (NC)	6.24 ± 0.53	0.025 ± 0.002	5.66 ± 0.18	71.67 ± 27.05
Fructose Control (FC)	6.98 ± 0.24[a]	0.029 ± 0.003	5.70 ± 0.39	79.67 ± 19.36
Silymarin Control (SC)	6.34 ± 0.41	0.026 ± 0.002	5.65 ± 0.19	71.83 ± 9.15
Fructose + 200 mg/kg silymarin (FTH)	6.71 ± 0.33	0.027 ± 0.003	5.81 ± 0.48	74.33 ± 17.67
Fructose + 400 mg/kg silymarin (FFH)	6.50 ± 0.36	0.026 ± 0.004	5.75 ± 0.28	72.67 ± 25.90

Values are mean ± SD; n=6 for each group. Subscript letters a only indicate significantly different with $P < 0.05$. a = compared with normal control.

3.3. Effect of Silymarin on Serum Lipid Profile

Fructose control group increased significantly in serum TC and LDL-C levels compared to normal control ($P < 0.05$). However, HDL-C tended to decline even though the difference was not statistically significant. In silymarin treated groups; TC, LDL-C and HDL-C levels showed improvement numerically however not statistically significant.

The Hepatic triglyceride (H-TG) and serum triglyceride (S-TG) values of the fructose consumed group increased significantly as compared to the normal control group and the

silymarin control group ($p < 0.001$). Both silymarin treated groups either the lower dose (200mg/kg) or higher dose (400mg/kg) significantly reduced H-TG and S-TG ($p < 0.05$).

Table 4: Effect of silymarin on serum and Liver Lipid Profile

Group	Lipid Profile of Rats				
	TG (mg/dl)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	H-TG (mmol/g tissue)
Normal Control (NC)	39.17 ± 5.52	39.00 ± 4.77	17.00 ± 2.28	5.50 ± 2.43	0.5 ± 0.01
Fructose Control (FC)	57.83 ± 8.10^{a2c2de}	53.83 ± 7.91^{a}	12.17 ± 3.54	9.00 ± 2.37^{a}	0.57 ± 0.03^{a3c3de}
Silymarin Control (SC)	42.83 ± 6.05	42.50 ± 6.95	15.83 ± 3.31	6.00 ± 1.79	0.52 ± 0.01
Fructose + 200 mg/kg (FTH)	45.50 ± 9.65	50.67 ± 7.89	12.67 ± 3.08	6.83 ± 1.94	0.54 ± 0.02
Fructose + 400 mg/kg (FFH)	44.17 ± 4.96	48.50 ± 10.84	15.33 ± 2.58	6.67 ± 1.21	0.53 ± 0.02

Values are mean ± SD; n=6 for each group. Subscript letters only without number on it indicate significantly different with $P < 0.05$. a = compared with normal control, c = compared with silymarin control, d= compared with fructose + silymarin (200 mg/kg) and e = compared with fructose + silymarin (400 mg/kg). Subscript numbers with letters indicate significant level: 3 = very highly significant ($p < 0.001$), 2 = highly significant ($p < 0.01$).

3.4. Effect of Silymarin on Liver Function Test

Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) levels increased in the fructose model group as compared to the normal control group ($p < 0.05$). ALT and AST in both silymarin treated groups were decreased as compared to the fructose model group even this difference was not statistically significant.

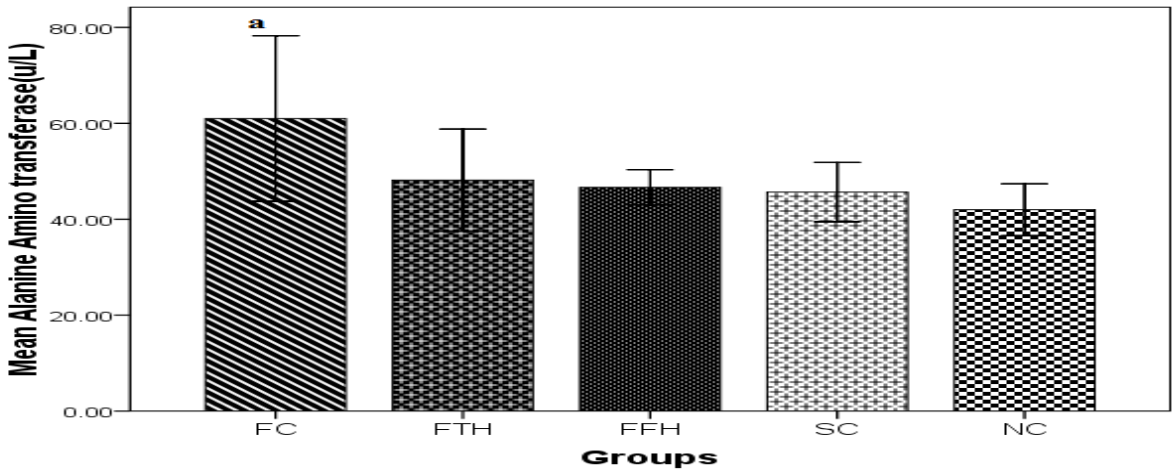

Figure 7: Effect of silymarin administration on serum alanine amino transferase

NC= normal control, SC= silymarin control, FFH= fructose + 400 mg/kg silymarin, FTH= fructose + 200 mg/kg silymarin, FC= fructose control. Values are mean ± SD; n=6 for each group. Subscript letter a only indicate significantly different with $P < 0.05$. a = compared with normal control.

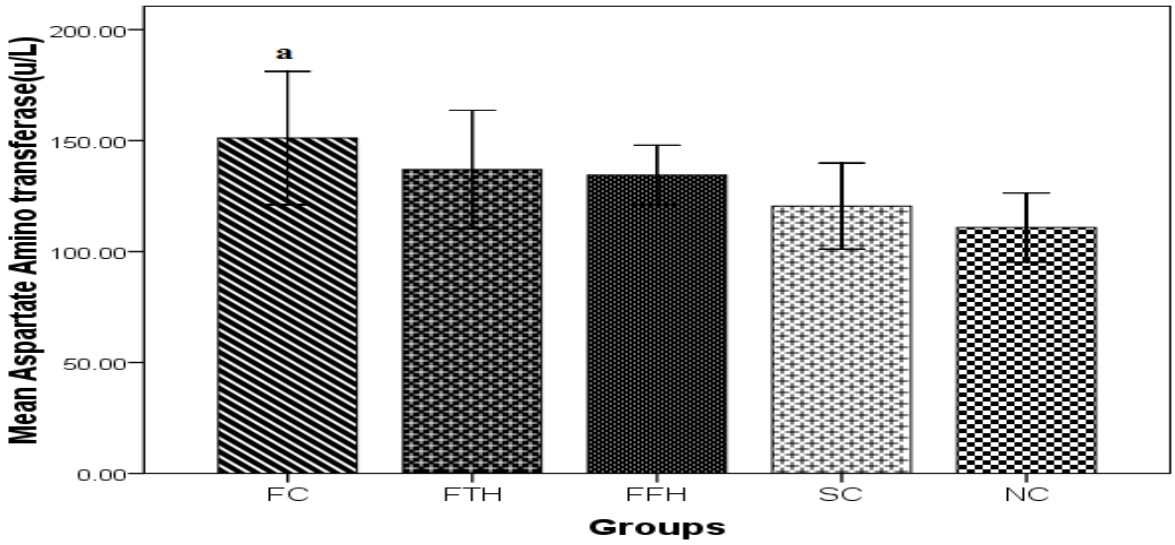

Figure 8: Effect of silymarin on serum aspartate amino transferase

NC= normal control, SC= silymarin control, FFH= fructose + 400 mg/kg silymarin, FTH= fructose + 200 mg/kg silymarin, FC= fructose control. Values are mean ± SD; n=6 for each group. Superscript letter a only indicate significantly different with $P < 0.05$. a = compared with normal control.

3.5. Effect of Silymarin on Hepatic MDA, GSH and Plasma TAC

The MDA level increased significantly in the fructose control group ($p < 0.001$) as compared to the normal control. Both the higher dose as well as the lower doses of silymarin significantly prevented the lipid peroxidation by the free radicals caused by fructose.

The hepatic GSH level of fructose control group drastically decreased as compared to normal control ($p < 0.01$). The group treated with 400 mg/kg silymarin increased GSH level significantly ($p < 0.01$). However, 200 mg/kg silymarin treated group showed increment in hepatic GSH but not statistically significant.

Plasma total antioxidant capacity (TAC) of fructose control group decreased as compared to normal control ($p < 0.01$). Silymarin either 200 or 400 mg/kg treated groups increased TAC of the plasma significantly ($p < 0.05$).

Table 5: Effects of silymarin on hepatic MDA, GSH and Plasma TAC

GROUP	H-MDA (nmol/g tissue)	GSH (µmol/g tissue)	P-TAC (nmol AAEAC/L)
Normal Control (NC)	52.65 ± 3.49	37.98 ± 1.07	0.933 ± 0.001
Fructose Control(FC)	67.60 ± 3.21^{a3c2de2}	33.93 ± 0.91^{a3c2e2}	0.927 ± .002^{a3c2de}
Silymarin Control (SC)	56.21 ± 4.99	36.53 ± 0.89	0.931 ± .002
Fructose + 200 mg/kg (FTH)	59.77 ± 5.40	35.49 ± 0.98	0.930 ± .002
Fructose + 400 mg/kg (FFH)	57.63 ± 4.48	36.35 ± 0.93	0.930 ± .001

Values are mean ± SD; n=6 for each group. Subscript letters only without number on it indicate significantly different with $P < 0.05$. a = compared with normal control, c= compared with treatment control, d = compared with fructose + silymarin (200 mg/kg) and e = compared with fructose + silymarin (400 mg/kg). Subscript numbers with letters indicate significant level: 3 = very highly significant ($p < 0.001$), 2 = highly significant ($p < 0.01$).

3.6. Effects of Silymarin on Gross Hepatic Manifestations

The liver of the normal control group was of moderate texture, with a smooth and red-brown surface. In contrast, hepatic volume was enlarged, and of bright red brown color and relatively hard texture in the fructose model group. The liver conditions of both groups treated with silymarin were intermediate between those of the above two groups with doomed red brown color and relatively smooth surface.

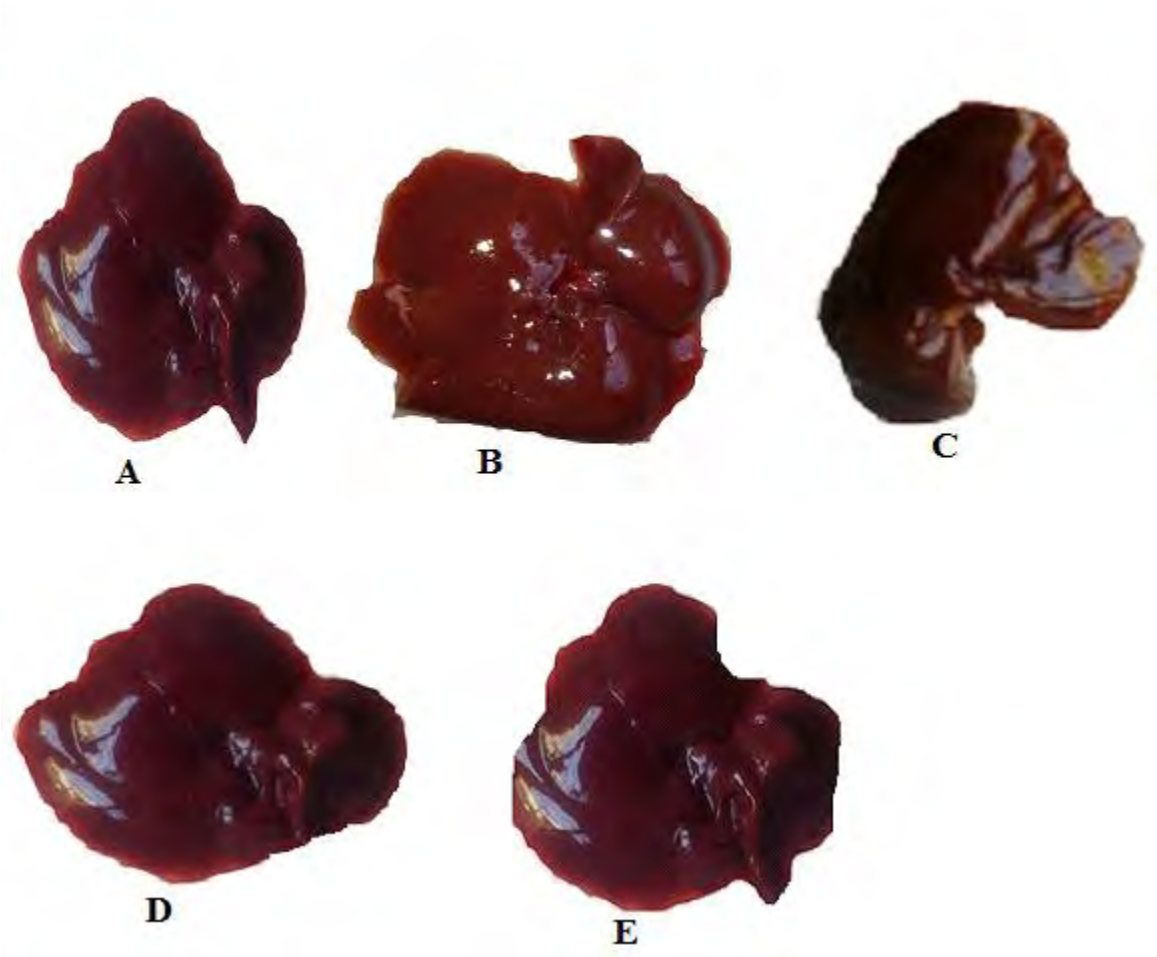

Figure 9: Rat gross hepatic characteristics; A) Normal control group; B) Fructose control group; C) Silymarin control group; D) fructose + 200 mg/kg silymarin treated group; E), fructose + 400 mg/kg silymarin treated group

3.7. Effect of Silymarin on Histopathological Manifestations

The liver lobules of the normal control group were distinct, and the liver cell cords were arranged regularly (Figure, A). However, the fructose model group (Figure, B) showed typical steatosis accompanied by a few infiltrate cells. The degree of hepatic injury including steatosis, cytological ballooning was attenuated by silymarin.

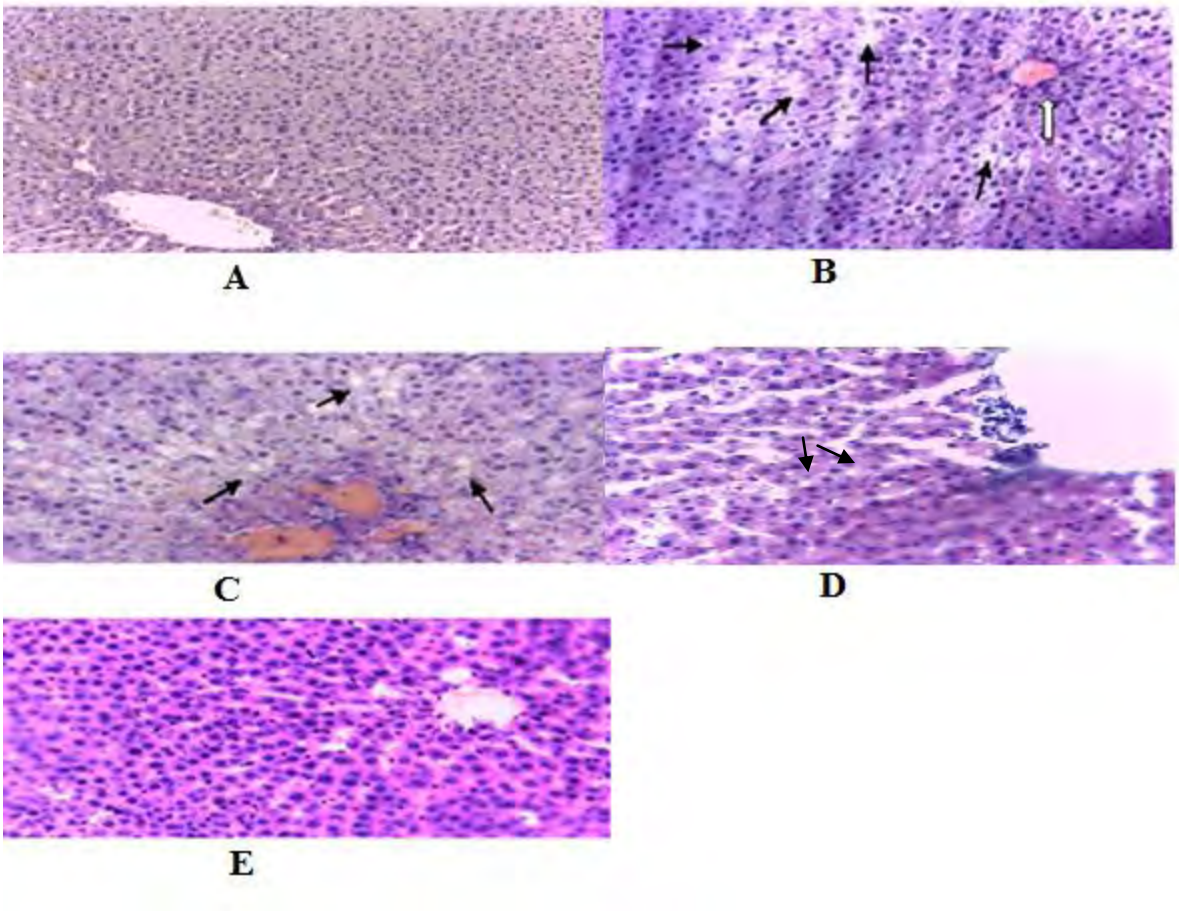

Figure 10: Photomicrographs of liver samples stained with Hematoxylin & Eosin; (→ indicate steatosis), (⇨ indicate lobular inflammation); A) No fatty change in normal control; B) the micrographs showed steatosis in the fructose control group. In contrast, fewer and smaller fat granules are observed from silymarin treated groups; C) Fructose + 200 mg/kg silymarin treated group; D) Fructose + 400 mg/kg silymarin treated group; E) no fatty change on Silymarin control group

3.7.1. Effect of Silymarin on Nonalcoholic Fatty Liver Disease Activity Scores (NAS)

Based on the NAS showed below, the fructose control group fulfilled the steatosis score of 1.84. The predominant distribution pattern of the steatosis observed in fructose control group were in zone 3 (3 rats) as well as in zone 1(3 rats). The silymarin treated groups prevent liver steatosis according to NAS.

Table 6: Histopathological nonalcoholic fatty liver disease activity scores

NAFLD Activity Score	**GROUPS**				
	NC	FC	SC	FTH	FFH
Steatosis grade	0	0.83[a]	0.17	0.50	0.33
Location of steatosis)	0	0.50	0	0	0
Microvesicular steatosis	0	0.17	0	0	0
Lobular inflammation	0	0.17	0	0	0
Fibrosis stage	0	0	0	0	0
Microgranulomas	0	0	0	0	0
Large lipogranulomas	0	0	0	0	0
Portal inflammation	0	0	0	0	0
Liver cell injury (ballooning)	0	0.17	0	0	0
Acidophil bodies	0	0	0	0	0
Pigmented microphages	0	0	0	0	0
Megamitochondria	0	0	0	0	0
Mallory's hyaline	0	0	0	0	0
Glycogenated nuclei	0	0	0	0	0
Total sum	0	1.84	0.17	0.50	0.33
Diagnostic classification for NASH	Not steatosis	Possible/border line	Not steatosis	Not steatosis	Not steatosis

NC= normal control, SC= silymarin control, FFH= fructose + 400 mg/kg silymarin, FTH= fructose + 200 mg/kg silymarin, FC= fructose control. Values are mean (n=6) for each group. Subscript letters a only indicate significantly different with $P < 0.05$ and a = compared with normal control,

CHAPTER FOUR

4. DISCUSSION

NAFLD is a common chronic liver disease worldwide and its incident increased in developing and developed countries. High fructose consumption and a high fat diet consumption or high fructose high fat combination diets for different period of time were believed to induce NAFLD model of rats with the metabolic syndrome features like dyslipidemia, insulin resistance, hypertension, extreme body weight gain (Aragno *et al*., 2009; Axelsen *et al*., 2010; Poudyal *et al*., 2010; Alisi *et al*., 2011; Poudya *et al*., 2012; Roth *et al*., 2012). Accumulating evidence suggests that fructose is distinct from glucose in its ability to cause intracellular phosphate depletion and uric acid generation in the liver. High levels of intracellular uric acid, advanced glycation end product (AGEs), and ROS can induce inflammatory effects and oxidative stress in the liver (Jurgens *et al*., 2005). The treatments of NAFLD currently include oral drug therapy and exercise therapy. All this existing drug therapy's seems to be inefficient therefore it is necessary to find drugs with better effect. Silymarin which is herbal based drug with flavolignan and flavonoid components was known very well for its liver protective effect. The active extract has antioxidant, anti-inflammatory, and antifibrotic properties; in addition, it stimulates protein biosynthesis and liver regeneration (Abenavoli *et al*., 2011).

In the present study, the fructose consumed control group had a significantly different value of stage of steatosis grade, liver weight, hepatic TG, serum TG, TC, LDL-C, ALT, AST and H-MDA concentration as compared to the normal control and contrariwise had a significantly low values of GSH, P-TAC and food consumption. The fructose model group had no significance difference on HDL-C, liver weight to body weight ratio, body mass index, weight gain and liquid intake as compared to other four groups. Both Silymarin treated groups ameliorated to some extent from fructose induced variations.

Any form of fructose consumption (along with the drinking water, high fat + fructose combination) were induced NAFLD in rats with the metabolic syndrome features like dyslipidemia, insulin resistance, hypertension, extreme body weight gain (Aragno *et al*., 2009; Axelsen *et al*., 2010; Poudyal *et al*., 2010; Alisi *et al*., 2011; Poudya *et al*., 2012;

Roth *et al*., 2012). Increased ALT and AST, lipid profiles such as; TC, TG, LDL-C, HDL-C in plasma as well as liver macrosteatosis and elevated level of liver TG also observed in mouse when fed with fructose alone or in combination with chow (Tetri *et al*., 2008; Sohet *et al*., 2009; Wada *et al*., 2010; Kohli *et al*., 2010).

The liquid intake was not statistically different even though numerical difference had been observed. This study was similar with the findings of Abdulla *et al*. (2011); however, the present finding disagreed with Mamikutty *et al*. (2015) who showed higher liquid intake in fructose solution drinking group than the water intake groups. Silymarin treated groups did not show any significance alterations in either fructose solution consumption or tap water drinking.

Fructose consumption decreased in food intake. This finding was in agreement with previously described findings in which fructose in either 20% or 10% showed significant reduction in food intake (Abdulla *et al*., 2011; Wang *et al*. 2013; Mamikutty *et al*., 2015). In contrary, another study showed that fructose either 5% or 10% did not alter the food consumption (Cardinali *et al*., 2013). Silymarin did not bring any significance change in chow consumption in either normal rats or fructose consumed rats. This result was in agreement with Ni and Wang (2016) finding. Fructose groups consumed less chow due to the fructose compensate the daily calorie requirement of the rats.

Fructose fed rats did not bring any significant change in body weight gain. This finding was similar with the previous study by Wang *et al*. (2013). This finding was also in agreement with another study done on C57BL/6 mouse model that showed the least impact of fructose on body weight gain as compared to other mono and disaccharide sugars (Bergheim *et al*., 2008). But some other studies revealed that fructose had positive impact on body weight gain (Jurgens *et al*., 2005; Abdulla *et al*., 2011; Cardinali *et al*., 2013). This might be due to the short period of the experiment. Silymarin did not bring any significant change in body weight gain in rats. This finding was similar with Ni and Wang, (2016) as previously described.

Liver weight of the fructose control group increased significantly as compared to normal control. This finding was supported by other findings which showed either fructose alone

or in combination with high fat diet brought significant increase on liver weight (Poudya *et al*., 2010; Alisi *et al*., 2011; Poudya *et al*., 2012; Roth *et al*., 2012). This might be due to ectopic lipid accumulation in the liver. In this study silymarin did not bring any significant alteration in fructose induced liver weight gain. But in another study showed that the 12 weeks liquid fat induced liver weight gain was reduced significantly by 5 weeks treatment of silymarin (Haddad *et al*., 2011).

In fructose control group serum TG, LDL-C and TC increased significantly in contrast no significant change in the HDL-C. This finding was in agreement with other studies done on either fructose or fructose with high fat diet revealed significant alteration in plasma TG, LDL-C and TC (Botezelli *et al*., 2010; Alisi *et al*., 2011; Panchal *et al*., 2012; Cardinali *et al*., 2013; Nassir *et al*., 2014; Senaphan *et al*., 2015 ; Liu *et al*., 2015). Silymarin in either dose brought numerical difference only in fructose altered serum lipid profile in rats but not statistically significant. Most of the studies were against our present finding. The high fat diet induced lipid profile alteration were significantly restored by silymarin (Haddad *et al*., 2011; Heidarian and Rafieian-Kopaei, 2012; Ni and Wang, 2016).

Hepatic triglyceride significantly increased in fructose consumed group. Silymarin in either dose significantly reduced the hepatic TG but could not reach the control value. Previous findings also supported our finding silymarins to reduce the hepatic TG accumulation (Ni and Wang, 2016; Haddad *et al*., 2009; Heidarian and Rafieian-Kopaei, 2012). The previous finding too strongly support the present finding who showed that flavonoid in *stellera chamsaesasme* L. is potentially prevent TG accumulation in liver of high fat induced NAFLD rats (Wang *et al*., 2015). The molecular mechanism is to down regulate the lipogenic enzymes gene expression in liver, hence, this molecular mechanism is taken into account for present finding in silymarin which contains a flavonoid taxifolin and quercetin.

Fructose consumed control group significantly increased in alanine amino transferase and aspartate amino transferase. Other findings were in agreement with this investigation (Aragno *et al*., 2009; Poudyal *et al*., 2010; Alisi *et al*., 2011; Panchal *et al*., 2012; Roth *et al*., 2012). Either dose of silymarin brought numerical reduction in serum ALT and AST

but not statistically significant. However, most of the finding showed that silymarin had a potential to reduce the serum AST and ALT in different toxic and high fat diet induced liver injury (Yao *et al*., 2011; Sabiu *et al*., 2015; Sayin *et al*. 2016; Ni and Wang, 2016).

Elevated level of hepatic MDA and the reduced level of GSH and TAC of the plasma were observed in the fructose consumed group. This finding was in lined with previous studies (Armutcu *et al*., 2005; Shaker *et al*., 2010; Patel *et al*., 2010). Other studies on fructose-sweetened liquid and fatty liver use products of lipid peroxidation as markers of oxidative stress and only few studies have evaluated enzymatic antioxidant systems (Du *et al* 2010). Fructose produces damaging effects in hepatocytes because it is highly reactive as a reducing agent and a precursor of advanced glycation end product (AGE). The liver promotes removal of high levels of fructose aggressively from the bloodstream to prevent the damaging effects of glycation/fructation on serum lipids and proteins. Glycated/fructated proteins not only show impaired functions but are also more susceptible to oxidative damage. Glycated/fructated proteins are ultimately converted into toxic AGEs (Takeuchi *et al*., 2010). Silymarin treated groups significantly reduced MDA while increased GSH and TAC of plasma and this finding was also supported by previous studies (Shaker *et al*., 2010; Heidarianl and Rafieian-Kopaei, 2012).

Hepatic protective effect of silymarin in general could be explained in four points: activity against lipid peroxidation as a result of free radical scavenging and the ability to increase the cellular content of glutathione, efficacy to increase the membrane stability and to regulate membrane permeability in the presence of liver damage, ability to regulate nuclear expression by means of a steroid-like effect and inhibition of transformation of stellate hepatocytes into myofibroblasts (Abenavoli *et al*., 2011). Silymarin can contribute to the antioxidant defenses in different ways. Firstly, by direct free radical scavenging. Secondly, by preventing free radical formation by inhibiting specific enzymes responsible for free radical production, or by maintaining the integrity of electron-transport chain of mitochondria in stress conditions and thirdly, by participating in the maintenance of optimal redox status of the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants (Surai, 2015). Silymarins antioxidant property has been investigated and it is a kind of chain breaking antioxidant

or free radical scavenger. The radical oxidation of silybin methylated 7-OH yields C-C dimers, which enable the molecular mechanism of their E-ring interaction with radicals. And this shows the importance of 20-OH group in its antioxidant property (Pei *et al*., 2009). Silymarin may inhibit lipid peroxidation by scavenging free radicals and increasing intracellular concentration of glutathione (Toklu *et al*., 2007). Due to its phenolic nature, it is capable of donating electrons to stabilize free radicals and reactive oxygen species (ROS). Silymarin also affects intracellular glutathione, which prevents lipid peroxidation of membranes (Karimi *et al.,* 2011).

The normal control group had red brown liver and relatively small in size as compared to the fructose consumed group which had large and bright red brown as well as relatively hard texture surface. Relative reduction in brightness of liver as well as in size was observed in both silymarin treated groups. This finding was similar with Yao *et al*. (2011). The size increment in liver might be due to the higher numbers of lipid vacuoles deposited in the hepatocyte cytoplasm.

The fructose consumed group fulfilled the steatosis grade with predominant distribution pattern in zone 3 which was significantly different as compared to normal control. The histopathological finding showed the ectopic lipid accumulation in the liver (steatosis) and this might be due to fructose over consumption and metabolism which led to *de novo* lipogenesis. Either dose of silymarin protected liver steatosis. This finding was in agreement with different diet combination induced steatosis potentially prevented by silymarin (Yao *et al*., 2011; Alisi *et al*., 2011; Roth *et al*., 2012). Silymarins protective effect on liver steatosis might be due to its antioxidant, membrane integrity and anti-inflammatory properties.

CHAPTER FIVE

5. Conclusion and Recommendation

5.1. Conclusion

The present study concluded that high fructose consumption caused the development of dyslipidemia, oxidative stress and steatosis which are the characteristics feature of NAFLD. These problems were reduced through silymarins treatment by improving the liver function and lipid profile tests. Fructose induced nonalcoholic fatty liver disease was prevented by silymarin via inhibition of lipid peroxidation through regulatory property of membrane integrity and their oxidant scavenging activity through increased intracellular glutathione level. Silymarin treatment with higher dose (400 mg/kg) has a better efficacy than lower dose (200 mg/kg) on NAFLD.

5.2. Recommendation

The present study further recommends the following investigations to be undertaken:

1. Long term investigation of high fructose consumption on rats should be done to see the body weight gain as well as the clear Histopathological findings that indicate NASH and amelioration effect by silymarin.

2. Further study should be done to investigate the combinatorial effect of high alcohol, high fructose and high fat consumption despite their individual roles to develop fatty liver disease model in rats since consumption of all these are common in day to day life in most of the people around the world.

3. Average amount of fructose per day per individual consumption in Ethiopia should be investigated to find out the susceptibility level of NAFLD due to fructose over consumption.

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